Reactive Oxygen Species–Activated Ca/Calmodulin Kinase IIΔ Is Required for Late $I_{Na}$ Augmentation Leading to Cellular Na and Ca Overload

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**Rationale:** In heart failure, Ca/calmodulin kinase (CaMKII) expression and reactive oxygen species (ROS) are increased. Both ROS and CaMKII can increase late $I_{Na}$ leading to intracellular Na accumulation and arrhythmias. It has been shown that ROS can activate CaMKII via oxidation.

**Objective:** We tested whether CaMKIIΔ is required for ROS-dependent late $I_{Na}$ regulation and whether ROS-induced Ca released from the sarcoplasmic reticulum (SR) is involved.

**Methods and Results:** 40 μmol/L H$_2$O$_2$ significantly increased CaMKII oxidation and autophosphorylation in permeabilized rabbit cardiomyocytes. Without free [Ca] (5 mmol/L BAPTA/1 mmol/L Br$_2$-BAPTA) or after SR depletion (caffeine 10 mmol/L, thapsigargin 5 μmol/L), the H$_2$O$_2$-dependent CaMKII oxidation and autophosphorylation was abolished. H$_2$O$_2$ significantly increased SR Ca spark frequency (confocal microscopy) but reduced SR Ca load. In wild-type (WT) mouse myocytes, H$_2$O$_2$ increased late $I_{Na}$ (whole cell patch-clamp). This increase was abolished in CaMKIIΔ−/− myocytes. H$_2$O$_2$-induced [Na] and [Ca] accumulation (SBFI [sodium-binding benzofuran isophthalate] and Indo-1 epifluorescence) was significantly slowed in CaMKIIΔ−/− myocytes (versus WT). CaMKIIΔ−/− myocytes developed significantly less H$_2$O$_2$-induced arrhythmias and were more resistant to hypercontracture. Opposite results (increased late $I_{Na}$, [Na] and [Ca] accumulation) were obtained by overexpression of CaMKIIΔ in rabbit myocytes (adenoviral gene transfer) reversible with CaMKII inhibition (10 μmol/L KN93 or 0.1 μmol/L AIP [autocamtide 2–related inhibitory peptide]).

**Conclusions:** Free [Ca] and a functional SR are required for ROS activation of CaMKII. ROS-activated CaMKIIΔ enhances late $I_{Na}$, which may lead to cellular Na and Ca overload. This may be of relevance in heart failure, where enhanced ROS production meets increased CaMKII expression. (Circ Res. 2011;108:555-565.)

**Key Words:** reactive oxygen species • Na$^+$ current • Ca$^{2+}$/calmodulin-dependent protein kinase II • sodium channels • sarcoplasmic reticulum

Reactive oxygen species (ROS) are generated during myocardial infarction and contribute to left ventricular remodeling and adverse outcomes.¹ ² In human failing myocardium, increased oxidative stress is associated with reduced left ventricular function.³ These detrimental effects involve impairment of Na and Ca homeostasis⁴–⁸ resulting in contractile dysfunction, electric instability and cell death.⁹ We and others have shown that H$_2$O$_2$, which generates ROS,² enhances the late, slowly-inactivating current through cardiac Na channels (late $I_{Na}$), thereby leading to action potential (AP) prolongation and arrhythmias.⁶ ¹⁰ Na entering the cell via late $I_{Na}$ leads to cytosolic Na accumulation, favoring Ca entry via sarcolemmal Na/Ca exchange (NCX) and cellular Ca overload, contractile dysfunction, and hypercontracture.⁵ ¹¹ ¹²

The mechanisms by which ROS increase late $I_{Na}$ and initiate this detrimental cascade are not clear. We have shown that Ca/calmodulin-dependent protein kinase δ (CaMKIIΔ) (CaMKII) can phosphorylate cardiac Na channels leading to enhanced late $I_{Na}$.¹³ The binding of Ca/calmodulin (Ca/CaM) to its regulatory domain results in conformational changes that expose the catalytic domain,¹⁴ which can phosphorylate numerous targets including threonine 287 at its own regulatory domain. This autophosphorylation renders the protein to remain active...
Non-standard Abbreviations and Acronyms

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AIP</td>
<td>autacamide 2–related inhibitory peptide</td>
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<tr>
<td>APD</td>
<td>action potential duration</td>
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<tr>
<td>βGal</td>
<td>β-galactosidase</td>
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<tr>
<td>BCL</td>
<td>basic cycle length</td>
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<tr>
<td>[Ca]i</td>
<td>intracellular calcium concentration</td>
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<tr>
<td>CaMKII</td>
<td>calmodulin-dependent protein kinase II</td>
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<tr>
<td>CaMKIIδ–/–</td>
<td>homozygous knockout of Ca/calmodulin-dependent protein kinase II</td>
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<tr>
<td>CaSpF</td>
<td>calcium spark frequency</td>
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<tr>
<td>DAD</td>
<td>delayed afterdepolarization</td>
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<tr>
<td>EAD</td>
<td>early afterdepolarization</td>
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<tr>
<td>FDHM</td>
<td>full-duration half-maximum</td>
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<tr>
<td>HF</td>
<td>heart failure</td>
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<tr>
<td>INa</td>
<td>Na current</td>
</tr>
<tr>
<td>[Na]i</td>
<td>intracellular sodium concentration</td>
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<tr>
<td>NCX</td>
<td>Na/Ca exchanger</td>
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<tr>
<td>NKA</td>
<td>Na/K ATPase</td>
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<tr>
<td>PK</td>
<td>protein kinase</td>
</tr>
<tr>
<td>RAN</td>
<td>ranolazine</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RyR2</td>
<td>ryanodine receptor type 2</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>THA</td>
<td>thapsigargin</td>
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<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
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It has been recently shown that ROS oxidize methionine 281/282 at the regulatory domain of CaMKII, and oxidized CaMKII has properties equivalent to autophosphorylated CaMKII in mediating angiotensin-induced apoptosis and ROS-induced afterdepolarizations. Moreover, there is evidence that Ca/CaM-CaMKII interaction is a prerequisite for ROS-dependent oxidation of CaMKII, and ROS have been shown to directly induce sarcoplasmic reticulum (SR) Ca release via thiol oxidation of the ryanodine receptor 2 (RyR2).

Thus, we hypothesized that (1) ROS-activated CaMKIIδ is responsible for the observed ROS effects on late INa; and (2) Ca released from SR Ca stores is involved in the ROS-dependent CaMKII activation process.

The results of the present study show that H2O2-induced augmentation of late INa requires CaMKIIδ, and the consequent cellular Na and Ca overload was significantly slowed in myocytes lacking CaMKIIδ. Moreover, we show that the H2O2-dependent CaMKII activation requires a functional SR. Finally, we establish CaMKIIδ as an important mediator of H2O2-dependent arrhythmogenesis and cellular death injury.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

CaMKIIδ Knockout Mice and CaMKIIδ Overexpression in Rabbit Myocytes

Ventricular myocytes were isolated from CaMKIIδ knockout mice (CaMKIIΔΔδ) and wild-type (WT) littermates. For CaMKIIδ overexpression, isolated rabbit ventricular myocytes were transfected with CaMKIIΔC adenovirus (β-galactosidase [βGal] as control).13,21

Patch-Clamp Experiments

Ruptured-patch whole-cell voltage-clamp or current-clamp was used to measure INa or membrane potential, respectively. Myocytes were held at -120 mV and INa was elicited using a train of pulses to -20 mV. APs were continuously elicited by square current pulses of 1 to 2 nA amplitude and 1 to 5 ms duration at basic cycle length (BCL) of 2 seconds.

Data Analysis and Statistics

All data are expressed as means±SEM. For longitudinal data, 2-way repeated measures analysis of variance (ANOVA) was run; otherwise, Student’s unpaired t test or 1-way ANOVA with Student–Newman–Keuls multiple comparison was used. Double-sided probability values of <0.05 were considered significant.

Results

H2O2-Derived CaMKII Activation Requires Functional SR Ca Stores

CaMKII autophosphorylation at serine 287 (p-CaMKII) and oxidation at methionine 281/282 (ox-CaMKII) were assessed using Western blotting in lysates of myocytes exposed to H2O2 (Figure 1; Online Table I). Increasing cytosolic [Ca] to 1 μmol/L significantly increased p-CaMKII levels and inhibition of catalytic CaMKII activity using autacamide 2–related inhibitory peptide (AIP) reversed this activation. In myocytes exposed to H2O2, p-CaMKII and ox-CaMKII levels increased in a concentration-dependent manner (40 versus 200 μmol/L H2O2) and AIP significantly reduced H2O2-induced autophosphorylation but not oxidation of CaMKII. This suggests that CaMKII oxidation is accompanied by a significant autophosphorylation of CaMKII. Interestingly, when SR Ca stores were depleted using caffeine and thapsigargin (THA), both H2O2-dependent oxidation and autophosphorylation of CaMKII were prevented. This suggests that Ca derived from SR Ca stores is required for substantial H2O2-dependent CaMKII activation to occur. To further investigate the Ca-dependency of CaMKII oxidation, permeabilized myocytes were bathed in a mock intracellular solution, in which Ca was heavily buffered (5 mmol/L BAPTA/1 mmol/L Br2-BAPTA). Figure 2 and Online Table I show that no significant H2O2-dependent CaMKII oxidation or autophosphorylation were observed in the absence of intracellular free [Ca]. Additionally, heavily buffered low free [Ca] of 20 nmol/L prevented a substantial increase in p-CaMKII and ox-CaMKII levels after H2O2 exposure. This suggests that even at increased Ca/CaM dissociation it is known that expression and activity of CaMKII are increased in heart failure (HF), and we have shown that transgenic CaMKIIδ overexpression results in left ventricular dysfunction and arrhythmias, whereas CaMKIIδ inhibition/knockout was shown to prevent cardiac remodelling and heart failure.16–18

Thus, we hypothesized that (1) ROS-activated CaMKIIδ is responsible for the observed ROS effects on late INa; and (2) Ca released from SR Ca stores is involved in the ROS-dependent CaMKII activation process.

The results of the present study show that H2O2-induced augmentation of late INa requires CaMKIIδ, and the consequent cellular Na and Ca overload was significantly slowed in myocytes lacking CaMKIIδ. Moreover, we show that the H2O2-dependent CaMKII activation requires a functional SR. Finally, we establish CaMKIIδ as an important mediator of H2O2-dependent arrhythmogenesis and cellular death injury.
We determined the effects of H₂O₂ on RyR2 function by analyzing the characteristics of SR Ca sparks. In permeabilized myocytes, the addition of 200 μmol/L H₂O₂ led to an immediate and massive SR Ca release throughout the whole cell, comparable to a caffeine transient, followed by an irreversible inhibition of RyR2 function (data not shown). Because intracellular H₂O₂ may reach only 1% to 15% of the applied exogenous concentration in case of an intact sarclemma, we reduced the H₂O₂ concentration to 40 μmol/L and set the exposure time to 30 s for all experiments in permeabilized myocytes. Addition of H₂O₂ caused an immediate significant increase in calcium spark frequency (CaSpF) and full-duration half-maximum (FDHM), and a slight reduction in Ca spark amplitude, leading to a 3.5-fold increase in SR Ca leak (Figure 3A through 3C). This ROS effect appeared to be transient. Indeed, 2.5 minutes after onset of H₂O₂ exposure CaSpF and amplitude had returned to baseline values, and SR Ca leak was significantly reduced despite a persistent prolongation of FDHM. Interestingly, at the time of maximal increased SR Ca leak, H₂O₂ exposure caused a significant reduction of SR Ca load that was not prevented by AIP (Figure 3D; 30 seconds of H₂O₂).

SR Ca sparks were also measured in intact mouse myocytes during electric field stimulation. Under baseline conditions, very few Ca sparks were detected (Figure 4). Similar to permeabilized myocytes, 200 μmol/L H₂O₂ increased CaSpF but reduced Ca spark amplitude. The resulting SR Ca leak was markedly (∼15-fold) increased (Figure 4A through 4C). This was accompanied by reduced SR Ca load (Figure 4D). Notably, inhibition of CaMKII (KN93) did not influence the H₂O₂-dependent changes in CaSpF, SR Ca leak or SR Ca load, although Ca spark amplitude was slightly reduced (Figure 4A through 4D). These results indicate that the H₂O₂-induced SR Ca leak does not require CaMKII.

CaMKII Is Required for H₂O₂-Enhanced Late Iₙa
Our group and others have shown previously that ROS enhance late Iₙa, and CaMKII exerts a strikingly similar effect. To test whether H₂O₂-activated CaMKII is required for the effects of ROS on late Iₙa, the current was measured in myocytes from WT and CaMKII⁰⁻ mice (Figure 5A). At baseline, no significant difference in late Iₙa integrals was detected between the 2 groups (Figure 5B and the Table). When WT myocytes were exposed to H₂O₂, a significant time-dependent increase in the late Iₙa integral, nearly doubling at 12 minutes after H₂O₂ treatment, was observed. This increase was absent in myocytes lacking CaMKII (Figure 5A and 5B; Table). Consistent with this, adenoviral CaMKII⁰ overexpression in rabbit myocytes exhibit signif-
H2O2-Induced Na and Ca Overload Is Mediated via CaMKII

In WT myocytes (stimulated at BCL 2 seconds), H2O2 increased [Na]i time-dependently, but this increase was much lower in CaMKIIδ−/− myocytes (Figure 6A and 6B; Online Table II). Similarly, the H2O2-induced increase in diastolic [Ca], was markedly reduced in CaMKIIδ−/− myocytes (versus WT; Figure 6F; Online Table II). In WT myocytes, the addition of reverse-mode NCX inhibitor KB-R7943 significantly blunted the H2O2-induced increase in diastolic [Ca], (Figure 6F; Online Table II). Likewise, CaMKIIδC overexpression enhanced H2O2-induced increases in [Na]i and diastolic [Ca], significantly (versus βGal), and KN93 slowed [Na]i and [Ca], rise in both CaMKIIδC overexpressing and control myocytes (Figure 6A, 6C, and 6G). KN93 can also reduce ICa,L availability. The analog KN92 (which does not inhibit CaMKII) shares this effect,23 but does not protect from H2O2-induced arrhythmogenesis.19 To control for possible CaMKII effects of KN93, experiments were also done with KN92, which did not alter the H2O2-induced increase of [Na], and diastolic [Ca], (Online Table II). Of note, at baseline there was no difference in [Na], and diastolic [Ca], between WT and CaMKIIδ−/− myocytes, or in control-transfected rabbit myocytes in the presence of KN93. This is consistent with the late [Na], data, arguing against an important role for CaMKII in the basal regulation of cellular Na and Ca homeostasis (Online Table II). Interestingly, at baseline, CaMKIIδC overexpression significantly increased [Na], (reversible with KN93) but did not significantly alter diastolic [Ca], (Online Table II).

To further investigate the importance of late [Na], for H2O2-induced increase in [Na], we conducted quantitative Na influx measurements in the presence of the Na/K ATPase (NKA) inhibitor strophanthin (Figure 6D and 6E). TTX (1 μmol/L) was used to specifically block Na influx via ICa,L. At baseline, the TTX-sensitive Na influx was not different between WT and CaMKIIδ−/− myocytes, in accordance with our late [Na], data. The addition of 200 μmol/L H2O2 increased the TTX-sensitive Na influx nearly threefold in WT but not in CaMKIIδ−/− myocytes (Figure 6E) again consistent with our late [Na], measurements (Table). These data are further supported by experiments using the late [Na], inhibitor ranolazine (RAN). RAN significantly reduced the H2O2-induced gain in [Na], and diastolic [Ca], after CaMKIIδC overexpression, but also in control (βGal; Figure 6A; Online Table II). Interestingly, RAN also reduced [Na], and diastolic [Ca], before H2O2 exposure (Online Table II), consistent with a relevant TTX-sensitive Na influx at baseline (Figure 6E).

To test the hypothesis that ROS-mediated SR Ca release is required for CaMKII activation leading to [Na], and diastolic [Ca], overload, SR Ca stores were depleted with THA. THA greatly slowed the H2O2-induced increase in [Na], and diastolic [Ca], for both CaMKIIδC overexpression and control myocytes (Online Table II), completely eliminating the difference between CaMKIIδC and βGal (at 12 minutes; Online Table II). Interestingly, THA significantly reduced [Na], in myocytes overexpressing CaMKIIδC not only after H2O2 exposure but also at baseline. THA may possibly interfere with a positive feedback loop involving CaMKII-dependent Ca spark augmentation26 leading to Ca-dependent CaMKII activation. On the other hand, THA did not alter [Na], in

Figure 2. ROS activation of CaMKII is Ca-dependent. Permeabilized myocytes bathed in heavy Ca buffers (5 mmol/L BAPTA/1 mmol/L Br2-BAPTA) were used for Western blots of p-CaMKII (A) or ox-CaMKII (B). All densitometric values are shown in Online Table I.
control-transfected myocytes, in line with our data in permeabilized myocytes showing no significant reduction of CaMKII activity with caffeine/THA at baseline. This supports the concept that baseline CaMKII activity is low and reduction of SR Ca release does not further reduce its activity.

H$_2$O$_2$-Induced Arrhythmias and CaMKII
To test whether CaMKII inhibition could prevent ROS-induced arrhythmias, mouse myocytes were exposed to H$_2$O$_2$ and membrane potential or sarcomere length were continuously monitored. H$_2$O$_2$ significantly prolonged action potential duration and depolarized the diastolic membrane potential in WT but not in CaMKII$^{+/−/−}$ myocytes (Figure 7A through 7C). At baseline, there was regular rhythmic activity for both WT and CaMKII$^{+/−/−}$. The addition of H$_2$O$_2$ induced early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs) (Figure 7D), as well as irregular contractile activity (Figure 7E). These arrhythmias were significantly more frequent and more severe in WT versus CaMKII$^{+/−/−}$ (Figure 7D and 7F). Cellular Ca overload results in the development of hypercontracture, which is a permanent reduction (<50%) of longitudinal cell length attributable to a persistent activation of myofilaments. Kaplan–Meier analysis (Figure 7G) revealed that myocytes lacking CaMKII$^{+/−}$ were more resistant to ROS-induced hypercontracture.

Discussion
We investigated the mechanisms by which the exposure of H$_2$O$_2$ enhances late $I_{Na}$ in isolated ventricular myocytes. We found that: (1) ROS-activated CaMKII$^{δ}$ is required for late $I_{Na}$ augmentation, which was abolished in the absence of CaMKII$^{δ}$ expression; and (2) ROS-induced SR Ca release is a prerequisite for ROS-dependent CaMKII activation, based on the observation that the latter was inhibited after SR depletion with THA and caffeine.

These novel findings add to the growing body of evidence that CaMKII is activated under pathophysiological conditions being involved in signaling cascades that lead to dysregu-
tion of cellular Na and Ca homeostasis, increased arrhythmogenesis, and ultimately cell death.

The Role of the SR Ca Stores in ROS-Dependent CaMKII Activation

In a fundamental study, Erickson et al have shown that ROS oxidize CaMKII at methionine 281/282, and this oxidation renders CaMKII autonomous of Ca/CaM levels similar to autophosphorylation at threonine 287.9 Direct oxidation of the kinase also produces a secondary increase in the fraction of autophosphorylated subunits.27,28 We showed here that exogenous H₂O₂ applied to permeabilized myocytes increased CaMKII oxidation and autophosphorylation. We also showed that oxidation and autophosphorylation critically depend on free cytosolic Ca levels. Without free [Ca], no significant H₂O₂-dependent CaMKII oxidation or autophosphorylation was observed (Figure 2). This is in accordance with the concept that H₂O₂ requires initial interaction of Ca/CaM with CaMKII to expose the oxidation and autophosphorylation sites of CaMKII.9

Moreover, we showed that a free [Ca], of 20 nmol/L in the presence of a strong and fast Ca buffer (BAPTA/Br₂-BAPTA) is not sufficient to result in H₂O₂-dependent CaMKII activation. In contrast, if fast localized increases in free [Ca] (during diastolic SR Ca leak or Ca sparks) are allowed by a slower buffer (EGTA), then the same free [Ca], of 20 nmol/L facilitates a robust H₂O₂-dependent CaMKII activation (Figure 1). This result is in accordance with Song et al,27 who showed that high concentrations of BAPTA but not EGTA could prevent H₂O₂-induced CaMKII-dependent Ca current facilitation. Further evidence comes from Xie et al showing that BAPTA loaded myocytes were resistant to H₂O₂-induced CaMKII-dependent formation of EADs.19 On the other hand, Palomeque et al showed that neither extracellular application of 1 μmol/L BAPTA-acetoxyxymethylester in cultured myocytes nor zero Ca-EGTA used for an in vitro phosphorylation assay were sufficient to inhibit H₂O₂-induced CaMKII autophosphorylation.28 Methodological differences may account for this discrepancy: although it is likely that BAPTA was properly loaded into their cultured myocytes, its concentration may not be sufficient to avoid very fast localized increases of [Ca]. Also, the isoosmotic homogenization buffer of their in vitro assay contained no detergent, thus leaving functional SR vesicles in the phosphorylation reaction. This suggests that SR Ca release may be involved in the rapid localized increases of free [Ca], required for H₂O₂-
dependent CaMKII activation. Here, we show that unloading the SR with caffeine and THA abolished the H$_2$O$_2$-induced CaMKII oxidation and autophosphorylation. Moreover, in the presence of THA, the H$_2$O$_2$-dependent cytosolic Na and Ca overload was markedly reduced. This relates mechanistically to work showing that preincubation with caffeine and THA overloads was markedly reduced. This relates mechanistically of H$_2$O$_2$ exposure. For some experiments, AIP (100 nmol/L) or TTX (1

ROS Enhance late I$_{Na}$ via CaMKII Activation

ROS have been shown to acutely enhance late I$_{Na}$, leading to AP prolongation and EADs. To date, the mechanism of ROS action on Na channels is not fully understood. Changes in the lipid environment or oxidation of multiple methionine residues of the channel may be responsible. Here we show that the acute H$_2$O$_2$-dependent increase in late I$_{Na}$ was absent in myocytes with genetic knockout of CaMKII$\delta$. Furthermore, subacate adenosival CaMKII$\delta$ overexpression enhanced whereas acute CaMKII inhibition with AIP slowed the H$_2$O$_2$-induced increase of late I$_{Na}$. Therefore, although redox modification of the Na channel or the lipid environment cannot be completely excluded, the dominating mechanism here involves acute CaMKII$\delta$ activation.

We have shown that CaMKII can phosphorylate cardiac Na channels $\alpha$ isoform (Na$_{\alpha}$1.5). Aiba et al showed that CaMKII phosphorylates the I-II linker but also to a lesser extent the C-terminus. Hund et al found that CaMKII phosphorylates serine 571 in the I-II linker contributing to altered $I_{Na}$ gating. Although phosphorylation does occur, ROS-activated CaMKII may also regulate late $I_{Na}$ independent of its catalytic activity, as it was shown for oxidation-dependent facilitation of I$_{Ca,L}$. Because the cardiac Na channel is a macromolecular complex, CaMKII interaction with proteins of this complex could result in altered Na channel gating. For example, coexpression of Na$_{\alpha}$1.5 with cardiac $\beta_1$ but not $\beta_2$ subunit increased late I$_{Na}$ and it may be possible that oxidized CaMKII is involved in the interaction of $\alpha$ and $\beta$ subunits.

**Table. Late $I_{Na}$ Integral Before (Baseline) and During Exposure to H$_2$O$_2$**

| AmsF$^{-1}$ (N) | Baseline | 12 Minutes of H$_2$O$_2$
<table>
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<tr>
<td>WT (15)</td>
<td>−203.5±21</td>
<td>−380.1±101.1*</td>
</tr>
<tr>
<td>CaMKII$\delta^-$/ (7)</td>
<td>−155.4±43.3</td>
<td>−204.1±93.1†</td>
</tr>
<tr>
<td>$\beta$Gal (17)</td>
<td>−99.3±7.4</td>
<td>−331.8±77.2*</td>
</tr>
<tr>
<td>$\beta$Gal+ AIP (18)</td>
<td>−105.9±7.8</td>
<td>−200.5±32.3†</td>
</tr>
<tr>
<td>$\beta$Gal+ TTX (4)</td>
<td>−66.4±22.6*</td>
<td>−54.5±32.1†</td>
</tr>
<tr>
<td>CaMKII$\delta$C (18)</td>
<td>−249.1±23.3*</td>
<td>−719.7±192.2††</td>
</tr>
<tr>
<td>CaMKII$\delta$C+ AIP (14)</td>
<td>−158.7±12.4‡</td>
<td>−277.0±37.4§</td>
</tr>
<tr>
<td>CaMKII$\delta$C+ TTX (8)</td>
<td>−101.9±29.4‡</td>
<td>−121.4±63.5$$</td>
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</table>

$P<0.05$ vs WT (baseline) or $\beta$Gal (baseline).

$P<0.05$ vs WT (12 minutes) or $\beta$Gal (12 minutes).

$P<0.05$ vs CaMKII$\delta$ (baseline).

$P<0.05$ vs CaMKII$\delta$C (12 minutes).

Late $I_{Na}$ integrals (50–500 ms) (AmsF$^{-1}$) at baseline and after 12 minutes of H$_2$O$_2$ exposure. For some experiments, AIP (100 nmol/L) or TTX (1 μmol/L) were used.
Beside CaMKII, protein kinase (PK)A and PKC are known to regulate Na channel gating. ROS have been shown to oxidize PKA regulatory subunit I, resulting in PKA translocation and activation.40 PKA increases peak \( I_{Na} \) density via acceleration of channel trafficking to the plasma membrane,41 but does not change inactivation kinetics.42,43 Mild oxidative stress has also been shown to activate PKC.44 The redox regulation of PKC is very specific for different isoforms and different cell types. Ward and Giles showed that the \( H_2O_2 \)-dependent slowing of \( I_{Na} \) open state inactivation was blocked in the presence of the PKC inhibitor Bisindolylmaleimide.10 In heterologous expression systems (rat and human NaV1.5), however, PKC activation did not change \( I_{Na} \) inactivation.45,46 The most consistent effect of PKC on cardiac Na channels is a reduction in peak \( I_{Na} \) density.47

Cellular Na and Ca Accumulation

The present study shows that ROS-induced Na and Ca overload are blunted in myocytes lacking CaMKII. We also
show that SR Ca unloading with THA similarly reduces ROS-induced Na and Ca overload, suggesting that the CaMKII activation depends on SR function. These data suggest that CaMKIIδ is critically involved in ROS-induced [Na] and [Ca] overload. Quantitative Na influx measurements showed that TTX-sensitive Na entry is markedly increased by ROS exposure in WT but not in CaMKIIδ−/− myocytes and the late $I_{\text{Na}}$ blocker RAN slowed the development of Na and Ca overload, thus suggesting that ROS-induced Na entry is mediated via Na channels. This is in line with data from us and others.6,8,48 We propose that the increase in [Na], precedes the major rise in [Ca], because reduction of extracellular [Na] or application of Na channel blockers slows the rise in [Ca], and inhibition of reverse mode NCX activity with KB-R7943 significantly blunts Ca overload in the present study. However, because RAN did not completely abolish the increase in [Na], and [Ca], other mechanisms may also be involved. For example, ROS can significantly impair NKA function,49 reduce SERCA2 activity and L-type Ca current ($I_{\text{Ca,L}}$),50 and increase NCX activity.51,52 To further understand these mechanisms we used numeric simulations of the cardiac action potential and Na and Ca handling.53 Incorporation of the measured ROS-induced late $I_{\text{Na}}$ into the model led to a rise in [Na] less than 1 mmol/L (Online Figure I, A: a versus b), much less than measured experimentally. ROS may also (via CaMKII) increase diastolic TTX-sensitive Na influx, as reported in HF myocytes.54 Increasing background Na conductance can bring the model to match the measured [Na], (Online Figure I, A, c), but inclusion of ROS-dependent NKA inhibition49 limits the extent of background Na current that is required to explain the measured [Na] increase (Online Figure I, A: c versus d). If we also incorporate the above mentioned ROS-dependent effects on SERCA2a, RyR2, $I_{\text{Ca,L}}$, and NCX function,50–52 then the model reasonably mimics the ROS-induced alterations observed here in [Na] and SR Ca content (Online Figure I, A, B, and C). Three aspects merit comment. First, the elevated [Na], and reduced Ca transient amplitude is predicted to cause substantial Ca influx via NCX during the action potential (ie, reverse mode NCX; Online Figure I, B and C) and less Ca extrusion during diastole. This is also seen in human and rabbit HF myocytes, which also exhibit elevated [Na], and reduced Ca transients.54,55 This agrees with our results that the NCX inhibitor KB-R7943 suppressed the ROS-induced increase in diastolic [Ca], (Online Table II; Figure 6F), that ROS-induced Ca overload depends on shifts of NCX activity,56 and that late $I_{\text{Na}}$ is responsible for diastolic Ca accumulation in HF where ROS production is augmented.57 Second, turning off only the late and diastolic $I_{\text{Na}}$ simulates reasonably the effects of ranolazine on [Na], (Online Figure I, A, g). Third, the model does not predict the extent of diastolic [Ca], elevation observed. Because intracellular Na and Ca are compartmentalized, part of the progressive measured rise in [Na], and [Ca], may be attributable to a consequent gradual rise in mitochondrial [Na] and [Ca].

**Arrhythmogenesis**

Here, we show that the propensity for ROS-induced EADs/DADs and cellular arrhythmias were significantly reduced in myocytes lacking CaMKIIδC, although they were not completely abolished. ROS effects in myocytes are clearly multifactorial, and so are arrhythmogenic mechanisms. Therefore, activated CaMKII may be an important factor for ROS-induced arrhythmias but does not explain all arrhythmias that develop on ROS or in HF. Possible mechanisms that contribute to the electric instability are EADs and DADs. It was shown previously that enhanced late $I_{\text{Na}}$ could prolong action potential duration (APD), leading to EADs.6 Moreover, transient inward $I_{\text{NCX}}$ ($I_{\text{L}}$), which leads to DADs may result from ROS-induced cellular Ca overload and increased RyR2 open probability. Here, we show that both EADs and DADs develop less often after H$_2$O$_2$ exposure in myocytes lacking CaMKIIδC. Although the reduction in the propensity for EADs in CaMKIIδ−/− myocytes may result from the lack in APD prolongation, less frequent DADs possibly indicate less cellular Na and Ca overload as shown here. The CaMKIIδ-dependent increase in late $I_{\text{Na}}$ may be responsible for the observed H$_2$O$_2$-induced APD prolongation. Although ROS can decrease outward currents like $I_{\text{Ko}}$,58 CaMKII activation tends to increase $I_{\text{Ko}}$.51 In the absence of confounding CaMKII activity (CaMKIIδ−/− myocytes), we found that H$_2$O$_2$ did not increase APD, which argues against a significant role for inhibition of $I_{\text{Ko}}$ in APD prolongation caused by H$_2$O$_2$. Interestingly, H$_2$O$_2$ rendered the diastolic membrane potential more positive increasing the likelihood that depolarizing currents would induce afterdepolarizations. This was not the case in CaMKIIδ−/− myocytes. Nevertheless, although we show here evidence that CaMKII may be involved in ROS-induced arrhythmias there was still H$_2$O$_2$-induced arrhythmogenic activity detectable in CaMKIIδ−/− myocytes suggesting that there are CaMKII-independent mechanisms as well. For instance, ROS can directly oxidize RyR2, leading to increased CaSpF.51 Although the present report establishes a clear mechanistic working hypothesis for the role of CaMKIIδ in ROS-dependent regulation of late $I_{\text{Na}}$ and its consequences, further studies are needed that aim at testing these mechanisms in HF.

In summary, we show that CaMKIIδ is required for the H$_2$O$_2$-induced augmentation of late $I_{\text{Na}}$ and secondary changes of [Na] and [Ca]. The present results also suggest that ROS-induced SR Ca release may be a prerequisite for ROS-dependent CaMKII activation. In addition, CaMKIIδ appears to be involved in H$_2$O$_2$-induced arrhythmogenesis. These results are important, because expression and activity of CaMKII have been shown to be increased in HF,59–61 where ROS generation is enhanced.6 In addition, HF is associated with enhanced late $I_{\text{Na}}$, leading to dysregulation of intracellular Na and Ca homeostasis, electric instability, and cell death.

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Disclosures
L.S.M. has a collaborative/research grant with Gilead Sciences. L.B. is an employee of Gilead Sciences.

References
What New Information Does This Article Contribute?

- 

What Is Known?

- Heart failure (HF) is associated with increased reactive oxygen species (ROS) and Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMKII) expression.
- ROS can activate CaMKII by oxidation.
- ROS and CaMKII can increase late \(I_{Na}\) and intracellular Na concentration.

What New Information Does This Article Contribute?

- Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) is required for ROS-dependent CaMKII oxidation and autophosphorylation.
- ROS-activated CaMKII enhances late \(I_{Na}\) leading to cellular Na\(^+\) and Ca\(^{2+}\) overload.
- ROS-activated CaMKII is arrhythmogenic.

In HF, intracellular ROS are increased and CaMKII expression and activity is upregulated, contributing to electrical and functional remodeling. CaMKII was previously shown to be activated by ROS through Ca\(^{2+}\)-dependent processes, but the origin of Ca\(^{2+}\) remained unclear. In addition, ROS and CaMKII have both been shown to increase late \(I_{Na}\), leading to proarrhythmogenic events, but a mechanistic link was missing. In this study, we show that SR Ca release in required for ROS-dependent CaMKII oxidation and autophosphorylation, which leads to increased late \(I_{Na}\) intracellular Na accumulation and Ca\(^{2+}\) overload. Moreover, ROS-activated CaMKII leads to action potential prolongation and cellular arrhythmias. These results provide novel insights into the Ca\(^{2+}\) dependence of the ROS activation process for CaMKII. Moreover, they highlight the importance of CaMKII for the ROS-induced disturbance of excitation–contraction coupling and arrhythmogenesis. Taken together, the present study suggests ROS-activated CaMKII as a promising new target for the treatment of heart failure.
Reactive Oxygen Species–Activated Ca/Calmodulin Kinase IIδ Is Required for Late \( I_{Na} \) Augmentation Leading to Cellular Na and Ca Overload

Stefan Wagner, Hanna M. Ruff, Sarah L. Weber, Sarah Bellmann, Thomas Sowa, Timo Schulte, Mark E. Anderson, Eleonora Grandi, Donald M. Bers, Johannes Backs, Luiz Belardinelli and Lars S. Maier

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**Detailed Methods**

**CaMKIIδ knockout mice and overexpression of CaMKIIδ in rabbit myocytes**

CaMKIIδ knockout mice (CaMKIIδ−/−) were compared with their age- and sex-matched wild-type (WT) litters. Ventricular myocytes were isolated and kept in modified Tyrode solution containing (mmol/L) 137 NaCl, 5.4 KCl, 1.2 MgSO4, 1.2 Na2HPO4, 20 HEPES, 15 glucose, and 1 CaCl2 (pH 7.4). For acute CaMKII overexpression, ventricular myocytes were isolated from female chinchilla bastard rabbits (1.3-2 kg). Transfection with CaMKIIδC adenovirus (Ad-CaMKIIδC) was performed and compared to β-galactosidase (Ad-βGal) as a control at a MOI of 100. Cells were cultured for 24 h with M199 and washed immediately prior to the experiment. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee.

**Permeabilization of myocytes**

Isolated rabbit cardiomyocytes were superfused for 5 min with relaxing buffer containing (mmol/L) 150 potassium aspartate, 1 EGTA, 10 HEPES, 0.25 MgCl2 (pH 7.4 KOH) followed by permeabilization buffer containing β-escin (Sigma, 50 µg/ml) for 30-60 s. After permeabilization, the bath solution was changed to a mock intracellular solution containing (mmol/L) 100 KCl, 25 HEPES, 5 Na2ATP, 5 U/ml creatine phosphokinase, 10 phosphocreatine, 4% dextrane, 5.5 MgCl2, (pH 7.2 KOH) either with 0.2 mmol/L EGTA (free [Ca2+]i 20 nmol/L) or heavy Ca buffers (5 mM BAPTA/1 mM Br2-BAPTA). Permeabilized cells were either used for Western blotting or Ca sparks measurements as described below. All experiments were conducted 2 min after permeabilization to minimize the loss of soluble proteins.

**Western blot analysis**

ROS were generated upon addition of 40 or 200 µmol/L H2O2 (for 10 min) in permeabilized rabbit myocytes placed in mock intracellular solution (see above) with PKA inhibitory peptide (PKI; 15 µM) and okadaic acid (2 µM) to prevent activation of PKA and dephosphorylation, respectively. For some experiments, cells were preincubated with thapsigargin (5 µmol/L) and caffeine (10 mmol/L) before start of H2O2 exposure or AIP (1 µmol/L) was present throughout the experiment. Cells were then harvested and lysed in Tris buffer (mmol/L) 20 Tris-HCl, 200 NaCl, 20 NaF, 1 Na3VO4, 1% Triton X-100, 1 DTT (pH 7.4) and complete protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail (both Roche Diagnostics) by trituration. Protein concentration was determined by BCA assay (Pierce Biotechnology). Denatured cell lysates (30 min, 37°C in 2% β-mercaptoethanol) were subjected to Western blotting (7.5% SDS-polyacrylamide gels) using primary antibodies against phospho-CaMKII (monoclonal mouse, 1:1500, Affinity Bioreagents), CaMKII (polycional rabbit, 1:12000, gift from D. M. Bers, University of California, Davis, CA, USA), and GAPDH (monoclonal mouse, 1:50000, BIORAD), respectively. For some experiments, after phospho-CaMKII immunoblotting the p-CaMKII antibody was "stripped" of the membrane followed by incubation with primary antibody against CaMKII. The membrane stripping was done using washing steps with Tris-buffered saline and 200 mmol/L NaOH followed by incubation with 5% non-fat dry milk for 1 h at room temperature. For the detection of oxidized CaMKII, an immune serum directed against oxidized M281/M282 (ox-CaMKII) was used (polycional rabbit, 1:2500, kind gift from M.E. Anderson, Carver College of Medicine, University of Iowa, Iowa City, IA, USA). To avoid unspecific reduction of oxidized CaMKII, β-mercaptoethanol was omitted during the denaturation process (5 min at 95°C). Primary antibodies were incubated at 4°C overnight. Secondary antibodies were horseradish peroxidase(HRP)-conjugated sheep anti-mouse IgG (Amersham Biosciences) for phospho-
CaMKII (1:5000) or GAPDH (1:20000), and HRP-conjugated donkey anti-rabbit IgG (1:15000, Amersham Biosciences) for CaMKII and ox-CaMKII, respectively (incubation for 1 h at room temperature). Chemiluminescent detection was done with SuperSignal West Pico Substrate (Pierce Biotechnology) or Immobilon\textsuperscript{TM} Western Chemiluminescent HRP Substrate (Millipore).

**Measurement of Ca Sparks via confocal microscopy**

Isolated myocytes were loaded with either 10 µmol/L fluo-4 acetoxyxymethylester (AM) or fluo-4 salt (Molecular Probes) for intact or permeabilized myocytes, respectively, and Ca sparks were measured using line-scan on a laser scanning confocal microscope (Zeiss Pascal 5). Ca sparks were counted and characterized manually by blinded observers. Ca spark frequency (CaSpF) was obtained by averaging the number of sparks in images recorded after 1 Hz stimulation and normalized to cell volume and scan rate (pL\textsuperscript{-1}s\textsuperscript{-1}); assuming voxel length and width of 0.2 µm, and depth of 1 µm. Ca spark duration was taken from the full-duration-half-maximum (FDHM). For some experiments, caffeine (10 mmol/L) was applied to induce rapid SR Ca release. [Ca\textsubscript{i}] was calibrated using the pseudo-ratio equation \([\text{Ca}_\text{rest}] = [\text{Ca}]/(K_d/[\text{Ca}]_\text{rest}+1)\) with \(K_{d} = 1100\) nmol/L (when [Ca]\textsubscript{rest} was known).\textsuperscript{6} The diastolic Ca leak was calculated as follows: CaSpF x Ca spark amplitude x FDHM and reported relative to caffeine transient amplitude.

**Patch-clamp experiments**

Ruptured-patch whole-cell voltage-clamp was used to measure \(I_{\text{Na}}\). Microelectrodes (2-3 M\text{\oh}) were filled with (mmol/L) 40 CsCl, 80 Cs-glutamate, 5 NaCl, 0.92 MgCl\textsubscript{2}, 5 Mg-ATP, 0.3 Li-GTP, 10 HEPES, 0.03 niflumic acid, 0.02 nifedipine, 0.004 strophanthidin (pH 7.2, CsOH). The bath solution contained (mmol/L) 130 NaCl, 10 tetraethylammonium chloride, 4 CsCl, 1 MgCl\textsubscript{2}, 10 glucose, 10 HEPES (pH 7.4, NaOH). Access resistance was <10 M\text{\oh}. Ruptured-patch whole-cell current clamp was used to measure membrane potential. Microelectrodes (10 M\text{\oh}) were filled with (mmol/L) 120 K-aspartate, 8 KCl, 7 NaCl, 1 MgCl\textsubscript{2}, 10 HEPES, 5 Mg-ATP, 0.3 Li-GTP (pH 7.2, KOH). The bath solution contained (mmol/L) 140 NaCl, 4 KCl, 1 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, 10 glucose, 5 HEPES (pH 7.4, NaOH). Access resistance was typically ~20 M\text{\oh} after patch rupture. In all experiments, myocytes were mounted on the stage of a microscope (Nikon Eclipse TE2000-U). Liquid junction potentials were corrected with the pipette in the bath. Fast capacitance was compensated in cell-attached configuration. Membrane capacitance and series resistance were compensated after patch rupture. Signals were filtered with 2.9 and 10 kHz Bessel filters and recorded with an EPC10 amplifier (HEKA Elektronik). Myocytes were held at -120 mV and \(I_{\text{Na}}\) was elicited using a train of pulses to -20 mV (1000 ms duration, 30 pulses, BCL 2s). To optimize voltage control, each pulse was preceded by a 5 ms pre-pulse to +50 mV. Recordings were started 5 min after rupture. Following baseline late \(I_{\text{Na}}\) measurement, 200 µmol/L \(\text{H}_2\text{O}_2\) was added to the bath and pulse series were elicited every 2 min. The measured current was integrated (between 50-500 ms) and normalized to the membrane capacitance (172.9±5.8 pF or 128.2±4.3 for mouse or rabbit, respectively). APs were continuously elicited by square current pulses of 1-2 nA amplitude and 1-5 ms duration at basic cycle length of 2 s. 200 µmol/L \(\text{H}_2\text{O}_2\) was added to the bath and the morphology of characteristic APs was analyzed every minute. All experiments were conducted at room temperature. For some experiments, CaMKII was inhibited using autocamtide 2-related autoinhibitory peptide (AIP, 100 nM) added to the pipette solution, or \(I_{\text{Na}}\) was blocked using tetrodotoxin (TTX, 1 µmol/L).

**Epifluorescence experiments**

Intracellular Na ([Na\textsubscript{i}]) and Ca ([Ca\textsubscript{i}]) concentration was measured as described.\textsuperscript{2} Briefly, myocytes on laminin-coated recording chambers were loaded with 10 µmol/L SBFI-AM or Indo-1-AM, respectively, in the presence of 0.02% (w/v) pluronic acid (Molecular Probes, Eugene, OR) for 2 h and 30 min, respectively, at room temperature in the dark. The chambers were
mounted on the stage of an inverted microscope (Nikon Eclipse TE2000-U) and superfused with Tyrode solution (37°C) containing (in mmol/L) 140 NaCl, 4 KCl, 1 MgCl₂, 5 HEPES, 10 glucose, 1 or 2 CaCl₂ (mouse or rabbit, respectively), pH 7.4. After 10 min of washing out the external dye, myocytes were field-stimulated (voltage 25% above threshold, BCL 2 s). Intracellular SBFi was excited at alternating wavelengths 340 and 380 nm, emitted epifluorescence was monitored at 510 nm (F₃₄₀ and F₃₈₀). Intracellular Indo-1 was excited at 360 nm and emitted epifluorescence was measured at 405 and 485 nm (F₄₀₅/F₄₈₅). All fluorescence emission was recorded using IonWizard software (IonOptix Corporation, Boston, MA). Background fluorescence was subtracted, the ratio F₃₄₀/F₃₈₀ or F₄₀₅/F₄₈₅, respectively, was calculated and converted to [Na]ᵢ or [Ca]ᵢ with calibration curves. 

In situ calibration of SBFi was accomplished by exposing myocytes to bath solutions containing 0, 5, 10 and 20 mmol/L [Na⁺]₀ in the presence of 10 µmol/L gramicidin D, 80 µmol/L monensin and 50 µmol/L strophanthidin. The solutions with various [Na⁺]₀ were prepared from two stock solutions of equal ionic strength containing (in mmol/L) 140 NaCl, 10 HEPES and 1 EGTA or (in mmol/L) 140 KCl, 10 HEPES and 1 EGTA, pH was 7.2 (with Tris Base). In situ calibration of Indo-1 was accomplished using the equation 

\[
[Ca^{2+}]_i = K_d \cdot \beta \cdot \left( \frac{R - R_{min}}{R_{max} - R} \right)
\]

with \(K_d=844\) nmol/L as previously described. After baseline measurement, 200 µmol/L H₂O₂ was added to the Tyrode solution and fluorescence was measured every 2 min. For some experiments, CaMKII was inhibited with KN93 (10 µmol/L) vs. the inactive analogue KN92 (10 µmol/L); late Iₙa was blocked with ranolazine (RAN, 10 µmol/L), sodium-calcium exchange (NCX) was inhibited with KB-R7943 (5 µmol/L), or SR Ca stores were unloaded with the SERCA-inhibitor THA (5 µmol/L). Na influx was taken as the initial rate of [Na]ᵢ rise after abrupt NKA inhibition with 100 µmol/L strophanthidin in Tyrode solution. The Na channel dependent influx was determined by subtracting the remaining Na influx in the presence of the selective Na channel blocker TTX (1 µmol/L) from the total Na influx (TTX-sensitive component). The experiments were then repeated in the presence of 200 µmol/L H₂O₂ and 100 µmol/L strophanthidin to determine the H₂O₂-induced TTX-sensitive Na influx.

**Measurement of Cellular Arrhythmias**

Freshly isolated mouse ventricular myocytes from CaMKIIδ⁻/⁻ and WT mice were electrical field stimulated (BCL 2 s) and sarcomere length was continuously monitored (IonOptix Corporation, Boston, MA). Cellular arrhythmias were evoked upon addition of H₂O₂ (200 µmol/L for 10 min). The propensity for cellular arrhythmias was analyzed using an arrhythmia score.

**Computational Modelling**

Action potential clamp simulations. The rabbit ventricular AP was simulated with the Shannon-Bers model. Model differential equations were implemented in Matlab (Mathworks Inc., Natick, MA, U.S.A.) and solved numerically with a variable order solver (ode15s). The late Na current \(I_{Na,L}\) was added to the original model with the formulation proposed by Hund et al.:

\[
I_{Na,L} = G_{Na,L} \cdot m_L^3 \cdot h_L \cdot (V - E_{Na})
\]

where late \(I_{Na}\) activation \(m_L\) mimics the activation of fast \(I_{Na}\), whereas inactivation \(h_L\) was formulated as follow:

\[
h_{L,s} = \frac{1}{1 + \exp((V_m + 91) / 6.1)}
\]

\[
\tau_{h,L} = 600 ms
\]
was constrained to reproduce the data in Table 1 on late $I_{\text{Na}}$ integral for βGal myocytes (under 1000 ms-long voltage step from -120 to -20 mV, integrated current from 50 to 500 ms) before and after 12 min H$_2$O$_2$. Setting of the maximal conductance to 0.0045 mS/μF at baseline and 3.3-fold higher (0.015 mS/μF) with ROS exposition, which led to simulated integrals of -100 and -333 A ms F$^{-1}$ respectively. The baseline cell model was stimulated at 0.5 Hz, and the AP was subsequently used for AP clamp simulations. Late $I_{\text{Na}}$ integrals were considerably smaller during a physiological AP (that is with more depolarized resting membrane potential and shorter depolarizing interval): -15.8 and -54.5 A ms F$^{-1}$ before and after H$_2$O$_2$ exposition.

**Data analysis and statistics**

All data are expressed as mean±S.E.M. For longitudinal data, two-way repeated measures analysis of variance (ANOVA) was run; else Student’s unpaired t test or one-way ANOVA with Student-Newman-Keuls multiple comparisons was used. Double sided P-values of P<0.05 were considered significant.
Supplemental Results and Discussion

Computational Modelling

We utilized computer modeling to predict ROS-dependent changes in $[Na]$, $[Ca]$, and NCX operating modes with the well established Shannon-Bers rabbit ventricular myocytes model. Incorporating the ROS-induced enhancement of late $I_{Na}$ alone, did not raise $[Na]$ very much (online fig. IA: a vs. b). However, it has been shown that there is a substantial TTX-sensitive Na influx that is present in resting myocytes and is enhanced in HF, contributing importantly to the increased $[Na]$ in HF. To produce the measured rise of steady state $[Na]$ in Fig 6C, we had to increase background Na conductance ~40-fold (Online fig. IA: c vs. b). This would be a very high background current (~1.7 A/F) although it only depolarizes resting $E_m$ by ~2 mV. However, decreased Na extrusion could also contribute to elevated $[Na]$, and ROS have been shown to potently inhibit NKA function. If we include a 50% reduction in NKA activity in our simulations, we need only 40% as much background Na conductance to achieve the measured $[Na]$ (~0.7 A/F, Online fig. IA, d). The high $[Na]$ would raise $[Ca]$ due to shifts in NCX function and dramatically increase Ca transient amplitude and SR Ca content (Online Fig IB-C). However, in our experiments SR Ca content was reduced and SR Ca leak was enhanced in accordance with previously published data also showing reduced Ca transient amplitude and slowed twitch $[Ca]$, decline. If we include an 80% reduction in SERCA function and a 10-fold increase in SR Ca leak, $[Na]$ increases further to 18.8 mmol/L (Online fig. IA, e). ROS have also been shown to dramatically reduce L-type Ca current ($I_{CaL}$) by ~75% and increase NCX function about 2.5-fold. Combining all of these effects predicts $[Na]$ to be 14.4 mmol/L, with reduced SR Ca content, increased diastolic Ca and reduced Ca transients (as observed experimentally). Of note there is also substantial Ca entry predicted via reverse mode NCX activity (about 65% of the total NCX activity) during the action potential (Online fig. I, f) as previously reported in HF myocytes where $[Na]$ is elevated and SR Ca release is depressed. Interestingly, under these conditions if we “turn off” the extra late $I_{Na}$ and Na background conductance, $[Na]$ decreases to 8.4 mmol/L (Online fig. I, g), which is consistent with our experimentally measured reversion of $[Na]$ elevation by ranolazine (Fig 6A and Online Table II). These modeling results are only approximations, but help put some of these complex changes into a useful interpretive framework.

As $[Na]$ rises, one might think that the Na/H exchange (NHE) might reduce intracellular pH. We think that this is unlikely to influence our results, for two reasons. First, at neutral pH NHE is not activated and would not be expected to function as an appreciable proton influx pathway (it is typically activated gradually at $pH \leq 6.9$). Second, the NKA predominates in Na efflux, so even if a cumulative Na efflux of 1 mmol/L were to have occurred via NHE, that could only lower intracellular pH by ~0.05 pH units (using the 20 mmol/L/pH unit buffering capacity at pH 7.2 reported by Zaniboni et al.), and even that would require abolition of other pH regulatory control mechanisms. On the other hand, if ROS were to cause cellular lactic acidosis and drive intracellular pH below 6.9, it could also contribute to the elevated $[Na]$. 


References


ROS activate CaMKII to regulate late $I_{Na}$

Online Table I. Densitometric values of Western blots (fold increase vs. vehicle or [Ca] 0 nmol/L)

<table>
<thead>
<tr>
<th></th>
<th>p-CaMKII/ GAPDH [N]</th>
<th>CaMKII/ GAPDH</th>
<th>p-CaMKII/ CaMKII</th>
<th>ox-CaMKII/ GAPDH</th>
<th>CaMKII/ GAPDH</th>
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<tr>
<td><strong>without heavy Ca buffering</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>1±0.26 [7]</td>
<td>1±0.15</td>
<td>1±0.14</td>
<td>1±0.2 [5]</td>
<td>1±0.14</td>
<td>1±0.17</td>
</tr>
<tr>
<td>Vehicle + AIP</td>
<td>0.75±0.34 [3]</td>
<td>1.02±0.14</td>
<td>0.92±0.52</td>
<td>1.1±0.17 [4]</td>
<td>1.08±0.15</td>
<td>0.96±0.09</td>
</tr>
<tr>
<td>Vehicle + Caffeine/THA [Ca] 1 µmol/L</td>
<td>0.97±0.05 [3]</td>
<td>1.36±0.36</td>
<td>0.96±0.3</td>
<td>1.18±0.3 [4]</td>
<td>1.16±0.15</td>
<td>0.79±0.11</td>
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<tr>
<td>[Ca] 1 µmol/L</td>
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<td>1.29±0.21</td>
<td>3.17±0.36 *</td>
<td>1.98 [1]</td>
<td>1.03</td>
<td>1.86</td>
</tr>
<tr>
<td>[Ca] 1 µmol/L+AIP</td>
<td>1.44±0.42 [3]</td>
<td>0.99±0.23</td>
<td>1.77±0.54 †</td>
<td>2.31±0.32 [4]</td>
<td>1.04±0.08</td>
<td>2.14±0.28 *</td>
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<td>2.39±0.23</td>
<td>1.81 [1]</td>
<td>1.14</td>
<td>1.53</td>
</tr>
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<td>H$_2$O$_2$ 200 µmol/L</td>
<td>4.05±0.89 [5]</td>
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<td>3.96±0.4 *</td>
<td>2.52±0.47 [4]</td>
<td>1.1±0.06</td>
<td>2.2±0.39 *</td>
</tr>
<tr>
<td>H$_2$O$_2$ 40 µmol/L + AIP</td>
<td>0.91±0.01 [2]</td>
<td>0.77±0.17</td>
<td>1.29±0.29</td>
<td>1.1±1</td>
<td>0.99</td>
<td>1.07</td>
</tr>
<tr>
<td>H$_2$O$_2$ 40 µmol/L + AIP</td>
<td>2±0.42 [5]</td>
<td>1.01±0.28</td>
<td>2.69±0.2 ‡</td>
<td>1.1±0.14</td>
<td>1.06±0.18</td>
<td>1.27±0.19 †</td>
</tr>
<tr>
<td>H$_2$O$_2$ 200 µmol/L + Caffeine/THA</td>
<td>1.1±0.08 [2]</td>
<td>0.75±0.17</td>
<td>1.19±0.02</td>
<td>1.43±0.36 [4]</td>
<td>1.06±0.18</td>
<td>1.27±0.19 †</td>
</tr>
</tbody>
</table>

|                  |                     |               |                 |                  |               |                  |
| **With heavy Ca buffering** |                     |               |                 |                  |               |                  |
| [Ca] 0 nmol/L    | 1±0.5 [4]           | 1±0.13        | 1±0.37          | 1±0.38 [3]       | 1±0.05        | 1±0.4            |
| [Ca] 0 nmol/L + H$_2$O$_2$ 40 µmol/L | 0.75±0.24 [2] | 0.91±0.34     | 0.84±0.15       | 0.51±0.23 [2]    | 0.87±0.11     | 0.61±0.34        |
| [Ca] 0 nmol/L + H$_2$O$_2$ 200 µmol/L | 1.03±0.51 [2] | 1.10±0.09     | 0.95±0.47       | 1.44 [1]         | 1.53          | 0.94             |
| [Ca] 0 nmol/L+ H$_2$O$_2$ 40 µmol/L+AIP | 1.02±0.51 [2] | 1.0±0.46      | 1.11±0.34       | 0.72±0.44 [2]    | 1.13±0.33     | 0.8±0.61         |
| [Ca] 0 nmol/L+ H$_2$O$_2$ 200 µmol/L+AIP | 0.92±0.87 [2] | 1.23±0.07     | 0.94±0.53       | 1.88 [1]         | 1.65          | 1.14             |
| [Ca] 20 nmol/L   | 1.56±0.67 [4]       | 1.06±0.17     | 1.41±0.71       | 0.85±0.39 [3]    | 1.24±0.38     | 0.82±0.38        |
| [Ca] 20 nmol/L + AIP | 0.69±0.1 [3] | 1.03±0.21     | 0.82±0.33       | 1±0.4 [3]        | 0.88±0.12     | 1.13±0.41        |
| [Ca] 20 nmol/L + H$_2$O$_2$ 40 µmol/L | 0.77±0.23 [2] | 0.67±0.16     | 1.03±0.35       | 0.67±0.54 [2]    | 0.8±0.14      | 0.95±0.62        |
| [Ca] 20 nmol/L + H$_2$O$_2$ 200 µmol/L | 1.9±1             | 1.56          | 1.2            | 2.27 [1]         | 1.72          | 1.31             |
| [Ca] 1 µmol/L    | 6.03±0.39 [4]       | 1.05±0.2      | 5.95±0.34 *     | 1.11±0.27 [2]    | 1.23±0.25     | 0.87±0.15        |
| [Ca] 1 µmol/L + AIP | 4.1±0.8 [3] | 0.91±0.13     | 4.35±0.67 †     | 1.2±0.23 [2]     | 0.95±0.11     | 1.33±0.25        |

Densitometric values of Western blots shown in figures 1 and 2. Values are reported as fold increase vs. vehicle or [Ca] 0 nmol/L, respectively. One way ANOVA: * - P<0.05 vs. vehicle; † - P<0.05 vs. [Ca] 1 µmol/L; ‡ - P<0.05 vs. H$_2$O$_2$ 200 µmol/L.
Online Table II. [Na]i and [Ca]i before (baseline) and during exposure to H2O2

<table>
<thead>
<tr>
<th></th>
<th>[Na]i mmol/L [N]</th>
<th>[Ca]i nmol/L [N]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>12 min H2O2</td>
</tr>
<tr>
<td>WT</td>
<td>11.2±0.7 [12]</td>
<td>22.6±4*</td>
</tr>
<tr>
<td>WT+KB-R7943</td>
<td></td>
<td>243.4±7.2 [11]</td>
</tr>
<tr>
<td>CaMKIIδc</td>
<td>11.1±1 [7]</td>
<td>13.6±1.5†</td>
</tr>
<tr>
<td>βGal</td>
<td>8.6±0.6 [26]</td>
<td>15.4±1.3*</td>
</tr>
<tr>
<td>βGal+KN92</td>
<td>7.5±1 [8]</td>
<td>15.3±1.5*</td>
</tr>
<tr>
<td>βGal+KN93</td>
<td>7.4±0.6 [12]</td>
<td>10.9±1†</td>
</tr>
<tr>
<td>βGal+RAN</td>
<td>7.2±0.5 [9] *</td>
<td>9.4±0.8†</td>
</tr>
<tr>
<td>βGal+THA</td>
<td>8.3±0.4 [5]</td>
<td>10.8±0.5†</td>
</tr>
<tr>
<td>CaMKIIδC</td>
<td>12.4±0.4 [26]*</td>
<td>24.1±1.7‡‡</td>
</tr>
<tr>
<td>CaMKIIδC+KN92</td>
<td>13.1±1 [11]*</td>
<td>22.9±1.6‡‡</td>
</tr>
<tr>
<td>CaMKIIδC+KN93</td>
<td>6.9±0.7 [9]‡</td>
<td>10.1±1.5#</td>
</tr>
<tr>
<td>CaMKIIδC+RAN</td>
<td>9.0±1.3 [9]‡</td>
<td>12±0.9#</td>
</tr>
<tr>
<td>CaMKIIδC+THA</td>
<td>9.3±1.2 [7]‡</td>
<td>10.6±1.1#</td>
</tr>
</tbody>
</table>

[Na]i and [Ca]i measured at baseline and 12 min after onset of exposure to H2O2. For some experiments, KB-R7943 (5 µmol/L), KN92 (10 µmol/L), KN93 (10 µmol/L), RAN (10 µmol/L) or THA (5 µmol/L) were used. *P<0.05 vs. WT (baseline) or βGal (baseline); †P<0.05 vs. WT (12 min) or βGal (12 min); ‡P<0.05 vs. CaMKIIδC (baseline); #P<0.05 vs. CaMKIIδC.
Online Figure legend

Online Figure I

Changes in Na and Ca handling predicted by a rabbit ventricular AP computer model. The digital cell was stimulated using a train of APs at a BCL of 2 s (AP clamp configuration; A, inset). A) Steady state levels of [Na] are shown at baseline (a, black), when enhancing late \( I_{Na} \) 3.33-fold as measured in the present study (b, light blue, inset), with additionally increased background Na conductance (Nabk) by 40-fold (c, dark blue), or with a 50% reduction in NKA function and only a 16-fold increase of Na conductance (d, magenta). Further simulations incorporated \( H_2O_2 \)-dependent changes in SR function (80% reduction in SERCA \( V_{max} \) and 10-fold increase in SR Ca leak: e, orange), and in L-type Ca-current (75% reduction) and NCX activity (2.5-fold increase; f, red) and when late \( I_{Na} \) and enhanced background Na influx were blocked (simulating ranolazine; g, white). B) Upper panel: simulated sodium pump current traces for baseline (a, black), enhanced late \( I_{Na} \) and background Na conductance (c, dark blue), and additionally diminished NKA function (d, magenta). Ca transients (middle panel), and NCX current (lower panel) for the indicated conditions (a, d and f). C) Predicted Ca transient amplitude (upper left), SR Ca content (upper right), diastolic [Ca], (lower left), and NCX current integral (lower right), where both the total outward current (upward) and net \( I_{NCX} \) (downward) are shown.
ROS activate CaMKII to regulate late \( I_{Na} \) 

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Online Figure I