Published in final edited form as:

Arch Biochem Biophys. 2019 May 15; 666: 31–39. doi:10.1016/j.abb.2019.03.016.

Dynamics of the Mitochondrial Permeability Transition Pore: Transient and Permanent Opening Events

Liron Boyman^{1,2}, Andrew K. Coleman^{1,2}, Guiling Zhao^{1,2}, Andrew P. Wescott^{1,2}, Humberto C. Joca^{1,2}, B. Maura Greiser^{1,2}, Mariusz Karbowski^{1,3}, Chris W. Ward⁴, and W. J. Lederer^{1,2}

¹Center for Biomedical Engineering and Technology

²Department of Physiology, University of Maryland School of Medicine 111 Penn Street Baltimore, MD 21201 USA

³Department of Biochemistry and Molecular Biology University of Maryland School of Medicine 111 Penn Street Baltimore, MD 21201 USA

⁴Department of Orthopedics, University of Maryland School of Medicine, Baltimore, MD, USA.

Abstract

A gentle optical examination of the mitochondrial permeability transition pore (mPTP) opening events was carried out in isolated quiescent ventricular myocytes by tracking the inner membrane potential (Ψ_M) using TMRM (tetramethylrhodamine methyl ester). Zeiss Airyscan 880 “super-resolution” or “high-resolution” imaging was done with very low levels of illumination (0.009% laser power). In cellular areas imaged every 9 seconds (ROI or regions of interest), transient depolarizations of variable amplitudes occurred at increasing rates for the first 30 minutes. The time to first depolarization events was 8.4 min ($\pm$ 1.1 SEM n=21 cells). At longer times, essentially permanent and irreversible depolarizations occurred at an increasing fraction of all events. In other cellular areas surrounding the ROI, mitochondria were rarely illuminated (once per 5 minutes) and virtually no permanent depolarization events occurred for over 1 hour of imaging. These findings suggest that photon stress due to the imaging itself plays an important role in the generation of both the transient mPTP opening events as well as the permanent mPTP opening events. Consistent with the evidence that photon “stress” in mitochondria loaded with virtually any photon absorbing substance, generates reactive oxygen species (ROS) [1–5], we show that cyclosporine-A (CsA, 10 mM) and the antioxidant n-acetyl cysteine (NAC, 10 mM), reduced the number of events by 80 % and 93% respectively. Furthermore, CsA and NAC

Corresponding Author: W. Jonathan Lederer, Center for Biomedical Engineering and Technology, University of Maryland School of Medicine, Baltimore, Maryland 21201 USA, Tel: 410-706-4895, FAX: 410-510-1545 jleder@som.umaryland.edu. **Co-corresponding Author:** Liron Boyman, Center for Biomedical Engineering and Technology, University of Maryland School of Medicine Baltimore, Maryland 21201 USA, Tel: 410-706-2663, LBoyman@som.umaryland.edu.

Data availability.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Disclosures

None.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

treatment led to the virtual disappearance of permanent depolarization events. Nevertheless, all transient depolarization events in any condition (control, CsA and NAC) appeared to repolarize with a similar half-time of 30 ± 6 s ($n=478$) at 37°C . Further experiments showed quantitatively similar results in cerebral vascular smooth muscle cells, using a different confocal system, and different photon absorbing reagent (TMRE; tetramethylrhodamine ethyl ester). In these experiments, using modest power (1% laser power) transient depolarization events were seen in only 8 out of 23 cells while with higher power (8%), all cells showed transient events, which align with the level of photon stress being the driver of the effect. Together, our findings suggest that photon-induced ROS is sufficient to cause depolarization events of individual mitochondria in quiescent cells; without electrical or mechanical activity to stimulate mitochondrial metabolism, and without raising the mitochondrial matrix Ca^{2+} . In a broad context, these findings neither support nor deny the relevance or occurrence of Ψ_{M} depolarization events in specific putatively physiologic mitochondrial behaviors such as MitoFlashes [6, 7] or MitoWinks [8]. Instead, our findings raise a caution with regards to the physiological and pathophysiological functions attributed to singular Ψ_{M} depolarization events when those functions are investigated using photon absorbing substances. Nevertheless, using photon stress as a tool (“Optical Stress-Probe”), we can extract information on the activation, reversibility, permanency and kinetics of mitochondrial depolarization. These data may provide new information on mPTP, help identify the mPTP protein complex, and establish the physiological function of the mPTP protein complex and their links to MitoFlashes and MitoWinks.

Keywords

Ventricular myocytes; Mitochondria; Mitochondrial Permeability Transition Pore; Mitochondrial Inner Membrane Potential

Mitochondria are intracellular organelles in cardiac myocytes and other cells that convert the energy stored in carbohydrates, lipids and proteins into the universal energy currency, ATP. The inner mitochondrial membrane (IMM) is normally polarized to the very negative potential of approximately -160 mV (Ψ_{M}) by the extrusion of protons by the electron transport chain (ETC) complexes. Dynamic and reversible changes of Ψ_{M} have been observed in a variety of cell types with an array of spatiotemporal features. For example,

Ψ_{M} changes have been shown to occur in the entire population of cellular mitochondria, all at once, along with changes of mitochondrial NAD(P)H[9–11]. Other reports show more localized events displayed by individual mitochondria. These localized events were linked to morphological changes of the cristae architecture[12, 13]. Dramatic depolarizations of Ψ_{M} can also occur through a mechanism called “permeability transition” and attributed to the opening of a mitochondrial permeability transition pore (mPTP). The mPTPs can be activated by elevated concentrations of reactive oxygen species (ROS) in the mitochondrial matrix or elevated mitochondrial matrix Ca^{2+} ($[\text{Ca}^{2+}]_{\text{M}}$), or both. Recent reports using primarily quiescent cardiac ventricular myocytes have presented new views suggesting a physiological role to the activation of mPTP and attribute a physiological function Ψ_{M} to depolarizations of individual mitochondria called “MitoFlashes” [6, 7] or “MitoWinks” [8].

Under normal physiological conditions, the hyperpolarized inner membrane potential of approximately -160 mV powers the production of ATP from ADP and inorganic phosphate by the ATP synthase protein. The Adenine Nucleotide Translocator (ANT) transports ATP out of the mitochondrial matrix once it is made and ADP into the matrix from the intermembrane space and the cytosol. The IMM is very tight, or minimally leaky, and thus significantly impermeable to protons and other charged substrates enabling Ψ_M to be maintained in an energy efficient manner. Protons are used to drive the ATP synthase located in the IMM. Protons in the intermembrane space can also be used in a highly regulated manner by uncoupling proteins (UCPs) found in the IMM to regulate temperature and by specific transporters in the IMM (e.g. the Na^+/H^+ transporter needed to extrude Na^+). Calcium (Ca^{2+}) is also largely impermeant to the IMM but can enter the matrix through the MCU (mitochondrial Ca^{2+} uniporter) which is a low abundance, low conductance (pS), highly selective Ca^{2+} channel in the IMM [14, 15]. The MCU Ca^{2+} flux is one of the mechanisms that link cellular activity to mitochondrial function [16–18]. Ca^{2+} is extruded from the matrix by the highly regulated mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCLX) [19–21]. Together these transporters and channels minimally degrade Ψ_M through their leak of charge. In contrast to the IMM, the outer mitochondrial membrane (OMM), readily conducts small cations like Na^+ , K^+ and Ca^{2+} through VDAC (the voltage dependent anion channel). Both ADP and ATP cross the OMM in a regulated manner through VDAC.

Activation of mPTP provides a dramatic increase in a non-selective conductance across the IMM and OMM and produces a rapid mitochondrial depolarization measured by the decrease in fluorescence of TMRM (tetramethyl rhodamine methyl ester) or TMRE (tetramethyl rhodamine ethyl ester). The activation of the mPTP is considered a rapid and fairly non-selective permeability event. Consistent with the sudden opening of a large conductance channel [22–26], this opening permits the 600 Da MW (i.e., calcein) or even larger molecules of up to 1500 Da to enter the mitochondria [27, 28]. The identification of the molecular component(s) of mPTP or the protein structure(s) responsible for the observed events has (have) been problematic. At one point, the mPTP was thought to include multiple components from the IMM and the OMM that would be assembled to produce mPTP that spanned both membranes. One set of candidate components included three elements - VDAC, ANT and mitochondrial matrix cyclophilin (Cyp-D) [29–31]. After that hypothesized set of components was ruled out by knockout experiments [32–35], several groups have suggested that mPTP is composed of reorganized subunits of ATP synthase [36–38]. However, this conclusion has remained controversial [39, 40]. Despite the unknown identity of mPTP, clues to its identity, and its behavior, can be studied by monitoring Ψ_M .

The work presented here was carried out in quiescent ventricular myocytes in which new instrumentation has enabled us to carry out an investigation of mPTP depolarizations and their links to mitochondrial matrix [ROS] with great sensitivity. With this approach we have investigated how ROS affects mPTP-dependent depolarization of spatially resolved individual mitochondria. This approach is similar to that undertaken to characterize MitoFlashes or MitoWinks. We used light to generate reactive oxygen species (ROS) in the mitochondrial matrix loaded with TMRM or TMRE. TMRM and TMRE are fluorescent indicators used by us and others to measure Ψ_M and, like other chemicals, produce ROS by absorbing photons [1–5]. These same dyes have been used by others to characterize

depolarization events of individual mitochondria including MitoFlashes and MitoWinks. However, with the combination of light intensity, dye concentration and low imaging rate, we have been able to precisely control the dose of ROS systematically without materially changing $[Ca^{2+}]_i$ or $[Ca^{2+}]_M$ [41]. Thus, under these conditions of $[Ca^{2+}]_i$ remaining about 100 nM, increased ROS dose by repeated imaging, activates mPTP opening events.

We conducted a detailed examination of single mitochondrial depolarization and recovery to learn more about the mechanisms that activate and regulate mPTP. In isolated quiescent ventricular myocytes, single mitochondria we identified by the TMRM (or TMRE) fluorescence that reports their membrane potential. Using confocal and super-resolution imaging we visualized a time-dependent increase in transient mPTP openings (i.e., transient depolarization and full recovery) that progressed to sustained depolarizations. Our observation that the frequency and properties of mPTP events tracked with the relative power of laser illumination suggested that level of ROS generated as the by-product of low power laser illumination of TMRM (or TMRE) may be a driver of the effect. Our evidence that scavenging ROS with NAC was equally effective in suppressing mPTP as inhibiting its activation by CsA, confirmed ROS as central to the activation of mPTP. Given that no electrical or mechanical stimuli has been applied to account for mPTP depolarizations in quiescent ventricular myocytes with nearly constant ~ 100 nM $[Ca^{2+}]_i$, our investigations support the hypothesis that mPTP openings are driven by the level of ROS in the matrix[23] under these conditions.

Material and Methods

Ψ_M measurements in cardiomyocytes.

Ventricular myocytes were isolated using standard enzymatic approaches [42] from 6 week old to 10 week-old Sprague-Dawley rats (250–300 grams). Ventricular myocytes were stored in mammalian cell culture medium (Media 199, Gibco/Thermo-Fisher, USA). The cell medium was supplemented with Insulin-transferrin-sodium selenite media supplement (Sigma-Aldrich), penicillin, streptomycin, 50 μ M Cytochalasin D, and 50 nM TMRM (tetramethylrhodamine methyl ester perchlorate). The isolated cardiomyocytes were allowed to settle on the glass bottom of a plastic imaging chamber (Lab-Tek™ Chambered No 1 Coverglass). The plastic imaging chamber was placed inside a microscope stage-top incubator (INU TIZW, TOKAI HIT, Japan). The temperature within the stage-top incubator was set to 37 °C and in normal air CO₂ was set to 5 %. Confocal measurements of Ψ_m (using TMRM) was carried out by scanning with the 561 laser-line (Zeiss 880 Airyscan, Germany, low illumination intensity). All procedures and protocols involving animal use were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. Airyscan 880 images were both standard confocal images (whole cell) and deconvolved Airyscan images (zoomed in high resolution images) that were further analyzed using the image-processing software *ImageJ* (Wayne Rasband, National Institutes of Health, Bethesda, MD). Transient changes of Ψ_M were analyzed up until the imaged cellular region (68 μ m by 68 μ m) lost 5% of its initial TMRM fluorescence ($t_{95}=31.71 \pm 5$ minutes, $n=7$ cells). Further statistical analysis and plotting were done with OriginPro 2018. All results are presented as mean $\pm$ s.e.m.

Tissue preparation and arterial smooth muscle cell isolation.

Rat cerebral arterial smooth muscle cells were isolated as previously described[43]. In brief, Sprague-Dawley rats of either sex were sacrificed by intraperitoneal injection of a lethal dose pentobarbital, and the brain was removed and placed into ice (4 ° C) cold HEPES-buffered saline solution containing (in mM): (134) NaCl, (6) KCl, (1) MgCl₂, (2) CaCl₂, (10) glucose, (10) HEPES (pH 7.4 adjusted with NaOH). Posterior and cerebellar cerebral arteries were dissected and cleaned of connective tissues. The arteries were then transferred into HEPES-buffered isolation solution containing (in mM) (55) NaCl, (80) sodium glutamate, (5.6) KCl, (2) MgCl₂, (10) HEPES, and (10) glucose (pH 7.3 with NaOH), which was supplemented with papain (0.7 mg/ml) and collagenase (1.0 mg/ml) for digestion. Isolated smooth muscle cells were maintained in ice-cold (4°C) HEPES-buffered isolation solution and used within 8 h after dissociation. All procedures and protocols involving animal use were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

Ψ_M measurements in smooth muscle cells.

Freshly isolated arterial smooth muscle cells were incubated in HEPES-buffered saline solution with tetramethylrhodamine ethyl ester (TMRE, 100 nM, to measure Ψ_m for 20 min, followed by 5 min wash. To prevent cell contraction, 10 μ M wortmannin was included in all the experimental solutions. Zeiss LSM510 microscopy was used for fluorescence imaging. Unless otherwise stated, 400 images sized 71.43 $\times$ 71.43 μ m (512 $\times$ 512 pixels) were acquired every 3 s at lower (1%) or higher (8%) laser power. Data were analyzed offline using Image J 1.42q. Statistics and figures were processed and prepared using OriginPro 2018. All results are presented as mean $\pm$ s.e.m.

Results

The data acquired in our investigation makes use of the Zeiss Airyscan 880 inverter confocal super-resolution microscope to image rat cardiac ventricular myocytes and the mitochondria therein. The myocytes were isolated and placed in an incubator imaging bath (37 ° C, 5% CO₂, 20% O₂, pH = 7.4). Mitochondria are densely packed within ventricular myocytes, occupying approximately 1/3 of the cell volume[44–46]. The mitochondria were regularly imaged every 9–12 seconds in a 68 micron by 68 micron square as shown in the Fig. 1A diagram. Every 5 minutes an image of the entire cell was also acquired. The timing of these events is shown in the time-line in Fig. 1B. The zoomed-in region of the whole cell image shows the mitochondria that are polarized (red, very negative Ψ_M). TMRM (tetramethyl rhodamine methyl ester) was used to image the mitochondria and used to report on the mitochondrial membrane potential, Ψ_M [47]. TMRM is an amphiphilic positively-charged cell-permeant fluorescent indicator that readily dissolves in aqueous solutions and distributes itself across membranes in a voltage-dependent manner[48–50]. The very low level of illumination enables us to minimally perturb the redox state of the cell and the mitochondria, thus enabling us to use this process as a tool to investigate mPTP behavior by what we call “Optical Stress-Probe” imaging.

Transient opening of mPTP.

Ventricular myocytes placed in the supplemented M199 media in the incubator adhere to the glass coverslip that forms the floor of the incubator. The solution contains TMRM at a concentration of 50 nM and this leads to the quasi-steady-state fluorescence signal in the mitochondria shown in Fig. 2A, Left. The estimated -80 mV membrane potential of the living adult ventricular myocyte at 37°C underlies the movement of TMRM from the extracellular solution into the cytosol and rapidly creates a steady-state distribution. The steady-state level of TMRM in the cytosol leads to a steady-state level of approximately 20-fold more than in the extracellular solution. Nevertheless, it still looks dark compared to the mitochondria which are quite bright. The TMRM is used to identify polarized mitochondria because they accumulate TMRM in the matrix at a very high levels (approximately 8,000-fold more than the extracellular level and 400-fold more than cytosol) because of the very negative Ψ_{M} . Fig. 2A, right panel, shows a super-resolution image of a normal rat ventricular myocyte using the Zeiss LSM 880 Airyscan technology to image the TMRM in the mitochondria. Further zooming in of the region identified in the small square shows three mitochondria (see Fig. 2B). The time-dependent changes in the TMRM fluorescence of the three mitochondria are shown in time-sequence (four items top to bottom). At the top all three mitochondria are bright yellow-red at $t=1529$ s. The pseudocolor relative fluorescence is shown to the right of the mitochondrial images. These three mitochondria are organized diagonally from the lower left to the upper right. Twelve seconds after the top most image, the second image from the top (at $t=1541$ s) shows that the middle mitochondrion is much darker (nearly black, marked by a white arrow). Thirty seconds later, the third image was acquired (at $t=1571$ s) and it shows that the middle mitochondrion has largely recovered its fluorescence. A discussion of the kinetics of the changes in fluorescence intensity follows below and in the Discussion. Much later (at time=1 hour), the mitochondria is very dark and, like its neighboring mitochondrion in the upper right corner of the zoomed frame, it has “permanently” depolarized while the mitochondrion in the bottom left is still fluorescent.

Photon stress.

Like all fluorescent indicators, illuminating the indicator with photons that are absorbed leads to the production of reactive oxygen species (ROS) [1–3, 41]. In the experiments shown here, every effort is taken to minimize the illumination level to keep the ROS production by photon stress low. Fig. 2A shows a zoomed-out view of the entire ventricular myocyte (Fig. 2A left) and the zoomed in view of about half of the cell (Fig. 2A, right). Fig. 2B shows the sequence of a mitochondrion before (top) it is transiently depolarized (second from top) and when it recovers (third from top) and much later when it is permanently depolarized (bottom). Fig. 2C shows the averaged relative fluorescence time-course of all transient mPTP opening events. Normalization was made to the fluorescence level of each mitochondrion before each transient mPTP opening event. For this average, the maximum depolarization time was used to synchronize the transient events. The maximum depolarization is shown to be associated with an average drop in TMRM fluorescence by $54 \pm 2\%$ and the half-time of recovery was 31 ± 6 s (478 events in 7 cells). The asymmetry of the transient depolarization kinetics and the recovery kinetics is striking. This asymmetry is probably due to a combination of a rapid depolarization (compared to the imaging interval) when the transient mPTP opening event occurs and the mitochondrial capacitance is

discharged. On the other hand, the much slower recovery time reflects the time it takes to repolarize the mitochondria as the ETC pumps out protons when mPTP closes coupled with the re-equilibration of TMRM as it diffuses across the mitochondrial membranes to faithfully reflect the repolarized Ψ_M . More experiments will be needed to sort out the detailed time-course and cause(s) of the asymmetry. It is still important to note that measuring the time-course of changes in TMRM fluorescence (Optical Stress-Probe imaging) does provide important detail surrounding the transient opening events.

What are the causes of the transient mPTP opening events and their recoveries?

The known ability of photon stress to produce ROS [1–3, 41] certainly appears to be a major factor in the development of transient mPTP opening events. Additionally, there is the historic evidence linking elevated mitochondrial ROS and elevated mitochondrial Ca^{2+} ($[\text{Ca}^{2+}]_M$) to the activation of mitochondrial permeability transition. Other factors may also play a role, such as mitochondrial matrix pH. Since ROS, $[\text{Ca}^{2+}]_M$, and pH_M have been argued to be important in the development of MitoFlashes[6, 7] and MitoWinks[8], the use of Optical Stress-Probe imaging with simultaneous measurements of $[\text{H}^+]_M$ and $[\text{Ca}^{2+}]_M$ would appear to be valuable to sort out the roles of these variable in future studies. Here we show that the fast onset of depolarization (~ 9 s or less) is contrasted with the longer period of recovery of polarization. Furthermore, the range of the extent of the depolarization shown in Fig. 2D and Fig. 2E during mPTP opening events is wide (from a reduction of TMRM fluorescence of about 20% to nearly 100%). It may possibly mean that there is a range of the open time of the putative channel that underlies the event. This is illustrated in the example shown in Fig. 2D (red trace) that lasts much longer than the typical event or than the average of all events. The half time of recovery, however, is about the same (20–30 s) for all events. These data are consistent with the opening of a channel to produce the onset of the transient depolarization of variable time and a repolarization period that is similar for all events. Repolarization then is consistent with the channel closure followed by a time-dependent extrusion of protons by the ETC and diffusion of TMRM into the matrix from the cytosol.

Are transient mPTP depolarization events spontaneous elements in quiescent heart cells?

The simple answer based on our findings is probably not. Fig. 3A supports our conclusion that transient mPTP depolarizing events almost never occur without photon illumination. The first event in an imaged cell occurs on average after 8.4 minutes of imaging (± 1.1 SEM $n=21$ cells). The first events for individual cells are identified in Fig. 3A to enable comparison with Fig. 5C. Importantly the cumulative number of transient mPTP events (Fig. 3B) is slow to begin and increases non-linearly with time as does the number of transient events per cell per minute (Fig. 3B). These observations, when taken together, suggest that imaging generated ROS (i.e. photon stress) underlies many of the mPTP opening events that we observe (both transient and permanent) in these experiments. Such conclusion is further supported by the findings shown in Fig. 3D–I. In these experiments, the occurrence of transient depolarization events is reduced by 80 % when the mPTP antagonist CsA (10 mM) is present. Furthermore, the occurrence of transient depolarization events is reduced by 93 % when the ROS scavenger NAC (10 mM) is present. As seen under control conditions, in the presence of CsA or NAC, a gradual increase in the occurrence of events still develops over time (see Fig. 3E and Fig. 3H) as one would expect if time-dependent accumulation of ROS

plays a central role albeit at a different rate under each condition. Our findings shown in Figs. 3 and 4 are consistent with the channel hypothesis for mPTP. This hypothesis posits that the mPTP depolarization event occurs when the mPTP “channel” opens due to ROS-dependent interaction with the mPTP protein complex. Fig. 4C shows that the number of events (N) scale with the ROS burden: $N_{\text{control}} > N_{\text{CsA}} > N_{\text{NAC}}$. These data are also consistent with the channel hypothesis when the mPTP terminates, a reaction that, if spontaneous, should not depend on the ROS burden. We observe that when the mPTP depolarization event is over and the hypothesized channel closes the rate of closure does not depend on how the opening was initiated (i.e. control conditions versus CsA treatment or NAC exposure). In all cases, the $t_{0.5}$ for recovery of Ψ_M should be similar. Fig. 4A shows that the time-course of recovery does not appear to depend on the ROS burden: $t_{\text{control}} \cong t_{\text{CsA}} \cong t_{\text{NAC}}$. As noted above Fig. 4C show the stark effect of CsA and NAC in reducing the number of depolarization events observed over a time period of 30 minutes in each cell. Fig. 4D shows that like heart mitochondria, smooth muscle mitochondria produce depolarization events more quickly when the illumination intensity is greater.

Transition from transient mPTP opening events to permanent mPTP opening events.

Fig. 5 shows the results of all ventricular myocytes that were imaged for one hour under control conditions and while treated with either CsA or NAC. While transient mPTP opening events were seen abundantly in the first 30 minutes in the region of interest (ROI) in all control cells, an increasing number of permanent depolarizations occurred only in the control cells. In all cells, the ROI was scanned every ~ 9 s. Fig. 5A shows a control cell and the uniformity of the polarized mitochondria at the beginning of the experiment. Fig. 5A left shows the whole cell and Fig. 5A right shows the zoomed-in ROI. After an hour of imaging, many mitochondria in the ROI were permanently depolarized. Mitochondria in the rarely imaged surround (imaged every 5 minutes) are virtually unchanged throughout the study (Fig. 5B acquired at 60 min). Fig. 5C shows the fluorescence of the ROI in the control cells and the variation cell-to-cell. Fig. 5D shows the large decrease in the average fluorescence within the ROI of control cells (black line) and the very small decrease (almost none) in CsA cells (red line) and NAC cells (blue line).

Discussion

We have examined mitochondria in healthy isolated rat ventricular myocytes at 37 °C for an hour to track the behavior of the mitochondrial permeability transition pore (mPTP) channels. The experiments took advantage of the fluorescent potentiometric indicator, TMRM (tetramethyl rhodamine methyl ester), to measure the inner membrane potential Ψ_M . We examined single mitochondria under near-normal conditions but in the absence of cyclic contraction. The exposure to low level ROS due to photon-TMRM interactions activated silent mPTP channels in mitochondria. Transient mPTP opening events appeared first and were followed by permanent mPTP opening events. Both transient and permanent mPTP depolarization events were antagonized by the antioxidant NAC and the mPTP blocker CsA. These observations about mPTP depolarizations suggest that they arise from the ROS generated by the imaging process itself and are not due to spontaneous functions of the mitochondria. These mPTP opening events were primarily observed in single

mitochondria and not in a highly interactive collection of mitochondria. This is clear evidence that there is not a large network of electrically coupled mitochondria under the conditions of these experiments. There are likely additional processes and mechanisms that can lead to reversible depolarization Ψ_M in intact cells that are beyond the scope of the current work. We highlight the components of the mechanism relevant to this work in discussion Fig. 6.

When considering our observations and findings in the context of published work, a number of questions are raised. First, how do the new data presented here relate to the transient depolarizations of individual mitochondria such as MitoFlashes and MitoWinks? Second, do the mitochondria in heart operate as a network of interacting partners? (We noted above that this is unlikely.) Third, does the work presented here provide one or more tools that may help in the quest to identify the molecular identity of mPTP channels?

MitoFlashes and MitoWinks.

The work reported here, focuses on the mitochondrial inner membrane potential or Ψ_M , and both transient and permanent depolarizations of individual mitochondria that occur while imaging Ψ_M with the fluorescent potentiometric indicator TMRM. We schematically illustrate the process in Fig. 6. We do not measure mitochondrially targeted cpYFP fluorescence (MitoFlashes) or formally examine MitoWinks in this paper. It is appreciated, however, that MitoFlashes have been seen in both isolated myocytes and in functioning tissue[51] and that MitoWinks have only been observed in isolated cells[6, 8]. While MitoFlashes and MitoWinks appear to have many similarities, they may still arise through different mechanisms. Note that MitoWinks have not been examined with cpYFP. Nevertheless, our findings are relevant to both. Both phenomena are reported to be associated with transient mPTP depolarizations (See Venn Diagram, Fig. 6B). Both MitoFlashes and MitoWinks can be observed and studied in isolated quiescent ventricular myocytes using photon-absorbing mitochondrially-localized indicators and imaged by confocal microscopy as we have done here. In this study, we examined all of the mitochondrial depolarizations that occurred in the region of interest (ROI) during the examination period. Importantly, we observed mitochondria in the ROI for a longer period of time but at a lower scan rate. We determined that virtually all depolarization events of the mitochondria (both transient and permanent) were due to photon stress (i.e. photon-generated ROS) during the observation period. We confirmed this by the huge reduction in the number of depolarization events when the antioxidant NAC (10 mM) was applied. We deduced that the depolarization events were due to mPTP because they were almost eliminated by treatment with CsA (10 μ M). Thus, we conclude that the depolarization events we observed were due to the generation of ROS that opened mPTP.

The rate of occurrence of depolarization events under the conditions of the experiments presented here was hugely lower than that reported by those investigating either MitoFlashes or MitoWinks. The depolarization events in our ROI was 24-fold less than that seen for MitoFlashes [6] and 20–50 fold less than for MitoWinks [8], when normalized to the scanned area of the ROI. Note that in our experiments we acquired images every 9–12 s under our extremely low illumination conditions in the presence of 50 nM TMRM. We

attribute the lower rate of depolarization events to the lower frequency of the imaging done in our experiments. Our imaging was still fast enough to resolve all of the events observed by others.

In our experiments, there appeared to be no ROS “spill-over” effect from depolarized mitochondria in the ROI affecting mitochondria within the cell but outside of the ROI. We conclude this because the mitochondria in the same cell that were outside the scanned ROI showed virtually no depolarizations contemporaneously with the depolarizations within the ROI. See Fig. 5. The considerable controversy surrounding the mt-cpYFP fluorescence signal and its interpretation[52–56] is not one that we have investigated.

Single mitochondria versus mitochondrial networks?

We present a compelling set of observations in the cardiac mitochondria that suggest virtually all of the mitochondria in ventricular myocytes (i.e. sub-sarcolemmal, intermyofibrillar, and perinuclear mitochondria) act as individual units. This is particularly clear when these mitochondria are examined during the development of transient or permanent mPTP depolarizations. Our finding here is different than the suggested network of interconnected mitochondria of significant size with unusual features [57]. Based on our data, the transient mPTP opening events occur largely in single mitochondrial units for the preponderance of observed events. Nevertheless, there were a small number of episodes where two to four mitochondria were observed to transiently depolarize (and/or repolarize) in synchrony. Consistent with this finding is our observation that the vast majority of permanent depolarization events appear to take place in single mitochondria.

Transient depolarizations in mitochondria versus oscillatory behavior.

Here we report of transient Ψ_M depolarization displayed largely by individual mitochondria. Other studies have described similar events as “oscillations” even when they only report a single transient depolarization. The physiology or pathophysiology of true oscillations have different mechanistic underpinnings. The opening and closing of a single channel produces a transient change in current or a transient depolarization. The activation of a system with some positive feedback or some regenerative signaling often produces oscillations. Thus, cyclic repetitive cell-wide oscillations of Ψ_M may occur with oscillations of mitochondrial NAD(P)H, both occurring with steady frequency [9–11]. These oscillations have been observed by multiple groups working on an array of cell types. In addition, other studies report of partially synchronized oscillations of Ψ_M in groups of mitochondria or ‘clusters’ of mitochondria observed in isolated cardiomyocytes [58]. In all cases, these are partially synchronized events where Ψ_M changes at once in numerous mitochondria and thus require a synchronizing mechanism. These types of observations are clearly different than those reported here. The changes of Ψ_M that we observe do not have a repetitive oscillatory frequency. The events that we observe include transient or more permanent depolarization events limited to individual mitochondria. The distinction between our findings here and the reports by others that show partially synchronized wide-spread oscillations of Ψ_M raises a number of interesting questions. For example, what is the synchronizing mechanism? Do changes of Ψ_M reflect oscillations of mitochondrial energy production or oscillations of cellular energy demands? There are likely additional important

questions raised by these findings, and future research along these lines certainly represents a fascinating direction to better understand mitochondrial biology. Nevertheless these events are fundamentally different from the transient depolarizations in mitochondria that we report here or the reports describing MitoFlashes [6, 7] or MitoWinks [8].

Origin of photon-stress induced mitochondrial depolarization.

We can only speculate on how photon-stress induced ROS production underlies the opening of mPTP. Our observations suggest that the initial event is attributable to the oxidation of a residue(s) on the mPTP protein complex that de-stabilizes the native protein complex. The destabilization enables the mPTP to open for a short period of time (milliseconds to seconds) and then close. Presumably, the closing is related to reduction of the oxidized residue(s) allowing the protein complex to assume its original stable conformation. Our methods (tracking the time-course of fluorescence intensity changes) do not provide much functional or temporal information. Nevertheless, as intensity of the effect progresses with greater or longer photon stress, perhaps a second tandem de-stabilization at a different site on the protein complex leads to more permanent open state. Perhaps the antioxidant NAC works by strengthening the native chemical reducing power of the mitochondrial matrix environment. If so, perhaps CsA works to protect the mPTP protein complex and shield the possible oxidation sites. More definitive molecular and quantitative biophysical information is needed to better identify and characterize mPTP.

Identity of the mPTP channel remains uncertain.

Recent reviews account for the demise of the tripartite mPTP channel (VDAC, ANT and Cyclophilin-D) [33–35, 39]. The essence of the argument is that mPTP events can still occur in the absence of one or more of the components. A new major candidate for the mPTP channel is ATP synthase or one or more parts of it [59, 60]. The essence of the arguments against this hypothesized mPTP channel is that mPTP opening events continue to exist when the putative key components of the ATP synthase are hobbled [39, 61]. The vacuum of additional candidates makes the use of Optical Stress-Probe imaging an attractive approach when searching for a new candidate and testing how well that new candidate works in heart cells. It also makes it possible to investigate the physiology and pathophysiology of the mPTP channel whether or not it acts as a transient mPTP channel or one that opens permanently.

What is the function of the mPTP protein complex?

Neither we in this paper nor others have been able to answer this question clearly. What we have presented here is evidence that under quiescent conditions with normal $[Ca^{2+}]_i$ and $[Ca^{2+}]_M$, the mPTP protein opens extremely rarely if ever as best we can determine. However, under conditions that generate ROS, such as photon stress, transient depolarizations occur more often. From our observations and those carefully delineated in the work by Shroff and co-workers[4] we need to proceed with care and caution when the process of observation may significantly affect our mechanistic investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by American Heart Association grants SDG 15SDG22100002 (to L.B.) and 16PRE31030023 (to A.P.W.); By NIH R01 HL106056 and R01 HL105239 and U01 HL116321 (to W.J.L.); By NIH R01 AR071618, R01 AR071614, and R01 HL142290 (to C.W.W); By the Medical Scientist Training Program and Training Program in Integrative Membrane Biology, NIH 2T32GM092237-06 and 5T32GM008181-28 (to A.P.W); By Interdisciplinary Training Program in Muscle Biology, NIH 5T32AR007592-23 (to AKC).

Abbreviations

$[Ca^{2+}]_i$	cytosolic free calcium
$[Ca^{2+}]_m$	mitochondrial matrix free calcium
IMM	inner mitochondrial membrane
OMM	outer mitochondrial membrane
Ψ_M	the voltage gradient across the inner mitochondrial membrane
mPTP	mitochondrial permeability transition pore
ROS	reactive oxygen species
CsA	cyclosporine-A
NAC	n-acetyl cysteine
ROI	region of interest

References

- [1]. Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ, Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes, *Journal of Experimental Medicine* 192(7) (2000) 1001–1014. [PubMed: 11015441]
- [2]. Jimenez-Banzo A, Nonell S, Hofkens J, Flors C, Singlet oxygen photosensitization by EGFP and its chromophore HBDI, *Biophys J* 94(1) (2008) 168–72. [PubMed: 17766345]
- [3]. Gandin E, Lion Y, Van de Vorst A, Quantum Yield of Singlet Oxygen Production by Xanthene Derivatives, *Photochem Photobiol* 37(3) (1983) 271–178.
- [4]. Laissue PP, Alghamdi RA, Tomancak P, Reynaud EG, Shroff H, Assessing phototoxicity in live fluorescence imaging, *Nat Methods* 14(7) (2017) 657–661. [PubMed: 28661494]
- [5]. Phototoxicity revisited, *Nat Methods* 15(10) (2018) 751. [PubMed: 30275585]
- [6]. Wang W, Fang H, Groom L, Cheng A, Zhang W, Liu J, Wang X, Li K, Han P, Zheng M, Yin J, Wang W, Mattson MP, Kao JP, Lakatta EG, Sheu SS, Ouyang K, Chen J, Dirksen RT, Cheng H, Superoxide flashes in single mitochondria, *Cell* 134(2) (2008) 279–90. [PubMed: 18662543]
- [7]. Wang X, Jian C, Zhang X, Huang Z, Xu J, Hou T, Shang W, Ding Y, Zhang W, Ouyang M, Wang Y, Yang Z, Zheng M, Cheng H, Superoxide flashes: elemental events of mitochondrial ROS signaling in the heart, *J Mol Cell Cardiol* 52(5) (2012) 940–8. [PubMed: 22405973]

- [8]. Lu X, Kwong JQ, Molkentin JD, Bers DM, Individual Cardiac Mitochondria Undergo Rare Transient Permeability Transition Pore Openings, *Circ Res* 118(5) (2016) 834–41. [PubMed: 26712344]
- [9]. Porat-Shliom N, Chen Y, Tora M, Shitara A, Masedunskas A, Weigert R, In vivo tissue-wide synchronization of mitochondrial metabolic oscillations, *Cell Rep* 9(2) (2014) 514–21. [PubMed: 25373899]
- [10]. Andersen AZ, Poulsen AK, Brasen JC, Olsen LF, On-line measurements of oscillating mitochondrial membrane potential in glucose-fermenting *Saccharomyces cerevisiae*, *Yeast* 24(9) (2007) 731–9. [PubMed: 17568453]
- [11]. Merrins MJ, Poudel C, McKenna JP, Ha J, Sherman A, Bertram R, Satin LS, Phase Analysis of Metabolic Oscillations and Membrane Potential in Pancreatic Islet beta-Cells, *Biophys J* 110(3) (2016) 691–699. [PubMed: 26840733]
- [12]. Lee H, Yoon Y, Transient contraction of mitochondria induces depolarization through the inner membrane dynamin OPA1 protein, *J Biol Chem* 289(17) (2014) 11862–72. [PubMed: 24627489]
- [13]. Rosselin M, Santo-Domingo J, Bermont F, Giacomello M, Demaurex N, L-OPA1 regulates mitoflash biogenesis independently from membrane fusion, *EMBO Rep* 18(3) (2017) 451–463. [PubMed: 28174208]
- [14]. Kirichok Y, Krapivinsky G, Clapham DE, The mitochondrial calcium uniporter is a highly selective ion channel, *Nature* 427(6972) (2004) 360–4. [PubMed: 14737170]
- [15]. Fieni F, Lee SB, Jan YN, Kirichok Y, Activity of the mitochondrial calcium uniporter varies greatly between tissues, *Nat Commun* 3 (2012) 1317. [PubMed: 23271651]
- [16]. Luongo TS, Lambert JP, Yuan A, Zhang X, Gross P, Song J, Shanmughapriya S, Gao E, Jain M, Houser SR, Koch WJ, Cheung JY, Madesh M, Elrod JW, The Mitochondrial Calcium Uniporter Matches Energetic Supply with Cardiac Workload during Stress and Modulates Permeability Transition, *Cell Rep* 12(1) (2015) 23–34. [PubMed: 26119731]
- [17]. Kwong JQ, Lu X, Correll RN, Schwaneckamp JA, Vagnozzi RJ, Sargent MA, York AJ, Zhang J, Bers DM, Molkentin JD, The Mitochondrial Calcium Uniporter Selectively Matches Metabolic Output to Acute Contractile Stress in the Heart, *Cell reports* 12(1) (2015) 15–22. [PubMed: 26119742]
- [18]. Wu Y, Rasmussen TP, Koval OM, Joiner ML, Hall DD, Chen B, Luczak ED, Wang Q, Rokita AG, Wehrens XH, Song LS, Anderson ME, The mitochondrial uniporter controls fight or flight heart rate increases, *Nat Commun* 6 (2015) 6081. [PubMed: 25603276]
- [19]. Palty R, Silverman WF, Hershfinkel M, Caporale T, Sensi SL, Parnis J, Nolte C, Fishman D, Shoshan-Barmatz V, Herrmann S, Khananshvili D, Sekler I, NCLX is an essential component of mitochondrial Na⁺/Ca²⁺ exchange, *Proc Natl Acad Sci U S A* 107(1) (2010) 436–41. [PubMed: 20018762]
- [20]. Boyman L, Williams GS, Khananshvili D, Sekler I, Lederer WJ, NCLX: the mitochondrial sodium calcium exchanger, *J Mol Cell Cardiol* 59 (2013) 205–13. [PubMed: 23538132]
- [21]. Kostic M, Ludtmann MH, Bading H, Hershfinkel M, Steer E, Chu CT, Abramov AY, Sekler I, PKA Phosphorylation of NCLX Reverses Mitochondrial Calcium Overload and Depolarization, Promoting Survival of PINK1- Deficient Dopaminergic Neurons, *Cell Rep* 13(2) (2015) 376–86. [PubMed: 26440884]
- [22]. Al-Nasser I, Crompton M, The reversible Ca²⁺-induced permeabilization of rat liver mitochondria, *Biochem J* 239(1) (1986) 19–29. [PubMed: 3099778]
- [23]. Crompton M, Costi A, Kinetic evidence for a heart mitochondrial pore activated by Ca²⁺, inorganic phosphate and oxidative stress. A potential mechanism for mitochondrial dysfunction during cellular Ca²⁺ overload, *Eur J Biochem* 178(2) (1988) 489–501. [PubMed: 2850179]
- [24]. Kinnally KW, Campo ML, Tedeschi H, Mitochondrial channel activity studied by patch-clamping mitoplasts, *J Bioenerg Biomembr* 21(4) (1989) 497–506. [PubMed: 2478535]
- [25]. Szabo I, Zoratti M, The giant channel of the inner mitochondrial membrane is inhibited by cyclosporin A, *J Biol Chem* 266(6) (1991) 3376–9. [PubMed: 1847371]
- [26]. Szabo I, Zoratti M, The mitochondrial megachannel is the permeability transition pore, *J Bioenerg Biomembr* 24(1) (1992) 111–7. [PubMed: 1380498]

- [27]. Bernardi P, Broekemeier KM, Pfeiffer DR, Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane, *J Bioenerg Biomembr* 26(5) (1994) 509–17. [PubMed: 7896766]
- [28]. Haworth RA, Hunter DR, The Ca^{2+} -induced membrane transition in mitochondria. II. Nature of the Ca^{2+} trigger site, *Arch Biochem Biophys* 195(2) (1979) 460–7. [PubMed: 38751]
- [29]. Szabo I, De Pinto V, Zoratti M, The mitochondrial permeability transition pore may comprise VDAC molecules. II. The electrophysiological properties of VDAC are compatible with those of the mitochondrial megachannel, *FEBS Lett* 330(2) (1993) 206–10. [PubMed: 7689984]
- [30]. Halestrap AP, Davidson AM, Inhibition of Ca^{2+} -induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase, *Biochem J* 268(1) (1990) 153–60. [PubMed: 2160810]
- [31]. Halestrap AP, The C Ring of the F1Fo ATP Synthase Forms the Mitochondrial Permeability Transition Pore: A Critical Appraisal, *Front Oncol* 4 (2014) 234. [PubMed: 25202683]
- [32]. Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP, MacGregor GR, Wallace DC, The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore, *Nature* 427(6973) (2004) 461–5. [PubMed: 14749836]
- [33]. Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, Robbins J, Molkentin JD, Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death, *Nature* 434(7033) (2005) 658–62. [PubMed: 15800627]
- [34]. Baines CP, Kaiser RA, Sheiko T, Craigie WJ, Molkentin JD, Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death, *Nat Cell Biol* 9(5) (2007) 550–5. [PubMed: 17417626]
- [35]. Kwong JQ, Molkentin JD, Physiological and pathological roles of the mitochondrial permeability transition pore in the heart, *Cell Metab* 21(2) (2015) 206–14. [PubMed: 25651175]
- [36]. Giorgio V, von Stockum S, Antoniel M, Fabbro A, Fogolari F, Forte M, Glick GD, Petronilli V, Zoratti M, Szabo I, Lippe G, Bernardi P, Dimers of mitochondrial ATP synthase form the permeability transition pore, *Proc Natl Acad Sci U S A* 110(15) (2013) 5887–92. [PubMed: 23530243]
- [37]. Bonora M, Morganti C, Morciano G, Pedriali G, Lebedzinska-Arciszewska M, Aquila G, Giorgi C, Rizzo P, Campo G, Ferrari R, Kroemer G, Wieckowski MR, Galluzzi L, Pinton P, Mitochondrial permeability transition involves dissociation of F1FO ATP synthase dimers and C-ring conformation, *EMBO Rep* 18(7) (2017) 1077–1089. [PubMed: 28566520]
- [38]. Alavian KN, Beutner G, Lazrove E, Sacchetti S, Park HA, Licznerski P, Li H, Nabili P, Hockensmith K, Graham M, Porter GA Jr., Jonas EA, An uncoupling channel within the c-subunit ring of the F1FO ATP synthase is the mitochondrial permeability transition pore, *Proc Natl Acad Sci U S A* 111(29) (2014) 10580–5. [PubMed: 24979777]
- [39]. Baines CP, Gutierrez-Aguilar M, The still uncertain identity of the channel-forming unit(s) of the mitochondrial permeability transition pore, *Cell Calcium* 73 (2018) 121–130. [PubMed: 29793100]
- [40]. He J, Ford HC, Carroll J, Ding S, Fearnley IM, Walker JE, Persistence of the mitochondrial permeability transition in the absence of subunit c of human ATP synthase, *Proc Natl Acad Sci U S A* 114(13) (2017) 3409–3414. [PubMed: 28289229]
- [41]. Boyman L, Chikando AC, Williams GS, Khairallah RJ, Kettlewell S, Ward CW, Smith GL, Kao JP, Lederer WJ, Calcium movement in cardiac mitochondria, *Biophys J* 107(6) (2014) 1289–301. [PubMed: 25229137]
- [42]. Boyman L, Hiller R, Lederer WJ, Khananshvilis D, Direct Loading of the purified endogenous inhibitor into the cytoplasm of patched cardiomyocytes blocks the ion currents and calcium transport through the NCX1 protein, *Biochemistry* 47(25) (2008) 6602–11. [PubMed: 18507397]
- [43]. Zhao G, Adebisi A, Xi Q, Jaggar JH, Hypoxia reduces K_{Ca} channel activity by inducing Ca^{2+} spark uncoupling in cerebral artery smooth muscle cells, *Am.J.Physiol Cell Physiol* 292(6) (2007) C2122–C2128. [PubMed: 17314264]

- [44]. Barth E, Stammer G, Speiser B, Schaper J, Ultrastructural quantitation of mitochondria and myofilaments in cardiac muscle from 10 different animal species including man, *J Mol Cell Cardiol* 24(7) (1992) 669–81. [PubMed: 1404407]
- [45]. Legato MJ, Cellular mechanisms of normal growth in the mammalian heart. II. A quantitative and qualitative comparison between the right and left ventricular myocytes in the dog from birth to five months of age, *Circ Res* 44(2) (1979) 263–79. [PubMed: 761308]
- [46]. Smith HE, Page E, Ultrastructural changes in rabbit heart mitochondria during the perinatal period. Neonatal transition to aerobic metabolism, *Dev Biol* 57(1) (1977) 109–17. [PubMed: 863101]
- [47]. Farkas DL, Wei MD, Febroriello P, Carson JH, Loew LM, Simultaneous imaging of cell and mitochondrial membrane potentials, *Biophys J* 56(6) (1989) 1053–69. [PubMed: 2611324]
- [48]. Loew LM, Tuft RA, Carrington W, Fay FS, Imaging in five dimensions: time-dependent membrane potentials in individual mitochondria, *Biophys J* 65(6) (1993) 2396–407. [PubMed: 8312478]
- [49]. Loew LM, Carrington W, Tuft RA, Fay FS, Physiological cytosolic Ca²⁺ transients evoke concurrent mitochondrial depolarizations, *Proc Natl Acad Sci U S A* 91(26) (1994) 12579–83. [PubMed: 7809081]
- [50]. Fink C, Morgan F, Loew LM, Intracellular fluorescent probe concentrations by confocal microscopy, *Biophys J* 75(4) (1998) 1648–58. [PubMed: 9746507]
- [51]. Gong G, Wang W, Confocal imaging of single mitochondrial superoxide flashes in intact heart or in vivo, *J Vis Exp* (81) (2013) e50818.
- [52]. Schwarzlender M, Wagner S, Ermakova YG, Belousov VV, Radi R, Beckman JS, Buettner GR, Demareux N, Duchon MR, Forman HJ, Fricker MD, Gems D, Halestrap AP, Halliwell B, Jakob U, Johnston IG, Jones NS, Logan DC, Morgan B, Muller FL, Nicholls DG, Remington SJ, Schumacker PT, Winterbourn CC, Sweetlove LJ, Meyer AJ, Dick TP, Murphy MP, The ‘mitoflash’ probe cpYFP does not respond to superoxide, *Nature* 514(7523) (2014) E12–4. [PubMed: 25341790]
- [53]. Quatresous E, Legrand C, Pouvreau S, Mitochondria-targeted cpYFP: pH or superoxide sensor?, *J Gen Physiol* 140(5) (2012) 567–70. [PubMed: 23071268]
- [54]. Schwarzlender M, Logan DC, Fricker MD, Sweetlove LJ, The circularly permuted yellow fluorescent protein cpYFP that has been used as a superoxide probe is highly responsive to pH but not superoxide in mitochondria: implications for the existence of superoxide ‘flashes’, *Biochem J* 437(3) (2011) 381–7. [PubMed: 21631430]
- [55]. Muller FL, A critical evaluation of cpYFP as a probe for superoxide, *Free Radic Biol Med* 47(12) (2009) 1779–80. [PubMed: 19778603]
- [56]. Schwarzlender M, Murphy MP, Duchon MR, Logan DC, Fricker MD, Halestrap AP, Muller FL, Rizzuto R, Dick TP, Meyer AJ, Sweetlove LJ, Mitochondrial ‘flashes’: a radical concept repHined, *Trends Cell Biol* 22(10) (2012) 503–8. [PubMed: 22917552]
- [57]. Glancy B, Hartnell LM, Combs CA, Femnou A, Sun J, Murphy E, Subramaniam S, Balaban RS, Power Grid Protection of the Muscle Mitochondrial Reticulum, *Cell reports* 19(3) (2017) 487–496. [PubMed: 28423313]
- [58]. Kurz FT, Aon MA, O’Rourke B, Armondas AA, Spatio-temporal oscillations of individual mitochondria in cardiac myocytes reveal modulation of synchronized mitochondrial clusters, *Proc Natl Acad Sci U S A* 107(32) (2010) 14315–20. [PubMed: 20656937]
- [59]. Bernardi P, Why F-ATP Synthase Remains a Strong Candidate as the Mitochondrial Permeability Transition Pore, *Front Physiol* 9 (2018) 1543. [PubMed: 30443222]
- [60]. Beutner G, Alanzalon RE, Porter GA Jr., Cyclophilin D regulates the dynamic assembly of mitochondrial ATP synthase into synthasomes, *Scientific reports* 7(1) (2017) 14488. [PubMed: 29101324]
- [61]. Chinopoulos C, Mitochondrial permeability transition pore: Back to the drawing board, *Neurochem Int* 117 (2018) 49–54. [PubMed: 28647376]

Fig. 1. Diagram of Imaging Procedure.

A, Diagram illustrating how a single isolated ventricular myocyte was imaged. A square (edge 136 μm , red frame) was imaged by standard confocal scan 1024 $\times$ 1024 pixels with low intensity (0.009 % laser power) 561 nm laser illumination. These images were acquired every 5 minutes. Within the large frame, a high-resolution square Airyscan image (edge 68 μm , green frame) was acquired every 9–12 s after the previous image, XY resolution was 120 nm using low intensity 561 nm light. A blue box within the Airyscan frame is used to illustrate that individual mitochondria can be viewed from within the Airyscan image.

TMRM (tetramethyl rhodamine methyl ester, 50 nM) was in the extracellular solution (see Methods) and thus entered the cytosol and the mitochondria. The TMRM was used to view the inner membrane potential (Ψ_m) of individual mitochondria. By this means Ψ_M of individual mitochondria was tracked dynamically at high spatial resolution. **B**, Cells were imaged for 60 minutes and the time-line illustrates the imaging tasks during that period. Imaging was done with a Zeiss Airyscan 880 inverted confocal microscope using a 63X oil PlanApo lens NA=1.4.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2. Transient depolarization of individual mitochondria.

A, left, confocal image of a cardiomyocyte showing the fluorescence of TMRM. Right, deconvolved Airyscan confocal image of the central region (green box is the Region of Interest or ROI) of cell shown on left. **B**, TMRM fluorescent of highly zoomed-in square (edge 2.1 μm) subcellular region from right panel of **A**. **C**, Time-course of transient mitochondrial depolarizations; data reflect the average of many events in multiple cells (478 events, n = 7 cells). The TMRM fluorescence was reduced by 54%. The half-time of recovery was 31 $\pm$ 6 s. **D**, Typical single transient depolarization shows TMRM reduction by 48% (blue trace), half-time of recovery 22 s; large transient depolarization shows TMRM reduction by 98% (red trace), half-time of recovery 25 s. **E**, the event amplitude distribution (478 events, n = 7 cells). See movie showing both transient mPTP opening events and permanent mPTP opening events in the supplementary information.

Fig. 3. The frequency of the transient mPTP opening events (depolarizations) of individual mitochondria.

A, the number of transient depolarizations events seen over the first 30 minutes of the imaging protocol. The initial transient mPTP opening event of each of the 7 cells studied is identified with an arrow. **B**, the cumulative number of transient mPTP opening events over the first 30 minutes of the imaging protocol (total number of events is 414). **C**, The number of transient mPTP opening events per cell per minute over the first 30 minutes of the imaging protocol. **D and G**, same as (A) but with the either CsA or NAC added to the M199

media at the indicated concentration. **E and H**, same as (B) but with the either CsA or NAC added to the M199 as indicated. **F and I**, same as (C) but with the either CsA or NAC added to the M199 as indicated. (n=7 cells for control group, n=5 cells for CsA group, n=5 cells for CsA group).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4. Transient depolarization of individual mitochondria in the presence of Cyclosporine A or N-Acetyl-Cysteine and in smooth muscle cells.

A, Time-course of transient mitochondrial depolarizations; data reflect the average of multiple events in multiple cells (478 events in the control group, $n = 7$ cells, 46 events in the CsA group, $n = 5$ cells, 14 events in the NAC group, $n = 5$ cells). The half-time of recovery is indicated for each group. **B**, the event amplitude distribution. **C**, The mean number of transient depolarization events per cell observed during the first 30 minutes of imaging (control group $n = 7$ cells, CsA group $n = 5$ cells, NAC group $n = 5$ cells). The * denotes p -value < 0.05 compared to the control group, Student's t -Test. **D**, The time at which the first depolarization event was observed in isolated smooth muscle cells. Green bar summarizes the results obtained when the laser power was set to 1% ($n=8$ cells) and the orange bar summarizes the results when the laser power was set to 8% ($n=23$ cells), the * denotes p -value < 0.05 , Student's t -Test.

Fig. 5. Transient mPTP opening events transition to permanent mPTP opening events as photon stress continues.

A, left, confocal image of a cardiomyocyte showing the fluorescence of TMRM at the beginning of the imaging protocol. Right, deconvolved high resolution Airyscan confocal image of the central region (ROI) of the same cell. **B**, left, confocal image of a cardiomyocyte showing the fluorescence of TMRM at the end of the imaging protocol (i.e., after 1 hour). Right, deconvolved high resolution Airyscan confocal image of the central region of the same cell. **C**, The time-dependent TMRM signal from the ROI. The

measurements from each cell (color coded and numbered 1 through 7) are shown. **D**, The average time-dependent TMRM signal from the ROI (n=7 cells for control group, n=5 cells for CsA group, n=5 cells for NAC group, s.e.m shown).

Fig. 6. Discussion figure.

A, Schematic diagram showing the core components of mitochondrial transient depolarization events. The mitochondrial permeability transition pore (mPTP, black oval) spans the inner mitochondrial membrane (IMM) and the outer mitochondrial membrane (OMM). The mPTP can be activated by reactive oxygen species (ROS) produced by multiple sources. The mPTP can also be activated by high levels of free Ca^{2+} in the mitochondrial matrix ($[\text{Ca}^{2+}]_m$). The $[\text{Ca}^{2+}]_m$ is set by the Ca^{2+} entry via the mitochondrial Ca^{2+} uniporter (MCU) and the extrusion of Ca^{2+} by the mitochondrial sodium calcium exchanger (NCLX).

The “Optical Stress-Probe” evokes mPTP-dependent depolarization events through the generation of ROS. **B**, Venn diagram illustrating the relation between different reported depolarization events. Red ellipse: all events that involve depolarization of the IMM (i.e., depolarization of Ψ_M). Grey ellipse: all events that involve opening of mPTP. Green circle: all events that involve MitoFlash (See Yu et. al., 2019 and [6]). Blue circle: all events that involve MitoWinks (see [8]). According to the defining papers, there are depolarization events that may be neither MitoWinks nor MitoFlash events; there are MitoFlash events that may also be MitoWink events.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript