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Visualization of cyclosporin A and Ca^{2+}-sensitive cyclical mitochondrial depolarizations in cell culture

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Abstract

Mitochondria not only facilitate chemiosmotic energy transduction, but also are excitable organelles that are important participants in intracellular Ca^{2+} signaling and are obligate participants in the active cell death cascade known as apoptosis. Underlying these functions is the cyclosporin A (CSA)-sensitive mitochondrial permeability transition pore (MTP), which can open transiently in a low conductance mode (MTPL) to relieve excess Ca^{2+}, and irreversibly during the initiation of apoptosis. Here we image for the first time CSA- and Ca^{2+}-sensitive cyclical mitochondrial depolarizations in cultures of the SH-SY5Y human neuroblastoma cell. In addition, we show that mitochondrial transmembrane potential ($\Delta\Psi$) increases in response to CSA, indicating a baseline channel activity. Moreover, networks of mitochondria are shown to behave as an excitable system that may use Ca^{2+} as a diffusible messenger to recruit neighboring mitochondria to depolarize. We propose that these depolarizations represent MTPL activity. Our data further reinforce the notion that mitochondria are excitable organelles and suggest coordinated activation of MTPL.

Keywords: Mitochondrial membrane potential; Permeability transition pore; Cyclosporin A; Calcium dependent depolarization

1. Introduction

The classical view of mitochondria simply as facilitators of chemiosmotic energy transduction [1] has evolved into a broader understanding that mitochondria are important participants in various intracellular processes. As participants in Ca^{2+} regulation, mitochondria are known to modulate the amplitude and pattern of Ca^{2+} transients due to physiological stimuli [2,3] and may even dominate over other mechanisms in the clearance of normal Ca^{2+} transients [4]. In many systems, mitochondria are necessary for the initiation of the active cell death process known as apoptosis [5], apparently through the release into the cytosol of molecules either loosely bound to mitochondrial membranes or residing in the intermembrane space. Species known to be released to initiate apoptosis include the electron transport chain (ETC) factor cytochrome *c* and 'apoptosis promoting activation factor' (APAF), which in combination with cytochrome *c* and dATP activates a proteolytic 'caspase' cascade [6].

Surprisingly, mitochondria are also excitable organelles that both exhibit spontaneous depolarization

Abbreviations: MTP, mitochondrial permeability transition pore; MTPL, low conductance mode of MTP; CSA, cyclosporin A; $\Delta\Psi$, mitochondrial membrane potential (delta psi); TMRM, tetramethylrhodamine; ROS, reactive oxygen species

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and are able to sustain waves of electrical depolarization and Ca^{2+} release in vitro [7]. Central to the mitochondrial role in initiation of apoptosis and mitochondrial excitability is the mitochondrial permeability transition pore (MTP). The MTP is a large proteinaceous channel which forms between the inner and outer membranes and can non-selectively pass solutes up to 1500 Da [8]. The MTP may have been identified electrophysiologically as the 1 nS mitochondrial multiconductance ion channel (MCC) [9]. Irreversible opening of the MTP results in a loss of the mitochondrial potential ($\Delta\Psi$) and the release of Ca^{2+}, metabolites and other small matrix molecules into the cytoplasm as well as osmotic swelling of mitochondria and disruption of outer membrane integrity [8]. The probability of pore opening is increased directly by intramitochondrial Ca^{2+} and by cellular reactive oxygen species (ROS) [10], and is decreased by matrix acidification [11] and by a strong transmembrane electrical potential [12]. MTP opening is by definition irreversibly inhibited by the immunosuppressant CSA [13], via its binding to a mitochondrial matrix cyclophilin [14].

Because it is regulated by ROS, $\Delta\Psi$ and Ca^{2+}, the MTP is ideally situated to be an important sensor of cellular and mitochondrial function. Although the MTP opens irreversibly as a part of the process of apoptosis [5], it also can open transiently in a low conductance mode, MTPL [15], that is not accompanied by osmotic swelling and may relieve excess $\Delta\Psi$ or Ca^{2+} [7,16]. Little is known about the regulation of MTPL, and it has only been observed in preparations of isolated mitochondria. Here we use the cationic fluorophore tetramethylrhodamine (TMRM) to image transient $\Delta\Psi$ and Ca^{2+}-sensitive mitochondrial depolarizations and repolarizations in cell culture. We propose that these depolarizations represent the reversible MTPL acting in a coordinated manner over the entire mitochondrial network.

2. Materials and methods

2.1. Image data

SH-SY5Y cells (gift of Dr. W.D. Parker) were grown on coverslips attached to 35-mm culture dishes (Mat-Tek Corp.) and were maintained in pyruvate free DMEM/10% FBS at 37°C and 5% CO_2. Cells were stained in the incubator for 15 min with 0.5 μM TMRM (Molecular Probes) suspended in serum and phenol red free RPMI 1640. Cells were rinsed and covered with the same medium containing 0.1 μM TMRM and data was collected with a Merlin image acquisition system (LSR) attached to an Olympus inverted microscope. Illumination monochromator was set to 548 nm and light was collected through a 570-nm dichroic/longpass 590-nm filter cube at 1 or 0.1 frames per second. CSA, BAPTA or BAPTA-control cells were pretreated with the indicated combination of BAPTA-AM (Molecular Probes), DMSO vehicle and CSA (Sigma) for 20 min, stained and imaged as described, with treatment compounds present at all steps other than rinse. Images were analyzed using Merlin software using 10-pixel diameter regions of interest, and depolarizations were scored manually. Data files were imported into Adobe Premiere software for compression into AVI or QuickTime movies.

2.2. Flow cytometry data

Cells were grown on 12-well plates and maintained as above. Cells were stained in the incubator for 30 min in maintenance medium containing the indicated combinations of treatments and 0.5 μM TMRM. Cells were then rinsed, trypsinized and collected in maintenance medium without TMRM. Cells were analyzed using a FACScan instrument (Becton Dickinson) with excitation at 488 nm and logarithmic data collection on FL2. 2500 cells were collected per sample, and three wells were analyzed per condition.

3. Results

We used TMRM, a rapidly redistributing cationic fluorophore selectively accumulated into mitochondria across $\Delta\Psi$ [17], to examine mitochondrial function in SH-SY5Y under a fluorescence microscope. Upon initial observation, in a field of stained cells at approximately 80% confluence, the mitochondria in several cells (estimated $\sim$1–2%) exhibited a much brighter fluorescence than surrounding cells. The signal from these brightly stained mitochondria would

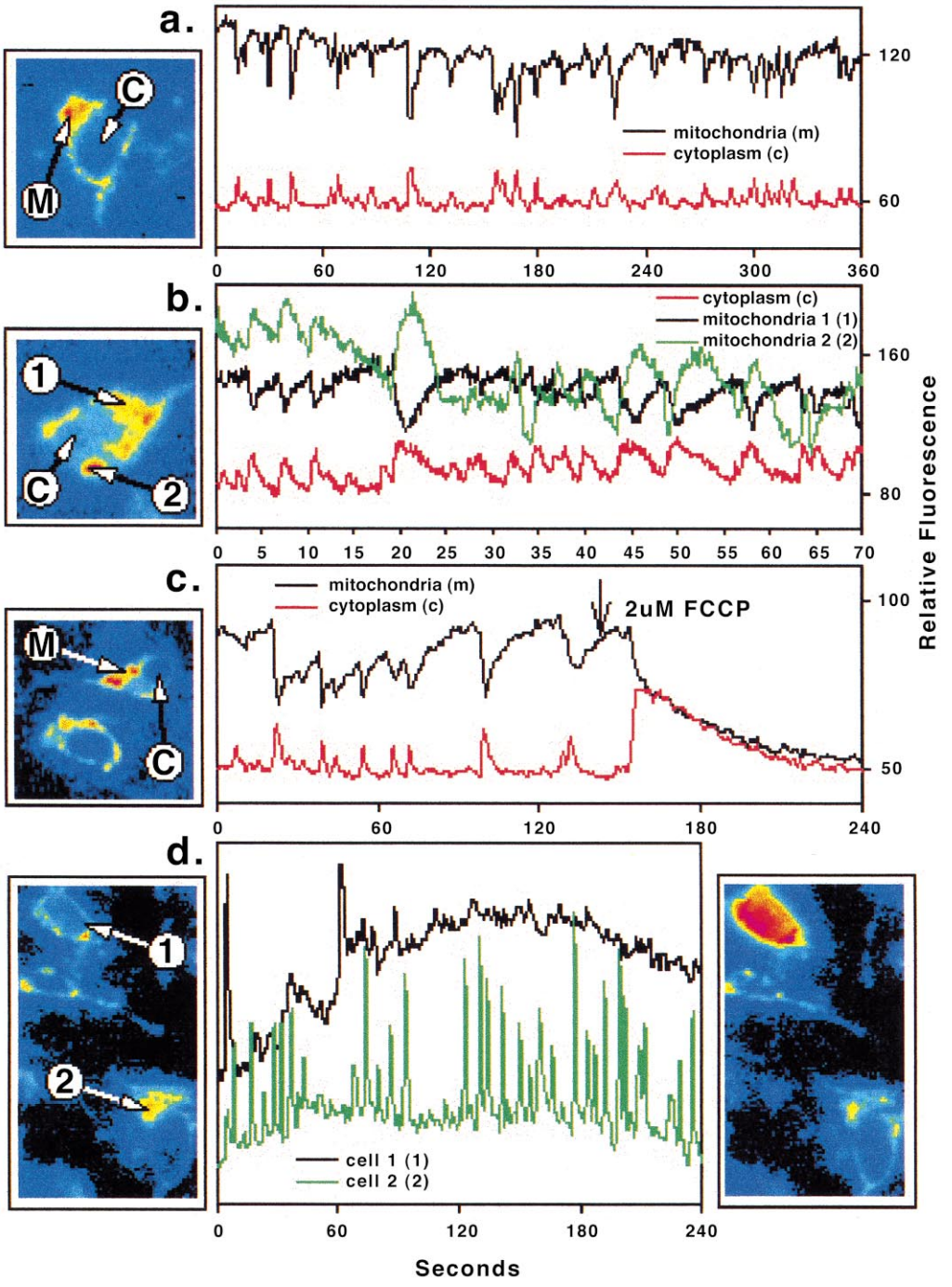

Fig. 1. Time series of mitochondrial depolarizations. (a) Control SH-SY5Y cell at 60× imaged at 1 frame/s, showing loss of TMRM signal under mitochondrial region of interest (ROI) and gain of signal in cytoplasmic/nuclear area. (b) CSA-treated cell at 90× imaged at 10 frames/s, showing increased frequency and both positive going and negative going traces. (c) Control cells at 40× imaged at 1 frame/s, showing FCCP (2 μM) induced cessation of depolarization and loss of TMRM signal. (d) CSA-treated cells at 40× imaged at 1 frame/s, showing pattern consistent with irreversible depolarization.

periodically disappear, leaving a more diffuse signal throughout the cell body, and then reappear in a manner suggestive of mitochondrial repolarization. The movie of this phenomenon can be viewed at http://dana.med.virginia.edu/csnd/MitoMovies as FIG1AQT.MOV (QuickTime format). Temporal quantitative analysis of the TMRM signals is presented in Fig. 1a, which shows relative fluorescence intensity for a region of interest centered on the mitochondrial staining and a region of interest centered on the non-stained cytoplasmic area. Note that discharge by the mitochondria results in an increase in signal by the cytoplasm.

We hypothesized that the MTP might be responsible for the observed spontaneous mitochondrial depolarization. In order to block pore opening and eliminate depolarizations, we pretreated SH-SY5Y cells with 1 μM CSA and visualized them as before. Surprisingly, all cells in CSA-treated fields exhibited bright mitochondrial staining and the entire population exhibited robust asynchronous mitochondrial depolarizations and repolarizations. A low-magnification movie of this process can be viewed as CSAQT.MOV at our web page. Fig. 1b is a plot of three regions of interest (ROI) from a CSA-treated cell imaged at higher temporal resolution (see also FIG1BQT.MOV at the web page). Perhaps because of the greater uptake of TMRM by CSA-treated cells and because we used widefield collection of fluorescence, positive going cytoplasmic regions could be accompanied by either positive or negative going mitochondrial ROIs. We believe that this phenomenon is due to the collection of light from mitochondria and adjacent cytoplasm, and that it would not be observed at the level of individual mitochondria [18]. Lacking the necessary equipment [17,18], we did not attempt to calibrate fluorescence intensity to $\Delta\Psi$. Fig. 1c shows that the addition of the protonophore FCCP, which eliminates the H^+ gradient, eliminates mitochondrial cycling of TMRM signal and results in a discharge transient qualitatively similar to preceding depolarizations (see also FIG1CQT.MOV at the web page). As with isolated mitochondria [19], we also observed occasional irreversible MTP opening in CSA-treated cells. Fig. 1d shows mitochondrial ROIs from both a cell that has irreversibly depolarized and from a cell in the same dish that continues to spike (see also FIG1DQT.MOV at the web page).

Because these findings suggested that CSA was increasing mean $\Delta\Psi$, we next used TMRM accumulation measured with flow cytometry (FCM) to determine the response to CSA in populations of SH-SY5Y neuroblastoma cells. Fig. 2 shows relative TMRM fluorescence population means ± S.E.M. for SH-SY5Y cells pretreated with increasing concentrations of CSA. The maximal increase in TMRM uptake induced by CSA is dramatic and appears to be a saturable phenomenon with an EC_{50} of 1–2 μM CSA. These data suggest that there is significant MTPL-like activity at baseline.

Because CSA can also inhibit the P-glycoprotein multidrug resistance (MDR) transporter, which transports TMRM and other lipophilic cations out

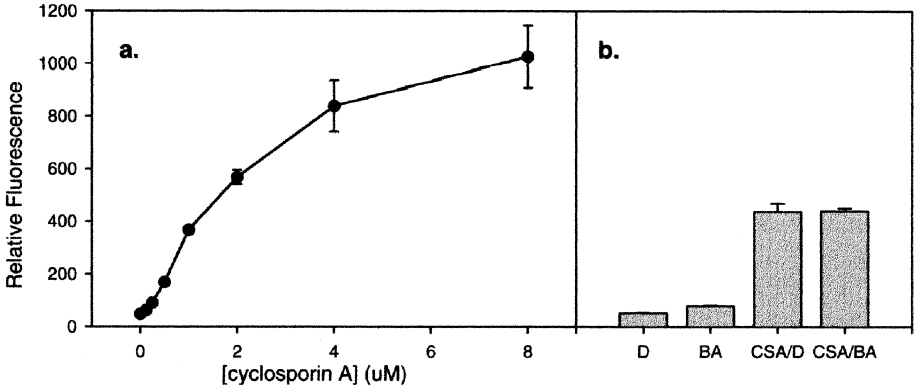

Fig. 2. CSA-induced changes in TMRM signal measured with flow cytometry. (a) Concentration–response curve for CSA induced increase in TMRM uptake, showing mean TMRM signal ± S.E.M. (b) Effect of BAPTA on TMRM signal, showing mean TMRM signal ± S.E.M. for DMSO vehicle (D), 5 μM BAPTA-AM (BA), 1 μM CSA with DMSO (CSA/D) and 1 μM CSA with 5 μM BAPTA-AM (CSA/BA). D and BA are not the same (unpaired *t*-test, $P < 0.01$); CSA/D and CSA/BA are not different.

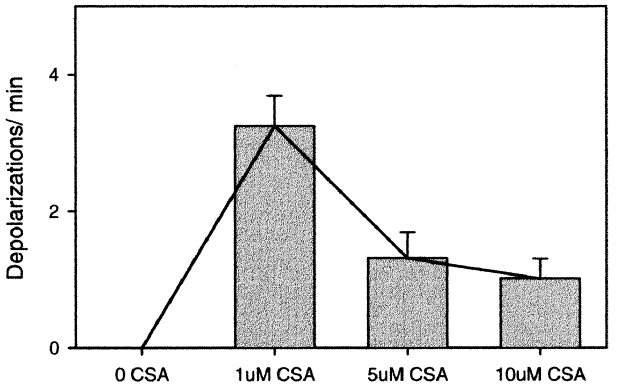

Fig. 3. Concentration–response curve for CSA-induced depolarizations. Shown is mean frequency (depolarizations/min ± S.E.M.) for all cells. ANOVA suggests an effect of CSA ($F=11.5$, $P<0.001$) and post hoc 99% Bonferroni confidence intervals suggest that 1 μM CSA differs from all other conditions, which do not differ from each other. Cells were imaged for an average of 4 min, and mitochondrial regions of interest in 0 of 39 cells in three dishes depolarized with no CSA, 26 of 26 cells in four dishes with 1 μM CSA, 13 of 20 cells in three dishes with 5 μM CSA and 10 of 17 cells in three dishes with 10 μM CSA.

of the cytoplasm, we analyzed the effects of the MDR inhibitor reserpine on TMRM accumulation. We found (data not shown) that 1 μg/ml reserpine approximately doubled TMRM accumulation into SH-SY5Y. TMRM uptake in the presence of reserpine and protonophore (50 μM CCCP) was slightly less than that with reserpine alone, and over twice that seen with 1 μM CsA and CCCP. We also could not demonstrate significant verapamil-sensitive calcein accumulation in our SH-SY5Y cells (Vybrant MDR Assay, Molecular Probes). Thus, if MDR activity is present in the SH-SY5Y used in these experiments, it is at a low level and will not account for the majority of the CSA-induced increase in TMRM accumulation, which takes place into a protonophore-sensitive compartment.

We then analyzed mitochondrial depolarization frequencies by using more dispersed cultures containing five to ten cells per field, and selected fields of interest based on phase contrast instead of TMRM intensity. In this series of experiments, none of the control cells that were imaged exhibited depolarization, though earlier experiments had revealed occasional depolarizing control cells in much more densely populated cultures. In contrast, SH-SY5Y cells pretreated with 1 μM CSA exhibited a marked increase in the mean rate of mitochondrial depolarization, with few dormant cells. Increasing the concentration of CSA to 5 μM reduced significantly mean mitochondrial depolarization frequency. Increasing the concentration of CSA to 10 μM did not result in a statistically significant further decrease. Fig. 3 shows these results graphically. Note that 1 μM CSA corresponds roughly to the EC_{50} for the increase in $\Delta\Psi$ described earlier.

Because MTP activity is sensitive to Ca^{2+} [10], we tested whether chelating intracellular Ca^{2+} might attenuate mitochondrial depolarization/repolarization

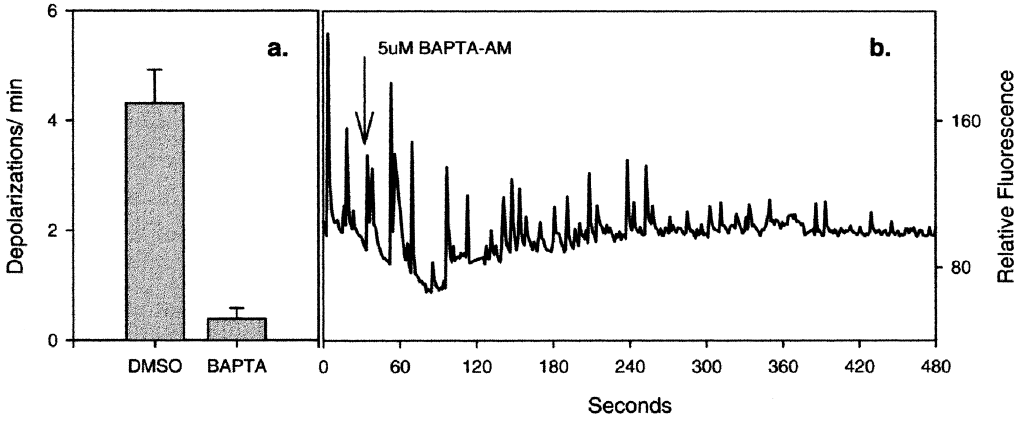

Fig. 4. BAPTA chelation of Ca^{2+} suppresses depolarizations. (a) Mean frequency (depolarizations/min ± S.E.M.) for cells treated with 1 μM CSA and DMSO vehicle or 5 μM BAPTA-AM. Cells were imaged for an average of 4 min, and 19 of 19 DMSO-treated cells in three dishes depolarized, while 6 of 22 BAPTA-AM-treated cells in three dishes depolarized. BAPTA-AM- and DMSO-treated cells are not the same ($P<0.001$, unpaired t-test). (b) Representative trace of SH-SY5Y cell treated with 1 μM CSA, imaged at 1 frame/s, and exposed to 5 μM BAPTA-AM as shown.

activity. As shown in Fig. 4a, cells preincubated with 5 μM BAPTA-AM, a cell-permeant calcium chelating agent, showed substantial lowering of mean depolarization frequency compared to cells preincubated with the DMSO vehicle. When we added 5 μM BAPTA-AM to cells showing spontaneous depolarization/repolarization, we observed a transient decrease in the baseline TMRM signal, a return to depolarization, and then a gradual attenuation over the course of 5 min (Fig. 4b). In order to test whether BAPTA-AM affected TMRM uptake, we measured TMRM uptake using FCM in populations of BAPTA-AM-treated cells with and without CSA. As shown in Fig. 2b, BAPTA alone caused a small but statistically significant increase in TMRM uptake, and the CSA induced potentiation of TMRM uptake was unaffected by BAPTA.

4. Discussion

Using temporal analysis of TMRM fluorescent signal redistribution within SH-SY5Y neuroblastoma cells, we have demonstrated CSA- and Ca^{2+}-dependent cyclical mitochondrial depolarizations in cell culture. Our data indicate that MTPL has a significant opening frequency under baseline conditions, because $\Delta\Psi$ can be increased by blocking MTPL with CSA at a fraction of available sites. Our results of spontaneous mitochondrial depolarization within cells are qualitatively similar but quantitatively different (faster) than those reported in metabolically starved myocytes [20], support the concept of MTP activation in a low conductance mode (MTPL [15]) and further demonstrate coordinated MTPL activity within individual cells. Modulating MTPL in cells appears to shift mitochondria into an excitable mode that has previously been observed in isolated mitochondria [7].

At least two questions immediately present themselves: how does CSA induce mitochondrial 'spiking', and how do we explain the spread of the depolarization to all or most mitochondria in a cell? Answers to these questions might force us to consider the possibility that the low conductance state of the transition pore, MTPL, and the irreversible high conductance state, MTP, operate under different criteria (see [15] for discussion).

The second question is easier to address. Isolated mitochondria have been demonstrated to support traveling waves of depolarization and Ca^{2+} waves [7], which the authors conclude are due to mitochondrial Ca^{2+}-induced Ca^{2+} release (CICR). They hypothesize that CICR arises from cyclical increases in matrix pH in response to mitochondrial Ca^{2+} uptake through the uniporter, driven by $\Delta\Psi$. Their study suggested that CICR is triggered by rapid mitochondrial entry of Ca^{2+}, with slower Ca^{2+} entry leading to mitochondrial Ca^{2+} storage but not spontaneous mitochondrial depolarization. The novel aspects of this formulation are that pH, not $[Ca^{2+}]$, is the major regulator of MPTL opening, and that rate of Ca^{2+} entry and resulting rate of compensatory rise in matrix pH are important factors in producing 'flickering' [15]. Our wave shapes and particularly our frequencies differ from the results using isolated mitochondria; however, these differences might be explained by the fact that events on the time scale of our results would not be resolvable in preparations of stirred isolated mitochondria [14].

BAPTA, which blocks our depolarizations, chelates cytosolic Ca^{2+}, thus reducing rate of Ca^{2+} entry into mitochondria, compensatory rise in matrix pH and increased probability of MTPL opening [14]. Alternatively, BAPTA might prevent the recruitment of neighboring organelles or pore sites due to a wave of diffusing Ca^{2+} [2,7]. At the limit of our temporal resolution ($\sim$0.15 s), we cannot discern the propagation of waves across groups of mitochondria. We cannot eliminate the possibility of Ca^{2+}-induced wave propagation; nonetheless, even if the kinetics of TMRM release did not distort the signal, Ca^{2+} traveling at a likely upper limit of 300 μm/s [21] would cross a 50-μM wide cell in about 0.2 s and would not be resolvable with our equipment. We can say that, if increased mitochondrial matrix pH is responsible for the propagation of depolarization, then the mitochondria shown here to depolarize together or in large subgroups are behaving as a syncytium instead of as individual organelles. This would be consistent with the recent report of complex, dynamic, three-dimensional mitochondrial syncytia in close proximity to endoplasmic reticula within cells, yielding temporally synchronous Ca^{2+} influx into mitochondria following pharmacological release of endoplasmic reticular Ca^{2+} stores [22].

In support of this possibility is the recent report of BAPTA-sensitive spontaneous mitochondrial depolarizations in cardiac myocytes [23]. These mitochondrial 'flickerings' were also substantially reduced by elimination of sarcoplasmic reticular Ca^{2+} stores, blockade of sarcoplasmic Ca^{2+} release, or inhibition of mitochondrial Ca^{2+} uptake [23].

How could CSA induce cyclical mitochondrial depolarizations that follow an inverted U-shaped dose–response curve? Recall that the EC_{50} for the CSA-induced increase in $\Delta\Psi$ and the maximum in CSA-induced depolarizations coincide at about 1 μM CSA. Consider a mitochondrial membrane with numerous pore locations, half of which are blocked by CSA at the EC_{50}, and half of which are free to open stochastically. Blocking half of the channels with CSA would have the net effect of increasing $\Delta\Psi$. The increased $\Delta\Psi$ would be predicted to increase rate of mitochondrial Ca^{2+} entry through the unregulated uniporter, yielding a greater probability of depolarization and CICR [15]. Once initiated, depolarization through remaining MTPL sites may be coordinated by Ca^{2+} released from sites that have already discharged and resulting increases in matrix pH, as described above. Further increasing [CSA] blocks the remaining pore sites and prevents any depolarization. This model provides a mechanism for a positive effect of $\Delta\Psi$ on the opening probability for MTPL. In contrast, the classical MTP is inhibited by increasing $\Delta\Psi$ [14].

As with the recent report describing the imaging of the MTP in single mitochondria [19], we observed but did not quantify an influence of illumination upon induction of depolarization. Upon initial acquisition of a field of cells, few are spiking. By the time the field is focused and data recording has started, cells are fully involved. It has been suggested that light-induced ROS production might increase pore open probability [19], and ROS may affect mean $\Delta\Psi$ [23]. Moreover, there is a precedent for the interaction of rhodamine derivatives with porphyrin groups such as might be found in the mitochondrial membrane [24]. While we cannot exclude the possibility that light-induced ROS production contributed to our observed depolarizations, the CSA-induced increase in $\Delta\Psi$ measured with FCM is independent of such processes. Furthermore, the subsequent suppression by higher CSA concentrations and Ca^{2+} dependency of depolarization implicate classical MTP(L) activity, independent of whether the light-induced ROS contribute to MTP opening.

In summary, we believe that these data demonstrate for the first time MTPL activity in whole cells. We show that MTPL has a significant probability of opening at baseline, and can be induced to function in a coordinated, excitable manner over most if not all mitochondria. As with isolated mitochondria, MTPL activity depends strongly on Ca^{2+}, but may differ from the classic MTP with respect to pH and $\Delta\Psi$. Cells under increased ROS burden appear to suffer from a reduced $\Delta\Psi$ [25]. Because MTPL has the potential to interact strongly with Ca^{2+} signaling systems and to be influenced by mitochondrial function and ROS, it would be most useful to study the potential changes quantitatively and to determine the effects of ROS on the induction of MTPL.

Acknowledgements

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