Individual Cardiac Mitochondria Undergo Rare Transient Permeability Transition Pore Openings

Xiyuan Lu, Jennifer Q. Kwong, Jeffery D. Molkentin, Donald M. Bers

Rationale: Mitochondria produce ATP, especially critical for survival of highly aerobic cells, such as cardiac myocytes. Conversely, opening of mitochondrial high-conductance and long-lasting permeability transition pores (mPTP) causes respiratory uncoupling, mitochondrial injury, and cell death. However, low conductance and transient mPTP openings (tPTP) might limit mitochondrial Ca2+ load and be cardioprotective, but direct evidence for tPTP in cells is limited.

Objective: To directly characterize tPTP occurrence during sarcoplasmic reticulum Ca2+ release in adult cardiac myocytes.

Methods and Results: Here, we measured tPTP directly as transient drops in mitochondrial [Ca2+] ([Ca2+]mito) and membrane potential (ΔΨm) in adult cardiac myocytes during cyclic sarcoplasmic reticulum Ca release, by simultaneous live imaging of 500 to 1000 individual mitochondria. The frequency of tPTPs rose at higher [Ca2+]mito, [Ca2+], with 1 μmol/L peroxide exposure and in myocyte from failing hearts. The tPTPs were suppressed by preventing mitochondrial Ca2+ influx, by mPTP inhibitor cyclosporine A, sanglifehrin, and in cyclophilin D knockout mice. These tPTP events were 57±5 s in duration, but were rare (occurring in <0.1% of myocyte mitochondria at any moment) such that the overall energetic cost to the cell is minimal. The tPTP pore size is much smaller than for permanent mPTP, as neither Rhod-2 nor calcein (600 Da) were lost. Thus, proteins and even molecules the size of NADH (663 Da) will be retained during these tPTP.

Conclusions: We conclude that tPTP openings (MitoWinks) may be molecularly related to pathological mPTP, but are likely to be normal physiological manifestation that benefits mitochondrial (and cell) survival by allowing individual mitochondria to reset themselves with little overall energetic cost. (Circ Res. 2016;118:834-841. DOI: 10.1161/CIRCRESAHA.115.308093.)

Key Words: calcium ■ cardiac myocytes ■ cyclosporine ■ mitochondria ■ metabolism

Mitochondria sustain cellular life through energy production but also mediate programmed cell death. ATP production is mainly via cellular respiration, which is driven by the voltage gradient (ΔΨm) across the inner mitochondrial membrane that drives proton flux through the F0F1–ATP synthase, and membrane potential (ΔΨm) in adult cardiac myocytes during cyclic sarcoplasmic reticulum Ca release, by simultaneous live imaging of 500 to 1000 individual mitochondria. The frequency of tPTPs rose at higher [Ca2+]mito, [Ca2+], with 1 μmol/L peroxide exposure and in myocyte from failing hearts. The tPTPs were suppressed by preventing mitochondrial Ca2+ influx, by mPTP inhibitor cyclosporine A, sanglifehrin, and in cyclophilin D knockout mice. These tPTP events were 57±5 s in duration, but were rare (occurring in <0.1% of myocyte mitochondria at any moment) such that the overall energetic cost to the cell is minimal. The tPTP pore size is much smaller than for permanent mPTP, as neither Rhod-2 nor calcein (600 Da) were lost. Thus, proteins and even molecules the size of NADH (663 Da) will be retained during these tPTP.

The molecular identity of mPTP is unknown, but F0F1–ATP synthase dimers have been proposed as a candidate. Much work has shown that mPTP inhibition by cyclosporine A (CsA) or by genetic ablation of a critical mPTP associated protein, cyclophilin D (CypD), protects against mPTP and cell death in response to ischemia-reperfusion injury and amyotrophic lateral sclerosis. Thus, mPTP is an attractive drug target to protect against cardiac injury. However, mPTP inhibition by CsA during ischemia preconditioning abolished the protective effect of ischemia preconditioning. Moreover, chronic mPTP inhibition leads to mitochondrial Ca2+ overload and cardiac dysfunction, raising the idea that some mPTP openings might be beneficial by allowing Ca2+ release and maintenance of normal physiological mitochondrial [Ca2+] ([Ca2+]mito). But how might that occur without the pathological consequences of mPTP?
Transient Mitochondrial PTP in Cardiac Myocytes

A transient mode of mPTP opening (tPTP), with lower conductance was proposed as protective against pathological Ca\textsuperscript{2+} overload.\textsuperscript{1,13} Unlike prolonged or permanent mPTP (pPTP) openings, these could limit metabolite loss and allow full mitochondrial recovery. Until now, evidence of tPTP openings is restricted to inferences from isolated in vitro mitochondria, mostly in suspensions.\textsuperscript{13–16} Here, we continuously monitor mitochondrial recovery. Until now, evidence of tPTP openings, these could limit metabolite loss and allow full mitochondrial recovery.

To further examine these events, we used Na\textsuperscript{+}-free internal solution to inhibit mNCX. Figure 1G shows images at times \((\approx)=57\) s, do not allow solutes \(>600\) Da through (eg, not NADH) and are modulated by Ca\textsuperscript{2+}, reactive oxygen species (ROS), CypD, and CsA like larger pPTP events.\textsuperscript{17,18} MitoWinks may serve a physiological role to protect cells against mitochondrial Ca\textsuperscript{2+} overload or alleviate the cells from accumulated ROS damage.

**Methods**

Detailed Methods are available in the Online Data Supplement.

**Results**

**Transient PTP Openings in Single Mitochondria During Cyclic Sarcoplasmic Reticulum Ca\textsuperscript{2+} Release**

To simultaneously assess mPTP-mediated transient Ca\textsuperscript{2+} release events in 500 to 1000 individual mitochondria in situ, we monitored [Ca\textsuperscript{2+}]\textsubscript{mito} using Rhod-2 and 2-dimensional confocal microscopy in cardiac myocytes during spontaneous sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} releases (Figure 1A and 1B). Our previously validated method\textsuperscript{19} uses acutely saponin-permeabilized adult ventricular myocytes with physiological intracellular solutions with light Ca\textsuperscript{2+} buffering (50 \(\mu\text{mol/L EGTA}\)). Raising [Ca\textsuperscript{2+}]\textsubscript{i} to 100 \(\mu\text{mol/L}\) (Figure 1B) induces spontaneous SR Ca\textsuperscript{2+} release events (Ca\textsuperscript{2+} waves) at 5 to 15 minutes\textsuperscript{−1}, which creates physiologically relevant Ca\textsuperscript{2+} releases in a well-controlled system. Because [Ca\textsuperscript{2+}]\textsubscript{i} rises and Ca\textsuperscript{2+} waves occur [Ca\textsuperscript{2+}]\textsubscript{mito} rises with each Ca\textsuperscript{2+} wave, but [Ca\textsuperscript{2+}]\textsubscript{mito} decline (mainly via Na/Ca exchange, mNCX) is slow, allowing progressive [Ca\textsuperscript{2+}]\textsubscript{mito} rise. During this protocol individual mitochondria stochastically and suddenly released Ca\textsuperscript{2+} (Figure 1C), and these events were suppressed by the mPTP inhibitor CsA (Figure 1D) and sanglifehrin A (Online Figure II). This demonstrates mPTP-mediated events under relatively physiological conditions.

To further examine these events, we used Na\textsuperscript{-}‘free internal solution to inhibit mNCX. Figure 1E shows images at times (*) during the [Ca\textsuperscript{2+}]\textsubscript{mito} trace. Although average [Ca\textsuperscript{2+}]\textsubscript{mito} rises progressively, the highlighted mitochondrion rapidly releases Ca\textsuperscript{2+} (likely PTP opening) and \(\approx 60\) s later Ca\textsuperscript{2+} reuptake resumes. This implies PTP closure and restored \(\Delta\Psi\textsubscript{m}\) (which drives Ca\textsuperscript{2+}uptake). Figure 1G shows similar results using a different protocol, where [Ca\textsuperscript{2+}]\textsubscript{i} was raised from 0 to 2 \(\mu\text{mol/L Ca}\textsuperscript{2+}\) with [Ca\textsuperscript{2+}]\textsubscript{mito} clamped using 0.5 \(\mu\text{mol/L EGTA}\), and SR function suppressed by 5 \(\mu\text{mol/L thapsigargin}\). This is a typically used protocol, and may simulate tonic cellular Ca\textsuperscript{2+} loading. The frequency of putative tPTP events was similar for both SR release and Ca-clamp protocols, and in both cases 10 \(\mu\text{mol/L CsA}\) inhibited the events, consistent with them being tPTP openings (Figure 1F). These events are rare (\(\approx 2\times 10^{-4}\) per mitochondria per min) under basal conditions, and our ability to monitor nearly 1000 individual mitochondria continuously for 10 minutes was critical for observing these events. The average duration of these tPTP openings is \(57\pm 5\) s (Figure 1I), and this allows us to quantify that only 0.02% of the myocyte mitochondria experience this tPTP at any moment under physiological conditions (Figure 1H). This means that 99.98% of the mitochondria are busy making ATP, whereas a tiny percent might be resting or resetting (and may be consuming ATP). But this is a quantitatively negligible energetic drain for the myocyte. This agrees with inferences about tPTPs in isolated mitochondrial suspensions where individual events cannot be seen.\textsuperscript{13}

In records where we saw 64 tPTP events we also observed 54 Ca\textsuperscript{2+} release events that never recovered, and we interpret those as likely permanent PTP events (pPTP). There was some tendency for both tPTP and pPTP to occur later in the 10 minutes observation period Figure 1J), especially for pPTP (81% were in the last 4 minutes). In some myocytes we saw no tPTPs, which is a logical consequence of their stochastic rarity, because those cells showed no difference in the [Ca\textsuperscript{2+}]\textsubscript{mito} reached at the end of the protocol (Figure 1K).

**Mitochondrial Depolarization Accompanies tPTP Opening**

Brief mPTP opening should depolarize \(\Delta\Psi\textsubscript{m}\) and cause mitochondrial Ca\textsuperscript{2+} release,\textsuperscript{13,20} but it was also proposed that Ca\textsuperscript{2+} enters mitochondria via a subconductance mPTP opening associated with partial \(\Delta\Psi\textsubscript{m}\) depolarization.\textsuperscript{21}

We monitored [Ca\textsuperscript{2+}]\textsubscript{mito} (with Fluo-8 AM) and \(\Delta\Psi\textsubscript{m}\) (with tetramethylrhodamine methylester) simultaneously, during protocols as in Figure 1E. Figure 2A and 2B shows a tPTP event in which rapid \(\Delta\Psi\textsubscript{m}\) depolarization is followed by [Ca\textsuperscript{2+}]\textsubscript{mito} decline, and then when tPTP closes, proton pumping via cytochromes restores \(\Delta\Psi\textsubscript{m}\) and that allows Ca\textsuperscript{2+} uptake to resume. Thus, tPTP opening rapidly dissipates \(\Delta\Psi\textsubscript{m}\) allowing electrochemically downhill Ca\textsuperscript{2+} efflux, but the mitochondrion retains functionality, as manifest by \(\Delta\Psi\textsubscript{m}\) recovery. Moreover, simultaneous monitoring of mitochondrial redox state (FAD autofluorescence) and \(\Delta\Psi\textsubscript{m}\) (with tetramethylrhodamine methylester) show FADH\textsubscript{2} oxidation on pore opening, with gradual reduction on closure (Online Figure I). Hence, during tPTP opening, the mitochondria retain key matrix metabolites that allow respiratory recovery and repolarization once the pore closes, and this differs from pPTP. One possibility is that tPTP openings have lower pore size versus sustained pPTP openings (in which molecules of 1500 Da can permeate).\textsuperscript{13}
Figure 1. Transient permeability transition pores (PTP) openings in single mitochondria. A, Images of permeabilized cardiac myocyte loaded with Rhod-2 (red) and Fluo-4 (green; left). Scale bar, 16 μm. B, Time course of [Ca\textsuperscript{2+}]\textsubscript{mito} and [Ca\textsuperscript{2+}]\textsubscript{i} signals in the same cardiac myocyte, showing mitochondrial Ca\textsuperscript{2+} uptake (red) induced by cyclic sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release (green) whose time course is not fully captured at this sampling rate. C, Enlarged images from the indicated myocyte region in A. The 3 frames are from the times indicated along the traces in B (arrows indicate individual mitochondria [or pairs] displaying [Ca\textsuperscript{2+}]\textsubscript{mito} release (scale bar, 4 μm)). D, Number of mitochondrial Ca\textsuperscript{2+} release events are decreased by presence of cyclosporine A (CsA; n=7). E and G, Transient Ca\textsuperscript{2+} release events in individual mitochondria, during SR Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} clamp protocols. Images are taken from times marked by * during time courses at right. Arrows mark individual (or pairs) of mitochondrial that displayed transient changes in [Ca\textsuperscript{2+}]\textsubscript{mito} (scale bar, 2 μm). F, Frequency of transient PTP (tPTP) events in the absence and in the presence of CsA. H, Percentage of mitochondria in tPTP openings at any time (based on tPTP frequency, mean open duration, and total number of mitochondria observed). Histograms of tPTP open duration (I) and time at which tPTP and permanent mPTP (pPTP) openings were observed (J). K, Amplitude of [Ca\textsuperscript{2+}]\textsubscript{mito} rise at 10 minutes for cells that exhibited tPTP vs those that did not (measured using SR Ca\textsuperscript{2+} release protocol).
Ca\(^{2+}\) and ROS Favor tPTP Activity

Permanent PTP openings in isolated mitochondria are known to be triggered by increasing matrix Ca\(^{2+}\) and by ROS.\(^{13,14}\) We tested the involvement of [Ca\(^{2+}\)]\text{mito} and H\(_2\)O\(_2\) in tPTP activation (Figure 3). The predominant Ca\(^{2+}\) influx pathway in mitochondria is the mitochondrial Ca\(^{2+}\) uniporter (MCU). When we inhibit mitochondrial Ca\(^{2+}\) uniporter either pharmacologically (Ru360) or by genetic deletion (mitochondrial Ca\(^{2+}\) uniporter-knockout) tPTP events are strongly suppressed during SR Ca\(^{2+}\) releases (Figure 3A). This is consistent with our observation that there are no detectable tPTP openings in Ca\(^{2+}\)-free solution in the absence of SR function (Figure 3D). CypD is known to be an important facilitator of mPTP opening.\(^{22}\) Pharmacological inhibition of CypD either by CsA or by genetic ablation of the CypD gene abolished tPTP openings during spontaneous SR Ca\(^{2+}\) release (Figure 3A). So [Ca\(^{2+}\)]\text{mito} is required, and CsD is essential for tPTP.

ROS, including H\(_2\)O\(_2\), can greatly sensitize the mPTP to Ca\(^{2+}\).\(^{23}\) Figure 3B shows that even low [H\(_2\)O\(_2\)] (1 \text{μmol/L}) increased tPTP opening frequency 6-fold (10.0±2.6 versus 1.6±0.6, H\(_2\)O\(_2\) versus CTL, **\(P<0.05\)). However, peroxide did not alter the average time point at which the pore opened (395±45 s versus 463±24 s, CTL versus H\(_2\)O\(_2\)) or the duration of pore opening (52±8 s versus 45±6 s, CTL versus H\(_2\)O\(_2\); Figure 3B–D). Moreover, the frequency of tPTP opening as a function of [Ca\(^{2+}\)] shows that H\(_2\)O\(_2\) increased mPTP sensitivity for Ca\(^{2+}\) (**\(P<0.001\), Figure 3D). Taken together, our data indicate that Ca\(^{2+}\) and moderate oxidative stress via H\(_2\)O\(_2\) synergize in tPTP opening.

To test whether similar tPTP openings could be observed in intact myocytes, we measured CsA-sensitive ΔΨ\(_{m}\) in electrically stimulated myocytes (1 Hz). The frequency and duration of tPTP openings were comparable in intact versus permeabilized myocytes (Figure 3B and 3F versus Figure 1F, 1H, and 1I). These results are consistent with our baseline permeabilized myocyte data reflecting basal physiological levels of tPTP openings. Increasing stimulation frequency from 2 to 4 Hz tended to increase tPTPs (1.0±0.6 versus 5.5±1.1, not significant in ANOVA; Online Figure IIIA), but β-adrenergic stimulation with isoproterenol significantly increased tPTP opening at 1 Hz (nearly 10-fold), and also reduced tPTP duration by ≈50% (Figure 3B; Online Figure III). Thus, increased work or stress can favor tPTP opening.

Ca\(^{2+}\)- and ROS-Induced tPTP Are Increased in Heart Failure

The failing heart exhibits dysregulation of myocyte Ca\(^{2+}\) handling and increased oxidative stress, which could favor tPTP opening. We measured tPTP in heart failure (HF) myocytes. HF was induced by transverse aortic constriction. Systolic function was substantially depressed after 6 to 8 weeks assessed by echocardiography (Figure 4A and 4B) and hearts were enlarged (heart: body weight) with pulmonary congestion (lung: body weight; Figure 4C). This is the stage at which we tested tPTP in myocytes.

Using the SR Ca\(^{2+}\) release protocol as in Figure 1E, HF versus sham myocyte exhibited many more tPTP openings, and these could be suppressed by CsA (Figure 4D). To test which factor might be responsible for higher tPTP during HF, we...
measured mitochondrial Ca\(^{2+}\) uptake and ROS in sham and HF myocytes. The amplitude of [Ca\(^{2+}\)]\(_{\text{in}}\) rise was the same between sham and HF (1.83±0.17 versus 1.92±0.14, HF versus Sham; Figure 4E). However, the ROS sensor DCF (2′,7′-dichlorodihydrofluorescein diacetate) indicated that the rate of ROS formation in HF cells was significantly higher than sham, both at rest and during 1 Hz pacing (Figure 4F). Given the potent effects of ROS on tPTP (Figure 3B and 3D), we suspect that the high tPTP rate in HF could be mediated by increased ROS production. It is possible that the increased number of tPTPs in HF is part of a physiological self-repair mechanism of mitochondria in HF that limits injury to individual mitochondria.

**Discussion**

The notion of tPTP as a lower conductance and more reversible manifestation of the well-studied and pathological permanent PTP has been suggested by previous work on isolated mitochondria populations,\(^8,13,14\) and subconductance mPTP
transient openings during in vitro voltage clamp. Mitochondrial superoxide flashes may also involve transient mPTP opening, but those events differ in allowing rhod-2 loss (900 Da molecules) and occur even at very low [Ca2+]i. Here, we directly demonstrate physiological tPTP openings in individual mitochondria in situ in cardiac myocytes, measure their duration (≈60 s) and pore size cutoff (not allowing 600-Da molecules), both of which are distinct from pPTP. However, we also found tPTP to have sensitivity to CsA, CypD, mitochondrial Ca2+ uniporter blockade, [Ca2+]i, [Ca2+]mito, and H2O2 that are similar to pPTP. Thus, our working hypothesis is that tPTP openings (or MitoWinks) share much of the molecular mechanism and machinery that is involved in the more extensively studied pPTP. Bernardi et al. suggested that dimeric F1F0–ATP synthase (complex V) serves also as the pPTP itself, but whether this applies to tPTP will require better resolution of detailed molecular basis of pPTP. Disruption of a reported matrix electric coupling between mitochondria and plasma membrane during exocytosis, and neurosecretion. We did not observe transitions from tPTP to pPTP, but those might only reasonably be seen as a delayed calcein or Rhod-2 release after ΔΨm depolarization. Because tPTP events are so rare, we cannot unequivocally assess this.

We infer that tPTP openings occur in single mitochondria, but confocal resolution limitations mean that fluorescence from other nearby mitochondria can influence signals in our mitochondrion-sized region of interest. Figure 1G is illustrative. At ≈220 s the local [Ca2+]mito rises steeply, which could reflect a burst of Ca2+ influx into one mitochondrion or another mitochondrion in close proximity. When the tPTP opening occurs (at 400 s) the [Ca2+]mito decline is incomplete. One likely interpretation is that the total fluorescence is from 2 (or 3) individual mitochondria, only 1 of which exhibits a MitoWink. This spatial constraint does not influence our conclusions.

A major point is that unlike pathological pPTP, these rare tPTP openings are somehow beneficial to a mitochondrion, by allowing a physiological reset by release of excess Ca2+ channel pore and greater reversibility. This could be functionally analogous to the kiss-and-run hypothesis of partial, low-conductance vesicle fusion to plasma membrane during neurosecretion. We did not observe transitions from tPTP to pPTP, but those might only reasonably be seen as a delayed calcein or Rhod-2 release after ΔΨm depolarization. Because tPTP events are so rare, we cannot unequivocally assess this.
and perhaps other accumulated harmful factors, but without losing key larger molecules and without harming cell-wide ATP production. That conclusion is clearly appropriate under our quasi-physiological resting conditions where only 0.02% of mitochondria are simultaneously depolarized in this tPTP mode. This percentage increases with mild ROS exposure (6-fold), 1 Hz pacing with isoproterenol (10-fold) and in basal HF myocytes (24-fold), such that nearly 0.5% of cellular mitochondria would be nonfunctional at any time. Note also that during a tPTP mitochondria would consume rather than make ATP (via F, F, -ATPase), which would increase the functional cost of tPTPs on ATP production. So, under in vivo high work loads combined with pathological stresses, tPTP frequency might become high enough to limit ATP production. Like in control myocytes, there were comparable numbers of tPTP and pPTP openings in HF during the 10 minutes observation. Thus with the parallel rise in pPTP events in HF or other pathologies, the functional consequences would be further exacerbated because the pPTP (versus tPTP) openings are permanent and cumulative.

So, in an individual cardiac mitochondrion how many tPTP events might occur during its normal turnover lifetime (estimated at 17 days)? Our measurements imply 1 tPTP every 3 to 83 hours (basal HF versus control), but again this could be more frequent under in vivo stress. Moreover, if these tPTPs represent the turning point between beneficial reversal versus a pPTP and mitochondrial death, it will be important to understand these events in more detail.

We were surprised that tPTP openings lasted ≈60 s, thinking that less time would be required to release Ca. Although it often required >10 s for [Ca] to reach a maximum, the longer duration might also allow other, beneficial effects to occur to help reset that mitochondrial. Another, possibly related surprise, was that [Ca] typically rose faster after tPTP closure than it had before. We propose that during tPTP openings mitochondria-free [Ca] drops rapidly, but Ca buffers diffuse out more slowly. Then on tPTP closure, the same low Ca influx rate would raise [Ca] faster (ie, with less intramitochondrial Ca buffering). This agrees with slow rises in mitochondrial Ca buffering power during Ca uptake in isolated mitochondrial, which was attributed to slow uptake of phosphates. We found that longer tPTP durations had faster subsequent [Ca] recovery (Online Figure IV), consistent with this idea. The lack of PTP reopening as [Ca] recovers might also be because of loss of mitochondrial Ca bound phosphate and polyphosphate, that may both promote PTP opening. Hence, tPTPs may also reset mitochondrial Ca buffering in a way that restores acute responsiveness of Ca-dependent dehydrogenase activation during cytosolic Ca changes, while limiting further PTP events.

This characterization of individual MitoWinks in cardiac myocytes demonstrates a potentially beneficial physiological restorative mPTP event, which contrasts functionally with pPTP openings (that lead to mitochondrial and cell death). These initial studies pave the way for future studies that may define the explicit molecular mechanism (eg, are tPTP and pPTP different functions of the same proteins?) and how tPTP openings integrate into normal mitochondrial function.

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Disclosures
None.

References


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**Novelty and Significance**

**What Is Known?**

- The high-conductance and permanent opening of mitochondrial permeability transition pore (pPTP) causes mitochondrial injury and cell death.
- Unlike permanent opening of PTP, transient and low-conductance opening of PTP (tPTP) was proposed as protective against pathological Ca2+ overload.
- Direct characterization of tPTP opening in cardiac myocytes still remained unclear.

**What New Information Does This Article Contribute?**

- tPTP opening in cardiac myocytes is rare (only 0.02% of mitochondria is in tPTP at any given time), last for ≈57 s, and respond to PTP regulatory factors, such as Ca2+, reactive oxygen species, cyclophilin D, and cyclosporine A.
- When tPTP opens, molecules >600 Da cannot pass through (smaller pore than full PTP), and mitochondria can retain small metabolic molecules (eg, NADH) during tPTP.
- tPTP opening frequency increased under higher work or pathological conditions, which could be a physiological mitochondrial self-repair mechanism.

- Characterization of tPTP in cardiac myocytes suggests a physiologically beneficial role of tPTP opening (versus full pPTP opening).

Metabolism in individual mitochondria is regulated during EC coupling. Opening of long lasting mitochondrial permeability transition pore (pPTP) causes mitochondrial injury; however, transient mPTP openings (tPTP) may protect against cardiac stress. In this study, we visualized the tPTP event in individual mitochondrion directly as transient drops in mitochondrial [Ca2+]mito and voltage (∆ψmito) by simultaneous live imaging of 500 to 1000 mitochondria in situ in adult cardiac myocytes. We quantitatively characterize for the first time key properties of these tPTP (open duration, pore size, and regulation) that may be physiologically beneficial in resetting mitochondria. The full recovery of [Ca2+]mito and (∆ψmito), and the small pore size are in striking contrast to the well-studied permanent PTP openings that lead to mitochondrial dysfunction and cell death. However, the Ca2+, ROS, cyclophilin D, and cyclosporine A sensitivity of tPTP resemble those of pPTP. We conclude that these new tPTP openings are mediated by the same molecular components as pPTP, but instead of being the harbinger of death, are beneficial for mitochondrial (and cell) survival by allowing individual mitochondria to reset themselves with negligible overall energetic cost.
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Supplementary Methods

Cardiac Myocyte isolation, dye loading and permeabilization

Cardiac ventricular myocytes were isolated from adult male C57b/6J mice and those lacking MCU or CypD were previously described routine methods and were approved by the UC Davis Institutional Animal Care and Use Committee (IACUC). Freshly isolated myocytes were plated on laminin-coated glass cover slip for >45 min before dye loading. All experiments were performed at room temperature (RT) (22-23°C). Cells were loaded with 10μM Fluo-8 AM for 45 min, or Rhod-2 AM or 1nM TMRM for 30 min, both at 23°C in nominally Ca²⁺ free Tyrode’s solution (in mmol/L; HEPES 5, NaCl 140, KCl 140, MgCl₂ 1, glucose 10; pH adjusted to 7.4 with NaOH), 30 min were allowed for de-esterification. The ΔΨₘ in intact myocyte was measured by TMRM under field stimulation at 1Hz with 1.8 mmol/L extracellular Na⁺. For plasma membrane permeabilization, cells were exposed to saponin (50 μg/ml; 30 sec) which was then washed off, in standard intracellular relaxing solution containing (in mmol/L) HEPES 10, K-aspartate 135, MgCl₂ 0.7, EGTA 2, reduced glutathione 10, MgATP 5, glucose 10, pH 7.2.

Solutions

A Ca²⁺-buffered, Ca²⁺-free, Na⁺-free internal solution contained (in mmol/L): EGTA 5, HEPES 20, K-aspartate 100, KCl 40, MgCl₂ 1, maleic acid 2, glutamic acid 2, pyruvic acid 5, K₂HPO₄ 0.5, pH 7.2 adjusted with trisma base. To control [Ca²⁺], 0.1 M CaCl₂ solution was added as calculated with MaxChelator. Free [Ca²⁺] was confirmed by Ca-sensitive electrode. Na⁺-free internal solution with low Ca²⁺ buffering capacity and 100 nM free [Ca²⁺] contained (in mmol/L): EGTA 0.05, CaCl₂ 0.0234, HEPES 20, K-aspartate 100, KCl 40, MgCl₂ 0.551, maleic acid 2, glutamic acid 2, pyruvic acid 5, K₂HPO₄ 0.5, MgATP 5, pH 7.2 adjusted with trisma base. When NaCl was added to these solutions, equal amounts of KCl were omitted. 80 μM cytochalasin D was included to inhibit myocyte contraction. Ca²⁺ calibration solutions contained 2 μM ionomycin, 10 μM FCCP and 20 μg/ml oligomycin.

Confocal imaging scanning

Mitochondrial Rhod-2 and TMRM fluorescence were measured (Zeiss LSM 5 live confocal microscope, 60X water-immersive objective) in 2-dimensional imaging mode with excitation at 532 nm, with emission at > 560 nm. Fluo-4, Fluo-8 and FAD autofluorescent signals were measured with same confocal (488 nm excitation, emission at 530 ± 15 nm). Calcien signals were collected using excitation at 488 nm and emission at 530 ± 15 nm. Time-lapse x,y images were acquired at 512 bit resolution and at the sampling rate of 507 ms per frame. Region of interest size for analysis of single (or two) mitochondria was ~1x2 μm, centered on an identifiable mitochondrion.

Chemicals and statistics

Indicators were obtained from Invitrogen (Eugene, OR), Ru360 from Calbiochem (La Jolla, CA). Data are presented as mean ± SE of n measurements. Comparisons between groups used Student’s t-test, One-Way ANOVO and Two-Way ANOVO (significant at p<0.05)

Heart failure model

We used transverse aortic constriction in mice to induce HF. Briefly, after a 5-mmol/L thoracotomy is made lateral to the left sternal border, two loose knots were tied around the transverse aorta, and a blunt 27-gauge needle was then placed parallel to the transverse aorta and inside the first loose knot. The first knot was then tied tightly, followed by the second. Immediately after, the needle was taken out, yielding a constriction of 0.4mm in diameter. With this amount of ligation, HF developed in 6-8 weeks after surgery. Experiments with cardiac cells were performed 8 weeks post-surgery.

Echocardiography

Mice were anesthetized in an isoflurane chamber prior to placing them in the supine position on an ECG platform. The paws of the mouse were taped down with electrode cream onto the ECG sensors, while the nose was connected to a nozzle that delivers 95% O₂ / 5% CO₂ with a concentration of isoflurane that maintains the mouse's heart rate at 550 ± 50 bpm. M-mode echocardiography was performed a day before surgery and eight weeks after surgery using the Visualsonics Vevo 2100 system.

Online Figure I. Transient PTP openings cause temporary mitochondrial depolarization (ΔΨᵣ) and increase oxidation of FAD. (A) Colocalization of ΔΨᵣ indicator TMRM (red) and auto-fluorescence of FAD (green) during 1Hz pacing in intact cardiac myocytes. (B) Traces were obtained from an individual mitochondrion (or pair), as indicated by white arrows. Depolarization preceded oxidation of FAD and then a gradual increase of reduction of FAD. (Scale bar = 4 μm)
Online Figure II. Frequency of tPTP events in the absence and presence of Sanglifehrin A in intact (at 1Hz pacing) and permeabilized cardiac myocytes (*P<0.05, vs Ctl, n=5-7).

Online Figure III. Frequency of transient PTP opening during pacing and β-adrenergic stimulation. (A) Frequency of tPTP openings (using TMRM to assess ΔΨm) in intact cardiac myocytes at different pacing frequency. And the influence of 50 nmol/L ISO on tPTP frequency at 1Hz pacing. (B) The time point at which tPTP opening occurred and its duration of opening (**P<0.01, vs 1Hz, n=6).

Online Figure IV. Correlation of Ca reuptake rate and duration time of the pore opening. (A) Rate of [Ca²⁺]mito rise before (Rate 1) and after tPTP opening (Rate 2). (B) Rate of [Ca²⁺]mito rise (Rate 2) as a function of duration of tPTP opening. (C) Rate of [Ca²⁺]mito rise after tPTP normalized to that before (Rate 2/Rate 1) vs. tPTP open time. Trace in A is the individual mitochondrion indicated by the red dot.
