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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 (H_2O_2)-induced oxidation, (2) RyR2 conformation change caused by oxidation, (3) CaM-RyR2 and FK506-binding protein (FKBP12.6)-RyR2 interaction upon oxidation, and (4) whether dantrolene affects 1–3. H_2O_2 was used to mimic oxidative stress. H_2O_2 significantly increased the frequency of Ca^{2+} sparks and spontaneous Ca^{2+} waves, and dantrolene almost completely blocked these effects. H_2O_2 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. H_2O_2 also accelerated F-DPc10-RyR2 association while dantrolene slowed it. Thus, H_2O_2 causes conformational changes (sensed by CaM and DPc10 binding) associated with Ca leak, and dantrolene reverses these RyR2 effects. In conclusion, in cardiomyocytes, H_2O_2 treatment markedly reduces the CaM-RyR2 affinity, has no effect on FKBP12.6-RyR2 affinity, and causes domain unzipping. Dantrolene can correct

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Disclosures

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domain unzipping, restore CaM-RyR2 affinity, and quiet pathological RyR2 channel gating. F-DPc10 and CaM are useful biosensors of a pathophysiological RyR2 state.

Keywords

Calmodulin; FKBP12.6; Ryanodine Receptor; Reactive oxygen species; Dantrolene

1. Introduction

The Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 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

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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 Figure IA–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–1B, 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 arrhythmogenic DADs, as measured by spontaneous Ca^{2+} waves (SCW) in intact cardiac myocytes. Fig 2A–2B shows

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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 (Fig 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 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_20_2 decreased affinity of F-CaM/RyR2 binding.

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However, because H_2O_2 was not removed before CaM addition, this effect might be due 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 H_2O_2-treated myocytes, we first incubated [F-CaM] (50 μM) for 60 min in media containing 50 μM H_2O_2 (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 H_2O_2 was present in the myocyte bath. As shown in Fig 5C–D (and Online Figure 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 pre-treated with 50 μM H_2O_2 (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 H_2O_2 (Balog used 1000 times higher H_2O_2 concentration for 24 hr; [37]).

As a further test of whether RyR2 oxidation alone inhibits subsequent CaM affinity, we removed H_2O_2 from myocytes by repeated 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 H_2O_2) 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 H_2O_2-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 H_2O_2-treated myocytes (Fig 6B). These results indicate that H_2O_2-induced loss of CaM binding can be restored by dantrolene. The lack of AIP effect suggests that any CaMKII activation by H_2O_2 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 H_2O_2 on Wash-In Kinetics of HF647-DPc10

Since CaM binding to RyR2 in myocytes is known to suppress Ca^{2+} sparks [15,38], the H_2O_2-induced increase in SR Ca^{2+} leak (measured as Ca^{2+} sparks in Fig 1) might be mediated mainly by the oxidation 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 H_2O_2 treatment enhances DPc10 access and whether that was sensitive to dantrolene (as shown above for CaSpF and CaM binding).

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.

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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 μM 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 control (Fig 8A–B). This indicates that RyR2 oxidation enhances DPc10 access (e.g. by causing domain unzipping). Furthermore, pre-equilibration with dantrolene in H_2O_2-treated myocytes reversed the H_2O_2-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 H_2O_2 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) H_2O_2 treatment increases both CaSpF and the occurrence of SCW, but dantrolene prevents this elevated Ca^{2+} leak; (2) RyR2 oxidation by H_2O_2 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 H_2O_2 (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 H_2O_2

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]. H_2O_2 can activate RyR2 function, but also alter function of other important Ca^{2+}-handing proteins,

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including SERCA and the Na^+-Ca^{2+} exchanger (NCX). Yan et al. [28] showed that CaSpF increased during 10 min of treatment with 50 μM H_2O_2 in intact myocytes. On the other hand, higher [H_2O_2] (200 μM) can reduce Ca^{2+} transient amplitude, CaSpF, and SR Ca^{2+} content, consistent with H_2O_2-dependent inhibition of SERCA activity [30]. To assess whether H_2O_2 activates the RyR2 function, we measured CaSpF under conditions where both amplitude and the rate of Ca^{2+} transient decline (reflecting SERCA activity), and the decay of caffeine-induced Ca^{2+} 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 H_2O_2-induced modulation than are SERCA or NCX. We also found a significant increase in the occurrence of arrhythmogenic SCW in H_2O_2-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 H_2O_2-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 Ca^{2+} signaling under control condition [32,44]. These are consistent with our observation that dantrolene significantly reduced the CaSpF and prevented potentially deleterious spontaneous arrhythmogenic Ca^{2+} waves in H_2O_2-treated myocytes, but did not alter the frequency of Ca^{2+} 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 make this an unlikely mechanism of dantrolene action.

4.3. H_2O_2 Reduced CaM, but not FKBP12.6 binding to RyR2

There are two main possible explanations for H_2O_2-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 H_2O_2 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 H_2O_2-treated myocytes, resulting in lower resting RyR2 leak, as has been seen for dantrolene in

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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 H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2 significantly accelerates F-DPc10 association rate in situ, indicating that H_2O_2 causes domain unzipping (Fig 8). We also found that dantrolene reduces access of F-DPc10 in either H_2O_2- or DPc10-treated myocytes, which suggests that H_2O_2 and DPc10 induce similar structural changes that are both corrected by dantrolene (Fig 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 Figure 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 Figure 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 H_2O_2 on Ca^{2+} 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 H_2O_2 was not prevented by KN-93, consistent with a CaMKII-independent effect of H_2O_2 on RyR2 dysfunction.

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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 H_2O_2 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 H_2O_2-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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

τ	time constant
$\tau_{wash\text{-}in}$	wash-in time constant
$\tau_{wash\text{-}out}$	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

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H_2O_2	hydrogen peroxide
K_d	dissociation constant
k_{in}	wash-in rate constant, $1/\tau_{wash\text{-}in}$
k_{out}	wash-out rate constant, $1/\tau_{wash\text{-}out}$
RyR2	cardiac ryanodine receptor
SR	sarcoplasmic reticulum
SCW	spontaneous Ca wave

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Highlights

- RyR2 oxidation reduces CaM binding affinity, but does not alter FKBP12.6 binding.
- Oxidation leads to defective domain interactions (unzipping) within RyR2.
- Dantrolene prevents oxidation-induced RyR2 unzipping and loss of CaM-RyR2 affinity.
- Dantrolene inhibits pathological RyR2 channel gating.

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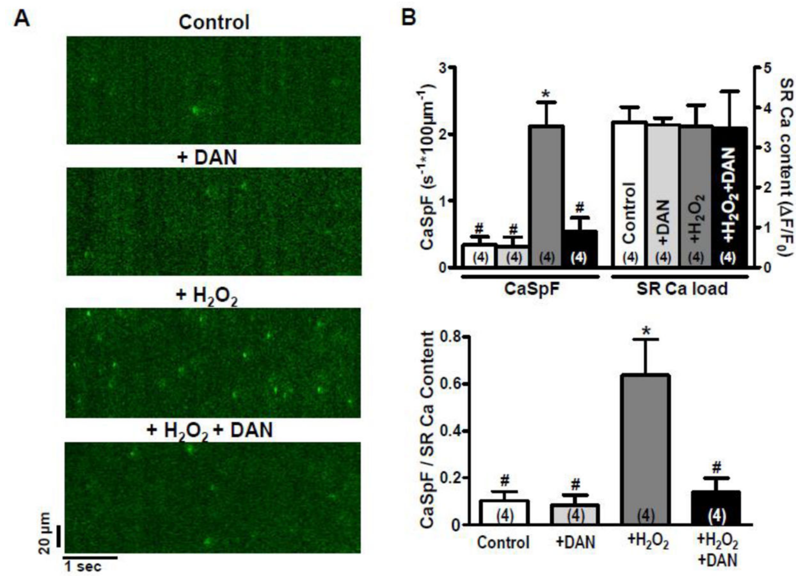

Fig 1.

Effects of H_2O_2 and dantrolene on Ca^{2+} sparks and SR Ca^{2+} content in intact myocytes. Ca^{2+} sparks and SR Ca^{2+} content were measured in myocytes which were treated or not for 5 min with 50 µM H_2O_2. Some myocytes were also pretreated for 2 hours with 1 µM dantrolene (DAN). (A) Representative line-scan images of Ca^{2+} sparks. (B) Summarized data of CaSpF and SR Ca^{2+} content. Data are reported as mean ± SE (n values on bars). *P<0.05 vs. Control, # P<0.05 vs. H_2O_2.

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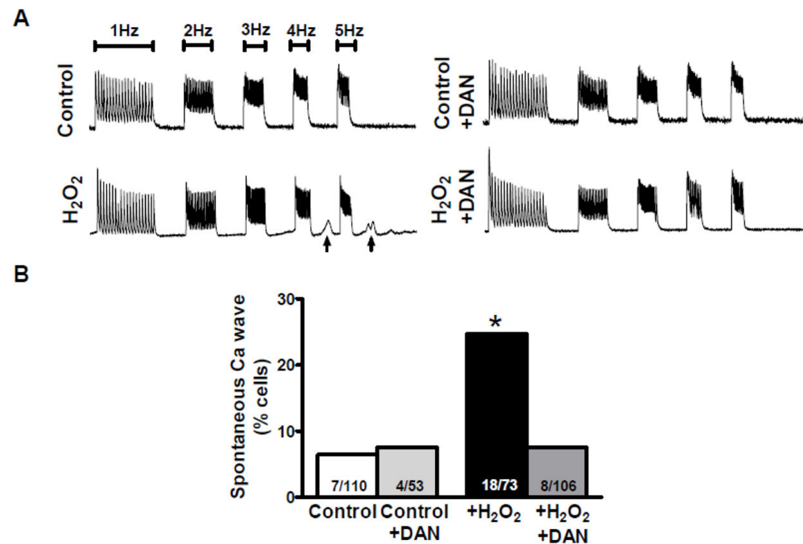

Fig 2.

Arrhythmogenic spontaneous Ca^{2+} waves (SCW) following treatment with H_2O_2 in intact myocytes. (A) Treatment with H_2O_2 (50 μM; 3min) led to an increased incidence of SCW (arrows), but dantrolene (DAN) prevented SCW. Representative time-plots of intracellular Ca^{2+} 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.

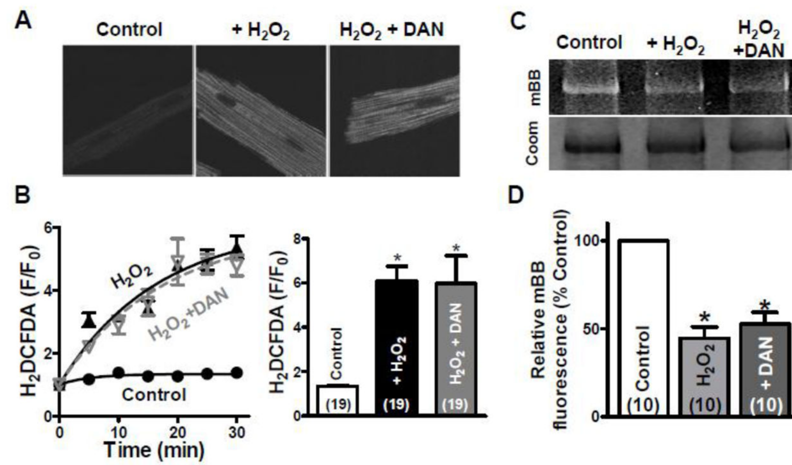

Fig 3.

Oxidation status of the intracellular environment and RyR2. (A) Representative confocal images of control, H_2O_2 (50 μM), and H_2O_2 (50 μM)+DAN (1 μM) loaded with an ROS-sensitive fluorescent indicator H_2DCFDA (10 μM). (B) Time course of H_2DCFDA fluorescence recorded under the same condition as indicated for panel A (left), and quantitative analysis of the H_2DCFDA 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, +H_2O_2 (50 μM), or +H_2O_2 (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.

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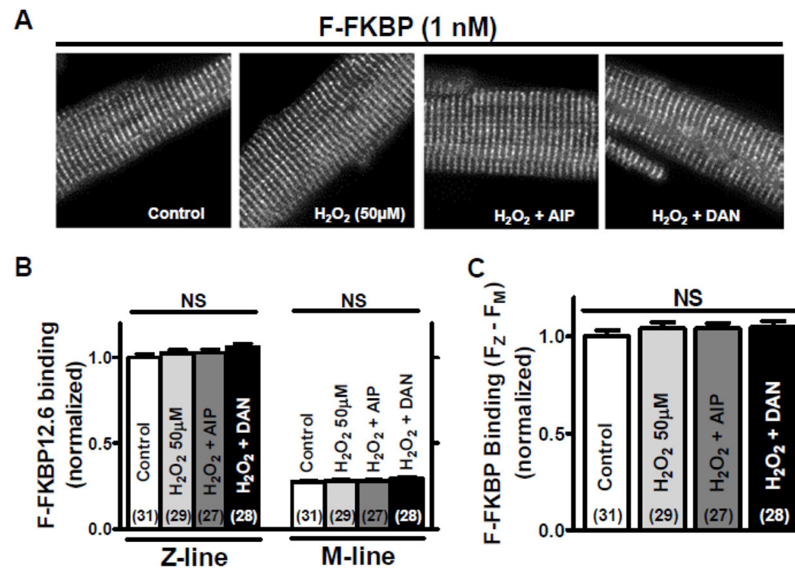

Fig 4.

Effect of H_2O_2 (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 H_2O_2 (50 μM), H_2O_2 (50 μM) +AIP (1 μM) or H_2O_2 (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.

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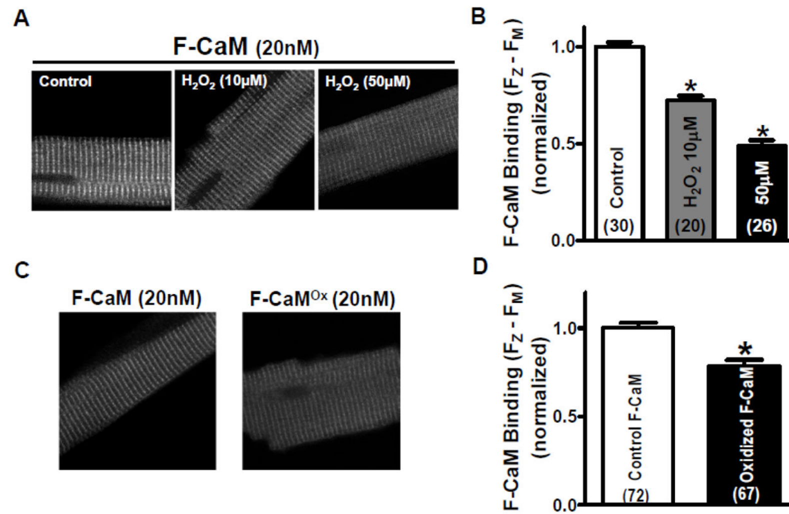

Fig 5.

Effects of myocyte or F-CaM oxidation by H_2O_2 on F-CaM binding at the Z-line. (A) Representative confocal images illustrating the effect of myocyte oxidation by H_2O_2 on F-CaM binding at the Z-lines ($F_Z – F_M$). H_2O_2 was applied to the bath 1 hour 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 H_2O_2, 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.

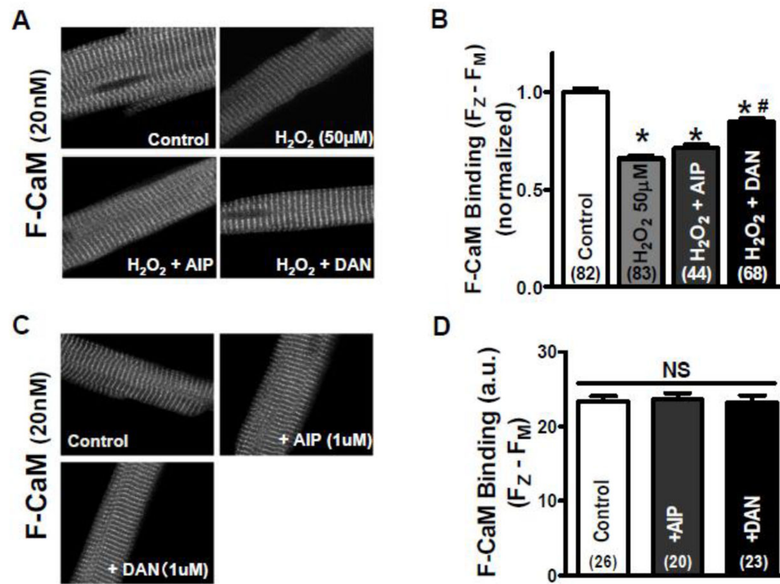

Fig 6.

Effects of RyR2 oxidation or AIP and DAN on calmodulin (CaM) binding at the Z-line. (A) H_2O_2 was applied to the myocyte bath 1 hour before beginning the recording, and was removed before the applying the F-CaM. Representative confocal images of the effects of H_2O_2, H_2O_2+AIP, and H_2O_2+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. H_2O_2. DAN: dantrolene.

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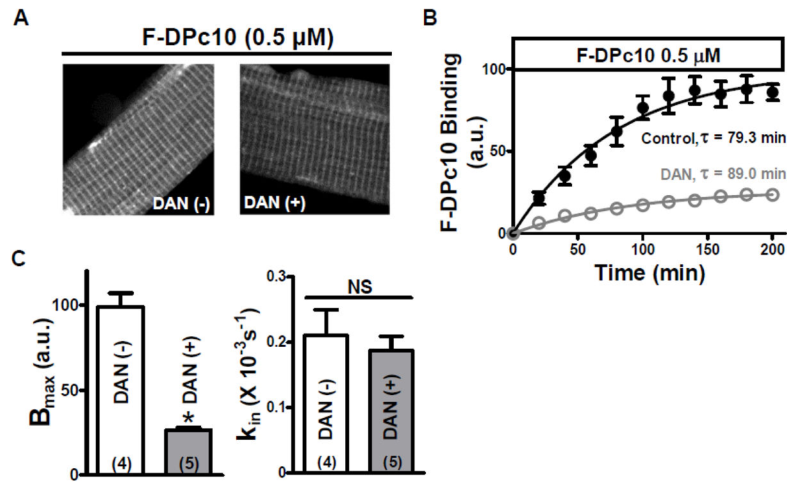

Fig 7.

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

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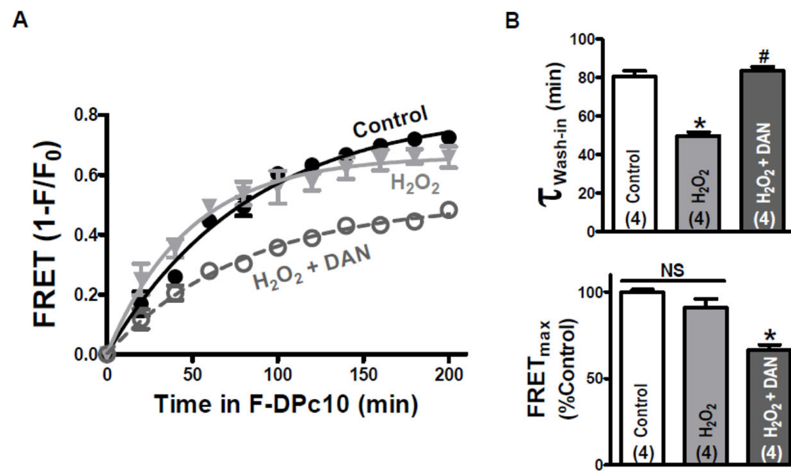

Fig 8.

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 μM) or H_2O_2 (50 μM) + DAN (1 μM). (B) Summary of $\tau_{wash-in}$ and $FRET_{max}$ corresponding to the data in panel A. Data are reported as mean ± SE (n values on bars). *p<0.001 vs. control, #p<0.001 vs. H_2O_2.