Synchronized whole-cell oscillations in mitochondrial metabolism triggered by a local release of reactive oxygen species in cardiac myocytes

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Summary

Reactive oxygen species (ROS) and/or Ca\(^{2+}\) overload can trigger depolarization of mitochondrial inner membrane potential (\(\Delta \Psi_m\)) and cell injury. Little is known about how loss of \(\Delta \Psi_m\) in a small number of mitochondria might influence the overall function of the cell. Here we employ the narrow focal excitation volume of the two-photon microscope to examine the effect of local mitochondrial depolarization in guinea-pig ventricular myocytes. Remarkably, a single local laser flash triggered synchronized and self-sustained oscillations in \(\Delta \Psi_m\), NADH, and ROS after a delay of \(\sim 40s\), in more than 70% of the mitochondrial population. Oscillations were initiated only after a specific threshold level of mitochondrially-produced ROS was exceeded, and did not involve the classical permeability transition pore or intracellular Ca\(^{2+}\) overload. The synchronized transitions were abolished by several respiratory inhibitors or a superoxide dismutase mimetic. Anion channel inhibitors potentiated matrix ROS accumulation in the flashed region, but blocked propagation to the rest of the myocyte, suggesting that an inner membrane, superoxide-permeable, anion channel opens in response to free radicals. The transitions in mitochondrial energetics were tightly coupled to activation of sarcolemmal K\(_{ATP}\) currents, causing oscillations in action potential duration, and thus might contribute to catastrophic arrhythmias during ischemia-reperfusion injury.

Introduction

Mitochondria play a multifunctional role as key arbiters of cell life and death. In addition to oxidative phosphorylation, mitochondria are involved in thermogenesis, free radical production, and intracellular Ca\(^{2+}\) homeostasis. Impairment of mitochondrial function during and after ischemia causes rapid energy depletion, contractile failure and loss of cellular integrity, which may lead to necrotic or apoptotic cell death (1-3).

ROS have been implicated in ischemic dysfunction; however, they play a dual role as determinants of cell survival, on the one hand contributing to Ca\(^{2+}\) overload and the induction of a
mitochondrial permeability transition, but on the other acting as second messengers that protect cells against injury (4-6). Mitochondria are a major site of physiological ROS production in the cardiomyocyte, with approximately 1 to 5% of the electrons flowing through the electron transport chain leaking into the production of ROS (7,8). The negative effects of ROS on metabolism are evident in several studies showing rapid and spatiotemporally heterogeneous discharge of $\Delta \Psi_m$ in response to oxidative stress (1,2) and/or $\text{Ca}^{2+}$ overload (9), including protocols employing laser-induced photo-oxidation (10,11) and mitochondrial ROS-induced ROS release (12). In light of our previous work showing that substrate-deprivation can initiate synchronized oscillations of mitochondrial redox and membrane potential (13), and that a diffusible cytoplasmic messenger may be involved (14), the present study tests whether similar global self-organizing behavior can be triggered by a highly localized perturbation of a few mitochondria among the thousands packed within the cardiac myocyte. We demonstrate that ROS release and mitochondrial depolarization in less than 1% of the volume of the cell can trigger spatiotemporally synchronized oscillations in $\Delta \Psi_m$, ROS production, and mitochondrial redox potential throughout the entire volume of the cell. Close coupling of the metabolic responses to cardiac electrical excitability highlights the importance of this form of intraorganellar communication in determining whole-cell function.

**Experimental Procedures**

*Cardiomyocyte isolation.* All experiments were carried out at 37°C on freshly isolated adult guinea pig ventricular myocytes prepared by enzymatic dispersion as previously described (13). After isolation, cells were stored in Dulbecco’s Modification of Eagle’s Medium (10-013 DMEM, Mediatech, Inc. Virginia) in laminin-coated petri dishes in a 5% CO$_2$ incubator at 37°C and used within 6-8 h of isolation.

Experimental recordings started after exchange of the DMEM with an experimental solution containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 10 mM HEPES, 1 mM CaCl$_2$, pH 7.5 (adjusted
with NaOH), supplemented with 10 mM glucose. 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.

Fluorescent probes for two-photon laser scanning microscopy. The cationic potentiometric fluorescent dye tetramethylrhodamine ethyl ester (TMRE) was used to monitor changes in \( \Delta \Psi_m \). The large potential gradient across the inner mitochondrial membrane results in the accumulation of TMRE within the matrix compartment according to its Nernst potential (15).

ROS production was monitored with the ROS-sensitive fluorescent probe 5-(6)-chloromethyl-2',7'-dichlorohydrofluorescein diacetate (CM-H2DCFDA). The acetate group of CM-H2DCFDA is hydrolyzed by esterases when it enters the cell and is trapped inside as the non-fluorescent 5-(6)-chloromethyl-2',7'-dichlorohydrofluorescein (CM-H2DCF). CM-H2DCF was chosen because, unlike underivatized dichlorofluorescein (H2DCFDA), it is well retained in cells (16) and, in our case, in the mitochondrial matrix. Similar results demonstrating matrix localization of ROS production were also obtained with the carboxy derivative, 5-(6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Cbx-DCF). Oxidation of CM-H2DCF by ROS, particularly by hydrogen peroxide (H2O2) and hydroxyl radical (17), yields the fluorescent product CM-DCF, and in an indirect manner, measures mitochondrially-produced O2\(^-\) that has dismutated to H2O2 through the action of mitochondrial Mn-dependent superoxide dismutase (8,18). To image the distribution of \( \Delta \Psi_m \) and ROS production simultaneously, 100 nM TMRE and 2-6 \( \mu \)M CM-H2DCFDA were added to the external solution and allowed to equilibrate for at least 20 min. Under these conditions, CM-DCF fluorescence was largely localized to the mitochondrial matrix space. The influence of partial fluorescence energy transfer (FRET) (19) between CM-DCF and TMRE is described in the online supplemental material.
Retention of calcein in the mitochondrial matrix was tested by loading myocytes for 20 min with 2 µM calcein-AM at room temperature. After dye loading the cells were resuspended in the experimental solution used for imaging. Intracellular esterase action then resulted in loading of both the cytoplasmic and mitochondrial compartments of the cell. Calcein-AM desterification was allowed to proceed for at least 1 h before imaging. To reduce the contribution of the cytoplasmic component to the fluorescence images, myocytes were patch-clamped with fluorophore-free pipet solution, which permitted diffusion of the cytoplasmic calcein into the large pipet volume.

**Image acquisition and analysis.** Images were recorded using a two-photon laser scanning microscope (Bio-Rad MRC-1024MP) with excitation at 740nm (Tsunami Ti:Sa laser, Spectra-Physics). Owing to the overlap in the cross sections for two-photon excitation of the three fluorophores of interest (NADH, CM-DCF, TMRE), this wavelength permitted recording of redox potential, ROS production and ∆Ψ_m simultaneously. The red emission of TMRE was collected at 605±25nm and the green emission of CM-DCF was recorded at 525±25nm. NADH emission was collected as the total fluorescence <490nm. At 3.5s intervals, 512 x 512 pixel 8-bit grayscale images of the three emission channels were collected simultaneously and stored. The total illumination time was 3.0-s per image, unless otherwise specified.

Average power from the Ti:Sa laser was 1000mW and the pulse bandwidth was ~12 nm, corresponding to <60 fs pulse duration at 80MHz repetition rate. This excitation was attenuated by the optical system and by a combination of neutral density filters such that the average intensity at the focal plane was <10 mW. Light-induced mitochondrial depolarization was applied in a small cytoplasmic volume by zooming the laser beam in on a 20x20 pixel (8.7 µm x 8.7 µm square, <1 µm focal depth) region of the cell. Since the total scan duration was the same as that used for full frame imaging, the laser dwell time in a given cell volume during a flash was ~655 times normal. This caused the local generation of ROS due to direct interaction with molecular O_2 to promote triplet
state excitation of local fluorophores (21,22). Local photon-induced ROS elaboration, reinforced by the restricted irreversible depolarization of $\Delta \Psi_m$, allowed us to perturb a small region of the myocyte to look for propagating effects. In the absence of the local perturbation, with few exceptions (see results), myocyte behavior was stable over the duration of the experiments.

**Image Analysis.** Images were analyzed offline using ImageJ software (Wayne Rasband, National Institutes of Health, [http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)) with customized plugins (B.O’R.).

For visualization of the spatio-temporal responses of TMRE and CM-DCF presented in figures 1,2,4,6,7 a 2 to 3 pixel wide line was drawn along the length of the myocyte (as shown in Fig. 1) and the average fluorescence profile along the line was determined for the entire time series of 2D images for a given experiment. A new image was then created, showing the line fluorescence as a function of time (time-line image).

To quantitatively determine whether mitochondrial depolarizations were spatially synchronized in the presence of glucose or inhibitors (Fig. 1), we applied grid analysis to the 2D images. A binary mask of the cell TMRE fluorescence was made and the cell area, excluding nuclei, was divided into small squares approximately the size of individual mitochondria (~2µm x 2µm). The average fluorescence within each grid object was measured and histograms were made of the distribution of fluorescence in polarized and depolarized mitochondria, giving two major peaks of fluorescence intensity. A cutoff value halfway between these peaks was then used to determine if a given grid object was classified as “polarized” and the fraction of polarized mitochondria with respect to the total number of objects at time zero was calculated for the image series. The initial value of ~80% in most experiments reflects an underestimation of the total number of polarized mitochondria due to overlap between the histogram distributions, causing some objects in the polarized population to fall below the cutoff.
Cardiomyocyte electrophysiological studies. Isolated ventricular myocytes were whole-cell patch-clamped using borosilicate glass pipettes (1-4MΩ tip resistance) and action potentials (current-clamp mode) or membrane currents (voltage-clamp mode) were recorded by means of an Axopatch 200A amplifier coupled to a Digidata 1200A interface (Axon Instruments, Union City, CA) using custom acquisition and analysis software. Myocytes were superfused with (in mmol/L) NaCl 140, KCl 5, MgCl₂ 1, CaCl₂ 2, glucose 10, and HEPES 10 (pH 7.4 with NaOH). Intracellular solutions contained (in mmol/L) potassium glutamate 130, KCl 9, NaCl 10, MgCl₂ 0.5, MgATP 5, EGTA 1, and HEPES 10 (pH 7.2 with KOH). Action potentials were evoked by brief (4 ms) current injections applied at 2 s intervals. Voltage ramps from –90 to +30 mV were applied in voltage-clamp mode over a pulse duration of 200 ms at a 2 s interval. Imaging was performed as described for the other experiments, except that a 40x lens was used instead of a 60x, to provide additional clearance between the cell and the lens for pipet access.

Statistical Analysis. Data were analyzed with the software GraphPad Prism (Ver. 2; San Diego, CA). The statistical significance of the differences between treatments (respiratory inhibitors, comparison between flashed cells and spontaneous ones) was evaluated with a t-test (small samples, paired t-test with two tail P-values). Summary statistics, presented as mean ± SEM, (95% confidence interval), were determined for periods of oscillation and rates of depolarization and repolarization. The statistical analysis of the rates of ∆Ψₘ depolarization or repolarization during mitochondrial oscillations was performed after nonlinear regression analysis performed with a Levenberg-Marquardt algorithm (Microcal(TM) Origin, Northampton, MA). The normality of the data was tested with a Kolmogorov-Smirnov test (GraphPad Prism).

Materials. TMRE, CM-H₂ DCFDA, carboxy-H₂ DCFDA and calcein AM were purchased from Molecular Probes, Inc, and BKA and TMPyP from Calbiochem. All other reagents were from Sigma-Aldrich. Stock solutions of cyclosporin A, rotenone, thapsigargin, oligomycin, antimycin A, DIDS
(4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt), 4'-chlorodiazepam (Ro5-4864), FGIN-I-27 and PK11195 (1-(2-Chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide) were prepared in DMSO and concentrated enough to avoid exceeding 0.1% DMSO (vol/vol) in the final solution. The stock solution of bongkrekic acid was prepared in 2N NH₄OH as recommended by the manufacturer. All stock solutions were stored at -20°C.

**Results**

*Global transitions of mitochondrial energetics triggered by local perturbation of mitochondrial function*

Under otherwise normal physiological conditions (normoxic cells in the presence of external substrates), we tested whether a highly localized metabolic perturbation could have widespread effects on the mitochondrial network of the myocyte. The thin optical section excited by the two-photon laser (23) was employed to perturb a small fraction (<0.3% of the total cell volume) of the mitochondrial population while monitoring the behavior of the remainder of the cell. After collecting 10-20 control images, an 8.7 x 8.7 µm region of the cell was excited in a single flash (Fig. 1A). This resulted in a rapid, but not instantaneous (exponential time constant 18.2s) (see Fig. 2C), depolarization of ΔΨᵣ in the flashed region (see white square in Fig. 1A). Thereafter, ΔΨᵣ remained depolarized in the flashed area throughout the experiment (Fig. 1A). Unflashed control cells in the same field remained polarized throughout the experiment (Fig. 1C, D).

In contradistinction to the response in the flashed region, mitochondria throughout the rest of the cell were initially unaffected, but began to oscillate in a synchronized manner after a substantial delay. The spatiotemporal pattern of mitochondrial depolarization and repolarization can be readily appreciated from the time-line images created from the entire image sequence (Fig. 1B; the time-line image is not a “line-scan” image, but is a 2D representation of the full 3D time series of the
experiment, as described in methods) and by inspection of the image montage for a single oscillation (Fig. 2A; from the oscillation marked with an asterisk in Fig. 1B).

Plots of whole-cell fluorescence illustrate that synchronized and periodic transitions in $\Delta \Psi_m$ and the NADH redox pool (Fig. 2D) were associated with bursts of mitochondrial ROS production (Fig. 2E), triggered by the initial depolarization and ROS accumulation in the flashed region (see white squares in Figs. 1A and flashed zone in Fig. 1B).

Quantitative analysis of mitochondrial oscillations

On average, $72 \pm 2.8\%$ of the total mitochondrial population depolarized during each cycle of oscillation ($n=9$ cells; 7 experiments; see methods for grid analysis), while in control cells, mitochondria remained polarized throughout the experiment (Fig. 2B; see also Fig. 1C, D). The probability of inducing cell-wide oscillations in response to a flash exceeded $80\%$ ($n=65$; 7 experiments), while in unflashed controls, oscillations were observed in less than $18\%$ of the cells ($n=244$ cells analyzed in 61 fields; 9 experiments). Furthermore, the few cells that oscillated in the absence of a flash did so only after a significantly longer delay ($448 \pm 64$ s; $n=13$; $p<0.001$) compared to those that had been flashed ($43 \pm 9$ s; $n=13$).

Close inspection of individual cycles of $\Delta \Psi_m$ depolarization-repolarization revealed that the rapid depolarization phase ($4.2\pm0.4$ s; $n=15$; 10 experiments) was followed by an exponential repolarization with fast ($14\pm1.34$ s; $n=14$) and slow ($111\pm4.9$ s; $n=6$) components, until another sudden depolarization initiated the next cycle (Fig. 2D). The average period of oscillation was determined to be $104\pm5$ s ($n=66$ periods analyzed in 15 cells; 10 experiments). By analyzing myocytes with fully polarized mitochondria before the flash, and almost fully depolarized during a transition, we calculated that the relative decrease in $\Delta \Psi_m$ during depolarization was $\sim60\%$. Assuming a $\Delta \Psi_m$ of $-150$ mV (e.g.,(2)), a $90$ mV depolarization was estimated.
**Role of mitochondrial ROS in the initiation and propagation of cell-wide mitochondrial oscillations**

The correlation between ROS and mitochondrial instability was supported by the observation that a distinct threshold of ROS accumulation was necessary to observe global oscillations in $\Delta \Psi_m$. Figure 3 shows the normalized ROS production as a function of time for representative cells. In the majority of myocytes exposed to a flash (Fig. 3A), total cellular CM-DCF fluorescence soon rose to a level more than ~20% above baseline, and oscillations in $\Delta \Psi_m$ occurred contemporaneously. In the few cells that were flashed, but showed no oscillations (Fig. 3A), or in unflashed cells (Fig. 3B), ROS accumulation remained below the 20% threshold level. Whether or not this ROS threshold was crossed was a predictor of global $\Delta \Psi_m$ depolarization across all experimental conditions (e.g., flashed cells, unflashed cells, metabolic inhibitors, ROS scavengers, etc.).

We next tested whether the mitochondrial electron transport chain was the major source of ROS production during the self-sustained oscillations of $\Delta \Psi_m$ by inhibiting specific sites in the redox chain. Rotenone, an inhibitor of NADH dehydrogenase (complex I; Fig. 4A), will inhibit electron transfer from complex I to ubiquinone and suppress superoxide ($O_2^-$) production from complex III(18), but may enhance $O_2^-$ produced at complex I(24). Acute addition of rotenone to myocytes undergoing triggered mitochondrial oscillations rapidly and completely abolished the synchronized transitions in $\Delta \Psi_m$ (Fig. 4B and 4D upper trace). Cells preincubated with rotenone also resisted depolarization of $\Delta \Psi_m$ when challenged with a flash, and had reduced ROS accumulation (Fig. 4E).

Consistent with the results obtained with rotenone, and in accord with previous studies (18,25,26), inhibition of the oxidation of ubiquinol to ubisemiquinone and electron transfer to the FeSIII center of complex III with myxothiazol (see Q cycle of Fig. 4A) also resulted in suppression
of triggered $\Delta\Psi_m$ oscillations (Fig. 4D), and significantly decreased of ROS production (Fig. 4E),
either added acutely or after preincubation.

Antimycin A, which is known to block normal electron flow through complex III, but
enhance $O_2^-$ production as a result of accumulation of ubisemiquinone(7,18) (Fig. 4A), markedly
increased ROS accumulation (by ~2-fold) in response to a flash (Fig. 4E). In the presence of
antimycin, the flash induced a rapid release of TMRE from the mitochondrial matrix into the
cytoplasm (Fig. 4C; evident as an increase in the diffuse distribution of the dye) and, within minutes,
caused rigor contracture, indicating a drop in cellular ATP to $\mu$M levels (27). This response supports
the abovementioned evidence that ROS generated at complex III can mediate mitochondrial
uncoupling, although in this case, the ROS concentrations were overwhelming and the
depolarization irreversible.

Through a mechanism similar to that of myxothiazol, inhibition of $O_2^-$ generated by the $Q$-
cycle can also be achieved by reduction of the FeSIII electron acceptor of complex III, which can
occur upon downstream inhibition of the electron transport chain (18,24,26). This was tested by
blocking cytochrome oxidase (complex IV) with cyanide (Fig. 4A). As predicted, acute addition of
cyanide abruptly stopped flash-triggered oscillations, allowed $\Delta\Psi_m$ to be restored (Fig. 4D), and
significantly decreased ROS production (Fig. 4E), again supporting the conclusion that complex III
was the major site of ROS production during metabolic oscillation.

The results obtained with cyanide indicated that flux through the electron transport chain,
and a ready supply of downstream electron acceptors were necessary for the mitochondrial ROS
bursts occurring during the oscillations. Hypothetically, other inhibitors that decrease electron
transport and push respiration toward state 4 should also decrease ROS production in this context.
This held true when respiration was slowed by inhibiting the $F_1F_0$ ATPase with oligomycin (Fig.
4A,E) or when the adenine nucleotide translocator (ANT) was inhibited with bongkrekic acid (BKA;
If ROS are the key messengers underlying propagation and synchronization of the mitochondrial transitions, then scavenging cytoplasmic ROS should suppress or alter the pattern of ∆Ψₘ oscillation in response to a flash. Flash-induced ∆Ψₘ, ROS, and NADH oscillations were suppressed by preincubation of myocytes with the superoxide dismutase (SOD) mimetic compound Mn(III)tetrakis(1-methyl-4-pyridil)porphyrin pentachloride (TMPyP), a membrane-permeant O₂⁻ scavenger(28,29) (Fig. 4F). Unlike inhibition of ROS production at its source, effective suppression of the response by increased cytoplasmic ROS buffering required a preincubation period of more than 30 minutes. In agreement with the results described above, TMPyP treatment kept ROS levels below the critical threshold.

Taken together, these findings suggest that the self-sustaining bursts of ROS production triggered by a prior local oxidant stress require O₂⁻ production at complex III, and that mitochondrial ROS are intimately involved in the oscillatory and propagation mechanisms. Consistently, BKA blocked the cell-wide mitochondrial oscillations by keeping ROS production below threshold levels. By inhibiting ANT, which may be part of a permeability transition pore (PTP) complex, BKA has been used previously as a PTP blocker. Therefore, we further investigated whether the PTP contributed to the observed mitochondrial ∆Ψₘ depolarizations.

Absence of PTP opening and mitochondrial Ca²⁺ overload in mechanism of cell-wide mitochondrial oscillation

The results described above indicate that beyond a threshold level, mitochondrialy-produced ROS released into the cytoplasm trigger regenerative ∆Ψₘ depolarization, perhaps mediated by the opening of an energy-dissipating mitochondrial ion channel. This finding begs the question of whether the ROS effect involves the classical PTP or if alternative mitochondrial channels are
implicated in the response. Cyclosporin A (CsA), a known inhibitor of the PTP (1,22,30) which can protect against PTP-mediated loss of \( \Delta \Psi_m \), had no effect on flash-induced or spontaneous synchronized transitions in mitochondrial activity. Cell-wide mitochondrial oscillations triggered by a flash were not abolished by either immediate addition or 2h preincubation with 1 \( \mu \)M CsA (Fig. 5A).

To rule out the possibility that a CsA-resistant PTP opening may have occurred, we also directly tested whether the PTP opened by determining if three different small molecular weight markers were released from the mitochondrial matrix. Both CM-DCF, and the similarly-sized carboxy derivative of DCF (~600 MW), were retained in the mitochondrial matrix throughout the experiments, despite repeated and large amplitude depolarizations of \( \Delta \Psi_m \) (Fig. 5B). Similarly, calcein (622 MW), an inert fluorophore commonly used to assess PTP opening, was not released from the mitochondrial matrix during \( \Delta \Psi_m \) oscillations (Fig. 5C), providing strong evidence that a full conductance PTP opening (allowing passage of molecules up to 1,500 MW) was not induced in these experiments.

The oxidation of CM-DCF by ROS should ostensibly cause an irreversible increase in fluorescence and record the history of ROS production in the cell. However, in some cases, we observed a decrease in CM-DCF fluorescence after each burst of ROS production (e.g., Fig. 2E). This anomalous behavior of the ROS signal was absent, and CM-DCF fluorescence increased in a stepwise manner, when TMRE was not loaded during the experiment, indicating that it was due to fluorescence resonance energy transfer (FRET) (19) between CM-DCF and TMRE as donor and acceptor fluorophores, respectively (see online supplemental material). This result also showed that the CM-DCF is retained inside the mitochondrial matrix during \( \Delta \Psi_m \) oscillations, while the TMRE moves in and out.
Ca\textsuperscript{2+} overload has been reported to be a cofactor in the opening of the PTP (see (1) for a review), and it also may be a messenger that can propagate mitochondrial transitions (9). Although no sarcomere shortening was evident in the present experiments, and the myocytes were studied under quiescent, minimally Ca\textsuperscript{2+}-loaded conditions, we tested whether suppression of sarcoplasmic reticulum (SR) or mitochondrial Ca\textsuperscript{2+} handling influenced flash-induced mitochondrial oscillations. Ruthenium red, an inhibitor of the Ca\textsuperscript{2+}-uniporter in mitochondria (31) and of the SR Ca\textsuperscript{2+}-release channel (32), did not prevent the flash-induced or spontaneous synchronized transitions in mitochondrial activity, either acutely, or when the flashes were given after 30 min of preincubation with the inhibitor (see supplemental material).

Thapsigargin, an inhibitor of the SR Ca\textsuperscript{2+}ATPase which effectively depletes intracellular Ca\textsuperscript{2+} stores in myocytes, also did not stop mitochondrial oscillations (see supplemental material). Finally, extensive buffering of intracellular Ca\textsuperscript{2+} with 1 mM EGTA did not affect flash-induced oscillations (see Fig. 7), confirming that Ca\textsuperscript{2+} was not a key factor in triggering the mitochondrial transitions in our experiments.

**Anion channel inhibitors block initiation of cell-wide mitochondrial oscillations, but enhance local matrix ROS accumulation in the flashed area**

To investigate whether a channel distinct from the classical PTP mediated mitochondrial depolarization and, perhaps, O\textsubscript{2}\textsuperscript{-} efflux from the matrix, we tested the effects of reported inhibitors of inner membrane anion channels (IMAC) on the response to local oxidant stress.

The isoquinoline carboxamide PK11195 specifically binds to the peripheral benzodiazepine receptor that localizes to mitochondria, and is one among several classes of amphipathic inhibitors of IMAC (33). DIDS, which also inhibits IMAC (34), is a stilbene-2,2\textsuperscript{-}disulfonate most frequently used as an inhibitor of Cl\textsuperscript{-} channels and/or anion transporters. Either of these inhibitors prevented the cell-wide synchronized $\Delta\Psi_m$ depolarizations and ROS bursts after a flash (Fig. 6A and 6C), despite
the fact that, within the flashed zone, mitochondria depolarized irreversibly and ROS production was accentuated (Fig. 6B and 6D). In fact, the normalized CM-DCF signal in the flashed region was 2.1±0.3 (n=3) and 1.8±0.2 (n=3)-fold higher in the presence of DIDS and PK11195, respectively, than in the absence of the IMAC blockers, indicating elevated levels of ROS trapped in the mitochondrial matrix.

In the presence of the IMAC inhibitors, CM-DCF fluorescence remained highly localized and retained a mitochondrial pattern in the flashed region and ∆Ψₘ polarization was well-maintained in mitochondria throughout the remainder of the cell, even directly adjacent to the flashed region (Fig. 6E and 6F).

Further pharmacological characterization of the entity involved in mitochondrial ∆Ψₘ depolarization showed that another ligand, the benzodiazepine 4'-chlorodiazepam (Ro5-4864), employed to characterize the mitochondrial benzodiazepine receptor (33, 43), also prevented the mitochondrial oscillations (Fig. 6F). Interestingly, the complete inhibition of mitochondrial depolarizations through IMAC was obtained with 64 µM of 4'-chlorodiazepam (Ro5-4864), whose IC₅₀ is 34 µM (35). Moreover, an agonist, FGIN-I-27 (36), had the inverse effect, i.e. it favored permanent mitochondrial ∆Ψₘ depolarizations (Fig. 6F).

Oscillations in mitochondrial energetics drive cyclical changes in the cardiac action potential

The oscillatory uncoupling of mitochondria depletes cellular ATP levels and drives the activation of ATP-sensitive K⁺ (Kₐₚ) channels in the sarcolemma. This will, in turn, produce cyclical changes in the action potential of the cardiomyocyte (13). We directly demonstrated this effect on cellular electrical excitability by recording action potentials or K⁺ currents using whole-cell patch clamp while simultaneously imaging myocytes during flash-triggered oscillations in ∆Ψₘ (Fig. 7). Action potentials (recorded in current clamp mode) shortened dramatically in coincidence
with cell-wide depolarization of $\Delta \Psi_m$ (Fig. 7A) and relengthened during the recovery phase of the cycle (Fig. 7B). Under voltage clamp conditions, the current-voltage relation of the oscillatory sarcolemmal current was consistent with the activation of $K_{ATP}$ current (i.e., weakly inwardly rectifying and having a reversal potential near the equilibrium potential for $K^+$) and was synchronized with the mitochondrial depolarizations (Fig. 7C and 7D). Localized transitions in $\Delta \Psi_m$ (e.g., limited to the flashed area) did not induce the drastic changes in action potentials (not shown).

**Discussion**

The main contributions of the present work are that: (i) periodic cell-wide synchronized oscillations in $\Delta \Psi_m$ can be triggered by the local release of ROS in a small volume of the cell (Figs. 1 and 2) after a threshold is reached (Fig. 3), (ii) mitochondrial ROS production by the electron transport chain at complex III (Fig. 4), and release of $O_2^{-}$ from the matrix (Figs. 4, 5 and 6), are required for cell-wide synchronized mitochondrial transitions, (iii) an inner membrane channel distinct from the classical PTP (Fig. 5 and supplemental material), which we propose is the IMAC, is involved in the depolarization of $\Delta \Psi_m$ and in the release of ROS from the mitochondrial matrix (Fig. 6), (iv) cytoplasmic scavenging of $O_2^{-}$ suppresses the cell-wide mitochondrial oscillations (Fig. 4F), and (v) synchronized transitions of mitochondrial energetics drive cyclic changes in the cardiac action potential (Fig. 7).

Our working hypothesis, supported by the present findings, is shown in Figure 8. ROS produced by leakage of electrons from the electron transport chain trigger the opening of the IMAC and subsequent release of $O_2^{-}$ to the cytoplasm. The channel has the dual role of both dissipating energy to depolarize $\Delta \Psi_m$ and transporting $O_2^{-}$ out of the matrix in a positive feedback loop. The local liberation of ROS from the mitochondria triggers propagating, regenerative ROS-induced ROS release in the entire mitochondrial network. Remarkably, a relatively small local increase of ROS
does not immediately spread to neighboring mitochondria, but pushes the system towards instability; eventually evolving into a synchronized oscillatory response.

*Events occuring during a metabolic oscillation*

The cyclical nature of the triggered mitochondrial response allowed us to examine the dynamic relationship between $\Delta \Psi_m$, NADH, and ROS production to gain insight into the underlying mechanism. Close inspection of the signals revealed that $\Delta \Psi_m$ depolarization and a rapid increase in the rate of mitochondrial ROS production occurred in concert with oxidation of the redox pool (Fig. 2D and 2E). The peak rate of ROS production occurred when mitochondria were rapidly uncoupling (Fig. 2D and 2E; and see supplement), but the NADH supply (and electron flow into the redox chain) had not yet been depleted. Once the pool of NADH was oxidized, ROS production slowed markedly and resumed only when the TCA cycle restored $\Delta \Psi_m$ and NADH during the recovery phase of the cycle. The cause of the recovery will require further investigation; however, we hypothesize that the IMAC closes after some time, allowing the TCA cycle and respiration-driven proton pumps to recharge the protonmotive force (Fig. 2D). The cycle repeats when the critical level of ROS is again attained, now in the context of a lower triggering threshold as a result of partial depletion of the pool of ROS scavengers. Preliminary studies which incorporate such a scheme into a computational model of mitochondrial metabolism(37) suggest that a Hopf bifurcation and sustained oscillations in $\Delta \Psi_m$ can occur under conditions in which either the $O_2^-$ scavenging capacity of the cell is decreased or the rate of mitochondrial ROS production is increased to critical levels (data not shown).

*Sites of mitochondrial ROS production and release*

Both the rate of ROS production and the synchronized cell-wide transitions in $\Delta \Psi_m$ were suppressed by interventions that would be expected to decrease $O_2^-$ generation from the Q-cycle at complex III of the respiratory chain. It is interesting to note that ROS generation from complex I appeared to
play little role in the setting of oxidative stress in the intact normoxic cells, based on the observation that rotenone or reduction of the electron transport chain by downstream inhibition (myxothiazol, cyanide, oligomycin, or ANT inhibition) suppressed ROS production (Fig. 4A and 4E). These findings are consistent with earlier studies demonstrating that reduction of the redox chain has opposing effects on ROS production at complex I versus complex III(18,24); i.e., reduction of complex I enhances ROS production from a highly electronegative component of this complex(38), while reduction of the FeSIII center of complex III prevents oxidation of ubiquinol to ubisemiquinone, the precursor to $O_2^-$(18). The latter effect also explains why ROS production increases during the rapid depolarization phase of the cycle - acceleration of electron flow through the chain during the rapid uncoupling/oxidation phase increases turnover in the Q-cycle (Fig. 4A) and $O_2^-$ production. This transient burst of ROS terminates when NADH is depleted and $\Delta \Psi_m$ is depolarized. Hence, both the site of $O_2^-$ generation and the balance between the supply of electrons to the respiratory chain and the respiratory flux will determine the steady-state ROS levels, and may account for apparently discrepant conclusions regarding the effect of uncouplers on ROS production(39,40). At complex III, ROS may be produced either at the cytoplasmic or matrix face of the inner membrane depending on the concentration of ubisemiquinone at the periplasmic ($UQ_p$) or the matrix ($UQ_m$) face of the $bc_1$ complex (41,42) (Fig. 4A). Our findings implicate the latter as the primary site of generation, with subsequent efflux of the $O_2^-$ apparently being involved in activating the energy dissipation pathway.

Since we used CM-DCF, which is preferentially oxidized by $H_2O_2$ or hydroxyl radical, we could only indirectly measure mitochondrially-produced $O_2^-$ that dismutates to $H_2O_2$ through the action of mitochondrial Mn-dependent superoxide dismutase(8,18,26,43). Nevertheless, the experimental evidence obtained with the specific $O_2^-$ scavenger TMPyP suggests that this free radical is involved in the synchronization mechanism (Fig. 4F). While $O_2^-$ appears to be strongly
implicated in the oscillatory mechanism, many other molecules are likely to be changing simultaneously (e.g., P, pH, ATP/ADP ratio, etc.), and may also be implicated in the response. This possibility, and the role of other cytoplasmic factors including substrate selectivity and ROS scavenging will require further investigation.

Since O$_2^-$ is membrane impermeant, a specific transport pathway in the mitochondrial inner membrane is suggested. This was supported by the observation that propagation and synchronization of $\Delta\Psi_m$ depolarization outside of the flashed region was suppressed by the anion channel inhibitors (if the membrane permeable H$_2$O$_2$ were the active species, then propagation would have still occurred). The marked accentuation of the stepwise jump in CM-DCF fluorescence observed in the flashed region of PK11195- or DIDS-treated cells is in accord with the idea that O$_2^-$ is trapped in the matrix when anionic pathways are blocked (Fig. 6B and 6D), a possibility that has been suggested previously (6). An alternative explanation for suppression of propagation of the response outside of the flashed region could be that the anion transport inhibitors blocked the target of ROS-induced depolarization (which we hypothesize is the IMAC) in the rest of the cell.

Inner membrane anion channels and $\Delta\Psi_m$ depolarization.

Our findings indicate that neither Ca$^{2+}$ nor the classical CsA-sensitive, large conductance PTP are responsible for the oscillatory depolarization of $\Delta\Psi_m$. (Figs. 5, 6 and supplemental material). Several lines of evidence led us to rule out a contribution of the PTP pore in the mitochondrial depolarizations under the present experimental conditions: (i) the inability of CsA to block the transitions (Fig. 5A); (ii) the absence of an effect of Ca$^{2+}$ (Fig. 7 and supplemental material); (iii) the lack of efflux of small (600 MW) fluorophores from the matrix upon depolarization (Figs. 5 and 6). It is interesting to note that previous studies of laser-induced depolarization of mitochondria often found that CsA (0.2-4\text{\mu}M) failed to block the transitions (10,12), but used BKA block as evidence that the PTP was involved. This argument may need to be
reexamined in light of the present findings. **Inhibiting the adenine nucleotide translocator with BKA and driving the mitochondria into state 4 significantly decreased the rate of mitochondrial ROS production, in a manner similar to inhibition of other sites in the electron transport chain with cyanide and oligomycin (Fig. 4E).**

We also reported a lack of effect of CsA on spontaneous oscillations in $\Delta \Psi_m$ in substrate-deprived myocytes (45), which were also shown to be reversibly suppressed by the benzodiazepine receptor ligand PK11195 (46), suggesting a mechanistic link with the present results. PK11195, along with many other amphipathic compounds (e.g., amiodarone, amitriptyline, dihydropyridines) and anion transport inhibitors, inhibit IMAC in isolated mitochondria (33,34). Patch-clamp studies of isolated mitoplasts demonstrated that an outwardly rectifying current was the predominant component of background conductance (47), and numerous single channel studies have provided evidence that anion channels are present on the inner membrane, the most common being the 108 pS (or “centum-picosiemen”) anion channel (48). Inhibitors of IMAC in isolated mitochondria(33,34) also block inner membrane anion channel activity in single channel recordings(48). In particular, Kinnally et al, have shown that both the 108 pS anion channel and the larger “multiconductance” channel (MCC), argued to be the single channel equivalent of the PTP, are blocked by mitochondrial benzodiazepine receptor (mBzR) antagonists(48). Discrimination between these channel types is complicated by the similar actions of the mBzR compounds; however, the MCC is exquisitely sensitive to cyclosporin A (49), which had no effect in our experiments. The mBzR has been reported to be an 18-kDa protein that is tightly associated with the outer membrane voltage-dependent anion channel (VDAC) and the adenine nucleotide translocator (ANT) at the mitochondrial contact site(50,51), but it is unclear how mBzR modulates inner membrane channel activity, or what proteins might form the inner membrane pores. The present findings, demonstrating that two structurally different ligands of the mitochondrial benzodiazepine receptor, 4'-
chlorodiazepam and PK11195 (51), in addition to DIDS, blocked the cell-wide ΔΨₘ depolarizations and potentiated ROS accumulation in the flashed region of the cell (Fig. 6), are consistent with a role for IMAC, rather than PTP, in both depolarization and O₂⁻ efflux. Induction of mitochondrial ΔΨₘ depolarization (Fig. 6F) by FGIN-I-27 reinforces a specific interaction with the mBzR, since this agonist binds selectively to the mBzR, but not to the GABAₐ receptor or to any other neurotransmitter receptors (36). Confirming the nature and the molecular identity of the channel components represents an important area for future investigation.

Significance of synchronized oscillations in mitochondrial energetics for cardiac physiology.

Previous evidence indicated the existence of oscillations in K_ΑΤΡ membrane currents and in the redox state in metabolically-compromised cardiac cells (13,14). Clearly, cell-wide transitions in mitochondrial energetics drive the cyclic activation of ATP-sensitive potassium currents, producing dramatic cyclic changes in the duration of the cardiac action potential during flash-induced oscillations in cardiomyocytes (Fig. 7A and 7C).

This effect will potentially introduce both temporal and spatial electrical dispersion in individual cells or regions of the myocardium, greatly increasing the susceptibility to fatal ventricular arrhythmias (13,46). Therefore, these results are likely to be relevant to the behavior of heart cells during ischemia/reperfusion, where ROS production plays a key role in cell injury. Conditions would particularly favor mitochondrial oscillation during reperfusion, when substrate is restored and a burst of ROS production occurs, and, in fact, oscillations in whole heart NADH have been observed upon reoxygenation (52). The results also point out that the opening of the IMAC channel under conditions of oxidative stress could possibly be a precursor to the opening of the PTP. If this early step can be interrupted or reversed, it could allow cells to avoid taking the irreversible path leading to necrotic or apoptotic cell death following ischemia and reperfusion.
In a broader context, our studies contribute to understanding how oxidative phosphorylation is coordinated among mitochondria during oxidative stress in spatially-structured metabolic networks (53,54). Since the mechanisms described should be active in all cell types containing mitochondria, the implications for normal as well as for pathophysiological cell function are universal (55-59).

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**Figure Legends**
**Figure 1.** Oscillations in mitochondrial metabolism triggered by a local perturbation.
Cardiomyocytes loaded with TMRE (ΔΨₘ indicator) and CM-H₂DCFDA (ROS-sensitive) (37°C). (A) Montage of TMRE and CM-DCF images immediately before and after the local laser flash. An 8.7 x 8.7 µm region of the cell was excited in a single flash given between the third and fourth image, resulting in rapid loss of ΔΨₘ and local generation of ROS (images in all figures have fluorescence intensity scaled in pseudocolor as shown, unless otherwise indicated; a.u.: arbitrary intensity units). The frame interval in panel A is 3.5s. (B) Time-line images of TMRE and CM-DCF (see Methods), created by analyzing a line drawn along the longitudinal axis of the cell (shown in panel A, leftmost images). Repetitive depolarizations of ΔΨₘ are evident as vertical blue bands (TMRE). Increased ROS generation is indicated by increased CM-DCF fluorescence (enhanced yellow-orange color), which is particularly evident during mitochondrial depolarizations. Brackets point out the flashed region. (C, D) Montage and time-line image, respectively, of an unflashed control from the same field as the flashed one. Bracket points out the nucleus (N).

**Figure 2.** Quantitative characterization of cell-wide mitochondrial oscillations.
(A) The asterisk on top of the TMRE time-line image shown in panel B of Figure 1 points out the cycle of ΔΨₘ depolarization and repolarization expanded as an image montage in panel (A). The frame interval in panel A is 7s. (B) Quantitative analysis of the fraction of polarized mitochondria from 2D TMRE images as a % of the total cell area (see Methods). Arrow indicates the timing of the flash. The trace labeled “Control” refers to a cell not exposed to a flash in the same microscopic field. (C) Simultaneous recordings of the temporal evolution of fluorescence intensity for TMRE, CM-DCF and the endogenous NADH signals in the flashed region before and after the laser flash (arrow). (D) Time course of average whole-cell fluorescence of TMRE and NADH and (E) CM-DCF and the derivative of CM-DCF signals (dF/dt, purple). The precise phase relationship between
all signals can be clearly appreciated from the vertical reference line drawn. The plots were from a different cell than that shown in panel A.

**Figure 3.** Threshold of ROS required for cell-wide mitochondrial oscillations.

CM-DCF fluorescence normalized to initial intensity (F/F₀) for flashed (A) or unflashed (B), cardiomyocytes, in which oscillations in ∆Ψₘ were initiated only when F/F₀ increased by more than 20% (dashed line) over the duration of the experiment. Color-coded arrows refer to the time of the first depolarization of ∆Ψₘ for the CM-DCF traces in a given experiment.

**Figure 4.** Effects of inhibitors of ROS production or scavengers on cell-wide mitochondrial oscillations.

Conditions as described in Figure 1. (A) Scheme of the possible pathway of ROS generation in the electron transport chain (adapted from (60)). The protonmotive Q-cycle of cytochrome bc1 (complex III) is also shown explicitly, along with the sites of action of its two inhibitors, myxothiazol (Myx) and antimycin (Ant). In this branched pathway of electron transfer, the ubisemiquinone anion (UQ⁻) can be generated from ubiquinol (UQH₂) at the periplasmic (UQₚ) or matrix (UQₘ) faces, respectively, of the mitochondrial membrane. High ROS production in Ant is due to accumulation of the precursors of O₂⁻, UQₚ and UQₘ. UQₚ anion reduces the b₅₆₆ and b₅₆₂ hemes, as indicated by arrows through the center of the Q-cycle, while reduction of the FeSIII center is required for the oxidation of UQH₂ to UQₚ (25,41). (B,C) Time-line images of TMRE for typical experiments of cells acutely exposed to rotenone (B, 15 µM) or antimycin A (C, 6 µM), respectively. Thick line above panels indicates presence of inhibitor. (D) TMRE traces of myocytes showing cell-wide mitochondrial oscillations after a laser flash and, subsequently, being subjected to an acute addition of rotenone (15 µM, Rot) (see also B), myxothiazol (5 µM, Myx), NaCN (5 mM, CN⁻) or bongkrekic acid (25 µM, BKA) while the image acquisition was briefly paused. After imaging
resumed (within ~3 min of inhibitor addition), the oscillations were abolished. Thick line in each panel indicates presence of inhibitor. (E) The effect of the different inhibitors on ROS production as measured by the normalized CM-DCF signal per unit time. Antimycin (Ant) and oligomycin (Olig) concentrations were 6 µM and 10 µg/ml whereas the other inhibitor concentrations were as described in (D). ROS production following a flash was quantified for cells preincubated in the presence of each inhibitor. Under these conditions, the cells did not exhibit oscillations after the laser flash. Each bar of the normalized CM-DCF signal corresponds to n=6 cells, obtained from two independent cell preparations. The n value exceeds that required according to retrospective power calculations based on the p value and the actual differences between the control and treatment means. *p<0.05, **p<0.01, ***p<0.001 vs. control. (F) The SOD mimetic TMPyP was acutely added to myocytes at a final concentration of 250 or 500 µM. Prolonged incubation (1h) with the scavenger completely suppressed the synchronized oscillations (n=10, 3 experiments). The control correspond to cells exhibiting cell-wide mitochondrial oscillations before addition of the scavenger. The time-dependent effect of TMPyP to suppress oscillations correlated with a significant decrease of ROS production to basal levels (not shown).

Figure 5. Experimental evidence showing the lack of involvement of the PTP.

Conditions as described in Figure 1. (A) Myocytes displaying flash-induced mitochondrial oscillations were subjected to an acute addition (top panel) or 2 h preincubation (lower panel) with 1 µM CsA. (B) Myocyte labeled with Cbx-DCF before (left panel) and after (right panel) the laser flash (white arrowhead) highlighting the mitochondrial localization of Cbx-DCF (green pseudocolor) after depolarization of ΔΨ_m; the TMRE probe localization (before depolarization) is shown in the right panel. The white bar corresponds to 20 µm. (C) Representative surface plot (n=10, 2 experiments) of a zoomed region of the mitochondrial network labeled with TMRE (top panels) and calcein (lower panels), before (left panels) and after (right panels) ΔΨ_m depolarization.
Arrows point to a mitochondrial cluster before and after $\Delta \Psi_m$ depolarization. Notice that calcein fluorescence stays in the mitochondria even after $\Delta \Psi_m$ depolarization. The white bar corresponds to 5 µm. Fluorescence in Cbx-DCF (B) and calcein (C) images is pseudocolor-scaled, where fluorescence below a threshold is shown in red and above the threshold is shown in yellow-green.

**Figure 6.** Blockage of mitochondrial inner membrane anion channels abolishes oscillations. Conditions as described in Figure 1. Either acute addition (not shown) or preincubation with PK11195 (panel A; 50 µM) or DIDS (panel C; 100 µM) abolished whole-cell mitochondrial oscillations triggered by a flash, but did not prevent local depolarization of $\Delta \Psi_m$ and ROS production in the flashed region, as shown in representative time-line images of TMRE (n=4) or CM-DCF (n=4) fluorescence. The time course plots of TMRE (red) and CM-DCF (green) demonstrate that matrix ROS still increases markedly during mitochondrial depolarization of the flashed region in the presence of PK11195 (B) or DIDS (D). (E) shows close-up views of $\Delta \Psi_m$ and CM-DCF distributions and their corresponding surface plots in the flashed and neighboring regions in the presence of the inhibitors. Fluorescence of CM-DCF remained localized within the mitochondria of the flashed region without affecting neighboring areas. PK11195 or DIDS had no apparent effect on unflashed controls (not shown). Brackets point out the flashed region. Prior to each experiment carried out in the presence of inhibitors, myocytes from the same cell isolation showed flash-triggered oscillations in $\Delta \Psi_m$ (not shown).

(F) The acute effect of 4′-chlorodiazepam (32 µM, left panel) on mitochondrial $\Delta \Psi_m$ is shown. Preincubation with 64 µM 4′-chlorodiazepam completely inhibited the flash-induced $\Delta \Psi_m$ depolarizations (n=10). Preincubation with the agonist FGIN-I-27 (92 µM, right panel) provoked permanent mitochondrial $\Delta \Psi_m$ depolarization in 80% of the flashed cells (n=10). Abbreviation: 40ChlDZP, 4′-chlorodiazepam.
Figure 7. Effects of mitochondrial oscillation on the electrical excitability of the cardiomyocyte.

(A) Action potentials (upper panel) evoked by brief current injections were recorded in current-clamp mode during whole-cell patch clamp while simultaneously imaging $\Delta \Psi_m$ with TMRE (lower panel). During a synchronized cell-wide depolarization-repolarization cycle, the action potential shortened in synchrony with fast mitochondrial depolarization, and the cell became inexcitable in the fully depolarized state (remaining upward spikes are from the stimulus only). Recovery of $\Delta \Psi_m$ coincided with restoration of the action potential. (B) Correlation between the action potential duration at 90% repolarization and $\Delta \Psi_m$. (C) Voltage-clamp ramps demonstrate that the current-voltage relationship of the oscillatory membrane current fits the profile of the sarcolemmal $K_{\text{ATP}}$ current (green trace: mitochondria polarized, red trace: mitochondria depolarized). (D) Correlation between sarcolemmal current measured at 0 mV (from voltage ramps) and $\Delta \Psi_m$.

Figure 8. Hypothetical scheme of the ROS-induced ROS release mechanism underlying the synchronization of mitochondrial activity during the oscillations.

According to the present experimental findings, the main chain of mitochondrial processes involved in ROS production and synchronization of mitochondrial activity are depicted (see discussion for further explanation).
Fig. 1. Aon et al., 2003
Fig. 2. Aon et al., 2003
Fig. 3. Aon et al., 2003
Fig. 4. Aon et al., 2003
Fig. 5 Aon et al., 2003
Fig. 6. Aon et al., 2003
Fig. 7. Aon et al., 2003
Fig. 8. Aon et al., 2003
Online supplemental material

Experimental Procedures

Fluorescence energy transfer between CM-DCF and TMRE. The two main requirements for the existence of FRET are that the fluorophores are in close proximity (<0.1 nm) and that the excitation spectrum of the energy acceptor must overlap the emission spectrum of the energy donor (1). The latter is indeed the case for the fluorescein/rhodamine pair. A decline in the CM-DCF signal was not expected due to the irreversibility of the oxidation of the probe, but was observed to correspond with TMRE reuptake into the mitochondria in some experiments. Thus, we tested for the existence of FRET between TMRE and CM-DCF by incubating cells with 6 µM CM-H2DCFH in the absence of TMRE (Fig. S1). In the absence of the acceptor fluorophore, TMRE, the CM-DCF signal increased in a stepwise manner with no declining phase (Fig. S1A, top panel) during metabolic oscillation (tracked by oxidation of the NADH pool), demonstrating that TMRE redistribution was responsible for the biphasic CM-DCF signal.

The serendipitous presence of FRET allowed us to extract additional information about the distribution of the fluorophores and sequelae of events occurring during mitochondrial oscillation. As shown in the experiment of Figure S1A (lower panel; frame sampling every 1.5 s instead of 3.5 s), at the onset of an oscillation the TMRE signal sharply increases. This could be interpreted as enhanced FRET in response to an early increase in ROS production just prior to the collapse of ∆Ψm (Fig. S1B: arrow 1; the alternative explanation that ∆Ψm was hyperpolarizing is less likely, since this transient was not typically observed in the absence of CM-DCF). During the ensuing TMRE loss from the matrix, CM-DCF fluorescence increases as a consequence of both additional
ROS production and relief of FRET until a maximum is attained (Fig. S1B: arrow 2). ROS production eventually slows in the depolarized state as the source of electrons (NADH) is depleted (Fig. S1B: arrow 3). During repolarization, the process then reverses as the TMRE reenters the matrix, and the CM-DCF signal declines since its fluorescence energy is transferred to the acceptor (Fig. S1B: arrow 4).

During large $\Delta \Psi_m$ depolarization, the exit of TMRE from mitochondria into the cytoplasm leads to quenching of the dye by an incompletely understood mechanism. The rapid redistribution of TMRE into the cytoplasm after depolarization of $\Delta \Psi_m$ can be transiently detected in the nucleus (used as a convenient location to measure the non-mitochondrial intracellular component) (Fig. S2).

In a control experiment, we triggered whole cell mitochondrial oscillations with a laser flash in isolated cardiomyocytes in the absence of TMRE and CM-DCF. The rationale of this control was to demonstrate that the observed oscillations are due to intrinsic metabolic mechanisms and rule out the putative role of artifacts due to the photosensitizing effects of the fluorophores. Figure S3 depicts the whole cell oscillations in NADH after a laser flash.

Figure S4 illustrates the absence of an effect of Ruthenium Red or Thapsigargin on the flash-triggered whole cell mitochondrial $\Delta \Psi_m$ oscillations.

The link activated by Figure S5 shows a movie of a typical experiment of a flash-induced whole cell mitochondrial oscillations.

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Legends for supplementary figures

**Figure S1.** Fluorescence resonance energy transfer between TMRE and CM-DCF, and the relationship between mitochondrial ROS production and ∆Ψ\textsubscript{m} depolarization. Cardiomyocytes loaded with TMRE (ΔΨ\textsubscript{m} indicator) and CM-H\textsubscript{2}DCFDA (ROS-sensitive) (37°C). (A, top panel) Cardiomyocytes loaded with CM-H\textsubscript{2}DCFH in the absence of TMRE. Cell-wide mitochondrial depolarizations, triggered after a flash, are evident as step increases in CM-DCF fluorescence (green line), or as a peak in the first derivative of the raw CM-DCF signal (black line). After the rapid rise in CM-DCF fluorescence, the signal plateaus until the next burst of ROS; no decline of the fluorescence is observed in the absence of TMRE. (B) The first derivative of TMRE (red) and CM-DCF (green) signals (from A, lower panel) monitored during mitochondrial oscillations by faster scanning (1.5 instead of 3.5s). A sharp peak in TMRE fluorescence was observed prior to the fast depolarization phase (A, lower panel; and labeled as peak 1 in B). See text for further explanation and the meaning of numbered arrows. Arrowheads point out the timing of the flash.

**Figure S2.** TMRE redistribution from mitochondria into the nucleus of a myocyte during mitochondrial ∆Ψ\textsubscript{m} depolarization.

The figure shows the dynamics of mitochondrial and nuclear TMRE simultaneously during depolarization of ∆Ψ\textsubscript{m} in a cell showing sustained mitochondrial oscillations (see movie in Fig. S5 below). Notice the spike of TMRE in the nucleus before quenching when mitochondrial ∆Ψ\textsubscript{m} depolarizes and releases the probe.
**Figure S3.** Flashed-triggered whole cell oscillations in NADH in an isolated cardiomyocyte in the absence of TMRE and CM-DCF. The arrow indicates the time at which the laser flash was given.

**Figure S4.** Lack of effect of inhibitors of sarcoplasmic reticulum or mitochondrial Ca\(^{2+}\) handling, on mitochondrial oscillations.

Cells were preincubated with 110 μM ruthenium red or 10 μM thapsigargin and examined for flashed-induced mitochondrial oscillations at the times indicated. Neither of the compounds prevented or interrupted the mitochondrial oscillations. The apparent changes in the oscillation period observed in the presence of the inhibitors were within the observed statistical variability (the oscillation period of 104±5 s exhibited a coefficient of variation of 43% in 10 independent experiments). Controls refer to cells exhibiting oscillations in the absence of inhibitors.

**Figure S5.** Global transitions of mitochondrial energetics triggered by local perturbation of mitochondrial function.

The linked movie is an accelerated playback of a typical experiment in which oscillations of ΔΨ\(_m\), ROS and NADH were triggered by a localized laser flash (given at 42 sec), evident as a loss of TMRE signal and increase in ROS in a square near the middle of the cell. The first global depolarization of ΔΨ\(_m\) is observed at approximately 360 sec followed by several self-sustained oscillations.
Fig. S1 Supplement. Aon et al., 2003
Fig. S2 Supplement. Aon et al., 2003
Fig. S3. Supplement. Aon et al., 2003
Ruthenium red

Thapsigargin

Fig. S4. Supplement. Aon et al., 2003
Fig. S5. Supplement. Aon et al., 2003
Synchronized whole-cell oscillations in mitochondrial metabolism triggered by a local release of reactive oxygen species in cardiac myocytes
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