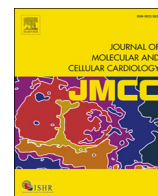




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Original article

Q1 Oxidation of ryanodine receptor (RyR) and calmodulin enhance Ca release and pathologically alter, RyR structure and calmodulin affinity

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ABSTRACT

Oxidative stress may contribute to cardiac ryanodine receptor (RyR2) dysfunction in heart failure (HF) and arrhythmias. Altered RyR2 domain–domain interaction (domain unzipping) and calmodulin (CaM) binding affinity are allosterically coupled indices of RyR2 conformation. In HF RyR2 exhibits reduced CaM binding, increased domain unzipping and greater SR Ca leak, and dantrolene can reverse these changes. However, effects of oxidative stress on RyR2 conformation and leak in myocytes are poorly understood. We used fluorescent CaM, FKBP12.6, and domain–peptide biosensor (F-DPc10) to measure, directly in cardiac myocytes, (1) RyR2 activation by hydrogen peroxide (H₂O₂)-induced oxidation, (2) RyR2 conformation change caused by oxidation, (3) CaM–RyR2 and FKBP12.6–RyR2 interaction upon oxidation, and (4) whether dantrolene affects 1–3. H₂O₂ was used to mimic oxidative stress. H₂O₂ significantly increased the frequency of Ca²⁺ sparks and spontaneous Ca²⁺ waves, and dantrolene almost completely blocked these effects. H₂O₂ pretreatment significantly reduced CaM–RyR2 binding, but had no effect on FKBP12.6–RyR2 binding. Dantrolene restored CaM–RyR2 binding but had no effect on intracellular and RyR2 oxidation levels. H₂O₂ also accelerated F-DPc10–RyR2 association while dantrolene slowed it. Thus, H₂O₂ causes conformational changes (sensed by CaM and DPc10 binding) associated with Ca leak, and dantrolene reverses these RyR2 effects. In conclusion, in cardiomyocytes, H₂O₂ treatment markedly reduces the CaM–RyR2 affinity, has no effect on FKBP12.6–RyR2 affinity, and causes domain unzipping. Dantrolene can correct domain unzipping, restore CaM–RyR2 affinity, and quiet pathological RyR2 channel gating. F-DPc10 and CaM are useful biosensors of a pathophysiological RyR2 state.

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1. Introduction

The Ca²⁺ release channel in cardiac sarcoplasmic reticulum (SR) is the type 2 ryanodine receptor (RyR2), which is pivotal in cardiac excitation–contraction coupling [1]. Several lines of evidence suggest that excessive Ca²⁺ leak through RyR2, in diastole, is seen in pathological conditions, such as heart failure (HF), leading to both systolic and diastolic dysfunction [2–7]. This abnormal RyR2 Ca²⁺ leak can cause

delayed afterdepolarization (DAD) that can lead to lethal arrhythmias, such as catecholaminergic polymorphic ventricular tachycardia (CPVT) that is linked to human RyR2 mutations. Stabilization of RyR2 closed state, resulting in suppression of abnormal SR Ca²⁺ leak, is a promising new therapeutic strategy against HF or lethal arrhythmias.

In our recent studies, we have proposed that altered RyR2 conformation, especially of an interaction between the N-terminal and central domains of the RyR2, destabilizes the RyR2 channel gating and contributes to the abnormal Ca²⁺ leak [4,8–12]. In normal conditions, the interaction between these domains is tight (zipped state), and stabilizes the closed RyR2 channel. On the other hand, in disease conditions, the interaction between N-terminal and central domains is weakened (unzipped state), resulting in an abnormally high Ca²⁺ leak via RyR2. This altered domain interaction (domain unzipping) may be diagnostic of a pathological RyR2 gating state that contributes to dysfunction.

A domain peptide corresponding to a sequence span within the central domain of RyR2 residues 2460–2495 (DPc10), that includes a CPVT mutation site (R2474S), can bind to the native N-terminal domain, competing with its normal interaction with the central domain. By inducing

Abbreviations: τ , time constant; $\tau_{\text{wash-in}}$, wash-in time constant; $\tau_{\text{wash-out}}$, wash-out time constant; AIP, autocamtide-2 related inhibitory peptide; B_{max} , binding maximum; CaM, calmodulin; CaSpF, calcium spark frequency; DAN, dantrolene; F, fluorescent; FDHM, full duration at half maximum; FWHM, full width at half maximum; FRET, fluorescence resonance energy transfer; HF, heart failure; H₂O₂, hydrogen peroxide; K_d , dissociation constant; k_{in} , wash-in rate constant, $1/\tau_{\text{wash-in}}$; k_{out} , wash-out rate constant, $1/\tau_{\text{wash-out}}$; RyR2, cardiac ryanodine receptor; SR, sarcoplasmic reticulum; SCW, spontaneous Ca wave.

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domain unzipping, DPc10 increases RyR2 leakiness in the normal cardiac myocytes or SR vesicles [4,11]. We have recently proposed that the binding kinetics of fluorescent DPc10 (F-DPc10) to the RyR2 in permeabilized cardiomyocytes is a sensitive measure of RyR2 conformational change to the pathological unzipped state [12].

Calmodulin (CaM) binds to and inhibits RyR2 opening at both high and low $[Ca^{2+}]$ [13,14]. We have shown that CaM–RyR2 binding affinity is reduced in HF [3,15] and that this increases RyR2 Ca^{2+} leak, as seen in HF or in knock-in (KI) mice carrying a human CPVT-associated RyR2 mutation (R2474S) or an RyR2 mutation that prevents CaM binding [15,16]. We also showed that CaM binding to RyR2 stabilizes the zipped state, suppresses DPc10 access and inhibits RyR2 leak. Reciprocally, domain unzipping inhibits CaM–RyR2 binding, which in turn causes abnormal Ca^{2+} leak from RyR2 [12].

Reactive oxygen species (ROS) are thought to be involved in a variety of cardiovascular diseases, including HF [17–19]. ROS can also cause RyR2 dysfunction by oxidation of thiol groups of cysteine residues in the RyR2 channel [20]. However, the relation between oxidation-induced RyR2 dysfunction and either CaM binding affinity or the pathological unzipped state is not clear, especially in the myocyte environment.

Dantrolene is the only specific and effective therapeutic agent for treatment of malignant hyperthermia that occurs in certain patients during or following surgery or anesthesia [21]. In HF or CPVT-KI mouse, dantrolene has been reported to correct domain unzipping by binding to the N-terminal (601–620) domain of RyR2, and thereby inhibit diastolic Ca^{2+} leak via RyR2 [11,22,23].

In the present study, we investigated directly in cardiac myocytes how moderate oxidative stress, as in HF, alters RyR2 conformation, as detected by measuring the fluorescently labeled DPc10 (F-DPc10) binding kinetics, and the binding affinity of fluorescent CaM and FKBP12.6. Furthermore, we assessed how dantrolene affects RyR2 conformation change and the binding affinity of CaM–RyR2 and FKBP12.6–RyR2.

2. Materials and methods

Rat ventricular myocytes were isolated and permeabilized as previously described [24]. The care of the animals and procedures were approved by the University of California, Davis Animal Research Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and Guidelines of the Animal Ethics Committee of Yamaguchi University School of Medicine. FKBP12.6 and CaM were labeled with Alexa Fluor 488 (F-FKBP12.6, F-CaM, respectively) and DPc10 was labeled with HiLyte Fluor 647 (F-DPc10, respectively) at AnaSpec as in our previous studies [12,25,26]. Online Fig. 1A–C shows hydrogen peroxide (H_2O_2) did not alter both AF488 and F-CaM fluorescence intensity. All experiments were performed at room temperature (25 °C). An expanded Materials and Methods section can be found in the Online Data Supplement.

3. Results

3.1. Activated RyR2 function by oxidation in myocytes

Oxidation induced by H_2O_2 is expected to activate SR Ca^{2+} leak through RyR2 at diastolic $[Ca^{2+}]_i$ [27,28]. We used line-scan images to measure the effects of 50 μM H_2O_2 on Ca^{2+} spark frequency (CaSpF) and SR Ca^{2+} content (evaluated via rapid caffeine application) in intact cardiomyocytes after 1 Hz electric field stimulation (Fig. 1). H_2O_2 treatment can also inhibit SR Ca^{2+} -ATPase (SERCA) activity [29,30]. Under our conditions neither H_2O_2 nor dantrolene significantly altered Ca^{2+} transient amplitude or time constant of $[Ca^{2+}]_i$ decline (Table SI). This indicates that 50 μM H_2O_2 had negligible effect on SERCA function. Fig. 1A–B, and Table SII show that H_2O_2 significantly increased the CaSpF and Ca^{2+} spark full duration at half maximum compared with control. Thus H_2O_2 may enhance diastolic RyR2 channel opening.

CaSpF depends on SERCA-dependent SR Ca^{2+} content [31], but H_2O_2 did not significantly alter SR Ca^{2+} content. So CaSpF normalized to SR Ca^{2+} content, was significant increase by H_2O_2 treatment of myocytes (Fig. 1C).

We also tested whether H_2O_2 enhanced the propensity for arrhythmic DADs, as measured by spontaneous Ca^{2+} waves (SCW) in intact cardiac myocytes. Fig. 2A–B shows that H_2O_2 treatment significantly increased the occurrence of SCW vs. control when the pacing rate increased from 1 to 5 Hz.

3.2. Dantrolene quiets oxidation-induced RyR2 activation

Dantrolene can prevent abnormal Ca^{2+} leak in CPVT KI and HF models [11,22,23,32]. To test whether dantrolene could prevent H_2O_2 -induced increase in CaSpF, dantrolene was added to myocytes before H_2O_2 treatment. Dantrolene pretreatment had no effect on control myocytes, but suppressed the H_2O_2 -induced increase in frequency of Ca^{2+} sparks and SCW (Figs. 1 and 2). To test whether dantrolene prevented these H_2O_2 effects on RyR2 by reducing the oxidative level in myocytes, we assessed oxidation levels in myocytes.

3.3. H_2O_2 -induced intracellular and RyR2 redox modification

To monitor intracellular ROS level, we used H_2DCFDA (10 μM). Fig. 3A shows confocal H_2DCFDA fluorescence images in control or H_2O_2 -treated myocytes with or without dantrolene. As shown in Fig. 3B, the intracellular ROS level was similarly increased after addition of 50 μM H_2O_2 , with or without dantrolene pretreatment. Thus, dantrolene does not alter the intracellular oxidative level overall. To assess whether dantrolene can attenuate the extent of RyR2 thiol modification in H_2O_2 -treated myocytes, we assayed monobromobimane (mBB) fluorescence-labeling. Fig. 3C–D shows that H_2O_2 treatment significantly decreased the content of free thiols on RyR2, regardless of dantrolene pretreatment. Taken together, these results indicate that dantrolene does not attenuate intracellular ROS or RyR2 Cys oxidation.

3.4. H_2O_2 does not alter FKBP12.6 binding at Z-line in permeabilized myocytes

We assessed the molecular mechanism by which RyR2 activity was increased by H_2O_2 -induced oxidative stress. FKBP12.6 can bind to and stabilize RyR2 channel gating, but details are controversial [33]. We measured the effect of H_2O_2 on FKBP12.6–RyR2 binding affinity in saponin-permeabilized myocytes using AF488-FKBP12.6 [15,25,26,34,35]. Fig. 4A shows confocal images of F-FKBP with or without H_2O_2 , and also with 1 μM of the specific Ca^{2+} /CaM-dependent kinase II (CaMKII) inhibitor autocamtide-2-related inhibitory peptide (AIP) or 1 μM dantrolene. We used 1 nM F-FKBP (near the K_d) [26], because at half-saturation it would be very sensitive to either an increase or decrease in RyR2 binding. Neither H_2O_2 nor AIP nor dantrolene altered Z-line (F_Z) and M-line (F_M) F-FKBP12.6 fluorescence (Fig. 4B), indicating that H_2O_2 failed to alter specific F-FKBP12.6 binding to the RyR2 (F_Z – F_M) in permeabilized myocytes, with or without AIP or dantrolene (Fig. 4C). Thus H_2O_2 did not alter FKBP12.6–RyR2 affinity.

3.5. Effect of H_2O_2 on CaM binding to Z-line in permeabilized myocytes

CaM also binds to RyR2 and reduces RyR2 open probability, and works as a regulatory protein for RyR2 channel gating [13–15,36]. We measured the effect of H_2O_2 on CaM–RyR2 binding affinity using F-CaM as in our previous reports [15,25]. Fig. 5A–B shows that both 10 and 50 μM H_2O_2 myocyte pretreatment reduced the F-CaM binding at the Z-line significantly, (using $[F-CaM] = 20$ nM, near its K_d [15]), but did not alter CaM at the M-line (Online Fig. IIA). This indicates that H_2O_2 decreased affinity of F-CaM/RyR2 binding. However, because H_2O_2 was not removed before CaM addition, this effect might be due

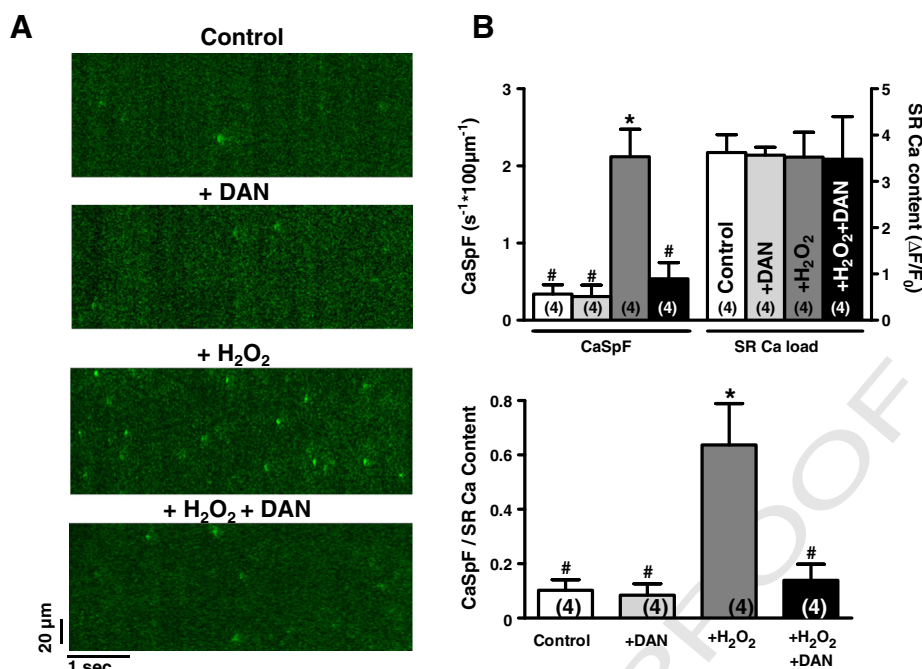


Fig. 1. Effects of H₂O₂ and dantrolene on Ca²⁺ sparks and SR Ca²⁺ content in intact myocytes. Ca²⁺ sparks and SR Ca²⁺ content were measured in myocytes which were treated or not for 5 min with 50 μM H₂O₂. Some myocytes were also pretreated for 2 h with 1 μM dantrolene (DAN). (A) Representative line-scan images of Ca²⁺ sparks. (B) Summarized data of CaSpF and SR Ca²⁺ content. Data are reported as mean ± SE (n values on bars). *P < 0.05 vs. Control, # P < 0.05 vs. H₂O₂.

to oxidation of either RyR2 or CaM, particularly because methionines on CaM can be oxidized and cause reduced binding to RyR2 [36].

To test whether CaM oxidation can explain the reduced RyR2 binding of H₂O₂-treated myocytes, we first incubated [F-CaM] (50 μM) for 60 min in media containing 50 μM H₂O₂ (the concentration used in pre-incubated myocytes). This allowed F-CaM oxidation (and we call this F-CaM^{ox}). This was then diluted 2500-fold to 20 nM F-CaM^{ox} and no H₂O₂ was present in the myocyte bath. As shown in Fig. 5C–D (and

Online Fig. IIC–D), F-CaM^{ox} binds less well to RyR2, but this 20% reduction is smaller than the 50% seen when F-CaM applied to myocytes pretreated with 50 μM H₂O₂ (Fig. 5A–B). This suggests that both CaM and RyR2 oxidation contribute to the reduced CaM binding. It also suggests that CaM was not fully oxidized, by our in vitro pre-exposure to 50 μM H₂O₂ (Balog used 1000 times higher H₂O₂ concentration for 24 h; [37]).

As a further test of whether RyR2 oxidation alone inhibits subsequent CaM affinity, we removed H₂O₂ from myocytes by repeated

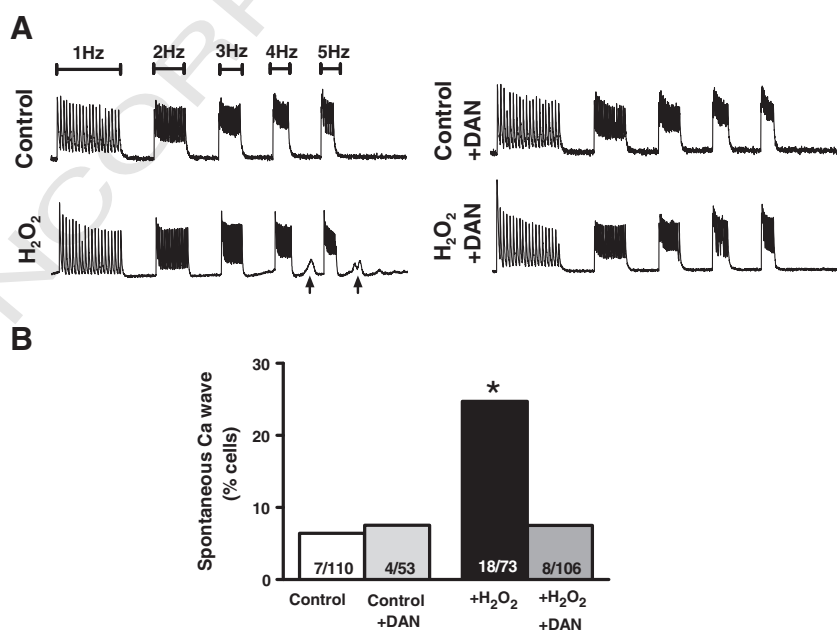


Fig. 2. Arrhythmogenic spontaneous Ca²⁺ waves (SCW) following treatment with H₂O₂ in intact myocytes. (A) Treatment with H₂O₂ (50 μM; 3 min) led to an increased incidence of SCW (arrows), but dantrolene (DAN) prevented SCW. Representative time-plots of intracellular Ca²⁺ during pacing. (B) Summary of % occurrence of SCW. Numbers inside the bars indicate cells with SCW/total cells studied for each group. *P < 0.05 vs. Control, Fisher's exact test.

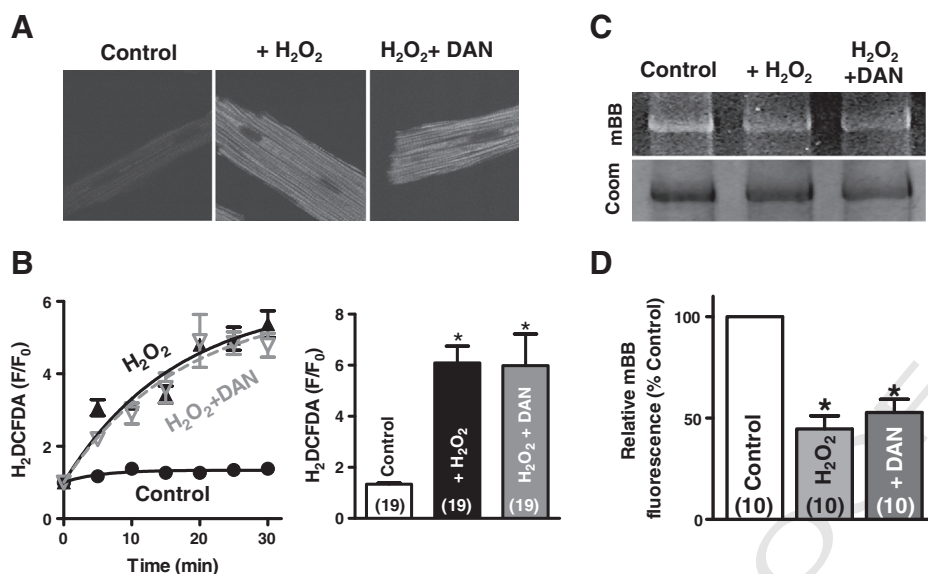


Fig. 3. Oxidation status of the intracellular environment and RyR2. (A) Representative confocal images of control, H₂O₂ (50 μM), and H₂O₂ (50 μM) + DAN (1 μM) loaded with an ROS-sensitive fluorescent indicator H₂DCFDA (10 μM). (B) Time course of H₂DCFDA fluorescence recorded under the same condition as indicated for panel A (left), and quantitative analysis of the H₂DCFDA fluorescence intensity at equilibrium for each condition (right). (C) Representative RyR2-bound mBB fluorescence intensity (upper strip) and Coomassie-stained gels (lower strip) of RyR2 measured under control conditions, + H₂O₂ (50 μM), or + H₂O₂ (50 μM) + DAN (1 μM) treatment. (D) Relative free thiol content of RyR2 (indicated by mBB fluorescence) normalized by the corresponding intensity of the Coomassie-stained RyR2 band. Data are reported as mean ± SE (n values on bars). DAN: dantrolene.

washing, prior to the addition of F-CaM. Fig. 6A shows confocal images of F-CaM binding at the Z-line. Myocyte oxidation (by 50 μM H₂O₂) dramatically reduced specific binding of F-CaM at the Z-line (F_Z-F_M) by 32% (Fig. 6B) without altering the M-line fluorescence (Online Fig. IIB). That is consistent with data in Fig. 5, and that ~60% of the reduced CaM binding in Fig. 5B was due to H₂O₂-induced changes at RyR2, with the remainder due to effects on CaM. Dantrolene (1 μM), but not the CaMKII inhibitor AIP, partially restored F-CaM binding in H₂O₂-treated myocytes (Fig. 6B). These results indicate that H₂O₂-induced loss of CaM binding can be restored by dantrolene. The lack of AIP effect

suggests that any CaMKII activation by H₂O₂ in this protocol does not contribute to the acute loss of CaM-RyR2 binding. Neither AIP nor dantrolene alter the CaM-RyR2 affinity under control conditions (Fig. 6D and Online Fig. III).

3.6. Effects of dantrolene or H₂O₂ on wash-in kinetics of HF647-DPc10

Since CaM binding to RyR2 in myocytes is known to suppress Ca²⁺ sparks [15,38], the H₂O₂-induced increase in SR Ca²⁺ leak (measured as Ca²⁺ sparks in Fig. 1) might be mediated mainly by the oxidation

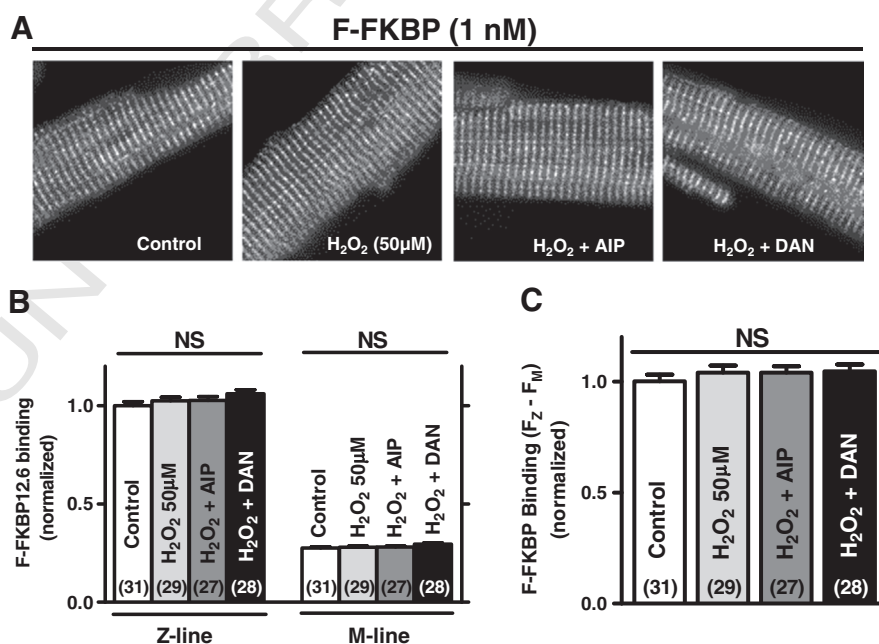


Fig. 4. Effect of H₂O₂ (50 μM) on FKBP12.6 binding at the Z-line. (A) Representative confocal images of saponin-permeabilized myocytes incubated with F-FKBP (1 nM), which were exposed to H₂O₂ (50 μM), H₂O₂ (50 μM) + AIP (1 μM) or H₂O₂ (50 μM) + DAN (1 μM). (B) F-FKBP fluorescence intensity (normalized to Z-line at control) from Z-line and M-line. (C) Summary of the specific binding of F-FKBP at Z-line (F_Z-F_M) corresponding to the experiment in panel A. Data are reported as mean ± SE (n values on bars). DAN: dantrolene.

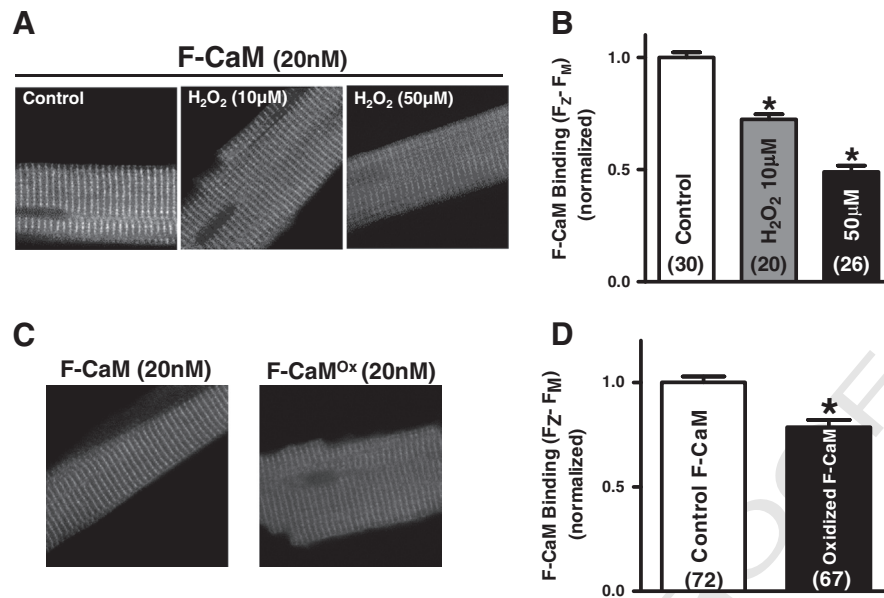


Fig. 5. Effects of myocyte or F-CaM oxidation by H₂O₂ on F-CaM binding at the Z-line. (A) Representative confocal images illustrating the effect of myocyte oxidation by H₂O₂ on F-CaM binding at the Z-lines (F_Z - F_M). H₂O₂ was applied to the bath 1 h before beginning to image and was present throughout the experiment. (B) Quantitative analysis of data from A, normalized to F_Z - F_M at control. (C) Representative confocal images of the effect of F-CaM oxidation on specific binding at the Z-line (F_Z - F_M). F-CaM was pre-incubated in H₂O₂, then applied to the myocyte bath under control conditions (F-CaM^{ox}). (D) Quantitative analysis of data from C. Data are reported as mean ± SE (n values on bars). *p < 0.001 vs. control.

of RyR2 and CaM and reduced CaM binding to the RyR2. The restoration of CaM binding (and normal Ca sparks) by dantrolene raised a connection to our recent work [12]. We had shown that RyR2 CaM binding exhibits negative allosteric coupling with the accessibility of the unzipping peptide DPc10 (DPc10 binding inhibits CaM binding, and CaM binding

inhibits DPc10 access [12]). Dantrolene may shift this balance toward the more normal state [22,23] by reducing DPc10 accessibility (zipping) and also increasing CaM affinity. We tested whether H₂O₂ treatment enhances DPc10 access and whether that was sensitive to dantrolene (as shown above for CaSpF and CaM binding).

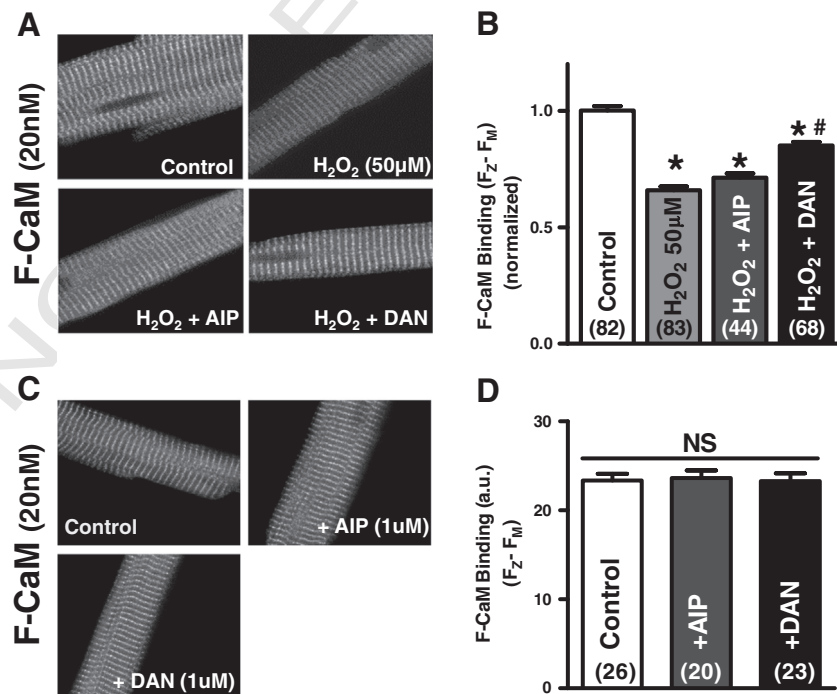


Fig. 6. Effects of RyR2 oxidation or AIP and DAN on calmodulin (CaM) binding at the Z-line. (A) H₂O₂ was applied to the myocyte bath 1 h before beginning the recording, and was removed before the applying the F-CaM. Representative confocal images of the effects of H₂O₂, H₂O₂ + AIP, and H₂O₂ + dantrolene on the specific binding of F-CaM binding at the Z-lines (F_Z - F_M). (B) Quantitative analysis of data from A. (C) Representative confocal image of the effects of AIP and DAN on F-CaM Z-line binding in control myocyte. (D) Quantitative analysis of data from C. Data are reported as mean ± SE (n values on bars; a.u., arbitrary units). *p < 0.001 vs. control, #p < 0.001 vs. H₂O₂. DAN: dantrolene.

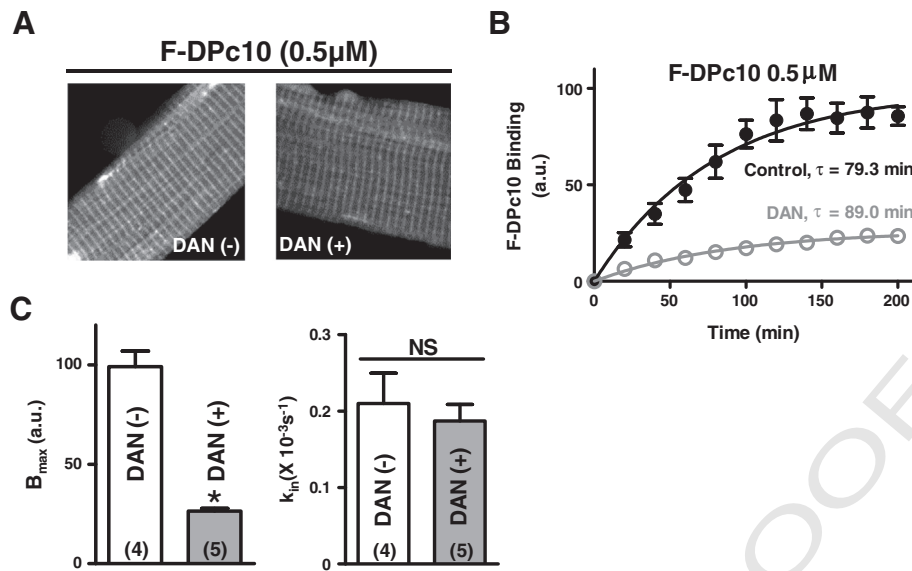


Fig. 7. Effect of dantrolene (DAN) (1 μ M) on F-DPc10 (0.5 μ M) binding at Z-line. (A) Representative confocal images illustrating the effect of dantrolene (1 μ M) on the F-DPc10 (0.5 μ M) binding at the Z-lines. (B) Time course of F-DPc10 (0.5 μ M) wash-in (full circles), and in the presence of dantrolene (1 μ M, open circles). (C) Summary of B_{max} and k_{in} for the data in panel B. Data are reported as mean $\pm$ SE (n values on bars).

We previously reported that the wash-in kinetics of DPc10 labeled with HyLite Fluor 647 (F-DPc10) were greatly slowed when CaM was bound to RyR2, which also prevented RyR2 activation by DPc10 exposure [12]. Our working model was that DPc10 access to its binding site was sterically blocked when CaM was bound. Here, we first tested whether dantrolene has the same effect as CaM in preventing DPc10 access.

Fig. 7A shows confocal images of F-DPc10 binding after 200 min of incubation. Fig. 7B shows the time course of F-DPc10 wash-in with or without 1 μ M dantrolene. Dantrolene reduced maximal F-DPc10 binding (B_{max}) by >70% (Fig. 7C), without altering M-line fluorescence (Online Fig. IV). This suggests that dantrolene keeps RyR2 conformationally closed with respect to access of DPc10. However, these results could also result from competition between F-DPc10 and dantrolene to a site on RyR2. To test this hypothesis, we measured the F-DPc10 wash-out kinetics, with or without dantrolene. Online Fig. VA–B shows that non-fluorescent DPc10 (NF-DPc10) can accelerate

the wash-out kinetics of F-DPc10. This is consistent with our previous report that F-DPc10 and NF-DPc10 bind at the same binding site [12]. However, dantrolene had no effect on F-DPc10 wash-out kinetics, indicating that F-DPc10 and dantrolene binding sites are separate (Online Fig. VC–D). This also suggests that the slow binding of F-DPc10 (and effect of dantrolene thereon) is due to on-rate effects, consistent with a RyR2 conformation that strongly limits access of DPc10 to its site on RyR2.

Next, we tested whether RyR2 oxidation would increase F-DPc10 access. Here, we used FRET between F-FKBP12.6 as a donor and F-DPc10 as an acceptor, to detect DPc10 that specifically binds at RyR2 [12]. Note that F-FKBP12.6 binds specifically at RyR2 with 1 nM affinity, and its binding is not influenced by CaM [26], H_2O_2 or dantrolene (Fig. 4). FRET was assessed as the decrease in F-FKBP12.6 donor fluorescence intensity by binding of F-DPc10 acceptor in its proximity, i.e. donor quench, as in our previous report [12]. H_2O_2 pre-incubation significantly accelerated the rate of F-DPc10 binding compared with

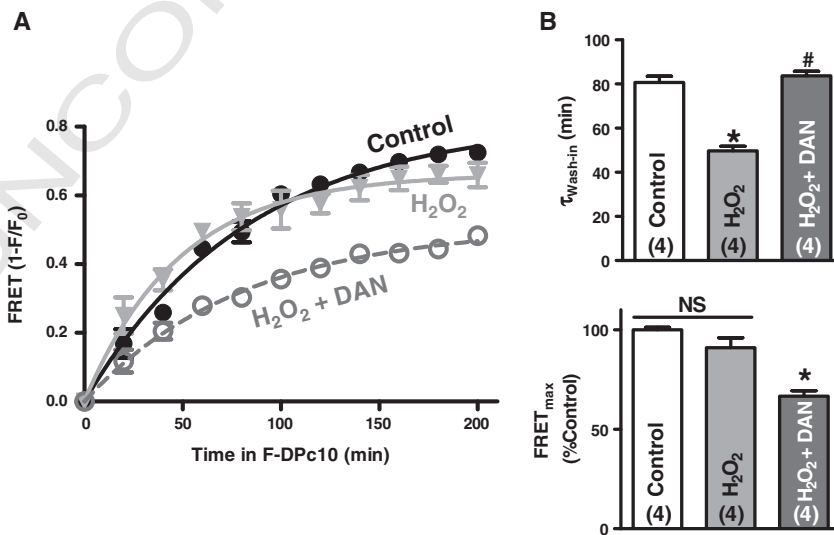


Fig. 8. Effects of H_2O_2 and dantrolene (DAN) on the F-DPc10 wash-in kinetics, detected via FRET between F-FKBP (donor) and F-DPc10 (acceptor). (A) FRET, detected as donor quenching in myocytes pre-equilibrated with H_2O_2 (50 μ M) or H_2O_2 (50 μ M) + DAN (1 μ M). (B) Summary of $\tau_{wash-in}$ and FRET $_{max}$ corresponding to the data in panel A. Data are reported as mean $\pm$ SE (n values on bars). * $p < 0.001$ vs. control, # $p < 0.001$ vs. H_2O_2 .

control (Fig. 8A–B). This indicates that RyR2 oxidation enhances DPc10 access (e.g. by causing domain unzipping). Furthermore, pre-equilibration with dantrolene in H₂O₂-treated myocytes reversed the H₂O₂-induced acceleration of access (Fig. 8B) and reduced the maximal extent of quench (Fig. 8C), which reflects a decrease in B_{max} of F-DPc10. Taken together, these findings show, for the first time in situ, that H₂O₂ leads to defective RyR2 domain unzipping and dantrolene can correct this conformational change as well as restoring CaM affinity.

4. Discussion

Novel findings of this study are the following: In permeabilized ventricular myocyte environment, (1) H₂O₂ treatment increases both CaSpF and the occurrence of SCW, but dantrolene prevents this elevated Ca²⁺ leak; (2) RyR2 oxidation by H₂O₂ decreases its binding affinity for CaM, but does not alter its FKBP12.6 affinity; (3) RyR2 oxidation leads to domain unzipping (high DPc10 access); and (4) dantrolene corrects domain unzipping, restores the CaM–RyR2 binding affinity, and inhibits pathological RyR2 channel gating. Our working model is that modest H₂O₂ (or ROS) levels cause a similar pathological change in RyR2 conformation as seen in HF, in which CaM affinity is reduced, DPc10 access is increased and SR Ca leak is elevated.

4.1. RyR2 function is activated by H₂O₂

Increased ROS production has been associated with pathological states, such as HF [39], and RyR2 activity in pathological states is increased by thiol oxidation [40,41]. H₂O₂ can activate RyR2 function, but also alter function of other important Ca²⁺-handling proteins, including SERCA and the Na⁺–Ca²⁺ exchanger (NCX). Yan et al. [28] showed that CaSpF increased during 10 min of treatment with 50 μM H₂O₂ in intact myocytes. On the other hand, higher [H₂O₂] (200 μM) can reduce Ca²⁺ transient amplitude, CaSpF, and SR Ca²⁺ content, consistent with H₂O₂-dependent inhibition of SERCA activity [30]. To assess whether H₂O₂ activates the RyR2 function, we measured CaSpF under conditions where both amplitude and the rate of Ca²⁺ transient decline (reflecting SERCA activity), and the decay of caffeine-induced Ca²⁺ transient (reflecting NCX activity), were similar. This indicates that both SERCA and NCX function were not appreciably altered under our specific conditions. Thus, RyR2 function may be more sensitive to H₂O₂-induced modulation than are SERCA or NCX. We also found a significant increase in the occurrence of arrhythmogenic SCW in H₂O₂-treated myocytes, when pacing rate increased from 1 to 5 Hz, indicating that hyperactivity of RyR2 by oxidation may contribute to triggering lethal arrhythmias.

4.2. Dantrolene reverses H₂O₂-induced RyR2 activation without altering cellular and RyR2 oxidation

Dantrolene has been shown to bind to amino acids 601–620 of RyR2 [42] and stabilize the RyR2 channel gating in pathological states, such as HF [32] or CPVT [43]. However, dantrolene has no effect on Ca²⁺ signaling under control condition [32,44]. These are consistent with our observation that dantrolene significantly reduced the CaSpF and prevented potentially deleterious spontaneous arrhythmogenic Ca²⁺ waves in H₂O₂-treated myocytes, but did not alter the frequency of Ca²⁺ sparks and SCW under control condition. To exclude the possibility that dantrolene attenuated the oxidation level to achieve this effect, we measured intracellular ROS production and RyR2 free thiol content in dantrolene-treated myocytes. Dantrolene influenced neither cellular nor RyR2 Cys oxidation level, suggesting that dantrolene directly stabilized RyR2, possibly by inhibiting domain unzipping [22]. Conceivably, dantrolene may indirectly affect RyR2 function via Met oxidation. However, the lack of significant effects on the overall cell oxidation and on the RyR2 Cys oxidation makes this an unlikely mechanism of dantrolene action.

4.3. H₂O₂ reduced CaM, but not FKBP12.6 binding to RyR2

There are two main possible explanations for H₂O₂-induced defective CaM binding to RyR2: (1) Oxidation of CaM inhibits the productive association of CaM with RyR2 or (2) Oxidation of RyR2 inhibits CaM binding. Balog et al. [37] reported that extensive in vitro oxidation of CaM abolishes the functional interaction between CaM and RyR2. That is consistent with our in situ observation that our much milder exposure to H₂O₂ caused some CaM oxidation and reduction of binding to RyR2 (Fig. 5C). On the other hand, it has been previously proposed [14] that RyR2 oxidation enhances RyR2 activity by decreasing CaM binding affinity. This also agrees with our myocyte result that RyR2 oxidation reduced subsequent CaM binding (Fig. 6). Taken together, these results strongly support the conclusion that oxidation of both CaM and RyR2 cause reduced CaM–RyR2 binding and this combined mechanism contributes to RyR2 dysfunction during oxidative stress. One of the interesting findings here is that dantrolene restores the CaM–RyR2 binding in H₂O₂-treated myocytes, resulting in lower resting RyR2 leak, as has been seen for dantrolene in HF or CPVT models [45,23], without changing intracellular and RyR2 oxidative level (Fig. 3).

FKBP12.6 binds to RyR2 with high affinity and can also influence RyR2 gating [26,46,47], and has been proposed to play an important role in stabilizing RyR2 function [46–48], although this issue is controversial [26,30,49,50]. Shan et al. reported [48] that 1 mM H₂O₂ combined with phosphorylation of Ser2808 by PKA could reduce FKBP12.6 binding to RyR2 by ~70%. In contrast, we find that neither PKA-dependent phosphorylation [26], DPc10-induced unzipping [12] nor the more moderate levels of H₂O₂ used here (plausibly reflective of HF myocyte) had any effect on FKBP12.6 binding to RyR2 in myocytes (Fig. 4). In our hands, CaM has much stronger effects on RyR2 function than does FKBP12.6, with more pronounced changes during pathophysiological conditions such as HF [15], oxidation or DPc10-induced unzipping.

4.4. Dantrolene corrects RyR2 conformation caused by either H₂O₂ or DPc10

We previously demonstrated that monitoring F-DPc10 binding kinetics is a powerful tool to evaluate functionally important RyR2 conformational changes, likely related to an interaction between the N-terminal and central domains of RyR2. Using this method, we now show that H₂O₂ significantly accelerates F-DPc10 association rate in situ, indicating that H₂O₂ causes domain unzipping (Fig. 8). We also found that dantrolene reduces access of F-DPc10 in either H₂O₂- or DPc10-treated myocytes, which suggests that H₂O₂ and DPc10 induce similar structural changes that are both corrected by dantrolene (Figs. 7 and 8). These findings are consistent with previous in vitro reports that oxidative stress of RyR2 (in SR vesicles) weakens domain interactions [51] and that dantrolene improves RyR2 function via correcting domain unzipping [22]. The B_{max} for F-DPc10 is lower in dantrolene-treated myocytes. That could have been a result of DPc10 and dantrolene competing at the same site. But we have ruled out that possibility. First, we measured the wash-out kinetics of F-DPc10 with or without NF-DPc10 in wash-out solution. Since F-DPc10 wash-out rate was faster with NF-DPc10 (Online Fig. VA–B), we infer that F-DPc10 and NF-DPc10 bind to RyR2 at the same site. In contrast, dantrolene did not alter the wash-out kinetics of F-DPc10. These results suggest that dantrolene prevents F-DPc10 access (drastically reducing on-rate) without altering F-DPc10 dissociation (off-rate) (Online Fig. VC–D). This observation supports the conclusions that F-DPc10 and dantrolene bind at separate sites on RyR2, and that dantrolene, like CaM [12], influences DPc10 access by an allosteric mechanism.

5. Limitations

CaMKII can also be activated by oxidation at methionine 281/282 [52] and can also phosphorylate and activate RyR2 in pathological states

[3,38,52–54]. This CaMKII pathway would be expected to exacerbate the direct ROS effects on CaM and RyR2 that alter RyR2 gating that has been our focus here. Here, we used the CaMKII inhibitors AIP (CaM binding experiments) and KN-93 (SCW experiments) specifically to assess CaMKII-independent effects of H₂O₂ on Ca²⁺ waves and CaM binding to RyR2. Wagner et al. [27] have shown that the increase in CaSpF and SR Ca leak observed with 200 μM H₂O₂ was not prevented by KN-93, consistent with a CaMKII-independent effect of H₂O₂ on RyR2 dysfunction. Future studies will be required to clarify the detailed relation between ROS effects on CaM and RyR2 directly and those mediated indirectly via CaMKII-dependent phosphorylation of RyR2.

6. Conclusion

Our results indicate that abnormal oxidative modification of RyR2 by H₂O₂ causes reduced CaM affinity of RyR2 (by oxidation of sites on both CaM and RyR2) and RyR2 conformation changes (domain unzipping) that lead to untimely and potentially arrhythmogenic RyR2 channel opening. Dantrolene restores normal CaM binding and conformational state, and quiets pathological RyR2 channel gating (but has no effect on RyR2 function under control normal conditions). This H₂O₂-induced structural unzipping, reduced CaM binding and more active RyR2 may represent a functionally integrated common pathological RyR2 state that is relevant for HF, oxidative stress and even CPVT-linked genetic mutations.

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Disclosures

The authors report no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yjmcc.2015.06.009>.

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