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 Ca^{2+} load and be cardioprotective, but direct evidence for tPTP in cells is limited.**

Objective: **To directly characterize tPTP occurrence during sarcoplasmic reticulum Ca^{2+} release in adult cardiac myocytes.**

Methods and Results: **Here, we measured tPTP directly as transient drops in mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_{mito}$) and membrane potential ($\Delta\Psi_m$) 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 $[Ca^{2+}]_{mito}$, $[Ca^{2+}]_i$, with 1 μmol/L peroxide exposure and in myocyte from failing hearts. The tPTPs were suppressed by preventing mitochondrial Ca^{2+} 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[1] but also mediate programmed cell death.[2] ATP production is mainly via cellular respiration, which is driven by the voltage gradient ($\Delta\Psi_m$) across the inner mitochondrial membrane that drives proton flux through the F_0F_1–ATP synthase. Extremely low resting inner mitochondrial membrane permeability is critical to maintain high $\Delta\Psi_m$ and ATP synthesis rate in living cells. However, under certain stresses, the inner mitochondrial membrane undergoes a permeability transition pore opening (mPTP), abolishing $\Delta\Psi_m$ and allowing molecules of ≤1500 Da in size to freely permeate.[3,4] This causes respiratory uncoupling, metabolite loss (eg, NADH), cessation of ATP synthesis, increased ATP consumption (via F_0F_1–ATP synthase), and mitochondrial and cell death.[5]

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The molecular identity of mPTP is unknown, but F_0F_1–ATP synthase dimers have been proposed as a candidate.[6] Much work has shown that mPTP inhibition by cyclosporine A (CsA)[7–9] 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.[8–11] 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.[10,11] Moreover, chronic mPTP inhibition leads to mitochondrial Ca^{2+} overload and cardiac dysfunction,[12] raising the idea that some mPTP openings might be beneficial by allowing Ca^{2+} release and maintenance of normal physiological mitochondria $[Ca^{2+}]$ ($[Ca^{2+}]_{mito}$). But how might that occur without the pathological consequences of mPTP?

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From the Department of Pharmacology, University of California, Davis (X.L., D.M.B.); Department of Pediatrics, Cincinnati Children's Hospital Medical Center, University of Cincinnati, OH (J.Q.K., J.D.M.).

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Correspondence to Donald M. Bers, PhD, Department of Pharmacology, University of California, 451 Health Sciences Drive, Davis, CA 95616. E-mail dmbers@ucdavis.edu

Nonstandard Abbreviations and Acronyms

$[Ca^{2+}]_{mito}$	mitochondrial-free Ca^{2+} concentration
CsA	cyclosporine A
CypD	cyclophilin D
mPTP	mitochondrial permeability transition pore
pPTP	permanent opening of mitochondrial permeability transition pore
ROS	reactive oxygen species
tPTP	transient mode of mitochondrial permeability transition pore opening

A transient mode of mPTP opening (tPTP), with lower conductance was proposed as protective against pathological Ca^{2+} overload.[2,13] 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.[13–16] Here, we continuously monitor ≈800 individual mitochondria in confocal imaging planes of adult cardiac myocytes in physiological conditions, and quantitatively characterize single tPTP openings in mitochondria by measuring $[Ca^{2+}]_{mito}$ and $\Delta\Psi_m$. These MitoWinks are rare, last for ≈57 s, do not allow solutes >600 Da through (eg, not NADH) and are modulated by Ca^{2+}, reactive oxygen species (ROS), CypD, and CsA like larger pPTP events.[17,18] MitoWinks may serve a physiological role to protect cells against mitochondrial Ca^{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^{2+} Release

To simultaneously assess mPTP-mediated transient Ca^{2+} release events in 500 to 1000 individual mitochondria in situ, we monitored $[Ca^{2+}]_{mito}$ using Rhod-2 and 2-dimensional confocal microscopy in cardiac myocytes during spontaneous sarcoplasmic reticulum (SR) Ca^{2+} releases (Figure 1A and 1B). Our previously validated method[19] uses acutely saponin-permeabilized adult ventricular myocytes with physiological intracellular solutions with light Ca^{2+} buffering (50 μmol/L [EGTA]). Raising $[Ca^{2+}]_i$ to 100 nmol/L (Figure 1B) induces spontaneous SR Ca^{2+} release events (Ca^{2+} waves) at 5 to 15 minutes^{-1}, which creates physiologically relevant Ca^{2+} releases in a well-controlled system. Because $[Ca^{2+}]_i$ rises and Ca^{2+} waves occur $[Ca^{2+}]_{mito}$ rises with each Ca^{2+} wave, but $[Ca^{2+}]_{mito}$ decline (mainly via Na/Ca exchange, mNCX) is slow, allowing progressive $[Ca^{2+}]_{mito}$ rise. During this protocol individual mitochondria stochastically and suddenly released Ca^{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^+-free internal solution to inhibit mNCX. Figure 1E shows images at times (*) during the $[Ca^{2+}]_{mito}$ trace. Although average $[Ca^{2+}]_{mito}$ rises progressively, the highlighted mitochondrion rapidly releases Ca^{2+} (likely PTP opening) and ≈60 s later Ca^{2+} reuptake resumes. This implies PTP closure and restored $\Delta\Psi_m$ (which drives Ca^{2+}uptake). Figure 1G shows similar results using a different protocol, where $[Ca^{2+}]_i$ was raised from 0 to 2 μmol/L Ca^{2+} with $[Ca^{2+}]_i$ clamped using 0.5 mmol/L EGTA, and SR function suppressed by 5 μmol/L thapsigargin. This is a typically used protocol, and may simulate tonic cellular Ca^{2+} loading. The frequency of putative tPTP events was similar for both SR release and Ca-clamp protocols, and in both cases 10 μmol/L CsA inhibited the events, consistent with them being tPTP openings (Figure 1F). These events are rare (≈2×10^{-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±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.[13]

In records where we saw 64 tPTP events we also observed 54 Ca^{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 $[Ca^{2+}]_{mito}$ reached at the end of the protocol (Figure 1K).

Mitochondrial Depolarization Accompanies tPTP Opening

Brief mPTP opening should depolarize $\Delta\Psi_m$ and cause mitochondrial Ca^{2+} release,[13,20] but it was also proposed that Ca^{2+} enters mitochondria via a subconductance mPTP opening associated with partial $\Delta\Psi_m$ depolarization.[21]

We monitored $[Ca^{2+}]_{mito}$ (with Fluo-8 AM) and $\Delta\Psi_m$ (with tetramethylrhodamine methylester) simultaneously, during protocols as in Figure 1E. Figure 2A and 2B shows a tPTP event in which rapid $\Delta\Psi_m$ depolarization is followed by $[Ca^{2+}]_{mito}$ decline, and then when tPTP closes, proton pumping via cytochromes restores $\Delta\Psi_m$ and that allows Ca^{2+} uptake to resume. Thus, tPTP opening rapidly dissipates $\Delta\Psi_m$, allowing electrochemically downhill Ca^{2+} efflux, but the mitochondrion retains functionality, as manifest by $\Delta\Psi_m$ recovery. Moreover, simultaneous monitoring of mitochondrial redox state (FAD autofluorescence) and $\Delta\Psi_m$ (with tetramethylrhodamine methylester) show $FADH_2$ 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).[13]

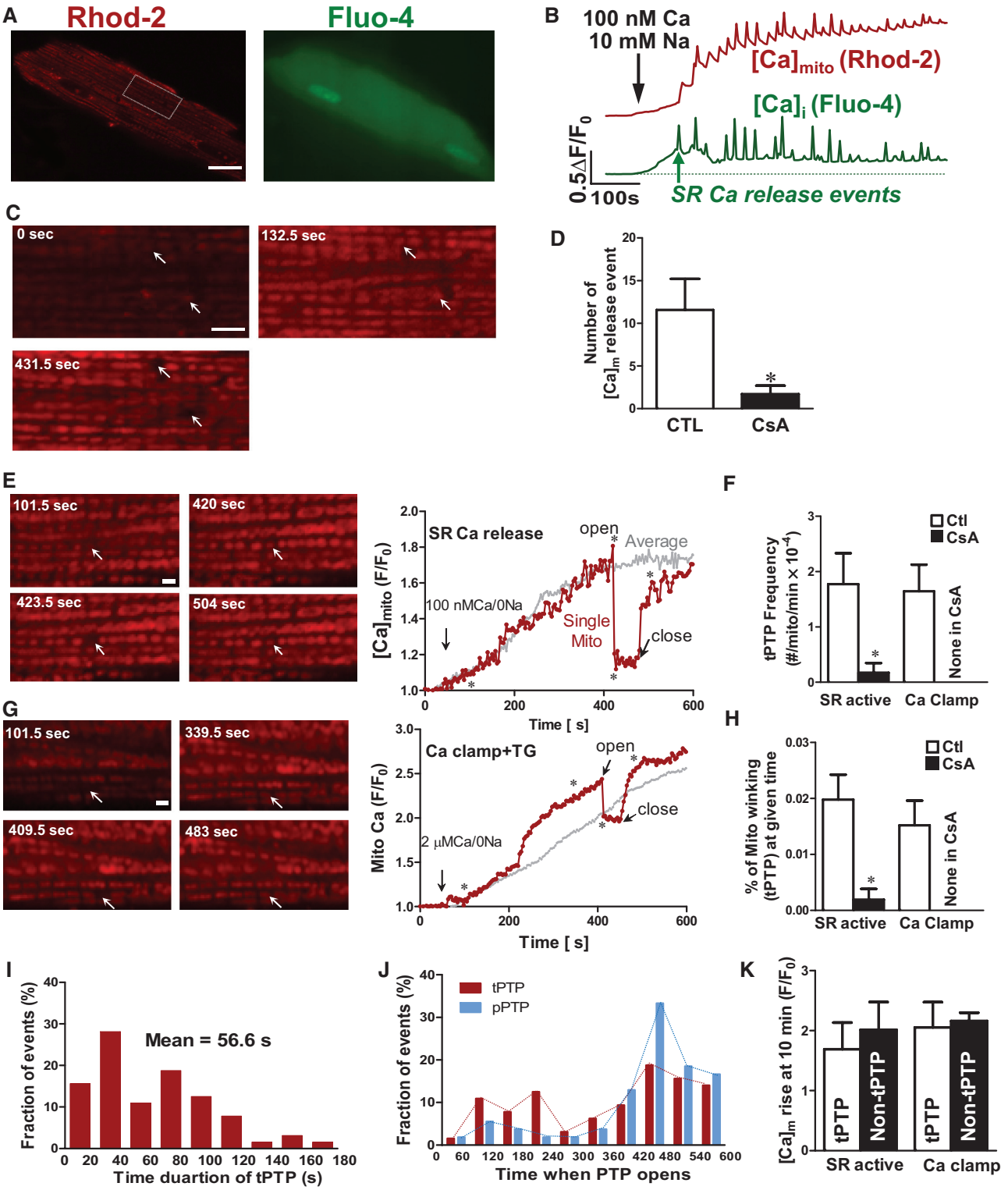

Figure 1. Transient permeability transition pores (PTP) openings in single mitochondria. A, Images of permeabilized cardiac myocyte loaded with Rhod-2 (red) and Fluo-4 (green; **left**). Scale bar, 16 μm. **B**, Time course of $[Ca^{2+}]_{mito}$ and $[Ca^{2+}]_i$ signals in the same cardiac myocyte, showing mitochondrial Ca^{2+} uptake (red) induced by cyclic sarcoplasmic reticulum (SR) Ca^{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 $[Ca^{2+}]_{mito}$ release (scale bar, 4 μm]). **D**, Number of mitochondrial Ca^{2+} release events are decreased by presence of cyclosporine A (CsA; n=7). **E** and **G**, Transient Ca^{2+} release events in individual mitochondria, during SR Ca^{2+} release and Ca^{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 $[Ca^{2+}]_{mito}$ (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 $[Ca^{2+}]_{mito}$ rise at 10 minutes for cells that exhibited tPTP vs those that did not (measured using SR Ca^{2+} release protocol).

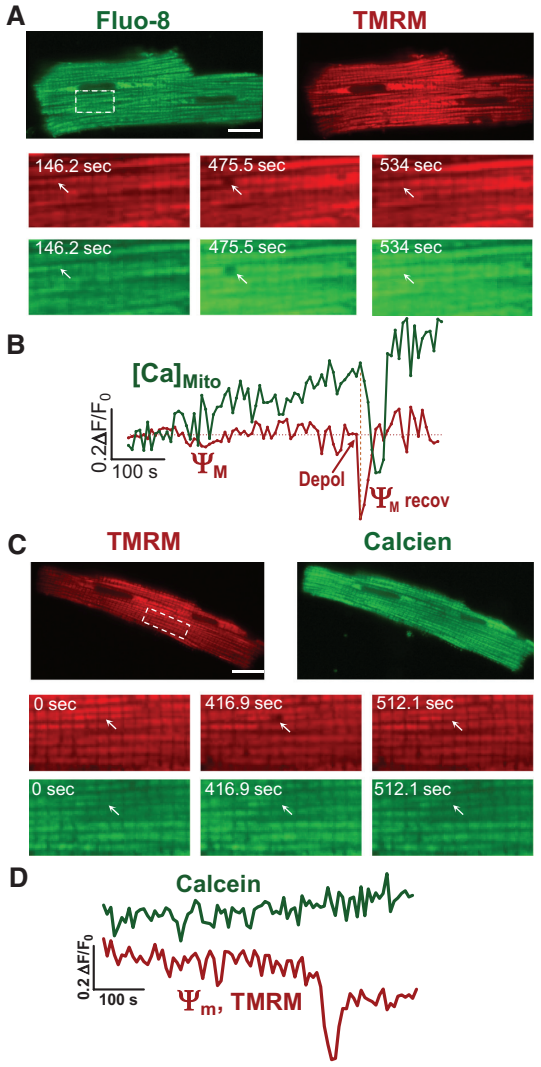

Figure 2. Transient permeability transition pores (tPTP) openings cause temporary mitochondrial depolarization ($\Delta\Psi_m$) and do not allow calcein permeation. A, Colocalization of the $\Delta\Psi_m$ indicator tetramethylrhodamine methylester (TMRM; red) and mitochondrial Ca^{2+} indicator Fluo-8 (green) in permeabilized cardiac myocytes. Enlarged images are from a portion of the above myocyte at times indicated. **B**, Traces were obtained from an individual mitochondrion (or pair), as indicated by white arrows. Depolarization preceded Ca^{2+} loss and gradual repolarization preceded Ca^{2+} refilling. **C**, Similar format for simultaneous mitochondrial $\Delta\Psi_m$ indicator (TMRM) and calcein in a permeabilized cardiac myocyte, with enlarged images showing individual mitochondrion (or pair; arrows) in which depolarization and calcein were simultaneously measured (**D**). Scale bar, 16 μm. The sarcoplasmic reticulum Ca^{2+} release protocol (Figure 1) was used.

Because we see full $[Ca^{2+}]_{mito}$ recovery, it is clear that Rhod-2 (MW 869 Da) does not leave the mitochondrial matrix during tPTP opening. To further test the molecular weight cutoff for tPTP, we loaded myocytes with calcein AM and tetramethylrhodamine methylester to monitor $\Delta\Psi_m$ and tPTP opening. Figure 2C shows that during tPTP opening, calcien (MW 623 Da) was retained. This indicates that tPTP pore size freely allows ions (protons and Ca^{2+}) to pass through, but prevents efflux of molecules >600 Da (eg, NADH, MW 663 and $FADH_2$, MW 786). This helps to explain why tPTP openings seem completely reversible and conserve mitochondrial functionality.

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 $[Ca^{2+}]_{mito}$ and H_2O_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 $[Ca^{2+}]_{mito}$ is required, and CypD is essential for tPTP.

ROS, including H_2O_2 can greatly sensitize the mPTP to Ca^{2+}.[23] Figure 3B shows that even low $[H_2O_2]$ (1 μmol/L) increased tPTP opening frequency 6-fold (10.0±2.6 versus 1.6±0.6, H_2O_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_2O_2) or the duration of pore opening (52±8 s versus 45±6 s, CTL versus H_2O_2; Figure 3B–D). Moreover, the frequency of tPTP opening as a function of $[Ca^{2+}]_i$ shows that H_2O_2 increased mPTP sensitivity for Ca ($^{***}P<0.001$, Figure 3D). Taken together, our data indicate that Ca^{2+} and moderate oxidative stress via H_2O_2 synergize in tPTP opening.

To test whether similar tPTP openings could be observed in intact myocytes, we measured CsA-sensitive $\Delta\Psi_m$ 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

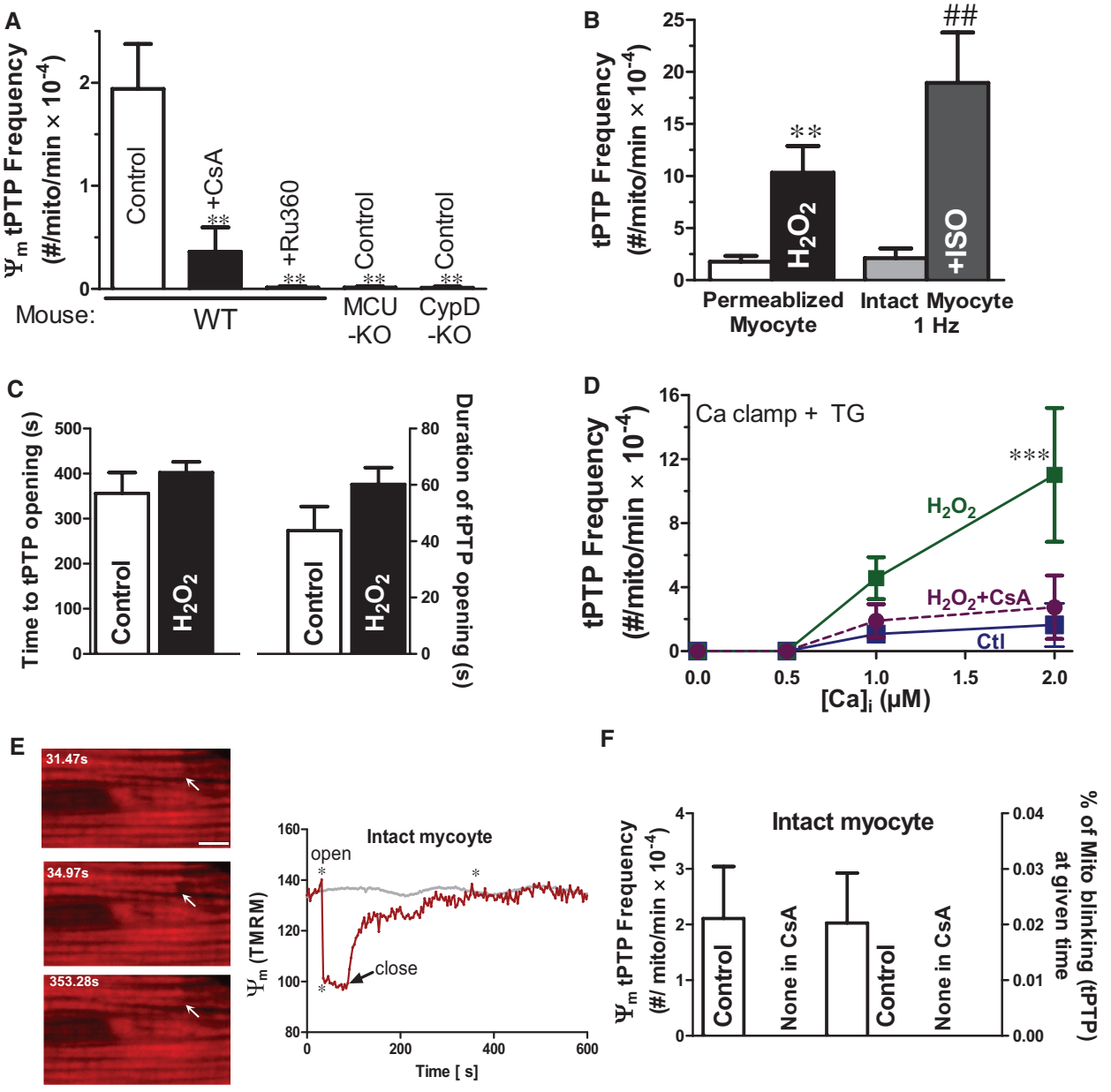

Figure 3. Ca^{2+}, reactive oxygen species, and cyclophilin D (CypD) are involved in transient permeability transition pores (tPTP) opening. A, Frequency of tPTP openings (using tetramethylrhodamine methylester [TMRM] to assess $\Delta\Psi_m$) in wild-type (WT) mice in the presence and in the absence of Ru360 or cyclosporine A (CsA), and in mitochondrial Ca^{2+} uniporter (MCU)- and CypD-knockout (KO) mice (**P<0.01, vs CTL, n=6–9). Data were acquired from both permeabilized (with the sarcoplasmic reticulum Ca^{2+} release protocol, as in Figure 1. Influence of 1 μmol/L H_2O_2 on tPTP frequency) and intact cells (at 1 Hz pacing frequency in the absence and in the presence of isoproterenol (ISO; **B**), and the time point at which tPTP opening occurred and its duration of opening (**C**; n=6–7). **D**, Frequency of tPTP openings as a function of $[Ca^{2+}]_i$ with and without 1 μmol/L H_2O_2 (±CsA; n=5–8 for each $[Ca^{2+}]_i$ and Ca^{2+}clamp treatment). **E**, Transient $\Delta\Psi_m$ depolarization in individual mitochondria during 1 Hz pacing in intact myocytes. Images are taken from times marked by * during time course at right. Arrows mark mitochondrion (or pair) that displayed transient $\Delta\Psi_m$ changes (scale bar, 4 μm). **F**, Frequency of intact myocyte tPTP events and percentage of mitochondria in tPTP openings at any time (±CsA).

measured mitochondrial Ca^{2+} uptake and ROS in sham and HF myocytes. The amplitude of $[Ca^{2+}]_m$ 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

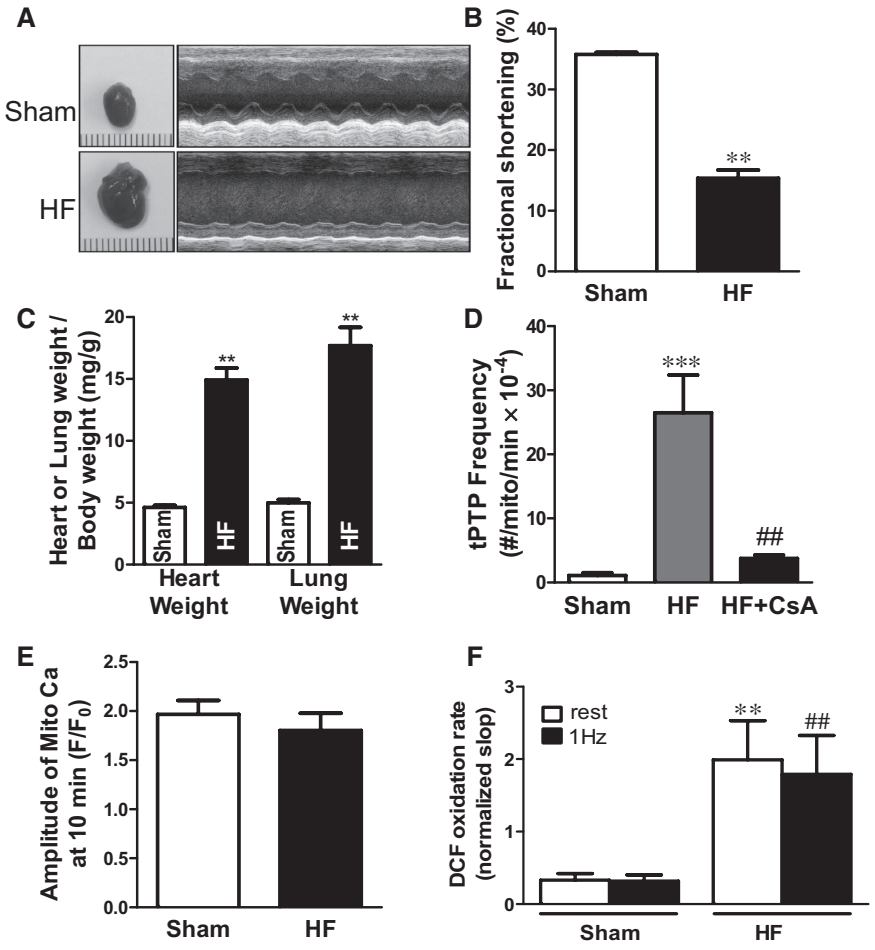

Figure 4. Heart failure (HF) increases transient permeability transition pores (tPTP) openings. A, Characterization of transverse aortic constriction induced HF model in mouse. Heart morphology and function were determined by measuring heart size, echocardiography indices. **B**, Fractional shortening, heart weight/body weight, and lung weight/body weight ratios (***P*<0.01, n=5 hearts; **C**). **D**, Frequency of tPTP openings increased in HF, and could be inhibited by applying cyclosporine A (CsA; 1.09±0.42 vs 26.5±5.9 vs 3.74±0.56, Sham, HF, HF+CsA, ****P*<0.001 vs Sham, ##*P*<0.01 vs HF treatment, n=5–7 cells). **E**, Amplitude of $[Ca^{2+}]_{mito}$ rise in sham and HF during 10 minutes (n=7). **F**, Peroxide generation in sham and HF during rest and 1 Hz pacing (***P*<0.01, n=10–11).

openings during in vitro voltage clamp.[24–26] Mitochondrial superoxide flashes may also involve transient mPTP opening,[27] but those events differ in allowing rhod-2 loss (900 Da molecules) and occur even at very low $[Ca^{2+}]$. 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 Ca^{2+} uniporter blockade, $[Ca^{2+}]_i$, $[Ca^{2+}]_{mito}$, and H_2O_2 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[6,28] suggested that dimeric F_0F_1–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[29,30] might contribute to tPTP, but individual mitochondrial function is the simplest interpretation of these MitoWinks.

An attractive possibility is that tPTP is simply an intermediate state on the transition to pPTP, exhibiting smaller 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 exocytosis, and neurosecretion.[31,32] 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 $\Delta\Psi_m$ 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 $[Ca^{2+}]_{mito}$ rises steeply, which could reflect a burst of Ca^{2+} influx into one mitochondrion or another mitochondrion in close proximity. When the tPTP opening occurs (at 400 s) the $[Ca^{2+}]_{mito}$ 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 Ca^{2+}

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_0F_1–ATPase),[33] 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[34])? 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 refreshment 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^{2+}. Although it often required >10 s for $[Ca^{2+}]_{mito}$ to reach a minimum, the longer duration might also allow other, beneficial effects to occur to help reset that mitochondrion. Another, possibly related surprise, was that $[Ca^{2+}]_{mito}$ typically rose faster after tPTP closure than it had before. We propose that during tPTP openings mitochondria-free $[Ca^{2+}]_{mito}$ drops rapidly, but Ca^{2+} buffers diffuses out more slowly. Then on tPTP closure, the same low Ca^{2+} influx rate would raise $[Ca^{2+}]_{mito}$ faster (ie, with less intramitochondrial Ca^{2+} buffering). This agrees with slow rises in mitochondrial Ca^{2+} buffering power during Ca^{2+} uptake in isolated mitochondrial, which was attributed to slow uptake of phosphates.[35] We found that longer tPTP durations had faster subsequent $[Ca^{2+}]_{mito}$ recovery (Online Figure IV), consistent with this idea. The lack of PTP reopening as $[Ca^{2+}]_{mito}$ recovers might also be because of loss of mitochondrial Ca^{2+} bound phosphate and polyphosphate, that may both promote PTP opening.[21,35] Hence, tPTPs may also reset mitochondrial Ca^{2+} buffering in a way that restores acute responsiveness of Ca-dependent dehydrogenase activation during cytosolic Ca^{2+} 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.

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Novelty and Significance

What Is Known?

- The highconductance 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 Ca overload.
- Direct characterization of tPTP opening in cardiac mycoytes 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 Ca, 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 mycoytes 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 mitochondria 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 $[Ca^{2+}]$ ($[Ca^{2+}]_{mito}$) and voltage (($\Delta\psi_{mito}$), 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 $[Ca^{2+}]_{mito}$ and ($\Delta\psi_{mito}$), 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 Ca^{2+}, 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.