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Redox-Mediated Reciprocal Regulation of SERCA and Na⁺/Ca²⁺-Exchanger Contributes to SR Ca²⁺-Depletion in Cardiac Myocytes

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Abstract

Myocardial failure is associated with increased oxidative stress and abnormal excitation-contraction coupling characterized by depletion of sarcoplasmic reticulum (SR) Ca²⁺-stores and a reduction in Ca²⁺-transient amplitude. Little is known about the mechanisms whereby oxidative stress affects Ca²⁺-handling and contractile function; however, reactive thiols may be involved. We used an *in vitro* cardiomyocyte system to test the hypothesis that short-term oxidative stress induces SR Ca²⁺-depletion via redox-mediated regulation of sarco-endoplasmic reticulum Ca²⁺-ATPase (SERCA) and the sodium-Ca²⁺-exchanger (NCX) and that this is associated with thiol oxidation. Adult rat ventricular myocytes paced at 5 Hz were superfused with H₂O₂ (100 μM, 15 min). H₂O₂ caused a progressive decrease in cell shortening followed by diastolic arrest, which was associated with decreases in SR Ca²⁺-content, systolic [Ca²⁺]_i and Ca²⁺-transient amplitude, but no change in diastolic [Ca²⁺]_i. H₂O₂ caused reciprocal effects on the activities of SERCA (decreased) and NCX (increased). Pretreatment with the NCX inhibitor KB-R7943 prior to H₂O₂ increased diastolic [Ca²⁺]_i and mimicked the effect of SERCA inhibition with thapsigargin. These functional effects were associated with oxidative modification of thiols on both SERCA and NCX. In conclusion, redox-mediated SR Ca²⁺-depletion involves reciprocal regulation of SERCA and NCX, possibly via direct oxidative modification of both proteins.

Keywords

SERCA; sodium/calcium exchanger; cardiac myocyte; oxidative stress; reactive thiols

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Introduction

Oxidative stress is increased in failing myocardium, and *in vitro* and *in vivo* studies have implicated redox-mediated mechanisms in several aspects of myocardial failure, including myocyte hypertrophy and apoptosis [1]. Failing myocardium is also characterized by abnormalities of excitation-contraction coupling that include depletion of the sarcoplasmic reticulum (SR) Ca^{2+} content and a secondary reduction in the amplitude of the Ca^{2+} transient [2-5]. While the mechanism whereby reactive oxygen species (ROS) affect Ca^{2+} -handling and contractile function in cardiac myocytes is poorly understood, there is evidence to suggest that oxidative protein modifications may be involved. For example, we found that myocyte contractile dysfunction and Ca^{2+} dysregulation in mice with Gq-induced heart failure is associated with oxidative modifications of SERCA [6]. Likewise, Györke and colleagues [7, 8] have shown that the SR Ca^{2+} release channel (RyR2) is oxidatively modified in the failing dog heart, leading to SR Ca^{2+} -leak and Ca^{2+} depletion.

Sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) and the $\text{Na}^{+}\text{Ca}^{2+}$ exchanger (NCX) play important roles in determining myocyte Ca^{2+} by regulating cytosolic Ca^{2+} -removal during diastole via reuptake into the SR (SERCA) or extrusion from the cell (NCX). Decreased SERCA and increased NCX expression and/or activity have both been observed in failing myocardium [2-5]. Of note, there is evidence that the activities of both SERCA and NCX may be regulated by redox mechanisms; however, knowledge on precisely how this regulation is effected is scarce. We demonstrated that oxidative thiol modifications can either increase or decrease SERCA activity in vascular smooth muscle cells [9-11]. Likewise, it is known that NCX has reactive thiols [12], and can be activated by H_2O_2 [13].

The goal of this study was to assess the *short-term* redox regulation of cardiac myocyte contractile phenotype, and in particular, to clarify the roles of SERCA and NCX in contributing to this phenotype. Because transcriptional (i.e. long-term) and post-translational mechanisms co-exist *in vivo*, an *in vitro* cardiac myocyte system was chosen. Prior *in vitro* studies of ROS in cardiac myocytes were mostly designed to examine the pathophysiology of ischemia/reperfusion, and consequently used relatively high concentrations of ROS (e.g., $\text{H}_2\text{O}_2 \geq 1 \text{ mM}$). However, we previously showed in adult rat ventricular myocytes (ARVM) that lower H_2O_2 concentrations (10 - 100 μM) activate intracellular signaling pathways coupled to cell growth and apoptosis [14]. Accordingly, we used an H_2O_2 concentration of 100 μM to superfuse cultured ARVM, and measured the effects on cell contraction/relaxation and intracellular Ca^{2+} over the ensuing 30 min. In parallel studies, the effects of H_2O_2 on SERCA and NCX were assessed under similar conditions by measuring a) activities and b) oxidative thiol modifications of SERCA and NCX. Because of the importance of contraction rate on the cellular effects of ROS [15], cells were field-paced at 5 Hz for both the physiologic and biochemical studies.

Methods

Adult rat ventricular myocytes

Adult rat ventricular myocytes (ARVM) were isolated from the hearts of adult (200 to 220 g) male Sprague-Dawley rats [16]. Cells were plated at a nonconfluent density of 50 to 75 cells/ mm^2 on plastic culture dishes or glass cover slips precoated with laminin (1 $\mu\text{g}/\text{cm}^2$) and kept at 37°C in ACCT medium (DMEM, BSA 2 mg/mL, L-carnitine 2 mmol/L, creatinine 5 mmol/L, taurine 5 mmol/L, penicillin 100 IU/mL, streptomycin 10 $\mu\text{g}/\text{mL}$) overnight. All experiments were performed the first day after cell culturing. This study was performed in accordance with the guidelines of the Animal Care and Use Committee of Boston University School of Medicine and the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Cell treatments

ARVM were treated with H_2O_2 (100 μM) for 15 and 30 min for biochemical measurements, or continuously superfused for up to 30 min during assessment of Ca^{2+} -transients and cell contractility, whereby H_2O_2 was added to 1.2 mmol/L Ca^{2+} -Tyrode solution. Under the conditions of these experiments, the ratio of H_2O_2 to cell is approx. 1.1×10^{-12} moles per cell under the static conditions of the biochemical measurements and approx. 4.3×10^{-13} moles per cell during the continuous superfusion conditions used to measure cell physiology. The NCX inhibitor KB-R7943 (10 μM) was added 15 minutes before superfusion with 1.2 mmol/L Ca^{2+} -Tyrode buffer alone or buffer containing H_2O_2 . Thapsigargin was added to the 1.2 mmol/L Ca^{2+} -Tyrode solution similar to as H_2O_2 so that the cells were superfused with thapsigargin at a concentration of 2 μM .

Measurement of myocyte contraction/relaxation and intracellular Ca^{2+} transients

Cell shortening and intracellular Ca^{2+} -transients were recorded in ARVM [17]. Cultured ARVM were incubated with membrane permeant fura-2 (1 $\mu\text{mol/L}$, Molecular Probes) and probenecid (500 $\mu\text{mol/L}$) to prevent leakage of fura-2 from cells. ARVM were maintained at 37°C , superfused with 1.2 mmol/L Ca^{2+} -Tyrode solution, and electrically paced at 5 Hz via platinum wires for 20 minutes to establish steady-state conditions. Cell shortening/relengthening, and Ca^{2+} -transients were measured using video edge detection and fluorescence measurements of the fura-2 ratio, respectively (SoftEdge Acquisition System and IonWizard, IonOptix Inc). Percent cell shortening (% CS) was calculated as diastolic cell length minus systolic cell length normalized to the diastolic cell length.

Caffeine-sensitive Ca^{2+} release

Caffeine-sensitive Ca^{2+} -release was assessed similar to as described by Bassani et al. [18]. ARVM were equilibrated for 20 minutes during which the cells were superfused with 1.2 mmol/L Ca^{2+} -Tyrode solution and paced at 5 Hz. Electrical stimulation was stopped 2 sec prior to rapid application of 10 mM caffeine in Tyrode buffer. Caffeine remained in the solution through the end of each trace after which the superfusate was changed to regular Tyrode. Electrical stimulation was resumed after a 1 minute wash-off period with regular Tyrode buffer. Rapid changes in superfusate were achieved using a quick switch system [19] to rapidly change the solution bathing the cell, thus allowing us to assess the immediate effect of caffeine on cell contracture.

SERCA activity

After various treatments, ARVM were homogenized on ice by sonication in Tris-sucrose homogenization buffer (8% (w/v) sucrose in (in mM) Tris-HCl pH 7.0 3, PMSF 1, DTT 2). The homogenate was centrifuged for 5 min at 4,000 rpm. The protein concentration of the supernatant was determined by Bradford assay. Samples were pre-treated with and without 10 μM of the SERCA inhibitor, thapsigargin. Ca^{2+} uptake was initiated by the addition of sample to assay buffer (in mM: KCl 100, NaN_3 5, MgCl_2 6, EGTA 0.15, CaCl_2 0.12, Tris-HCl pH 7.0 30, oxalate 10, ATP 2.5) containing 1 μCi $^{45}\text{CaCl}_2$ (New England Nuclear, Boston, MA) in a 37°C degree water bath. Aliquots of each sample taken at 30, 60, 90 s were vacuum filtered on glass filters (Whatman GF/C, Fisher Scientific, Pittsburgh, PA), washed 3 times with wash buffer (in mM: imidazole 30, sucrose 250, EGTA 0.5), and counted with a scintillation counter. SERCA activity is expressed as the initial rate of thapsigargin-sensitive ^{45}Ca uptake as nmol/mg protein/min.

NCX activity

NCX activity was measured using a modification of the method described by Reeves et al. [12]. ARVM were incubated with ouabain (50 μM , 2 hrs) to load cells with sodium before the

addition of H_2O_2 . ARVM were then homogenized on ice using a cell scraper in Tris-sucrose homogenization buffer and sonicated. The lysate was centrifuged (~1000g, 15 min). Sodium-driven Ca^{2+} uptake was measured at 37°C by adding the supernatant (50 μg) to assay buffer (200 μl) containing either 160 mM NaCl or 160 mM KCl; plus 20 mM MOPS, 1 mM MgCl_2 and 40 μM $^{45}\text{CaCl}_2$ at pH 7.4.

Biotinylated iodoacetamide (BIAM)-labeling

Free reactive thiols on SERCA and NCX were measured with biotinylated iodoacetamide (BIAM, Molecular Probes) by a modification of the technique of Kim et al. [20] as previously described [21]. Briefly, cells were lysed in buffer (1% NP 40, 0.25% DOC, 50 mmol/L PIPES, 100 $\mu\text{mol/L}$ DTPA, 150 mmol/L NaCl, pH 6.5) containing 100 $\mu\text{mol/L}$ BIAM. The lysates were separated by centrifugation at 14,000 rpm, β -mercaptoethanol (50 mmol/L) was added to stop further thiol labeling; and the proteins were passed through a PD-10 Sephadex-G25 column to eliminate excess free BIAM. Protein concentration of the lysates was determined by Bradford assay. BIAM-labeled proteins were gathered from 500 μg of total protein with streptavidin-sepharose beads (50 μL) overnight, washed 4 times with lysis buffer, and separated from the beads by adding Laemmli buffer containing 5 mol/L urea. BIAM-labeled SERCA and NCX were detected by Western blotting with anti-SERCA and anti-NCX antibodies, respectively. Bands were detected with horse radish peroxidase conjugated secondary antibodies and chemiluminescence substrate then exposed to film and quantified with a densitometer. Alternately, bands were detected with near-infrared dye conjugated secondary antibodies and quantified with the Odyssey Two-Color Infrared Imaging System (Licor). Data is reported as arbitrary densitometry units.

Statistical analysis

All data are presented as mean $\pm$ SEM. Differences across multiple conditions were tested by 1-way ANOVA and time courses by repeated measures ANOVA, whereby data propagation was applied when the endpoint (diastolic cell arrest) occurred before elapse of the 15 minutes experiment duration. Comparisons between conditions were tested by Student's unpaired *t* test with Bonferroni correction for multiple comparisons. A value of $P < 0.05$ was considered significant.

Results

H_2O_2 causes diastolic arrest and attenuates the Ca^{2+} -transient

ARVM were paced at 5 Hz, and following attainment of a stable baseline, were superfused with 100 μM H_2O_2 . H_2O_2 caused a progressive decrease in cell shortening beginning at approximately 10 min, and culminating after 15 - 20 min in cell arrest (Figure 1A). Decreased cell shortening was preceded by decreases in peak systolic Ca^{2+} and the amplitude of the Ca^{2+} -transient (Figure 1B). However, at the time of cell arrest, diastolic Ca^{2+} was unchanged (Figure 1B) and cell length was unchanged from baseline (Baseline = 118 ± 5 μM ; Cell arrest = 117 ± 5 μM ; $p = \text{ns}$; $n = 10$).

To assess the respective contributions of SERCA and NCX, early phase Ca^{2+} decay (time to 50% baseline, reflecting Ca^{2+} reuptake via SERCA) and late phase Ca^{2+} decay (time 50-80% baseline, reflecting Ca^{2+} extrusion via NCX) were measured. H_2O_2 increased time to 50% of baseline from vs. 55 ± 2 to 69 ± 6 ms ($p < 0.05$), but decreased time from 50-80% of baseline from 45 ± 2 to 32 ± 3 ms ($p < 0.005$), suggesting decreased Ca^{2+} reuptake via SERCA but increased Ca^{2+} extrusion via NCX.

H₂O₂ causes SR calcium depletion

The decreases in systolic Ca²⁺ and the amplitude of the Ca²⁺-transient suggest depletion of SR Ca²⁺ stores. To test this possibility, caffeine-induced SR Ca²⁺ release was measured. Superfusion with H₂O₂ for 15 min almost totally abolished caffeine-induced Ca²⁺ release (Figure 2), indicating severe depletion of SR Ca²⁺ stores.

H₂O₂ inhibits SERCA but activates NCX

To test the effects of H₂O₂ on SERCA and NCX activities, paced ARVM were treated with H₂O₂ for 15 or 30 minutes, and SERCA and NCX activities were determined by measuring the flux of Ca²⁺ in SR and sarcolemmal membranes, respectively. H₂O₂ decreased maximal Ca²⁺ stimulated SERCA activity (−52%, p<0.03, N=7, Figure 3A), but increased NCX activity (+199%, p<0.03, N=6, Figure 3B). Treatment with H₂O₂ for 15 minutes decreased SERCA activity by 42% (p<0.04, N=3).

Both SERCA and NCX contribute to the H₂O₂-induced Ca²⁺ phenotype

Because inhibition of SERCA activity alone would be expected to increase diastolic [Ca²⁺]_i, the lack of such an increase with H₂O₂ suggests that the increase in NCX activity may play a role. Under conditions of normal intracellular sodium concentration, NCX may also contribute to the extrusion of cytosolic Ca²⁺ during late diastole. To test the possibility that the failure of diastolic Ca²⁺ to increase with H₂O₂ reflects active extrusion via H₂O₂-mediated activation of NCX, we examined the effects of the SERCA inhibitor thapsigargin and the NCX inhibitor KB-R7943. As expected, thapsigargin alone caused increases in both systolic and diastolic [Ca²⁺]_i (Figure 4A) and failed to modify the effect of the H₂O₂ (data not shown).

To test the contribution of NCX to the effect of H₂O₂, we used the NCX inhibitor KBR7943. KB-R7943 (10 μM) inhibits bi-directional Na⁺/Ca²⁺ exchange current in guinea pig ventricular myocytes by approx. 80% under bi-directional ionic conditions [22]. KB-R7943 (10 μM) alone had no effect on the basal Ca²⁺ transient or myocyte contraction. However, pretreatment with KB-R7943 modified the H₂O₂-mediated Ca²⁺ phenotype by increasing diastolic [Ca²⁺]_i in a manner similar to that caused by thapsigargin (Figure 4B). These data suggest that increased NCX-dependent extrusion of cytosolic Ca²⁺ contributes to the effect of H₂O₂ by maintaining diastolic [Ca²⁺]_i at low levels, despite inhibition of SERCA.

H₂O₂ induces oxidative modification of reactive thiols on SERCA and NCX

To determine whether the H₂O₂-mediated changes in activity are associated with oxidative post-translational thiol modifications, we examined reactive thiols on SERCA and NCX under conditions similar to the functional experiments (100 μM H₂O₂; 15 and 30 minutes; paced at 5 Hz). Reduced reactive thiols on SERCA and NCX were assessed using biotinylated iodoacetamide (BIAM) labeling, as we have described [16]. With this technique, proteins with reduced reactive thiols are labeled by BIAM, separated using streptavidin-sepharose beads, and immunoblotted for SERCA or NCX. H₂O₂ (30 min) decreased the abundance of BIAM-sensitive free thiols on SERCA by 38 ± 9% (p<0.03, Figure 5A) and on NCX by 43 ± 6% (p<0.02, Figure 5B). Treatment with H₂O₂ for 15 minutes decreased SERCA BIAM labeling by 40% (p<0.05, N=3).

Discussion

In this study we demonstrate in cardiac myocytes *in vitro* that exposure to a modest oxidative stress (100 μM H₂O₂) causes a contractile phenotype characterized by a) reduced contractile amplitude; b) reductions in systolic [Ca²⁺]_i and the amplitude of the [Ca²⁺]_i transient, and c) depletion of the SR Ca²⁺ store. We further show that this phenotype is mediated, in part, by

reciprocal inhibition of SERCA and activation of NCX; and is associated with oxidative thiol modifications of both SERCA and NCX.

Perfusion of adult rat myocytes with H_2O_2 led to a progressive decrease in contractile amplitude, culminating in diastolic cell arrest. The decrease in contractile amplitude was preceded by a progressive decrease in peak systolic $[\text{Ca}^{2+}]_i$ resulting in a decrease in the amplitude of the $[\text{Ca}^{2+}]_i$ transient, and was associated with profound depletion of the SR Ca^{2+} store. Prior studies in cardiac myocytes and myocardium have demonstrated similar, although more rapid, decreases in contractile amplitude and SR Ca^{2+} stores [23].

Surprisingly, cell arrest was not associated with myocyte contracture, and likewise, was not associated with an increase in diastolic $[\text{Ca}^{2+}]_i$. This finding is in contrast to most [23-26], but not all [23], prior studies which generally observed an increase in diastolic $[\text{Ca}^{2+}]_i$ and contracture consistent with cytosolic Ca^{2+} overload. However, most prior studies focused on the role of ROS in ischemia / reperfusion, and thus, used relatively high concentrations of H_2O_2 , on the order of 1 mM and higher. Therefore, the lack cytosolic Ca^{2+} overload in our study may relate to the lower concentration of H_2O_2 (100 μM) that we used, or other differences related to cell type and experimental conditions. Intracellular H_2O_2 concentrations on the order of 100 μM have been observed in diseased tissues, with levels on the order of 1 – 15 μM in normal tissues [27]. It has been suggested that the intracellular concentrations achieved with exogenous H_2O_2 may be on the order of 10% of the extracellular concentration. Thus, our use of 100 μM H_2O_2 would be expected to lead to an intracellular concentration that is relevant to pathophysiologic, and possibly, physiologic conditions.

When we inhibited NCX, H_2O_2 caused an increase in diastolic $[\text{Ca}^{2+}]_i$, indicating that under the conditions of these experiments H_2O_2 stimulates Ca^{2+} efflux via the NCX. This conclusion is supported by the kinetics of $[\text{Ca}^{2+}]_i$ after H_2O_2 , which showed an accelerated rate of decline in $[\text{Ca}^{2+}]_i$ during late diastole (i.e., time for 50 to 80 % fall in $[\text{Ca}^{2+}]_i$). Taken together, these observations suggest that activation of NCX by H_2O_2 plays a role in preventing diastolic Ca^{2+} overload under these conditions. This conclusion is further supported by the demonstration that H_2O_2 increased NCX activity, as measured by sodium-driven Ca^{2+} uptake. NCX activation can not be attributed to an increase in intracellular Ca^{2+} , as diastolic $[\text{Ca}^{2+}]_i$ was not elevated and there was overall SR Ca^{2+} depletion. Likewise, intracellular sodium accumulation (e.g., due to Na^+K^+ -ATPase inhibition) would have favored Ca^{2+} influx, and would not explain the activation observed in sodium-loaded myocytes in the activity assay. The findings are thus consistent with direct redox activation of NCX. In this regard, disulfide bond formation has previously been implicated in the activation of NCX [12], although subsequent mutational analysis of cysteines involved in disulfide formation did not abolish redox-stimulated activation [28]. We found that H_2O_2 decreases the abundance of BIAM-labeled thiols on NCX. At pH 6.5 BIAM interacts with reduced reactive thiols that have a low pKa and are therefore susceptible to redox regulation under intracellular conditions. Therefore, these data suggest the presence of reactive cysteine thiols that undergo oxidative modification in response to H_2O_2 . Further study will be required to identify target thiols and to determine whether redox-mediated NCX activation contributes to the pathophysiology in failing myocardium. In this regard, it is noteworthy that increased NCX activity due to protein overexpression led to increased forward function, Ca^{2+} extrusion and SR Ca^{2+} depletion [29].

Our findings also suggest that H_2O_2 can inhibit SERCA activity. Inhibition of SERCA is expected to prevent SR refilling, thereby depleting SR Ca^{2+} and leading to cytosolic Ca^{2+} accumulation. As expected, we found that SERCA inhibition with thapsigargin markedly reduced the Ca^{2+} transient amplitude and increased diastolic $[\text{Ca}^{2+}]_i$ (see Figure 4A). As noted above, exposure to H_2O_2 in the presence of NCX inhibition likewise increased diastolic

$[Ca^{2+}]_i$ (see Figure 4B). In parallel experiments, H_2O_2 inhibited maximal Ca^{2+} -stimulated SERCA activity, as assessed by Ca^{2+} uptake. Finally, H_2O_2 slowed the initial rate of diastolic $[Ca^{2+}]_i$ decline (time to 50 % of baseline $[Ca^{2+}]_i$), supporting the functional significance of SERCA inhibition.

The mechanism responsible for SERCA inhibition remains to be determined. H_2O_2 decreased SERCA BIAM-labeling, indicating oxidative modification of reactive cysteine thiols. In vascular smooth muscle cells we have shown that oxidative thiol modifications can either increase or decrease SERCA activity: reversible S-glutathiolation of cysteine 674 increased SERCA activity [9], whereas irreversible sulfonylation was associated with reduced activity [10]. Thus, it is possible that H_2O_2 inhibits SERCA via an irreversible oxidative modification.

These observations further our understanding of the redox regulation of cardiac myocyte contractile phenotype. While we have focused on the roles of NCX and SERCA, it is likely that redox regulation of other Ca^{2+} -handling proteins, particularly the RyR2, also contribute to SR Ca^{2+} depletion in response to H_2O_2 . SR Ca^{2+} leakage via the RyR2 has been shown to contribute to SR Ca^{2+} depletion in dogs with heart failure [7], and has been attributed to disulfide formation [30]. Consistent with this thesis, we have found that H_2O_2 decreased BIAM-labeling of RyR2 by approximately 45% (data not shown). Given the demonstration of oxidative stress in failing myocardium, our findings support the thesis that redox mechanisms contribute to the abnormal contractile phenotype observed in failing myocardium.

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List of Abbreviations

ACCT	albumine-carnitine-creatine-taurine
ARVM	adult rat ventricular myocyte(s)
BIAM	biotinylated iodoacetamide
BSA	bovine serum albumin
Ca^{2+}	calcium
CS	cell shortening
DTT	di-thiol-3-thione
DMEM	Dulbecco's modified eagle medium
H_2O_2	hydrogen peroxide
Na^+	sodium
NCX	sodium-calcium exchanger
ROS	reactive oxygen species
RyR	ryanodine receptor
SEM	standard error of the mean
SERCA	sarco-endoplasmic reticulum calcium ATP-ase

SR sarcoplasmic reticulum

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Figure 1. Hydrogen peroxide causes cardiac myocyte contractile failure, diastolic arrest and dissipation of the Ca²⁺-transient

Panel A. Cardiac myocytes paced at 5 Hz were superfused with H₂O₂ (100 μM) for up to 30 min. H₂O₂ caused a progressive decrease in cell shortening followed after > 15 min by contractile arrest (BL = baseline; *p<0.01 vs. baseline). At the time of arrest, cell length was unchanged from baseline (see Results). **Panel B.** In parallel, H₂O₂ caused decreases in systolic Ca²⁺ and the amplitude of the Ca²⁺ transient, but no change in diastolic Ca²⁺. The top of the bar represents the peak Ca²⁺ (± SEM) and the bottom of the bar the minimum Ca²⁺ (± SEM). The size of the bar is proportional to the Ca²⁺ transient amplitude. P-values vs. baseline: †<0.05 for systolic Ca²⁺ and <0.01 for amplitude; ‡<0.05 for systolic Ca²⁺ and <0.001 for amplitude; §<0.001 for both systolic Ca²⁺ and amplitude. Data are the mean ± SEM for 9 - 10 cells from 6 different cell preparations.

Figure 2. Hydrogen peroxide causes sarcoplasmic reticulum Ca^{2+} -depletion

Myocytes were paced (5 Hz) and superfused with H_2O_2 (100 μM ; 15 min) as per Figure 1. Pacing was stopped and SR Ca^{2+} content was assessed by measuring the amplitude of the Ca^{2+} transient immediately after rapid exposure to caffeine (10 mM). Tracings are representative of 3 independent experiments each with 1-2 cells per condition.

Figure 3. Hydrogen peroxide inhibits SERCA but enhances NCX activity

Cardiac myocytes paced (5 Hz) as per Figures 1 and 2 were exposed to H_2O_2 (100 μ M; 30 min) and SERCA (**Panel A**) and NCX (**Panel B**) activities were assessed by measuring radiolabeled Ca^{2+} -flux in SR or sarcolemmal membranes, respectively. Data are the mean $\pm$ SEM for 6 - 7 experiments (* p <0.03).

Figure 4. Role of NCX in mediating the effect of hydrogen peroxide on the Ca^{2+} transient
Panel A. Cells (paced, 5 Hz) were exposed to the SERCA inhibitor thapsigargin (2 μ M) and the Ca^{2+} -transient was monitored for 10 min. SERCA inhibition increased systolic and diastolic Ca^{2+} , leading to a marked decrease in Ca^{2+} -transient amplitude. BL = baseline. P-values vs. baseline: * <0.01 for systolic Ca^{2+} , † <0.05 for diastolic Ca^{2+} and <0.01 for transient, and ‡ <0.01 and § <0.001 for diastolic Ca^{2+} and transient. Data are the mean $\pm$ SEM for 3 cells from 3 different cell preparations. **Panel B.** Cells (paced, 5 Hz) were pretreated with the NCX inhibitor KB-R7943 (10 μ M) for 15 min prior to superfusion with H_2O_2 (100 μ M). In contrast to the effect of superfusion with H_2O_2 alone (Figure 1B), after NCX inhibition H_2O_2 caused an increase in diastolic Ca^{2+} and attenuation of the Ca^{2+} transient amplitude. BL = baseline. P-values vs. KB-R7943: * <0.05 and † <0.01 for diastolic Ca^{2+} , ‡ <0.05 and § <0.001 for transient. Data are mean $\pm$ SEM; n = 4 cells from 3 different preparations. For details see Figure 1B.

Figure 5. Hydrogen peroxide causes oxidative modifications of free reactive thiols on SERCA and NCX

Cells (paced, 5 Hz) were treated with H₂O₂ (100 μM; 20 min) and free reactive thiols were assessed using biotinylated iodoacetamide (BIAM) labeling. H₂O₂ decreased the abundance of BIAM-labeled free thiols on both SERCA (**Panel A**) and NCX (**Panel B**). Data are mean ± SEM for 3 - 4 experiments. *p<0.03, †p<0.02.