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## Dynamic modulation of $\text{Ca}^{2+}$ sparks by mitochondrial oscillations in isolated guinea pig cardiomyocytes under oxidative stress

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### ABSTRACT

Local control of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) depends on the spatial organization of L-type  $\text{Ca}^{2+}$  channels and ryanodine receptors (RyR) in the dyad. Analogously,  $\text{Ca}^{2+}$  uptake by mitochondria is facilitated by their close proximity to the  $\text{Ca}^{2+}$  release sites, a process required for stimulating oxidative phosphorylation during changes in work. Mitochondrial feedback on CICR is less well understood. Since mitochondria are a primary source of reactive oxygen species (ROS), they could potentially influence the cytosolic redox state, in turn altering RyR open probability. We have shown that self-sustained oscillations in mitochondrial inner membrane potential ( $\Delta\Psi_m$ ), NADH, ROS, and reduced glutathione (GSH) can be triggered by a laser flash in cardiomyocytes. Here, we employ this method to directly examine how acute changes in energy state dynamically influence resting  $\text{Ca}^{2+}$  spark occurrence and properties. Two-photon laser scanning microscopy was used to monitor cytosolic  $\text{Ca}^{2+}$  (or ROS),  $\Delta\Psi_m$ , and NADH (or GSH) simultaneously in isolated guinea pig cardiomyocytes. Resting  $\text{Ca}^{2+}$  spark frequency increased with each  $\Delta\Psi_m$  depolarization and decreased with  $\Delta\Psi_m$  repolarization without affecting  $\text{Ca}^{2+}$  spark amplitude or time-to-peak. Stabilization of mitochondrial energetics by pretreatment with the superoxide scavenger TMPyP, or by acute addition of 4'-chlorodiazepam, a mitochondrial benzodiazepine receptor antagonist that blocks the inner membrane anion channel, prevented or reversed, respectively, the increased spark frequency. Cyclosporine A did not block the  $\Delta\Psi_m$  oscillations or prevent  $\text{Ca}^{2+}$  spark modulation by  $\Delta\Psi_m$ . The results support the hypothesis that mitochondria exert an influential role on the redox environment of the  $\text{Ca}^{2+}$  handling subsystem, with mechanistic implications for the pathophysiology of cardiac disease.

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### 1. Introduction

Structural and functional evidence suggests that a mitochondrial microdomain exists near the dyad in cardiac myocytes (reviewed in [1]). Electron micrographs show that t-tubules and  $\text{Ca}^{2+}$  release junctions are typically sandwiched between mitochondria at the z-line, with the mesh-like corbular SR (containing the SR  $\text{Ca}^{2+}$  pumps) wrapping around the mitochondria [2]. The large spike in junctional  $\text{Ca}^{2+}$  (50–100  $\mu\text{M}$ ) during  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) and the close proximity of mitochondria to the  $\text{Ca}^{2+}$  release sites (40–300 nm) are thought to promote rapid mitochondrial  $\text{Ca}^{2+}$  uptake, leading to matrix  $\text{Ca}^{2+}$  accumulation and the consequent stimulation of oxidative phosphorylation. This mechanism is essential for matching energy supply with demand [2,3].

In addition to the feedforward effect of  $\text{Ca}^{2+}$  on bioenergetics [4], feedback of mitochondrial function on  $\text{Ca}^{2+}$  cycling is also expected. Ion pumps in the sarcolemma ( $\text{Na}^+/\text{K}^+$  ATPase,  $\text{Ca}^{2+}$  ATPase) and the

$\text{Ca}^{2+}$  ATPase of the sarcoplasmic reticulum (SERCA2a) depend on the free energy of ATP hydrolysis [5], and the L-type  $\text{Ca}^{2+}$  channel [6,7], the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger [8] and the RyR [9] are also modulated by ATP, ADP and  $\text{Mg}^{2+}$ . In general, mitochondrial  $\Delta\Psi_m$  depolarization and decreased ATP production tend to suppress the activity of these  $\text{Ca}^{2+}$  transport proteins.

Another important functional role of mitochondria is that they are both a source and a target of ROS [10–12]. Under normal conditions, up to 1% of the electrons flowing to  $\text{O}_2$  through the electron transport chain may be diverted to form superoxide ( $\text{O}_2^-$ ), which is subsequently dismutated to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) by superoxide dismutase and then converted to  $\text{H}_2\text{O}$  by glutathione peroxidase and/or catalase [13]. Under normal conditions, there is a balance between ROS formation and antioxidant activity; however, under pathological conditions, oxidative stress can be initiated by either an increase in ROS production or by depletion of the antioxidant pool [14,15], leading to collapse of the mitochondrial membrane potential, an effect that is amplified by the mechanism of mitochondrial ROS-induced ROS release (RIRR) [16]. Studies from our laboratory have demonstrated that local oxidative stress produced by a laser flash in a few mitochondria can trigger self-sustaining cell-wide oscillations in  $\Delta\Psi_m$  and the redox states of the pyrimidine nucleotide [15] and glutathione pools [16] in cardiomyocytes.

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The dramatic changes in  $\Delta\Psi_m$  and intracellular redox state are likely to have important effects on CICR, especially considering that the RyR has been described as a redox sensor with a well-defined midpoint owing to the presence of highly reactive clusters of cysteines [17]. Oxidation of these reactive thiols markedly increases RyR open probability, whereas reduction suppresses channel activity. Here, we utilize the stereotypical pattern of triggered oscillations in mitochondrial energy state to determine if RyR channels are dynamically regulated through changes in cytoplasmic redox potential governed by mitochondria in the proximity of the  $\text{Ca}^{2+}$  release sites. We show that the resting  $\text{Ca}^{2+}$  spark frequency is dynamically modulated by the mitochondrial energetic and redox status. We also demonstrate that the oscillatory mechanism involves benzodiazepine-sensitive mitochondrial ion channels but not the permeability transition pore (mPTP).

## 2. Material and methods

All protocols involving animals conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Johns Hopkins Animal Care and Use Committee.

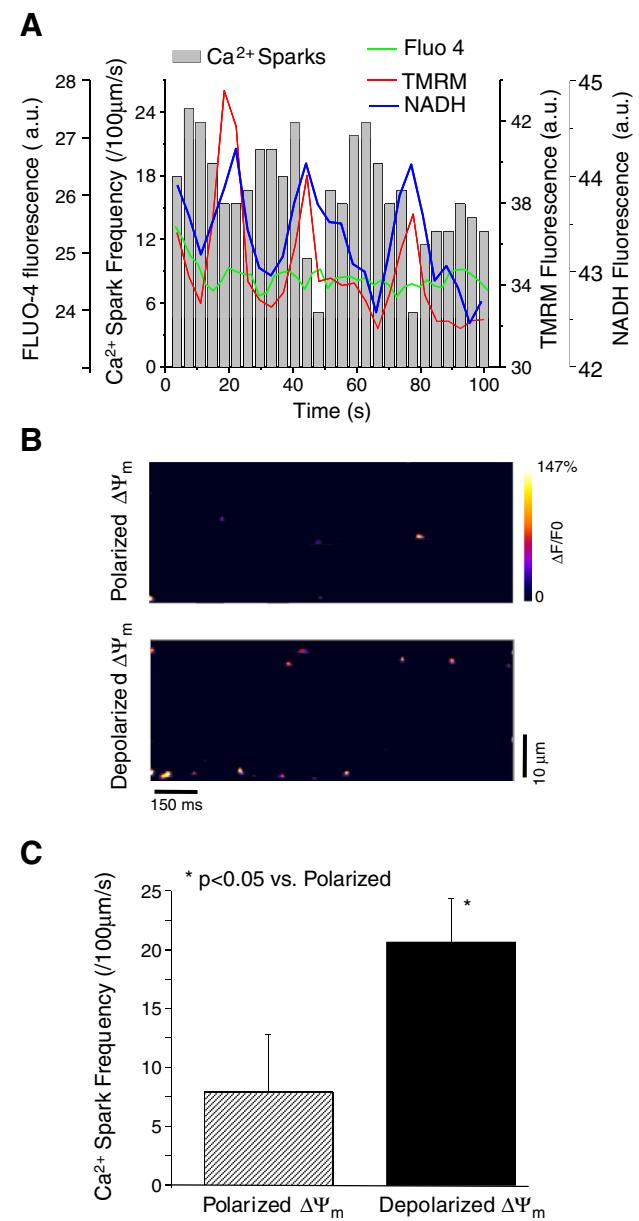
### 2.1. Cardiomyocyte isolation and loading of fluorescent probes

All experiments were carried out at 37 °C on freshly isolated adult guinea pig ventricular myocytes prepared by enzymatic dispersion as previously described [18]. Briefly, animals of either sex were anesthetized with sodium pentobarbital (30 mg/kg I.P.). Following thoracotomy, hearts were quickly excised, mounted on a Langendorff apparatus, and perfused with collagenase-containing solution at 37 °C. After isolation, cells were stored in a high K<sup>+</sup> solution (in mmol/L: 120 Glutamate, 25 KCl, 1 MgCl<sub>2</sub>, 10 HEPES, 1 EGTA, and pH 7.4 with KOH) temporarily. The cationic potentiometric fluorescent dye tetramethyl rhodamine methyl ester (TMRM) was used to monitor changes in  $\Delta\Psi_m$ . ROS production was monitored with MitoSOX (Invitrogen), a superoxide-sensitive fluorescent indicator. The colocalization of TMRM and MitoSOX within the mitochondria is shown in the supplemental materials (Fig. S1). To image the distribution of  $\Delta\Psi_m$  (or ROS) and  $\text{Ca}^{2+}$  simultaneously, 100 nM TMRM (or 2 μmol/L MitoSOX) and 4 μmol/L fluo-4 AM were added to the external solution and allowed to equilibrate for at least 25 min at 37 °C. After loading, the cells were resuspended in the experimental solution for 20 min to permit deesterification of the dye before recording images. To monitor the intracellular reduced glutathione (GSH) and  $\Delta\Psi_m$  simultaneously, cells were loaded with 50 μmol/L monochlorobimane (MCB) and TMRM as described previously [14].

### 2.2. Image acquisition and analysis

The dish containing the cardiomyocytes was equilibrated at 37 °C with unrestricted access to atmospheric oxygen on the stage of a Nikon E600FN upright microscope. Images were recorded using a two-photon laser scanning microscope (Bio-Rad MRC-1024MP) with excitation at 760 nm (Tsunami Ti:Sa laser, Spectra Physics) as described previously [15]. Because of the overlap in the cross-sections for two-photon excitation of the three fluorophores of interest (NADH or GSB, Fluo-4, and TMRM or MitoSOX), this wavelength permitted recording of redox,  $\text{Ca}^{2+}$ , and  $\Delta\Psi_m$  or ROS simultaneously. A three channel photomultiplier detector assembly with appropriate dichroic mirrors and bandpass filters was used to separate the fluorescence emissions of the blue (<500 nm for NADH or 480 nm for GSB), green (500–550 nm; Fluo-4) and red (580–630 nm; TMRM or MitoSOX) indicators with no crossover between the signals. For fast time resolution of  $\text{Ca}^{2+}$  sparks, the line-scan mode of the two-photon microscope was used. 512 × 512 pixel line-scan images (2 ms/line) were acquired every 3.7 s and the three emissions were collected and

stored simultaneously as 8-bit/channel grayscale images. The frequency of false event detection was determined by measuring  $\text{Ca}^{2+}$  spark frequency in the absence of an SR  $\text{Ca}^{2+}$  load (i.e., after 3–5 min of 1 Hz pacing and blocking SR  $\text{Ca}^{2+}$  uptake with 1 μmol/L thapsigargin). The frequency of  $\text{Ca}^{2+}$  sparks with a fully loaded SR was also measured after pacing during isoproterenol exposure. The average  $\text{Ca}^{2+}$  spark frequency was 0.28 with the SR depleted (false event rate) and 27.5 sparks/100 μm/s for a highly loaded SR (Fig. S2). Mitochondrial depolarization was induced either by applying a localized laser flash (e.g. the result shown in Fig. 1) to a small (~64 μm<sup>3</sup>) region of the cell volume [15] or by repeated line scanning at the selected mitochondrial row (e.g., Fig. 2). Images were analyzed offline using ImageJ



**Fig. 1.** Influence of oxidative stress on mitochondrial  $\Delta\Psi_m$  and  $\text{Ca}^{2+}$  spark frequency. (A) Phase relationship among oscillations in  $\Delta\Psi_m$ , NADH, and cyclic changes of  $\text{Ca}^{2+}$  sparks as a result of oxidative stress induced by a localized laser flash (a.u.: arbitrary units of fluorescence). The green line indicates Fluo-4 fluorescence intensity that reflects average cytosolic  $\text{Ca}^{2+}$ ; (B) representative contour plots showing increased  $\text{Ca}^{2+}$  spark frequency when mitochondria depolarized; and (C) quantitative comparison of  $\text{Ca}^{2+}$  spark frequency between  $\Delta\Psi_m$  polarization and depolarization states. \* p < 0.05 vs. Polarized.

software (Wayne Rasband, National Institutes of Health) and  $\text{Ca}^{2+}$  sparks were analyzed using a recently developed ImageJ plug-in [19].

Wide-field fluorescence images of TMRM (shown in Fig. 3B) were acquired using a high-sensitivity CCD camera (Cascade II: Photometrics).

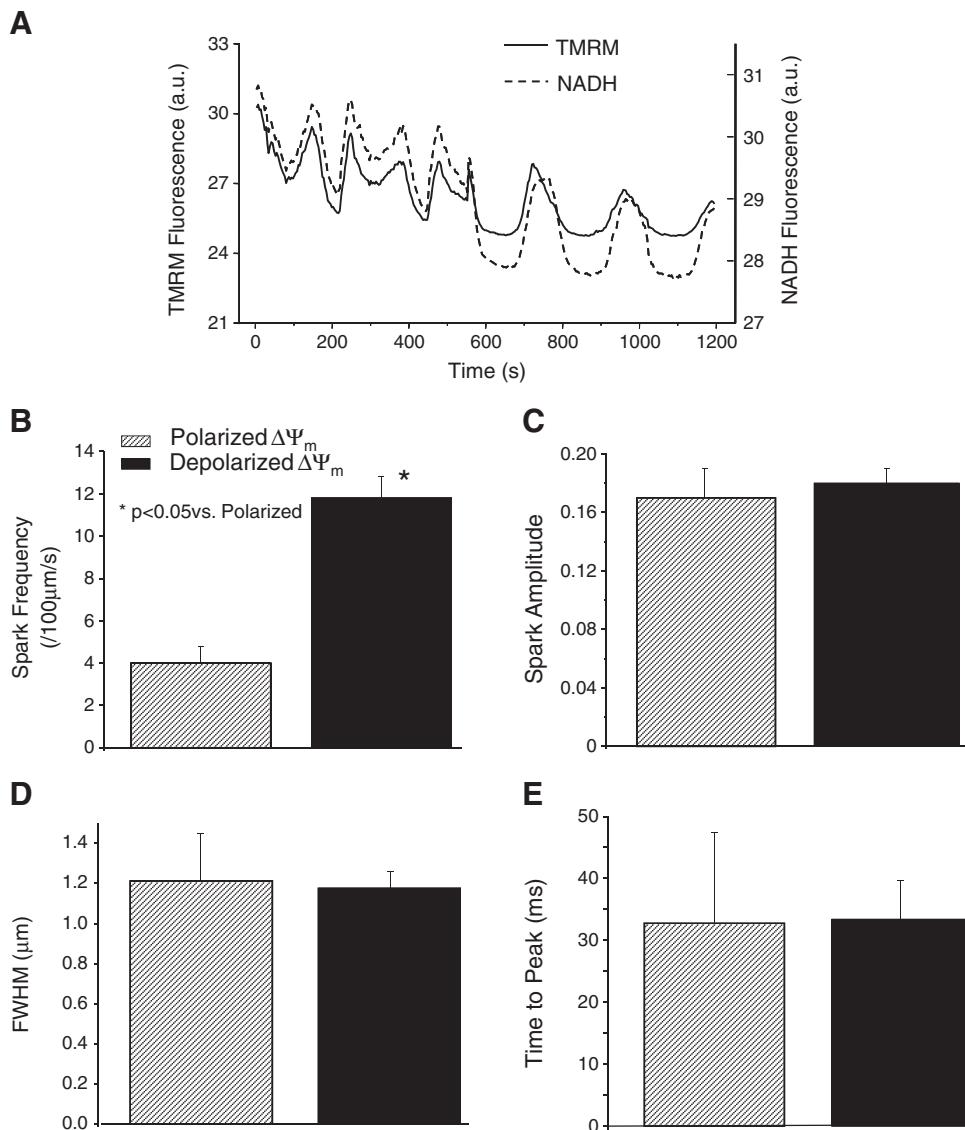
### 2.3. Experimental protocol

In the present study, four groups of non-beating cells were studied under different experimental conditions in order to investigate the effects of mitochondrial energy state on resting  $\text{Ca}^{2+}$  spark frequency. CON group: image recording was started immediately after cell loading in modified Tyrode's solution containing (in mmol/L) 140 NaCl, 5 KCl, 1  $\text{MgCl}_2$ , 10 HEPES, 1  $\text{CaCl}_2$ , pH 7.4 (adjusted with NaOH), supplemented with 10 mmol/L glucose; TMPyP group: 200  $\mu\text{mol/L}$  Mn (III)tetrakis(1-methyl-4-pyridil) porphyrin pentachloride (TMPyP) was added to the Tyrode's solution and the cell suspension was pre-incubated for 2 h with gentle shaking before image recording; 4'Cl-DZP group: experiments were started with normal Tyrode's, and after mitochondrial oscillations were observed, 20  $\mu\text{mol/L}$  4'-chlorodiazepam

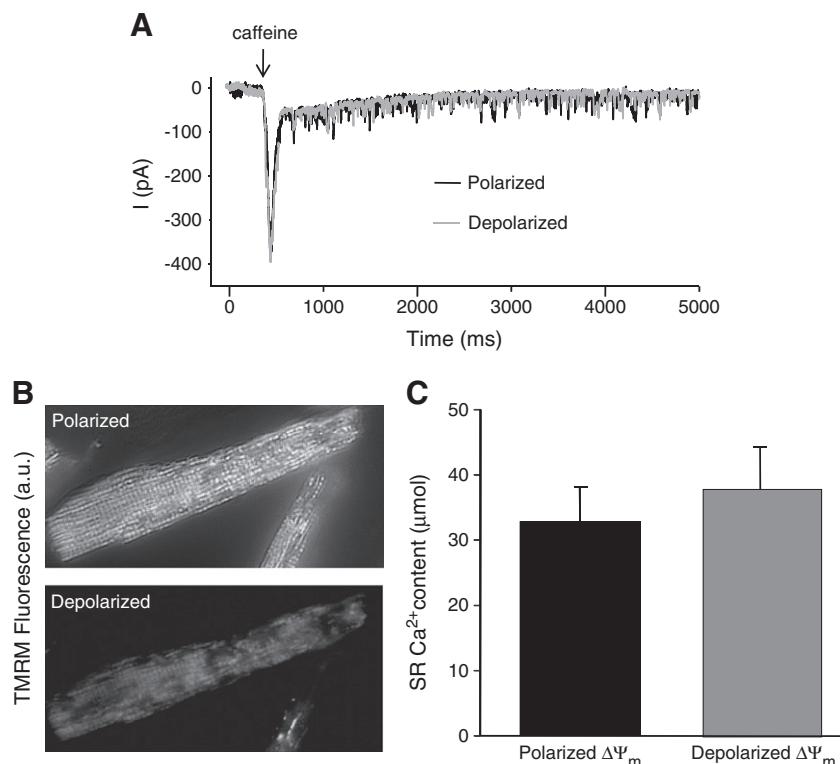
(4'Cl-DZP) was added to the perfuse before resuming the image acquisition; CsA group: experiments were started with normal Tyrode's, and after mitochondrial oscillations were detected, 1  $\mu\text{mol/L}$  cyclosporin A (CsA) was added to the perfuse, and image acquisition was continued.

### 2.4. SR $\text{Ca}^{2+}$ load measurements

Myocytes were whole-cell patch-clamped at 37 °C as previously described [3]. Briefly, cells were placed in a heated chamber on the stage of an inverted fluorescence microscope (Eclipse TS100 inverted microscopes, Nikon, Inc.) and superfused with solution containing (in mmol/L) 140 NaCl, 110 CsCl, 1  $\text{MgCl}_2$ , 10 HEPES, 10 Glucose, and 2  $\text{CaCl}_2$ , pH 7.4. Internal solution contained (in mmol/L) 138 potassium glutamate, 10 Na-HEPES, and 5 Mg-ATP, pH 7.2. Borosilicate glass pipettes of 2–3 MΩ tip resistance were used for whole-cell recording with an Axopatch 200A amplifier coupled to a Digidata 1200 personal computer interface (Axon Instruments, Foster City, CA) using custom-written software. After whole-cell configuration was established,



**Fig. 2.** Analysis of  $\text{Ca}^{2+}$  spark properties. (A) Oscillations in  $\Delta\Psi_m$  and NADH triggered by repeated laser line scanning; (B) Influence of  $\Delta\Psi_m$  energy state on  $\text{Ca}^{2+}$  spark frequency; and (C–E) modulation of frequency, but not amplitude, FWHM, or time-to-peak of  $\text{Ca}^{2+}$  sparks by mitochondrial energy state.



**Fig. 3.** SR Ca<sup>2+</sup> loading and mitochondrial energetic states. (A) Representative sarcolemmal NCX current recorded before and after UV light induced mitochondrial membrane potential depolarization; and (B) comparison of SR Ca<sup>2+</sup> content before and after mitochondrial membrane potential depolarization.

10 mmol/L caffeine was introduced with a custom-built heated rapid-switching device as described previously and NCX current evoked by caffeine-induced SR Ca<sup>2+</sup> release was measured following a 3 minutes train. SR Ca<sup>2+</sup> contents were calculated by integrating the area under the curve (AUC) of NCX current. Then the cell was exposed to a UV light (exposure time: 1 s), which depolarized the mitochondrial network in ~3–5 min (Fig. 3B). The NCX current was measured again and compared with that obtained before mitochondrial depolarization.

## 2.5. Materials

TMRM, MCB and Fluo-4-AM were purchased from Invitrogen Corp (Carlsbad, CA). 4'Cl-DZP and CsA were obtained from Sigma-Aldrich (St. Louis, MO). TMPPyP was purchased from Calbiochem (San Diego, CA). All other reagents were from Sigma. Stock solutions of these reagents were prepared in DMSO and concentrated enough to avoid exceeding 0.1% DMSO (v/v) in the final solution.

## 2.6. Statistics

Comparisons were performed using paired or unpaired 2-tailed Student's *t* test. Data were considered significantly different at  $p < 0.05$ . Results are presented as mean  $\pm$  SEM.

## 3. Results

### 3.1. Oscillations in mitochondrial energetics drive cyclical changes in resting Ca<sup>2+</sup> spark frequency

In order to analyze the dependence of resting Ca<sup>2+</sup> spark frequency on the mitochondrial energy state, experiments were performed on isolated guinea pig cardiomyocytes in the absence of electrical stimulation. As expected from our previous work, a localized laser flash in freshly isolated cardiomyocytes triggered cell-wide

oscillations in  $\Delta\Psi_m$  and NADH, which was confirmed by monitoring full field images (data not shown) at the onset of the experiment. The imaging system was then switched to line-scan mode across the longitudinal axis of the cell and  $\Delta\Psi_m$ , Ca<sup>2+</sup>, and NADH signals were collected simultaneously. Analysis of the Fluo-4 images revealed that resting Ca<sup>2+</sup> spark frequency increased with each depolarization of  $\Delta\Psi_m$  and decreased upon  $\Delta\Psi_m$  repolarization over several cycles of  $\Delta\Psi_m$  oscillation (Fig. 1A). A representative contour plot of Ca<sup>2+</sup> sparks during mitochondrial polarization or depolarization is shown in Fig. 1B. Average Ca<sup>2+</sup> spark frequency was  $8.2 \pm 4.6$  (sparks/100  $\mu\text{m}^2/\text{s}$ ) when  $\Delta\Psi_m$  was polarized versus  $21.4 \pm 3.4$  (sparks/100  $\mu\text{m}^2/\text{s}$ ) when  $\Delta\Psi_m$  was depolarized, showing a 2.5 fold increase in Ca<sup>2+</sup> spark frequency (Fig. 1C). Whole cell diastolic Ca<sup>2+</sup> did not change during mitochondrial membrane potential oscillation, as revealed by the average Fluo-4 fluorescence intensity (Fig. 1A, green line).

Repeated line scanning in the absence of a laser flash was also shown to trigger  $\Delta\Psi_m$  and NADH oscillations in some cells that were under highly stressed conditions (Fig. 2A). In this case, Ca<sup>2+</sup> spark frequency oscillated between  $4.1 \pm 0.8$  and  $12.2 \pm 1.1$  sparks/100  $\mu\text{m}^2/\text{s}$  ( $p < 0.05$ ) when mitochondria were polarized and depolarized, respectively (Fig. 2B). However, no significant changes were detected in other properties of the Ca<sup>2+</sup> sparks such as amplitude, full width at half-max (FWHM), and time-to-peak (Fig. 2C–E).

To determine whether SR Ca<sup>2+</sup> content is involved in the regulation of Ca<sup>2+</sup> spark frequency, the SR Ca<sup>2+</sup> content before and after mitochondrial depolarization were measured, respectively. For the example presented in Fig. 3, we observed only a modest increase in the NCX current (from 378 to 392 pA), reflecting a minor, statistically insignificant, change in SR Ca<sup>2+</sup> content (in  $\mu\text{mol}$ ,  $33.0 \pm 5.0$  versus  $37.7 \pm 6.5$ ) immediately after mitochondria depolarization (Fig. 3B). Small spikelike inward currents were also observed during the caffeine exposure; these were due to the opening of caffeine-sensitive channels of unknown identity in the guinea-pig sarcolemma

which were independent of SR  $\text{Ca}^{2+}$  load (still present after thapsigargin treatment). They were excluded from the NCX integral analysis (see supplemental materials, Fig. S5). There were no apparent changes in cellular morphology during the experiments.

### 3.2. Effects of ROS scavenging or mitochondrial ion channel inhibitors on resting $\text{Ca}^{2+}$ spark frequency

As we have previously proposed [15,16], the mechanism of the ROS-dependent mitochondrial oscillator involves the activation of a benzodiazepine-sensitive inner membrane anion channel (IMAC) whose opening is triggered by mitochondrial  $\text{O}_2^-$  production. To demonstrate the common mechanistic basis of the modulation of  $\text{Ca}^{2+}$  sparks in the present work, we employed the  $\text{O}_2^-$  sensor MitoSOX, which preferentially localizes to mitochondria (supplemental materials, Fig. S1), the superoxide dismutase mimetic TMPyP, and the peripheral benzodiazepine receptor ligand 4'Cl-DZP. During mitochondrial oscillation induced by oxidative stress, MitoSOX fluorescence increased in a stepwise manner in association with each oxidation of the NADH pool (Fig. 4A), as expected because the oxidized product is not readily reduced in the cell. The increased rate of  $\text{O}_2^-$  production correlated with each cycle of oxidation, as indicated by plotting the first derivative of the raw MitoSOX fluorescence signal along with the NADH signal (Fig. 4A). Accompanying mitochondrial oscillations, intracellular GSH concentration changes cyclically, with GSH depleted during the depolarization phase (Fig. 4B). The TMPyP treatment decreased the overall rate of  $\text{O}_2^-$  production and prevented mitochondrial oscillation (Fig. 4C), in accordance with its ability to keep ROS levels in the mitochondrial network below the threshold for mitochondrial criticality [20]. Again,  $\text{Ca}^{2+}$  spark frequency increased when mitochondria were

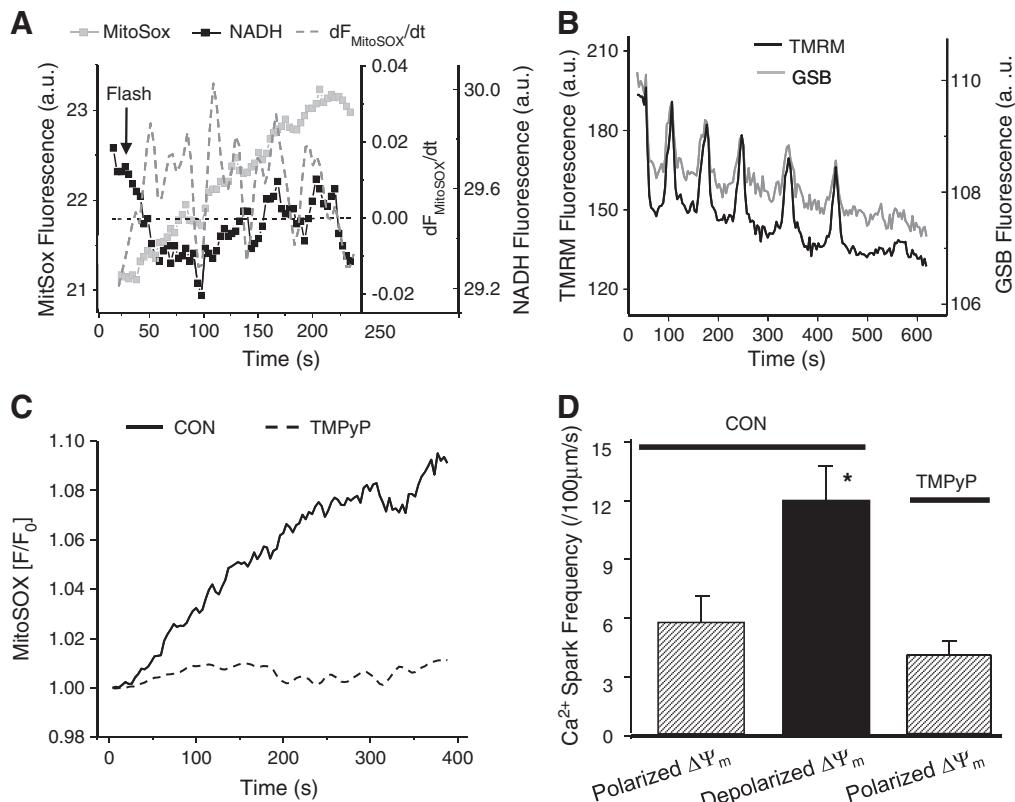
depolarized (NADH oxidized) and this increase was prevented by pretreatment with 200  $\mu\text{mol/L}$  TMPyP for 2 h (Fig. 4D).

The mitochondrial benzodiazepine receptor ligand 4'Cl-DZP inhibits IMAC [21] and prevents or reverses mitochondrial oscillation [15,22]. In depolarized cells displaying enhanced  $\text{Ca}^{2+}$  spark activity, the acute application of 4'Cl-DZP (20  $\mu\text{mol/L}$ ) rapidly recovered the NADH pool and stabilized  $\Delta\Psi_m$ , and  $\text{Ca}^{2+}$  spark frequency decreased concomitantly (Figs. 5A and 6). The average  $\text{Ca}^{2+}$  spark frequency significantly decreased after 4'Cl-DZP treatment from  $22.5 \pm 1.2$  to  $11.8 \pm 0.8$  sparks/100  $\mu\text{m}^2$  (Fig. 5B).

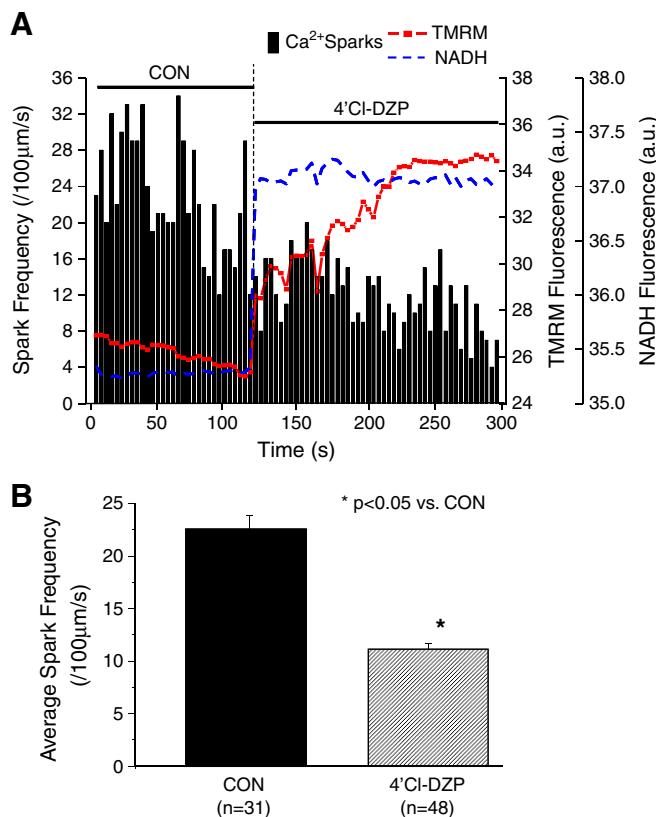
Acute addition of the mitochondrial permeability transition pore (mPTP) blocker cyclosporine A (CsA) did not eliminate the  $\Delta\Psi_m$  oscillations (Fig. 6A), nor did it prevent the modulation of the  $\text{Ca}^{2+}$  spark frequency by  $\Delta\Psi_m$ . However, in the presence of CsA (1  $\mu\text{mol/L}$ ), the peak  $\text{Ca}^{2+}$  spark frequency during  $\Delta\Psi_m$  depolarization ( $19.7 \pm 3.8$  sparks/100  $\mu\text{m}^2$ ) was less than in the absence of CsA in the same cell ( $35.2 \pm 2.1$  sparks/100  $\mu\text{m}^2$ ; Fig. 6B). We also observed reduced  $\text{Ca}^{2+}$  spark frequency in non-oscillating myocytes upon high concentration (10  $\mu\text{mol/L}$ ) CsA treatment (supplemental materials, Fig. S2). 4'Cl-DZP (20  $\mu\text{mol/L}$ ) applied at the end of the experiment, restored and stabilized  $\Delta\Psi_m$  and decreased resting  $\text{Ca}^{2+}$  sparks to control levels (Figs. 5A and 6B).

## 4. Discussion

The central finding of the present study is that the  $\text{Ca}^{2+}$  spark frequency in quiescent, non-beating cardiomyocytes is dynamically modulated by the mitochondrial energy state. During cell-wide oscillations of  $\Delta\Psi_m$ , mitochondrial depolarization was correlated with increased  $\text{Ca}^{2+}$  spark frequency, while repolarization suppressed



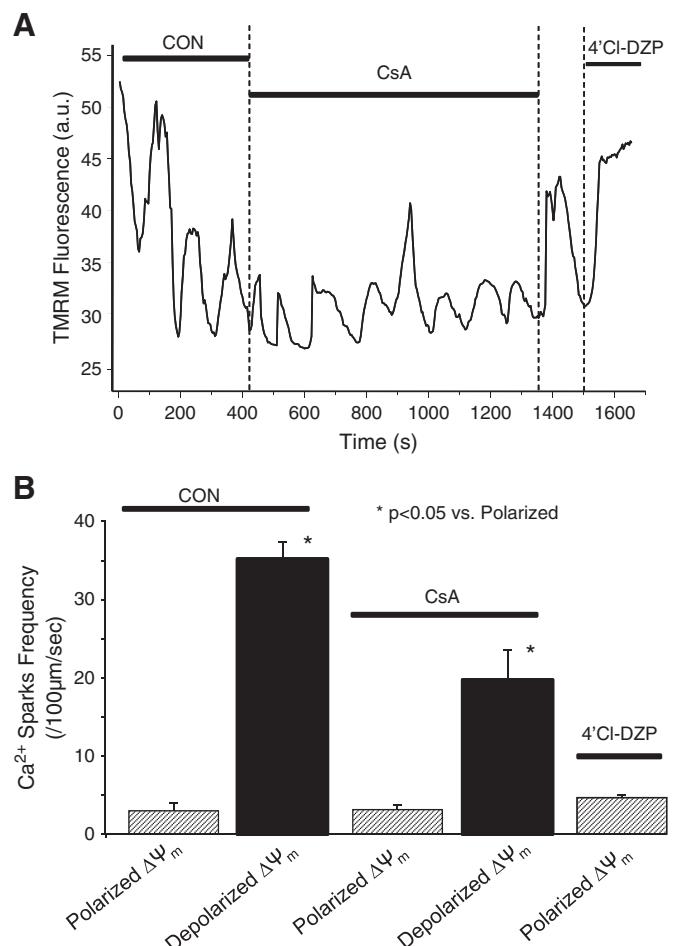
**Fig. 4.** Suppression of  $\Delta\Psi_m$  oscillations and enhanced  $\text{Ca}^{2+}$  spark frequency by the superoxide scavenger TMPyP. (A) Dynamics of the rate of  $\text{O}_2^-$  production (MitoSOX) and NADH fluorescence during mitochondrial oscillations (TMRM was not loaded in this experiment due to overlap with the MitoSOX emission); (B) dynamic changes of  $\Delta\Psi_m$  and GSH during mitochondrial oscillations; (C) effect of TMPyP on ROS production; and (D) effect of  $\text{O}_2^-$  scavenger on  $\text{Ca}^{2+}$  spark frequency.



**Fig. 5.** Effect of the mitochondrial benzodiazepine receptor ligand 4'Cl-DZP on the occurrence of  $\text{Ca}^{2+}$  sparks in the presence of oxidative stress. (A) Immediate increase of NADH, recovery of membrane potential, and suppression of  $\text{Ca}^{2+}$  sparks after addition of 4'Cl-DZP; and (B) comparison of average  $\text{Ca}^{2+}$  spark frequency before and after the addition of 4'Cl-DZP.

$\text{Ca}^{2+}$  spark frequency. The cyclical enhancement of  $\text{Ca}^{2+}$  spark frequency corresponded to the phase of high superoxide production by the mitochondrial electron transport chain, NADH oxidation, and depletion of the intracellular glutathione pool, consistent with our model of the ROS-dependent mitochondrial oscillator described previously in theoretical [16] and experimental [15] studies. The common mechanistic link between  $\Delta\Psi_m$  oscillations and  $\text{Ca}^{2+}$  sparks was demonstrated by concomitant suppression of mitochondrial oscillations and  $\text{Ca}^{2+}$  spark changes with the IMAC inhibitor 4'Cl-DZP, or by pretreatment of the cardiomyocytes with the superoxide scavenger TMPyP.

Over the past two decades, through ryanodine binding studies, SR  $\text{Ca}^{2+}$  release assays, and RyR channel reconstitution, it has been established that oxidation of critical sulphydryl groups on the RyR increases the open probability of the channel, while thiol reducing agents have the opposite effect [23–27]. Moreover, it has been demonstrated the RyR is a redox sensor with a well-defined midpoint potential with respect to the glutathione redox state (GSH:GSSG) [17], the largest capacity antioxidant system of the cell. This sensitivity to the thiol state has been attributed to ~20–40 hyperreactive cysteines present on each monomer of the tetrameric RyR channel assembly [24,28]. While a direct link between cysteine oxidation and RyR activity is undeniable, it has not been well established how this mechanism might come into play in intact cells or muscles. Several recent studies have examined the influence of mitochondrial function on  $\text{Ca}^{2+}$  sparks. For example, Isaeva et al. [29] reported an increase in  $\text{Ca}^{2+}$  spark activity in skinned skeletal muscle fibers that correlated with oxidation of the mitochondrial NADH pool, but not SR  $\text{Ca}^{2+}$  content, and could be suppressed by treatment with ROS scavengers or mimicked by  $\text{H}_2\text{O}_2$  treatment. The onset of enhanced spark activity depended on the muscle type, with mitochondria-rich slow muscles



**Fig. 6.** Effect of mitochondrial permeability transition pore (mPTP) blocker cyclosporine A (CsA) on the occurrence of  $\text{Ca}^{2+}$  sparks during mitochondrial  $\Delta\Psi_m$  oscillation. (A) Effect of acute addition of CsA on  $\Delta\Psi_m$  oscillations and  $\text{Ca}^{2+}$  spark frequency; and (B) Summary of  $\text{Ca}^{2+}$  spark frequency as a function of  $\Delta\Psi_m$  during control (CON), after adding CsA or in the presence of 4'Cl-DZP after CsA washout.

having the longest delay. Mitochondrial ROS production has also been implicated in  $\text{Ca}^{2+}$  spark activation in response to treatment with mitochondrial K<sup>+</sup> channel openers in cerebral arteries [30]. This effect can be inhibited by TMPyP.

Local activation of  $\text{Ca}^{2+}$  sparks close to sites of mitochondrial  $\Delta\Psi_m$  depolarization was first observed by Zorov et al. [31] after laser-induced mitochondrial permeability transition pore activation. This study established the concept of mitochondrial ROS-induced ROS release, whereby a certain amount of oxidative stress leads to a regenerative burst of ROS production as the mitochondria depolarize and uncouple. Similarly, we have reported that ROS-induced ROS release plays a role in the scaling of local  $\Delta\Psi_m$  depolarization to the entire mitochondrial network of mitochondria of the cardiomyocyte when a critical fraction of mitochondria reach a threshold level of oxidative stress [20], albeit through the activation of IMAC rather than the mPTP. Using a method similar to ours, another recent study also supported a link between mitochondrial ROS production and increased  $\text{Ca}^{2+}$  spark activity. In a study by Yan et al. [32], laser-induced ROS production initiated a wave of oxidation of the ROS probe (CM-DCF) associated with an increase in  $\text{Ca}^{2+}$  spark frequency (measured using the  $\text{Ca}^{2+}$  indicator Rhod-2), first near the illumination site and later more widespread throughout the cell. The enhancement in  $\text{Ca}^{2+}$  spark frequency was prevented by treatment with either TMPyP or myxothiazol, which (as we have shown) inhibit mitochondrial ROS production and  $\Delta\Psi_m$  oscillations [15]. Although

mitochondrial NADH or  $\Delta\Psi_m$  signals were not measured in that study, some cells showed oscillatory changes in ROS production with a mixed effect on spark frequency: early in the experiment spark frequency was enhanced, but later, spark frequency decreased when ROS production increased.

In the present work, we could directly correlate changes in  $\text{Ca}^{2+}$  spark frequency with indices of the mitochondrial energy and redox states ( $\Delta\Psi_m$ , NADH and GSH), enabling us to assess the roles of mitochondrial superoxide production, IMAC, mPTP, and the mitochondrial benzodiazepine receptor in the response. During whole-cell mitochondrial oscillations, we have shown that  $\Delta\Psi_m$  depolarizes and NADH oxidizes rapidly, with a concomitant burst of ROS production, which is then followed by a slower recovery phase with the cycle repeating approximately every 100 s. Cellular GSH and  $\Delta\Psi_m$  oscillate in parallel, with the oxidation phase slightly preceding the rapid  $\Delta\Psi_m$  depolarization (Fig. 4B). The present findings reveal that  $\text{Ca}^{2+}$  spark frequency increases by more than two-fold during each depolarization cycle and decreases to basal levels upon mitochondrial repolarization. The dynamic relationship between mitochondrial superoxide production,  $\text{Ca}^{2+}$  spark frequency and NADH oxidation was directly elucidated, confirming that high rates of mitochondrial ROS production correspond to increased  $\text{Ca}^{2+}$  spark frequency. We observed no significant change in  $\text{Ca}^{2+}$  spark amplitude or time-to-peak, nor the cytosolic  $\text{Ca}^{2+}$  during mitochondrial oscillations.

Suppression of both the mitochondrial oscillations and the effects on  $\text{Ca}^{2+}$  sparks by TMPyP indicates a common mechanism consistent with the ROS-dependent oscillator we have previously studied. Importantly, this was verified by observing that  $\text{Ca}^{2+}$  spark frequency declined in association with reduction of the NADH pool and  $\Delta\Psi_m$  stabilization in the presence of 4'Cl-DZP. Furthermore, the oscillations in  $\Delta\Psi_m$  were not prevented by CsA, an inhibitor of the mPTP, although the peak  $\text{Ca}^{2+}$  spark rate during  $\Delta\Psi_m$  depolarization in CsA was less than the peak rate in the absence of CsA. The latter was unlikely to be due to mPTP opening contributing to the mitochondrial depolarization since CsA apparently impaired  $\Delta\Psi_m$  recovery during each cycle (cf Fig. 6A). Rather, the CsA effect may be attributed to its effects on the RyR multiprotein complex [33,34]; nevertheless, all mitochondrial effects on  $\text{Ca}^{2+}$  spark frequency were still completely reversed by treatment with 4'Cl-DZP after CsA washout.

Because loss of mitochondrial function will change a number of intracellular modulatory factors, and modify a number of ion transport proteins, further investigation will be required to assess the total effect of mitochondrial depolarization on the  $\text{Ca}^{2+}$  handling subsystem. As we have previously reported [15], sarcolemmal  $K_{\text{ATP}}$  channels are activated during the oscillations in  $\Delta\Psi_m$ , indicating that the ATP/ADP ratio decreases significantly, which would also increase free  $Mg^{2+}$ . These changes would tend to suppress the activity of the SR  $\text{Ca}^{2+}$ -ATPase, the L-type  $\text{Ca}^{2+}$  channel, and the RyR, so it is not likely to account for the increased  $\text{Ca}^{2+}$  spark frequency. Numerous reports have revealed that cysteine oxidation events can have important functional consequences for an array of signal transduction cascades, making it apparent that multiple classes of regulatory proteins are reversibly oxidized by  $H_2O_2$ , among other oxidants [35]. The increase in resting  $\text{Ca}^{2+}$  spark activity described herein is consistent with the effects of thiol oxidation on RyR, consistent with our earlier studies showing that oxidation of the glutathione pool occurs during the metabolic oscillations [16], but the precise modulator of the SR  $\text{Ca}^{2+}$  release channel is difficult to select from among several candidates. The most straightforward interpretation would be that oxidation of the redox pool (including GSH and NADH), depleted during the sequential reactions of superoxide dismutase and glutathione peroxidase and supported by the oxidation of NADPH, directly modifies reactive cysteines on the cytoplasmic face of the RyR. However, we cannot exclude direct effects of individual reactive molecules on channel open probability: for example,  $H_2O_2$  (and hydroxyl radicals) [36], NO [37], nitroxyl [38] and  $NAD^+$  [39] have been shown to increase

RyR activity in channel reconstitution experiments, and the skeletal RyR reportedly contains an oxidoreductase-homology domain with an  $NAD^+$  binding site [40]. With respect to a possible direct effect of superoxide on RyR, Zima et al. [39] reported that superoxide suppressed  $\text{Ca}^{2+}$  spark activity when generated by the xanthine/xanthine oxidase reaction, yet one must also consider that superoxide plays a central role in the autocatalytic feedback mechanism of the mitochondrial oscillator [15,41] and thus will alter the levels and redox status of all other factors.

Although covalent modifications of RyR, including S-glutathionylation [42] and S-nitrosylation [43], are known to modify channel gating properties, these processes would have to be very quickly reversed (within seconds) to account for the phasic changes in  $\text{Ca}^{2+}$  spark frequency described here: this may be possible based on current paradigms [44]. Another interesting alternative could be that the RyR open probability is responding to oxidation of the channel from the luminal side of the SR, as suggested by several studies [45–47]. At present, we have no information about how the changes in mitochondrial energy state might affect intraluminal redox potential, which is thought to be much more oxidized than the cytoplasm [48], however,  $H_2O_2$  readily diffuses through membranes and the SR surrounds, and is in direct contact with, the mitochondrial outer membrane.

## 5. Conclusions

We conclude that during oxidative stress, the amplifying effect of mitochondrial RIRR to activate IMAC not only evokes oscillations in the energy state to influence sarcolemmal KATP currents, but also modulates the  $\text{Ca}^{2+}$  handling system directly by governing the local redox environment near the dyad. During acute  $\Delta\Psi_m$  depolarization, mitochondria elicit oxidation of the redox environment, favoring modification of critical thiol groups on the RyR receptor, increasing its open probability independent of changes in SR  $\text{Ca}^{2+}$  load in the short term. The results demonstrate that there is a dynamic mechanistic link between mitochondrial polarization state and resting  $\text{Ca}^{2+}$  spark rate. Defining all of the metabolic factors affecting  $\text{Ca}^{2+}$  release in beating cells will, however, require further investigation.

## Disclosure statement

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jmcc.2011.05.007.

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