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Review

Mitochondrial ROS-induced ROS release: An update and review

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Abstract

Unstable mitochondrial membrane potential and redox transitions can occur following insults including ischemia/reperfusion injury and toxin exposure, with negative consequences for mitochondrial integrity and cellular survival. These transitions can involve mechanisms such as the recently described process, "Reactive Oxygen Species (ROS)-induced ROS-release" (RIRR), and be generated by circuits where the mitochondrial permeability transition (MPT) pore and the inner membrane anion channel (IMAC) are involved. The exposure to excessive oxidative stress results in an increase in ROS reaching a threshold level that triggers the opening of one of the requisite mitochondrial channels. In turn, this leads to the simultaneous collapse of the mitochondrial membrane potential and a transient increased ROS generation by the electron transfer chain. Generated ROS can be released into cytosol and trigger RIRR in neighboring mitochondria. This mitochondrion-to-mitochondrion ROS-signaling constitutes a positive feedback mechanism for enhanced ROS production leading to potentially significant mitochondrial and cellular injury. This review and update considers a variety of RIRR mechanisms (involving MPT, IMAC and episodes of mitochondrial transient hyperpolarization). RIRR could be a general cell biology phenomenon relevant to the processes of programmed mitochondrial destruction and cell death, and may contribute to other mechanisms of post-ischemic pathologies, including arrhythmias.
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Keywords: Oxidative stress; Membrane potential; Permeability transition pore; Redox; Cardiac myocytes; Oscillation

1. Introduction

Unstable mitochondrial membrane potential ($\Delta\psi$) and redox transitions can occur as a result of diverse pathological states such as ischemia/reperfusion injury and toxin exposure, and can have negative consequences for mitochondrial integrity and cellular survival [1–4]. These transitions have been described and can occur via mechanisms involving the recently described process named, "Reactive Oxygen Species" (ROS)-induced ROS release (RIRR) [2]. RIRR is generated by circuits requiring mitochondrial membrane channels including the mitochondrial permeability transition (MPT) pore [5–7] and the inner membrane anion channel (IMAC) [8]. Under conditions that lead to RIRR, such as the exposure to an excessive oxidant stress burden, the increase in ROS reaches a threshold level that triggers the opening of one of the requisite mitochondrial channels (i.e., MPT pore or IMAC), which in turn leads to the simultaneous collapse of mitochondrial $\Delta\psi$ and a transient increased ROS generation by the electron transfer chain (Fig. 1) [2]. Release of this ROS burst to the cytosol could potentially function as a "second messenger" to activate RIRR in neighboring mitochondria. Thus, mitochondrion-to-mitochondrion RIRR constitutes a positive feedback mechanism for enhanced ROS production leading to potentially significant mitochondrial and cellular injury.

The discovery of the RIRR phenomenon to some extent modifies the "dogma" that mitochondrial ROS production is exponentially increased as $\Delta\psi$ rises [9,10]. Although the latter concept is correct in this context, proving its validity in a major number of cases, the recent data confirmed that mitochondria are also capable of producing a massive ROS release (at least transiently) when $\Delta\psi$ is nulled after MPT pore opening. That means that mitochondrial ROS can be generated due to some

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processes occurring together with the MPT when the components of the mitochondrial respiratory chain are going from the reduced to the oxidized state. Apparently, the source of the ROS burst involves the diversion of electrons from the electron transport chain. According to our data, oxidation of NAD(P)H (indicating the redox state of Complex I) lags seconds behind the $\Delta\psi$ loss (Fig. 2) [2], which is compatible with the time course of the ROS burst (representing single-electron donation to oxygen) and the cessation of normal flow of respiratory chain electrons. This striking conclusion that ROS can be generated after MPT induction has also recently been confirmed in vitro. Once isolated mitochondria were supplemented with NADH, MPT induction resulted in a massive ROS release [11]. Leak of NADH from *isolated* mitochondria due to the MPT pore opening explains the lack of such findings in earlier experiments. Fontaine and co-workers [11] suggested that apparent conformational changes in mitochondria (specifically, in Complex I) under conditions of the megachannel activation [12,13] were involved. The detailed mechanism of this phenomenon, however, remains to be elucidated.

The real importance of RIRR is obvious, considering the relevance of the MPT phenomenon to the process of programmed mitochondrial destruction and cell death (mitoptosis and apoptosis) [14–17] when mitochondria may become a significant generator of pathological amounts of ROS which likely play a key role in cell death. In addition to the many known mitochondrial functions (reviewed in [18]), mitochondria can serve the critical role of a cellular "signal amplifier" when a low amount of ROS in or around mitochondria can be significantly amplified by mitochondria and converted into a pathological ROS signal. Previously, mitochondria have been considered to be excitable organelles capable of generating and conveying electrical [19] and Ca^{2+} [20] signals. The RIRR phenomenon extends this important excitable organelle function to include the ability to modulate and amplify ROS signaling. In this review we will provide perspectives concerning the varieties of RIRR mechanisms including those involving: (1) the MPT pore; (2) the IMAC; and (3) episodes of mitochondrial transient or "flickering" hyperpolarization. We will discuss the possible importance and consequences of RIRR and MPT induction in cell physiology and cell death.

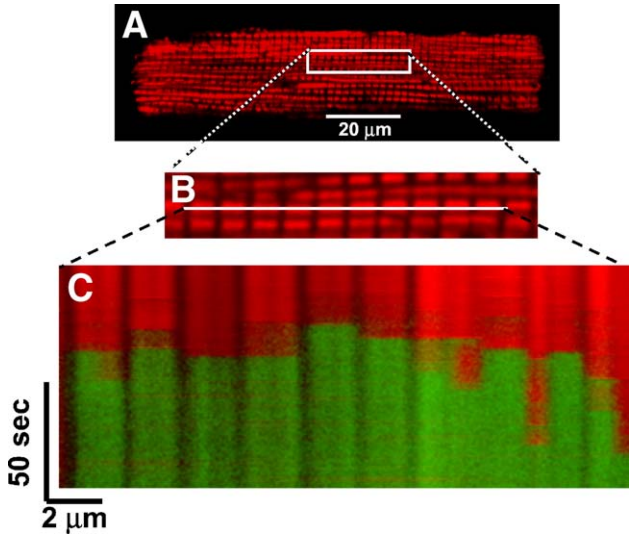

Fig. 1. ROS-induced ROS-release (RIRR) and MPT induction. Confocal microscope fluorescence imaging of cardiac myocytes loaded with $\Delta\psi$ probe, tetramethylrhodamine methyl ester (125 nM TMRM; RED) and ROS probe, 2,7 dichlorodihydrofluorescein diacetate (10 μM DCF, GREEN). (A) Upper panel, fluorescence image (Ex 543 nm, Em >560 nm) of TMRM loaded cardiac myocyte with energized mitochondria arranged in a regular lattice-like array; the box depicts the region enlarged in (B). (B) Enlarged portion of the TMRM loaded cardiac myocyte. Line drawn on image shows position scanned for experiment in bottom panel. (C) Row of mitochondria were line-scan imaged at 2 Hz with excitation at both 488 for DCF, and 543 nm for TMRM, and collecting simultaneous fluorescence emission at 510–550 nm and >560 nm, respectively. The sudden dissipation of TMRM fluorescence indicates loss of mitochondrial potential, $\Delta\psi$. ROS generation in each mitochondrion beginning at the moment of $\Delta\psi$ loss is indicated by the increase in DCF fluorescence. For details, see [2].

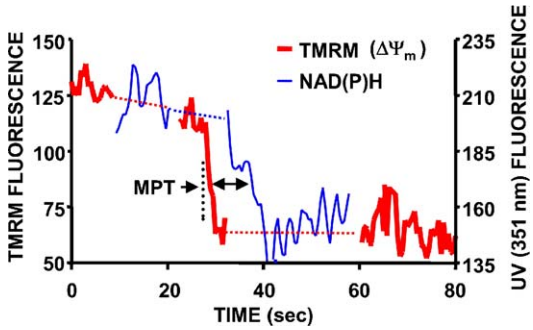

Fig. 2. Relationship between $\Delta\psi$ and NAD(P) redox state during the MPT. $\Delta\psi$ and the MPT are assessed by changes in the TMRM fluorescence (red trace) and the intrinsic NAD(P)H autofluorescence (blue trace) acquired simultaneously during 2 Hz line-scan imaging. (Adapted from [2]).

2. MPT pore-associated RIRR

The main mitochondrial ROS generators are located in the inner membrane (Complexes I–III) [21–24], in the matrix (dehydrogenases) [25] and in the outer membrane (monoamine oxidase) [26]. Mitochondrial NO synthase, like all other constitutional isoforms (***n***, ***e*** and ***i***) of NO synthase, is probably also able to generate superoxide anion radical [27–29]. Apart from enzymatic ROS formation, mitochondria can generate ROS non-enzymatically (i.e., via Fenton reaction). The determination of the level of contribution of each mitochondrial compartment to the net ROS release phenomenon is a difficult task to decipher because of the extremely complicated regulation of each process by a variety of environmental factors.

The RIRR phenomenon likely originates from the mitochondrial inner membrane since it can be significantly reduced by the inhibitor of the electron transfer chain, rotenone [2,30]. The original demonstration of RIRR was via the photodynamically-induced mitochondrial depolarization in cardiac myocytes loaded with mitochondrial cationic fluorescent probes such as TMRM (Fig. 1) [2]. Under these conditions RIRR occurs when controlled, incremental photo-excitation produced ROS in mitochondria is converted to a pathological self-amplified ROS burst. We hypothesized that RIRR is a general cell biology mechanism and is independent of the signaling ROS source [2].

The accumulated exposure to ROS leads to an oxidant stress burden in mitochondria that can reach a threshold level capable of inducing the MPT pore which we described as the "MPT ROS threshold" [1]. We presented several lines of evidence to prove that the observed depolarization is the result of the MPT: (1) the efflux rate from mitochondria of the $\Delta\psi_m$ probe, TMRM, upon MPT induction is extremely rapid and rate-limited by its diffusion; (2) the depolarization as well as the large-amplitude ROS burst are prevented by pretreatment with the MPT inhibitor, bongkrekic acid; (3) the fluorescent marker molecule, calcein, enters the mitochondrial matrix coincident with the very beginning of the depolarization phase; (4) significant, rapid mitochondrial swelling can be observed in situ and in vitro [1,2]. In contrast to the conventional belief (based on data obtained in in vitro models using isolated mitochondria) that Ca^{2+} is an obligatory factor involved in MPT induction which is delayed by cyclosporine A, neither of these two were found to be direct MPT effectors in an in situ model (in isolated cardiac myocytes with photo-dynamic MPT induction) [1,2,30]. Indeed, we showed that elevating Ca^{2+} to >micromolar levels does *not* open the MPT pore (nor alter its ROS threshold) in intact cardiac myocytes, whereas permeabilized cardiomyocytes as well as isolated mitochondria readily underwent MPT induction at submicromolar Ca^{2+} levels (see [1]; online Supplement).

RIRR likely contributes both to the spatial propagation of mitochondrial depolarization and to the programmed cell death. We observed that the myocyte death is correlated to the number of mitochondria undergoing MPT-induction and that scavenging both the triggering and propagation ROS by an α-tocopherol derivative, Trolox, prevented MPT induction and reduced cell death [1].

3. IMAC-associated RIRR

We have demonstrated that instability of the intracellular redox state may cause the oscillation of the mitochondrial $\Delta\psi$ [2] (as an example, see Fig. 3 A, B). The propagation model of RIRR has also been extensively studied in B. O'Rourke's lab [3,4,31–33]. These oscillations represent the pathological spreading of mitochondrially-generated ROS over the chain of closely situated mitochondria which forms a 3-dimensional lattice of ROS-oscillators involving RIRR that could potentially cover the entire intracellular volume, and may even extend

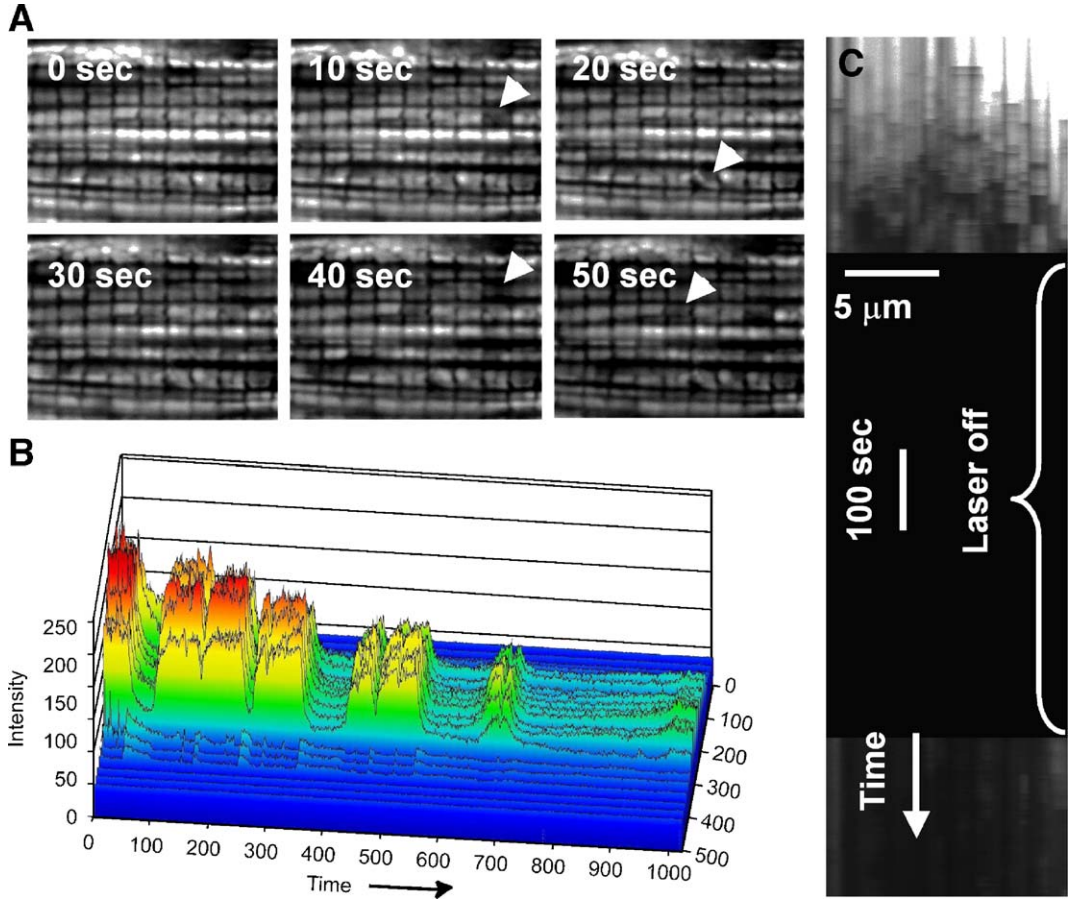

Fig. 3. Oscillations of $\Delta\psi$ in individual mitochondria over time in a portion of a cardiac myocyte induced by 10 μM clotrimazole. (A) Panel of consecutive fluorescence frames with an interval of 10 s acquired from a 18×15 μm region of a cardiac myocyte. Arrows indicate individual mitochondria or mitochondrial clusters displaying periodic changes in the membrane potential. Myocytes loaded with TMRM were line-scanned with the laser excitation light at 543 nM and emission >560 nM. (B) 3-dimensional presentation of the mitochondrial membrane potential oscillations shows different kinetics of the rise and the drop of the membrane potential. (C) Discontinuation of the excitation light after the oscillations are triggered (off during the time shown as the black rectangle in the image) does not have any effect on the oscillatory behavior once it has been initiated.

beyond the boundaries of individual cells. A local area of photo-induced ROS generation inside cardiac myocytes caused propagation of mitochondrial depolarizations in the cells which did not involve significant changes in global Ca^{2+} homeostasis. While the local photo-dynamically-induced RIRR process is linked to MPT induction in that local region [1,31], the propagation of the depolarization outside the original zone of photo-activation was prevented by IMAC inhibitors indicating that the mitochondrial ROS release there occurs through IMAC [31]. These authors introduce the application of "percolation" and "criticality" theory where the synchronization of ROS-release events can occur in a spanning cluster of oxidatively stressed mitochondria (i.e., ROS being the key messenger) [3,32]. The critical threshold when the mitochondrial network transforms into an oscillating regime is determined by the sensitivity of mitochondria to external ROS (e.g., via levels of endogenous ROS scavengers [33], etc.). In turn, this may have a strong impact on the electric excitability of the cell through "destabilization of the action potential (AP)" and in particular, during ischemia, may lead to AP shortening: in cardiac myocytes undergoing the stress of the ischemia/reperfusion transition, these mitochondrial oscillations may cause pathological disturbances in excitability in the whole heart and potentially initiate deadly cardiac arrhythmias [3,4]. This process could contribute to the mechanisms of post-ischemic arrhythmias pointing to the underlying critical role of mitochondria and RIRR. Recently, the same group [3] determined an important role of a peripheral (mitochondrial) benzodiazepine receptor [3,34,35] modulating the IMAC channel implicated in some forms of RIRR in cardiac myocytes. Thus, preventing RIRR via peripheral mitochondrial benzodiazepine receptor inhibition could alleviate these destabilizing effects on the AP and reduce the risk of arrhythmias.

4. RIRR mechanisms: instability beyond photo-dynamic triggering

In studies devised to reveal the nature of *unstable* mitochondrial $\Delta\psi$ oscillations in cardiac myocytes, we have tested a wide range of compounds which have lead us to the conclusion that these oscillations are dependent on ROS release requiring the MPT. The kinetics of these fluctuations (instant discharge with the transient rapid $\Delta\psi$ rise (Fig. 3), as well as the sensitivity of the MPT-related RIRR to bongkrekic acid provide evidence for the proposed mechanism. Similar photo-induced $\Delta\psi$ fluctuations were also detected in cultured astrocytes [36]. These unstable mitochondrial $\Delta\psi$ oscillations were significantly retarded by cyclosporine A, bongkrekic acid, and ligands of the mitochondrial benzodiazepine receptor (PK 11195 and Ro5-4864) [36,37]. Comparable MPT oscillations were also demonstrated in suspensions of isolated mitochondria [38]. In cells, occasionally (see below) the MPT-related RIRR process can initiate in the mitochondrial network a self-organized oscillator (repetitive $\Delta\psi$ oscillations) which sustains itself even after removal of the photo-excitation trigger (i.e., discontinuation of the laser excitation), and finally terminates by an apparently irreversible MPT (Fig. 3C). In cardiac myocytes, we observed mitochondrial $\Delta\psi$ oscillations caused by diethylmaleate (oxidant which depletes GSH and reacts with other sulfhydryl groups [2]), clotrimazole (dissociates hexokinase from mitochondria [39]), 2,4 dinitrofluorobenzene (creatine kinase inhibitor [40]) and cytochalasin B (F-actin disrupting agent [41,42]) (see Fig. 4 A–E). All these compounds can potentially target the MPT pore protein complex [43]. Cellular injury due to reoxygenation after prolonged hypoxia causes $\Delta\psi$ fluctuations in cardiomyocytes as well (Fig. 4B).

Another important consequence of RIRR is that it can result in a higher probability of spontaneous Ca^{2+} release events from the sarcoplasmic reticulum (i.e., Ca^{2+} sparks, Fig. 5) which we explained by a possible ROS- or NO-induced Ca^{2+} release mechanism [2,44] from the Ca^{2+} stores. Since enhanced

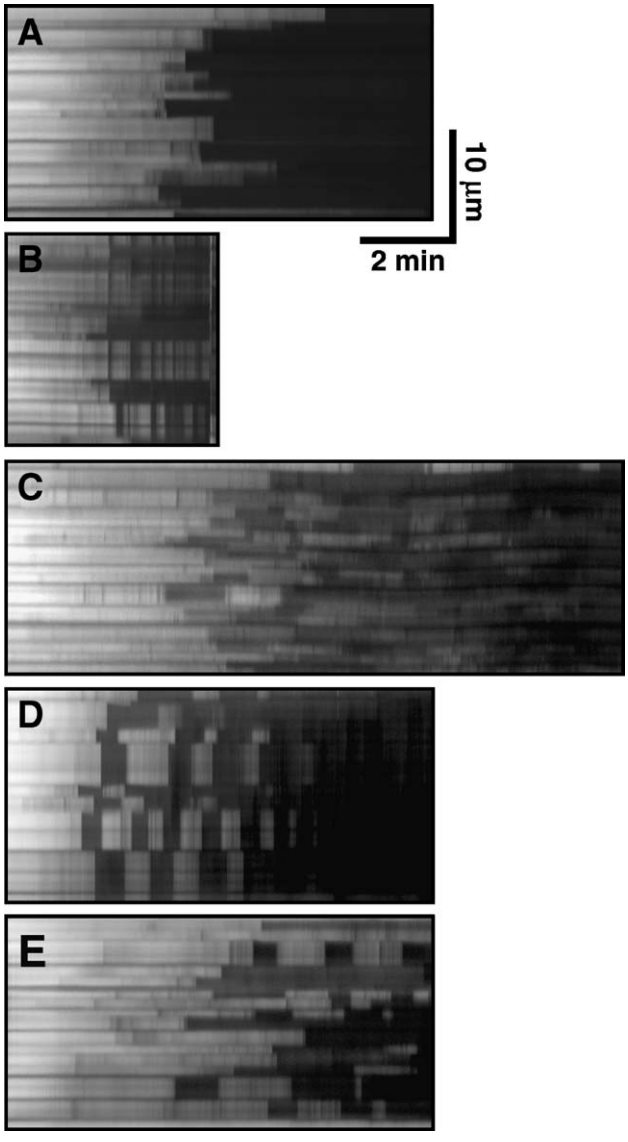

Fig. 4. Mitochondrial membrane potential oscillations in individual cardiac myocytes loaded with TMRM exposed to various compounds potentially affecting the MPT pore structure and function. (A) Control myocyte; a 22 μm line, scanned at 2 Hz. (B) Cell immediately after exposure to reoxygenation that followed a prolonged (2 h) hypoxic phase. (C) Cell treated with 10 μM dinitrofluorobenzene. (D) Cell treated with cytochalasin B. (E) Cell treated with 10 μM clotrimazole.

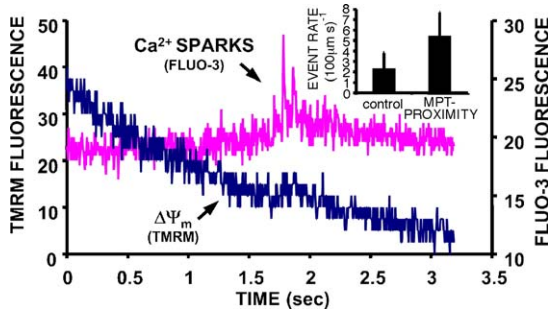

Fig. 5. Induction of Ca^{2+} sparks after MPT induction. The cardiac myocyte is dual-loaded with TMRM ($\Delta\psi$) and fluo-3 (Ca^{2+}) and line-scan imaged at 230 Hz. Representative example showing the dissipation of TMRM fluorescence from a single mitochondrion and a cluster of Ca^{2+} sparks in the immediate vicinity, within seconds of MPT induction. Inset: comparison of Ca^{2+} spark rate in proximity of MPT occurrence (adapted from [2]).

spontaneous Ca^{2+} release can potentially initiate arrhythmias [45], this is another mechanism by which RIRR may contribute to post-ischemic arrhythmias [3].

5. Mitochondrial transient-hyperpolarization-associated RIRR

The processes required for mitochondrial ROS amplification apparently reside within mitochondria themselves. As RIRR can be blocked by rotenone, it confirms the respiratory chain involvement in RIRR [2]. Quite often we observed transient mitochondrial hyperpolarization event preceding the MPT pore opening, which are generally seen as a single flicker (sometimes multiple events) during 2 Hz line-scanning (for details, see [2]). Careful analysis proved that the hyperpolarization phase, while preceding mitochondrial depolarization,

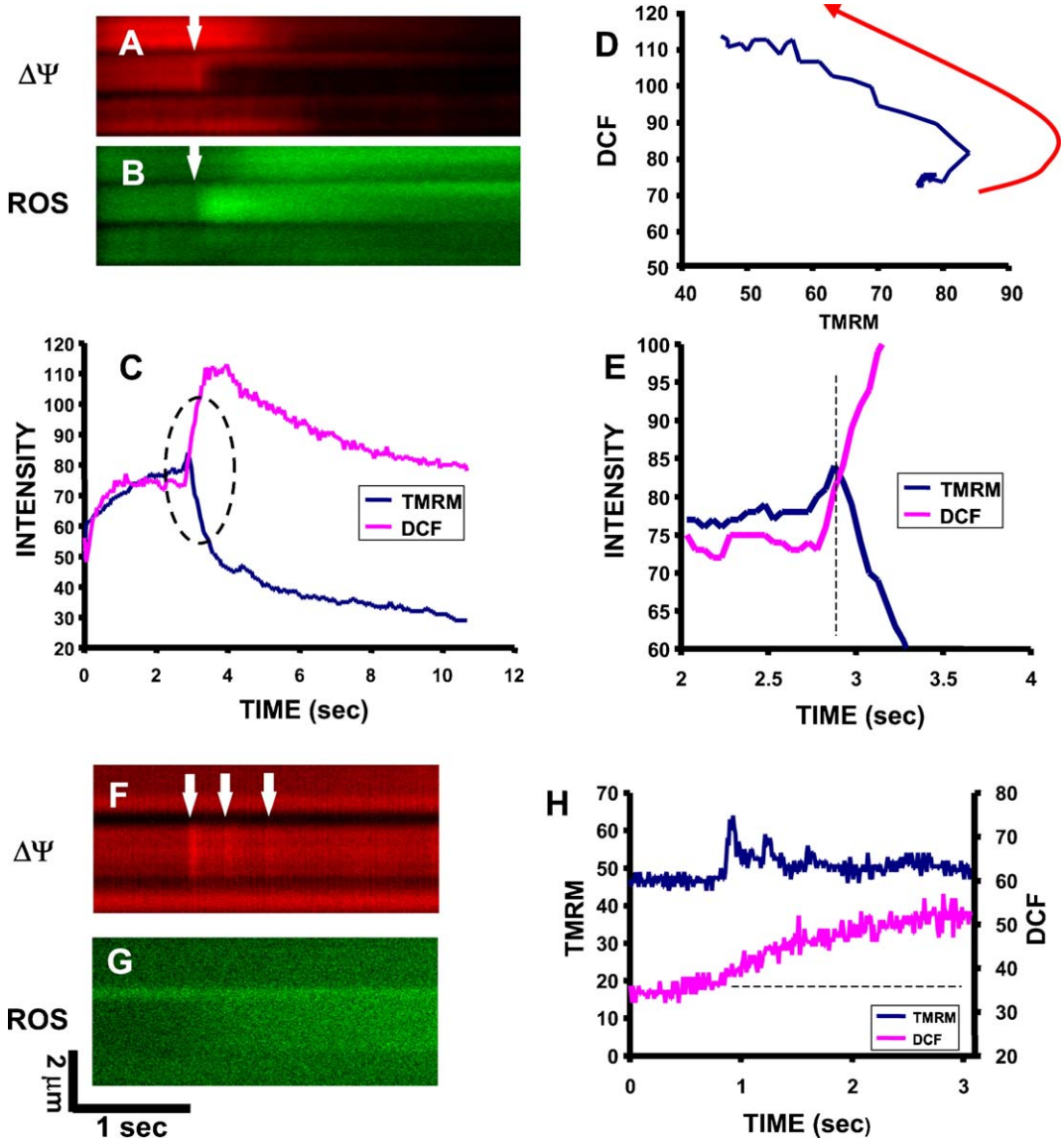

Fig. 6. Role of transient $\Delta\psi$ hyperpolarization (flickering) in RIRR. (A–B) Transient hyperpolarization precedes $\Delta\psi$ loss (A) and coincides with ROS-burst (B) in myocyte dual-loaded with TMRM (red) and DCF (green) and line-scanned at 2 Hz. Transient hyperpolarization preceding the membrane potential collapse is shown by arrow. (C) Hyperpolarization phase (oval in C, enlarged in (E)) initiates ROS burst (see phase diagram in (D); red arrow shows direction of time). (F–H) ROS production (green) occurs during transient mitochondrial hyperpolarization flickering (red); arrows in (F), a process *not* accompanied by MPT-related depolarization as in panels A–C.

coincides with the start of the excessive ROS generation (Fig. 6). Since the hyperpolarization phase is not always observed before MPT induction, it is not obligatory, but rather is a mitochondrial ROS-producing epiphenomenon of photo-excitation whose occurrence would be a likely MPT-trigger in an already unstable system. On the other hand, it is possible that this phase could be sufficiently rapid and be below the kinetic resolution of the mitochondrial probe (TMRM) response and/or substantially shorter than the 500 ms acquisition interval and thus not apparent in certain experiments. This flickering mitochondrial hyperpolarization pattern is a frequently observed phenomenon during TMRM photo-excitation in the course of line-scanning. While not every hyperpolarization flicker was able to initiate the MPT, nevertheless each flicker initiates a small additional burst of ROS production (Fig. 6 G, H). The MPT inhibitor, bongkrekic acid (by "freezing" adenine nucleotide translocator in the m-conformation), prevents the MPT in cardiac myocytes, but since it does not block flickers (Fig. 7) we have surmised that these flickers do not necessarily originate at the level of the MPT pore [2]. Fig. 8 demonstrates that during the hyperpolarization phase the neutral fluorescent molecule, calcein (mw=620), which was shown to readily enter mitochondria *after* MPT induction, in contrast is not able to enter mitochondria during the transient flicker providing additional evidence that the MPT pore remains closed during this transient hyperpolarization phase.

This transient mitochondrial hyperpolarization can potentially be explained by: (1) a transient block of electron transfer in mitochondria, (2) a transient block of the $\Delta\mu H^+$ use by endergonic reactions in the cell, (3) a transient conversion of mitochondrial ΔpH into $\Delta\psi$, or (4) a higher proton pumping activity by respiratory complexes. Of those four mechanisms, (1) is favored, and we excluded (2) and (3) since the transient hyperpolarization phase could still be observed in the presence of oligomycin and nigericin. This hyperpolarization phase also cannot be explained by an artifact such as fluorescence dequenching of the mitochondrial probe (sometimes observed when it is used in high concentration) since these events were still observable at as low as 12 nM of TMRM (Fig. 9), which is 1–2 orders less than that needed to observe fluorescence dequenching of mitochondrially-sequestered dye (reviewed in [16]). It also appears unlikely that the observed mitochondrial hyperpolarization is somehow determined by a transient change in activity of a P-glycoprotein (multi-drug-resistance) pump, for which mitochondrial dyes are substrates, since the extramitochondrial (cytosolic) TMRM signal (measured inside the nucleus in adult cardiac myocytes) remains constant. It has been proposed that a higher mitochondrial membrane potential results in a more reduced state of electron carriers responsible for single-electron leak to oxygen in turn producing the superoxide anion radical which can be converted into other ROS [9,10,46]. Regardless of the nature of the transient mitochondrial hyperpolarization phase, during this phase the triggering ROS "signal" is converting into a small, mitochondrially-generated ROS burst thus making this process another member of the family of RIRR processes.

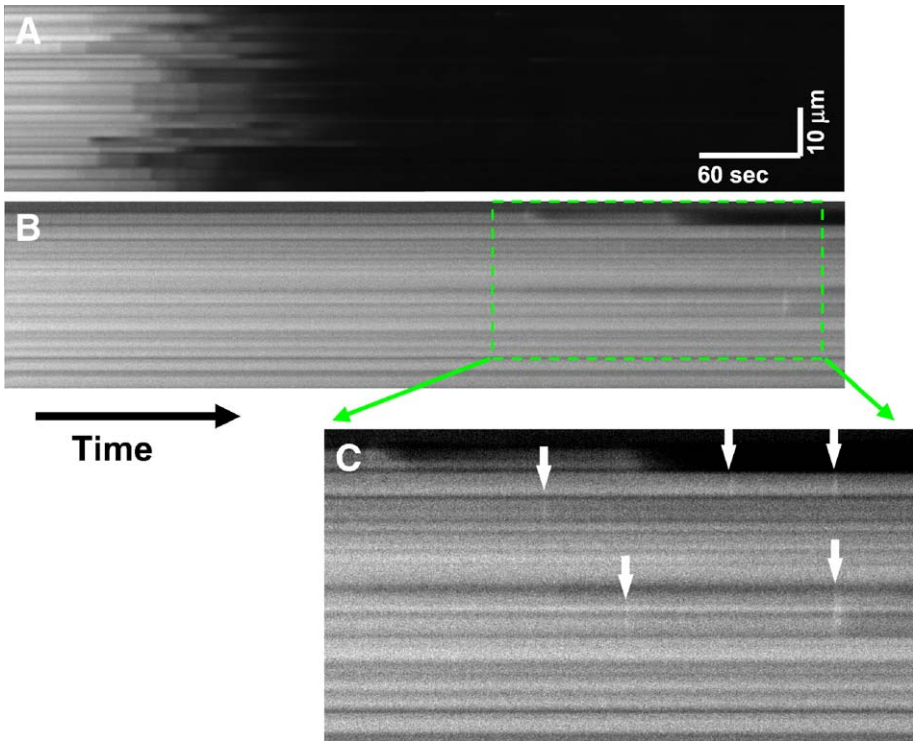

Fig. 7. Bongkrekic acid prevents MPT induction but does not prevent transient $\Delta\psi$ hyperpolarization flickering. (A) Control cardiac myocytes stained with TMRM and line-scanned along a mitochondrial row; (B) As (A) in the presence of 100 μM bongkrekic acid. (C) Zoomed area of the green box in B. Arrows show multiple $\Delta\psi$ flickering events.

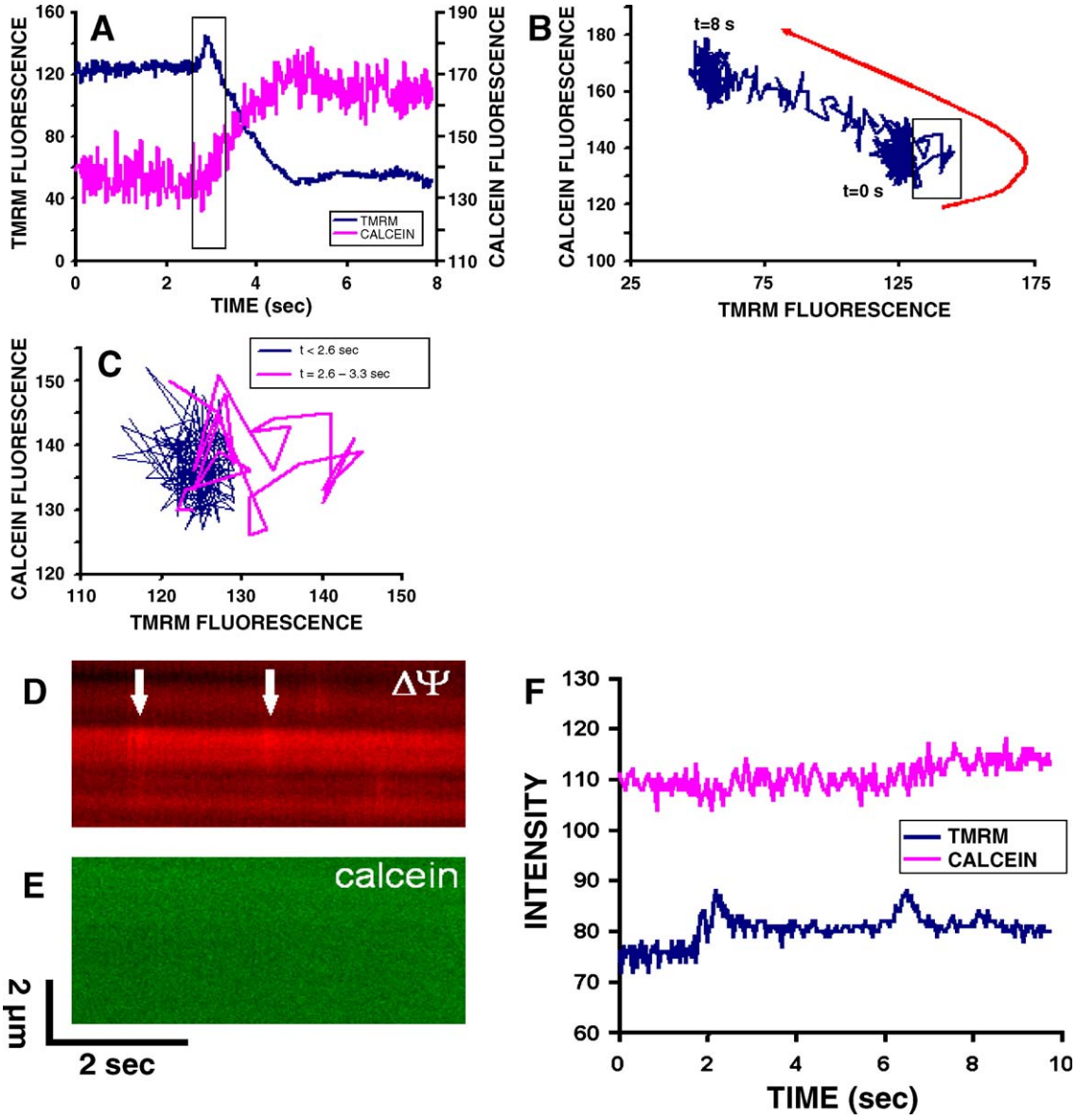

Fig. 8. Cytosolic calcein enters mitochondrion after MPT induction. (A) Cardiac myocyte dual-loaded with TMRM (inside mitochondria) and calcein-AM (localized to cytosol) and line-scanned (blue, TMRM fluorescence; pink, calcein fluorescence) showing transient $\Delta\psi$ hyperpolarization and MPT induction at ~3 s (B) Phase diagram of data in A; red arrow represents direction of time; box shows hyperpolarization phase. (C) Zoomed area of box region from the phase diagram in B; blue line represents the data before hyperpolarization occurs (<2.6 s); pink line represents the hyperpolarization phase (2.6–3.3 s) shown in the box in A. (D) A region in a line-scanned image with $\Delta\psi$ transient hyperpolarization phases. (E) Image of the calcein fluorescence corresponding to D. (F) Demonstration that calcein does not enter mitochondria during $\Delta\psi$ flickering.

Mitochondrial RIRR has been extensively studied in adult and neonatal cardiac myocytes. The adult myocyte is a good model for studying this phenomenon since mitochondria are of uniform size and are arranged in a regular lattice-like array convenient for the confocal microscopy methods we have developed [2]. Although mitochondria in neonatal cardiac myocytes, as well as in the hippocampal and granule neurons, and fibroblasts, are quite scattered in the cell, the methodological approach does work for them too, and in each case we have been able to observe RIRR. As we have discussed, the photo-excitation of mitochondria in situ with accumulated fluorescent probes results in the predictable and "controlled" MPT induction over time [1,2]. While individual mitochondria in the healthy cell normally vary to a small degree in the duration of photo-excitation needed to induce the MPT, after an external ROS insult, large mitochondrial groups often display an extended synchronous MPT pore opening pattern apparently indicating some form of direct mitochondrial communication [2,18]. The MPT ROS threshold averaged over a population of cellular mitochondria is quite characteristic reflecting the resistance of these mitochondria to oxidative stress. Based on our experiments, and by our definition, stressed mitochondria have a lower MPT ROS threshold while those resistant to

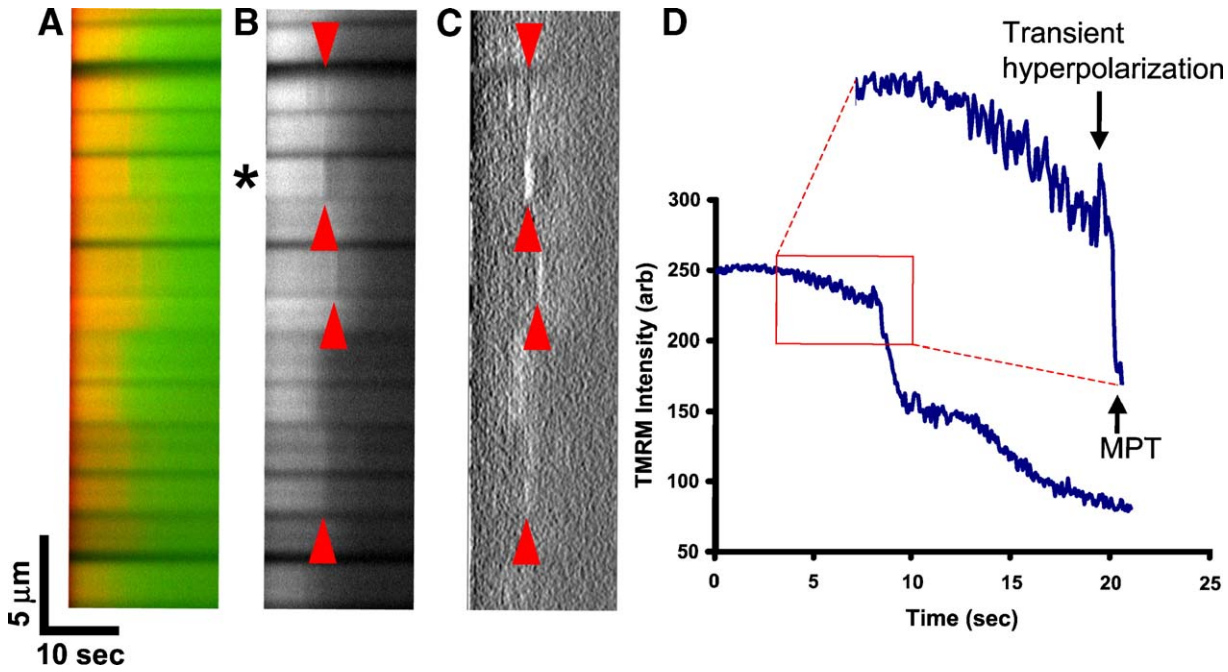

Fig. 9. Transient Δψ hyperpolarization (flickering) preceding MPT induction can be observed at extremely low levels of TMRM loading. (A) Overlay showing the 20 Hz linescan image in a cardiomyocyte loaded with 12 nM TMRM (RED) and DCF (GREEN). (B) The TMRM channel from Panel A; arrows indicate examples of transient (relative) hyperpolarization immediately preceding Δψ loss (MPT induction), as shown in Figs. 6 7 8. (C) The directional derivative (i.e., gradient) of the TMRM intensity with respect to time, to enhance the identification of intensity transients (transient hyperpolarization prior to MPT induction); arrows indicate examples of transient hyperpolarization (*positive* slope maxima) immediately preceding MPT. (D) Intensity plot of the TMRM responses of the mitochondrial pair indicated by the asterisk in Panel B; inset shows an enlargement of the area inside the red box, and shows transient (relative) hyperpolarization immediately preceding MPT.

oxidative stress have a higher threshold [1,2]. Using this approach we have characterized the deleterious effect of hypoxia/reoxygenation on mitochondria in cardiac myocytes. This method enabled us to study the regulatory mechanisms of cell survival (where mitochondria, RIRR and MPT play key role) in order to develop better therapeutic strategies for cardioprotection against pathologic injury [1,47].

Acknowledgments

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