Spontaneous mitochondrial depolarizations are independent of SR Ca\(^{2+}\) release

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O’Reilly, Catherine M., Kevin E. Fogarty, Robert M. Drummond, Richard A. Tuft, and John V. Walsh, Jr. Spontaneous mitochondrial depolarizations are independent of SR Ca\(^{2+}\) release. Am J Physiol Cell Physiol 286: C1139–C1151, 2004. First published January 7, 2004; 10.1152/ajpcell.00371.2003.—The mitochondrial membrane potential (ΔΨ\(_m\)) underlies many mitochondrial functions, including Ca\(^{2+}\) influx into the mitochondria, which allows them to serve as buffers of intracellular Ca\(^{2+}\). Spontaneous depolarizations of ΔΨ\(_m\), flickers, have been observed in isolated mitochondria and intact cells using the fluorescent cationic lipophile tetramethylrhodamine ethyl ester (TMRE), which distributes across the inner mitochondrial membrane in accordance with the Nernst equation. Flickers in cardiomyocytes have been attributed to uptake of Ca\(^{2+}\) released from the sarcoplasmic reticulum (SR) via ryanodine receptors in focal transients called Ca\(^{2+}\) sparks. We have shown previously that an increase in global Ca\(^{2+}\) in smooth muscle cells causes an increase in mitochondrial Ca\(^{2+}\) and depolarization of ΔΨ\(_m\). Here we sought to determine whether flickers in smooth muscle cells are caused by uptake of Ca\(^{2+}\) released focally in Ca\(^{2+}\) sparks. High-speed three-dimensional imaging was used to monitor ΔΨ\(_m\) in freshly dissociated myocytes from toad stomach that were simultaneously voltage clamped at 0 mV to ensure the cytosolic TMRE concentration was constant and equal to the low level in the bath (2.5 nM). This approach allows quantitative analysis of flickers as we have previously demonstrated. Depletion of SR Ca\(^{2+}\) not only failed to eliminate flickers but rather increased their magnitude and frequency somewhat. Flickers were not altered in magnitude or frequency by ryanodine or xestospongin C, inhibitors of intracellular Ca\(^{2+}\) release, or by cyclosporin A, an inhibitor of the permeability transition pore. Focal Ca\(^{2+}\) release from the SR does not cause flickers in the cells employed here.

mitochondria; mitochondrial membrane potential; intracellular calcium; permeability transition pore; sarcoplasmic reticulum

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It has been suggested that flickers result from mitochondrial uptake of Ca\(^{2+}\). Duchen and coworkers (17) demonstrated that flickers in rat cardiomyocytes are almost abolished by inhibition of Ca\(^{2+}\) release from SR, by the intracellular Ca\(^{2+}\) chelator BAPTA-AM, and by inhibition of the mitochondrial Ca\(^{2+}\) uniporter. They concluded that flickers are transient depolarizations caused by mitochondrial Ca\(^{2+}\) uptake after Ca\(^{2+}\) sparks in cardiomyocytes (17). There are several mechanisms by which mitochondrial uptake of Ca\(^{2+}\) from a microdomain may result in loss of \(\Delta \Psi_m\). As described above, the Ca\(^{2+}\) uniporter is an electrogenic transporter, and thus Ca\(^{2+}\) uptake into mitochondria may be expected to cause depolarization of \(\Delta \Psi_m\) (31). Moreover, should the mitochondrial inner membrane have a high electrical resistance, then very little Ca\(^{2+}\) uptake is required to cause significant mitochondrial depolarization (17). Thus Ca\(^{2+}\) uptake alone may be sufficient to cause flickers. Alternatively, an increase in [Ca\(^{2+}\)]\(_i\) may activate Ca\(^{2+}\)-sensitive ion channels in the mitochondrial membrane. One such transporter is the mitochondrial permeability transition pore (PTP), a nonspecific ion channel in the inner mitochondrial membrane. Flickers have frequently been attributed to PTP stimulation after phototoxicity- or oxidative stress-induced increases in reactive oxygen species (ROS) within mitochondria (12, 13, 18, 25, 28, 49). PTP activation may also be achieved in the absence of an inducer (such as ROS) by an increase in [Ca\(^{2+}\)]\(_i\) (21). In some studies where flickers were attributed to PTP activation, the transient mitochondrial depolarizations were attenuated by inhibition of Ca\(^{2+}\) signaling (18, 28).

Our purpose here was to examine the relationship between focal Ca\(^{2+}\) release from SR and mitochondrial flickers. We used a preparation of freshly dissociated smooth muscle cells where both Ca\(^{2+}\) sparks and mitochondrial flickers have been examined in some detail using new quantitative methods that we have devised (36, 47). In a previous study (36), we have shown that in smooth muscle cells, nearly all mitochondria flicker and do so independently of one another and not as part of a network. The quantitative method used in that study and employed again here allowed us to demonstrate that mean flicker amplitude is \(< 20 \text{ mV}\) although magnitudes range from \(< 10 \text{ mV}\) to \(> 100 \text{ mV}\). Moreover, the data indicated that an ion channel or electrogenic exchanger in the mitochondrial inner membrane caused these transient depolarizations in \(\Delta \Psi_m\) (36). In the current study we wished to investigate the mechanism by which flickers occur. In particular, we sought to determine whether flickers can be elicited by uptake of Ca\(^{2+}\) released focally by RyRs during a spark and, if so, are they caused by dissipation of \(\Delta \Psi_m\) simply due to Ca\(^{2+}\) influx. PTP activation was thought to be unlikely under the experimental conditions employed (see RESULTS). Nonetheless, we also assessed the possibility that either Ca\(^{2+}\)-dependent or Ca\(^{2+}\)-independent PTP activation caused flickers.

High-speed three-dimensional imaging of TMRE-loaded cells revealed spontaneous oscillations in the fluorescence from individual mitochondria that signal transient mitochondrial depolarizations. Flickers were apparent both in cells that exhibited spontaneous transient outward currents (STOCs), which are elicited by sparks, and in cells without STOCs. Mitochondrial flickers were also observed under conditions that are known to inhibit Ca\(^{2+}\) sparks. Flickers were not affected by either ryanodine (100 \(\mu M\)) or xestospongin C (0.5 \(\mu M\)), inhibitors of Ca\(^{2+}\) release from ryanodine- and IP\(_3\)-sensitive intracellular Ca\(^{2+}\) stores, respectively. Store depletion induced by caffeine and thapsigargin failed to inhibit flickers; in fact, both flicker size and frequency increased after emptying of the stores. Neither cyclosporin A (a PTP inhibitor) nor trolox (a ROS scavenger) abolished flickers. This study provides evidence that transient changes in \(\Delta \Psi_m\) occur in the absence of Ca\(^{2+}\) release from intracellular stores or PTP activation in smooth muscle cells. Some of these findings have been reported previously in abstract form (34, 35).

**MATERIALS AND METHODS**

**Cell preparation.** Adult toads (Bufo marinus) were killed by decapitation as approved by the University of Massachusetts Medical Center Animal Care Committee according to the guidelines of the US Departments of Agriculture and Health and Human Services. Single stomach smooth muscle cells were isolated by enzymatic dissociation with trypsin and collagenase as described previously (19).

**TMRE loading protocol.** Freshly isolated myocytes were incubated with TMRE (25 nM) for 10 min at room temperature. Cells were transferred to a tissue bath, where the final concentration of TMRE was 2.5 nM. Cells were allowed to equilibrate for 10 min before whole cell patch-clamp recording was established.

**Electrophysiology.** All cells were patch clamped in the whole cell configuration at room temperature using thin-walled borosilicate patch electrodes (~5 MΩ) (World Precision Instruments). The membrane potential was voltage clamped to 0 mV, and cells were allowed to equilibrate for 5–10 min before data acquisition. Thus cytosolic [TMRE] ([TMRE]c) was constant and equal to that in the bath solution, i.e., 2.5 nM.

**Imaging.** Three-dimensional images of TMRE-labeled mitochondria were obtained using a high-speed, wide-field digital imaging microscope and CCD camera (Lincoln Laboratory, MIT), which have been described in detail elsewhere (48). TMRE was excited at 514 nm using an argon-krypton laser (widefield illumination flux \(\sim 4 \times 10^{10}\) photons/cm\(^2\)·s\(^{-1}\)). A shutter was used to limit the total light exposure of the cells. TMRE emission was detected with a 550-nm long-pass filter. The Nikon 60×, NA 1.4, oil immersion objective was employed to focus light to its point of origin and enable accurate data analysis. Twenty image stacks were acquired at 5-s intervals to generate image sequences of 95-s duration. The pixel size was 333 × 333 nm, and the total area imaged was 42.6 × 42.6 \(\mu m\). Three-dimensional imaging made it possible to resolve the fluorescence of individual mitochondria reliably without contamination of the signal by fluorescence from mitochondria in other planes. It also ensured that movement into and out of the plane of focus (due to movement of the entire cell or contraction in a portion of the cell) was not mistaken for changes in mitochondrial FI.

**Image processing and data analysis.** Image processing and data analysis were performed using custom software on a SiliconGraphics work station. Due to the wide-field illumination, blurred fluorescence from out of focus objects contributes to the sum of the fluorescence at any given point within the acquired images. The data were processed using an iterative constrained deconvolution algorithm to restore out of focus light to its point of origin and enable accurate data analysis (6, 7). Mitochondria were easily identified in the restored three-dimensional images (see RESULTS). Voxels were selected over the entire length of the mitochondrion, the number of voxels used depending on the length of the mitochondrion. The central z-plane of each mitochondrion was identified in each three-dimensional image stack; each voxel was centered in the midpoint of mitochondrial depth and width. The average fluorescence of these voxels was designated as the FI. Mitochondria that drifted out of the field of view due to small

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movements of the cell were not included in the analysis, avoiding false identification of changes in $\Delta \Psi_m$. The percent noise in the signal was calculated to be 5.9%. A change in FI of $>20\%$ (3.4-fold the mean %noise) in a 5- to 10-s period was considered a change in mitochondrial potential and designated a “flicker.”

As we have previously reported, significant levels of TMRE bind to the inner mitochondrial membrane; thus absolute $\Delta \Psi_m$ cannot be calculated using the Nerst equation (36). However, because [TMRE], is constant, changes in $\Delta \Psi_m$ can be calculated as follows:

$$\text{flicker amplitude} = 58 \times \log \frac{FI_{pol}}{FI_{depol}}$$

where “pol” and “depol” are the state of mitochondrial polarization before and during a flicker, respectively. Thus flicker magnitude can be calculated directly from the FI (36).

The percentage of mitochondria that flickered in each cell was calculated as follows: $100 \times$ number of flickering mitochondria/total number of mitochondria in the field. Flicker frequency in flickering mitochondria was calculated as follows: total number of flickers observed during an image sequence/number of flickering mitochondria.

**Image preparation.** Custom software was used to prepare the images shown throughout the paper. Figure legends indicate whether a single plane from the three-dimensional image stack or a composite image (formed by projecting the 10 planes of a three-dimensional image stack onto a single plane) is displayed. Polarized mitochondria appear brighter than depolarized mitochondria.

**Solutions and reagents.** Experiments were carried out using the following solutions (in mM): pipette, 137 KCl, 3 MgCl$_2$, 20 HEPES, 3 Na$_2$-ATP, pH 7.2 with KOH/bath, 130 NaCl, 3 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, pH 7.4 with NaOH. TMRE was purchased from Molecular Probes; ryanodine, thapsigargin, and xestospongin C were from Calbiochem. All other reagents were from Sigma. Stock solutions of TMRE (10 mM) and xestospongin C (500 $\mu$M) were made up in DMSO. Thapsigargin stock (1 mM) was prepared in ethanol. Ryanodine stock (50 mM) was made up in ultrapure water. Stocks were stored at $-20^\circ$C. Caffeine (20 mM) was made up in solution and applied directly to the cells using a picospritzer II (General Valve).

**Statistical analysis.** The data result from an examination of 243 individual mitochondria and 1,862 flickers. Data are presented as means ± SE. Statistical significance was assessed using Graphpad Instat (3.05) software. Kruskal-Wallis nonparametric ANOVA was utilized to test the data, which did not follow a normal distribution. $P \leq 0.05$ was considered statistically significant.

**RESULTS**

Mitochondrial flickers are found in isolated smooth muscle cells. Figure 1A is a composite image showing a projection of the 10 planes of a three-dimensional image stack taken of a freshly isolated smooth muscle cell loaded with TMRE. TMRE enters the mitochondrial matrix along its electrochemical gradient so that its free concentration in the matrix is an exponential function of $\Delta \Psi_m$. The pattern of TMRE labeling in Fig. 1A is consistent with mitochondrial accumulation of dye. The mitochondria are distinguished as rod-shaped organelles of varying length arranged in alignment with the long axis of the cell. Differences in mitochondrial FI are apparent and indicate variations in resting $\Delta \Psi_m$ between organelles.

Flickers, transient fluctuations of $\Delta \Psi_m$, can be seen in Fig. 1B. The arrows indicate two mitochondria that flicker during the 95-s image sequence. The traces in Fig. 1C show differences in the magnitude and duration of the mitochondrial flickers. The graph displays changes in $\Delta \Psi_m$ during flickers in these mitochondria rather than absolute $\Delta \Psi_m$. In theory $\Delta \Psi_m$ can be calculated easily using the Nerst equation, but this approach is complicated by two important factors. First, in previous studies high concentrations of TMRE have often been used so that this probe becomes self-quenching within the mitochondrial matrix, disrupting the simple exponential relationship between mitochondrial [TMRE] ([TMRE]$_{m}$) and $\Delta \Psi_m$ (4, 10, 17, 28). We overcame this by employing a much lower cytosolic concentration than used heretofore (2.5 nM), which was made possible with a high-sensitivity, low-noise, digital imaging system. Such a low concentration also minimizes phototoxicity (see below). Second, there appears to be partitioning of TMRE into the inner mitochondrial membrane (36, 41), but as we have demonstrated, such partitioning is not an impediment to the calculation, in millivolts, of changes in $\Delta \Psi_m$, i.e., flickers (36). Results are presented on a log scale as the change in $\Delta \Psi_m$ is proportional to the log of the ratio of the free [TMRE]$_{m}$ before and during depolarization. As previously observed, mitochondria flicker independently of their neighbors, indicating that mitochondria operate individually rather than as part of a network (36).

Two successive image sequences were obtained from each cell before experimental manipulations and used as controls. There was no significant difference in flicker amplitude, percentage of flickering mitochondria, or frequency of flickers within flickering mitochondria between these two consecutive image sequences (Table 1).

Mitochondrial flickers are independent of ryanodine-sensitive Ca$^{2+}$ release from SR. The cells used in this study generate Ca$^{2+}$ sparks via release of Ca$^{2+}$ from SR through RyRs. The sparks in turn activate large-conductance Ca$^{2+}$-activated K$^+$ channels, causing STOCs that occur at a frequency of 2.5/s (48). Ryanodine inhibits Ca$^{2+}$ efflux via RyRs at concentrations in the micromolar range (8). Cells were treated with 100 $\mu$M ryanodine to inhibit Ca$^{2+}$ release from SR. At the start of the experiment, STOCS were apparent in two of the three cells studied, but 15 min after ryanodine addition STOCs were almost completely abolished, indicating that sparks had been inhibited. Image sequences were taken at 0.5, 5, and 15 min after addition of ryanodine to the bath. Flickers are evident 15 min after the application of ryanodine (Fig. 2A). Ryanodine was without effect on the percentage of mitochondria that were flickering, the number of flickers per flickering mitochondrion, and the flicker amplitude (Fig. 2B).

Mitochondrial flickers are not affected by xestospongin C, a blocker of IP$_3$Rs. IP$_3$-mediated SR Ca$^{2+}$ release has been linked to elevation of global cytosolic Ca$^{2+}$ in several smooth muscle cell types. Mitochondria take up Ca$^{2+}$ after release through IP$_3$Rs in rat pulmonary artery myocytes (15). In colonic myocytes, localized Ca$^{2+}$ transients and STOCs were inhibited by xestospongin C (a membrane-permeable blocker of IP$_3$-induced Ca$^{2+}$ release (20)) but not by ryanodine (1). To investigate IP$_3$R involvement, 0.5 $\mu$M xestospongin C was added to the bath, and cells were imaged at 10 s, 5 min, and 10 min after its application. Flickers can be seen 10 min after the addition of xestospongin C to the bath (Fig. 3A). A small inward shift in the holding current was seen after application of xestospongin C. Xestospongin C did not inhibit the percentage of mitochondria that flickered, the flicker frequency, or amplitude (Fig. 3B).
Mitochondrial flickers are increased by SR store depletion. Chelation of cytosolic Ca\(^{2+}\) should block Ca\(^{2+}\)-mediated cellular events regardless of whether the Ca\(^{2+}\) has an intra- or extracellular origin. Flickers were still observed in the presence of the membrane-permeable Ca\(^{2+}\) chelator BAPTA-AM (10 \(\mu\)M). Flickers were not expected to be dependent on Ca\(^{2+}\) influx. Had they been, they would have been observed primarily in those mitochondria in close proximity to the plasma membrane.
membrane, whereas they were detected in almost all mitochondria. Nevertheless, lack of inhibition by BAPTA indicates that uptake of Ca2+ after influx across the cell membrane does not cause flickers. However, it has been suggested that mitochondrial Ca2+ uptake after intracellular Ca2+ release in microdomains is resistant to inhibition by BAPTA (28). Thus another approach was required to investigate release of store Ca2+ as the cause of the flickers. Cells were incubated with 1 μM thapsigargin and ~7 min later exposed to 20 mM caffeine for 5 s. This procedure has been shown to deplete SR stores in the cells used here (48). STOCs were abolished after exposure to caffeine, indicating that sparks were inhibited. In contrast, flickers were observed in all three cells throughout the experiment (Fig. 4). Store depletion slightly but significantly increased the percentage of mitochondria that flickered ($P = 0.01$), the frequency of flickers ($P = 0.03$), and the flicker amplitude ($P = 0.009$) (Fig. 4B).

Mitochondrial flickers are independent of phototoxicity and PTP activity. Flickers were not inhibited by any measures taken to prevent Ca2+ release from intracellular stores. Thus neither Ca2+-dependent dissipation of $\Delta \Psi_m$ nor Ca2+-dependent activation of the PTP causes flickers. However, in many previous studies, flickers were attributed to repetitive transient openings of the PTP, caused by phototoxicity-induced accumulation of ROS in the mitochondria (10, 12, 13, 25, 26, 49). In each of those studies, flickers culminated in complete dissipation of $\Delta \Psi_m$ in the entire mitochondrial population. In cardiac myocytes, PTP inhibitors and ROS scavengers prevented loss of $\Delta \Psi_m$, but neither Ca2+ chelation nor inhibition of mitochondrial Ca2+ uptake had any effect (49). Thus Ca2+-independent PTP activation, and consequently mitochondrial depolarization, can occur as a result of phototoxicity. Phototoxic effects are dependent on the concentration of TMRE and the strength and duration of light exposure (10, 12, 13).

The high-sensitivity CCD camera used in the present study made it possible to limit [TMRE], and photon exposure to low levels. Thus the likelihood of ROS generation and PTP activation was minimized. Moreover, the pipette solution included Mg2+ and ATP, known to inhibit or reverse PTP activation (2, 22, 24, 33).

The data presented in Table 1 indicate that phototoxic activation of PTP was not the cause of the flickers, as there was no significant difference in amplitude or frequency of flickering with additional light exposure over the two consecutive control sequences. Indeed, with a single exception (Fig. 4), the size and frequency of flickers did not change over the entire course of the experimental manipulations that followed these initial control sequences. Furthermore, the degree of progressive mitochondrial depolarization over the course of the experiments detailed above (Figs. 2–4) was evaluated. The maximum FI of each individual mitochondrion during control 1 was compared with the maximum FI of each mitochondrion in the final image sequence taken. The change in mitochondrial FI represents a 15.2 ± 0.9 mV decrease in $\Delta \Psi_m$. This does not correspond to the complete loss of $\Delta \Psi_m$ ultimately observed in all other studies after PTP activation. These findings are contrary to those expected if flickers were caused by phototoxic activation of the PTP.

Qualitative examination of the images also indicates that phototoxicity does not account for the flickers. Mitochondria of similar resting FI (i.e. similar [TMRE]m) imaged simultaneously do not depolarize in concert (Fig. 5A). Strikingly, in several instances mitochondria depolarized between imaging sequences in the absence of illumination and repolarized subsequently on renewed exposure to light, as imaging resumed (Fig. 5B).

To further assess PTP activity, we applied cyclosporin A, a PTP inhibitor, to the cells. In astrocytes it has been shown that the rate of progressive global depolarization is significantly delayed in the presence of cyclosporin A (28). Caffeine (20 mM) was applied to the cells for 5 s to elicit a rise in global [Ca2+]c, and FI was monitored for 10 min after the caffeine pulse. Caffeine alone had no effect on the flickers (Fig. 6B). Cyclosporin A (10 μM) was then added to the bath, and mitochondria were imaged at 0.5, 5, and 10 min after its application. Flickers were still apparent 10 min after the addition of cyclosporin A to the bath (Fig. 6A). Cyclosporin A did not change the percentage of flickering mitochondria, the rate of flickering, or flicker amplitude (Fig. 6B). In addition, cells incubated in 10 μM cyclosporin A alone for periods ranging from 45 to 90 min continued to flicker. Finally, the ROS scavenger trolox (1 mM for up to 10 min) did not abolish mitochondrial flickering. The data indicate that PTP activation is not the mechanism underlying spontaneous transient mitochondrial flickers.

DISCUSSION

Mitochondrial flickers and the Ca2+ microdomain hypothesis. We have previously demonstrated that global elevations of $[Ca2+]_c$ result in an increase in $[Ca2+]_m$ and mitochondrial depolarization in two types of smooth muscle cells, including the gastric myocytes used in the present study (14, 15). Because caffeine-stimulated SR Ca2+ release caused a global Ca2+ transient and an increase in $[Ca2+]_m$, we postulated that Ca2+ sparks might also result in mitochondrial uptake of Ca2+ and mitochondrial depolarization, thereby causing mitochondrial flickers. This idea was strengthened by the fact that, in cardiomyocytes, mitochondrial flickers have been attributed to Ca2+ sparks by Duchen et al. (17) and caffeine-induced, localized increases in $[Ca2+]_m$ result in transient elevations of $[Ca2+]_m$ called “marks” by Pacher et al. (38). (However, in the latter study the elevations in $[Ca2+]_m$, extended over a large area and persisted for a longer time than true Ca2+ sparks, and thus they might be more appropriately considered in the same class of events as a global increase in $[Ca2+]_c$.) Thus it was surpris-

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<th>Table 1. Comparison of flickers from consecutive image sequences</th>
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<td>Control 1</td>
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<td>Flicker amplitude, mV</td>
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*Values are means ± SE. Flickers were compared during the first (control 1) and second image sequences (control 2) that were used as controls. Control 1: no. of flickers ($n_{flicker}$) = 161, no. of flickering mitochondria ($n_{flickering_{m}}$) = 89; control 2: $n_{flicker}$ = 199, $n_{flickering_{m}}$ = 104. There was no significant difference (NS) in any of the flicker parameters studied. †Mann-Whitney test; †paired t-test.
Fig. 2. Mitochondrial flickering in the presence of ryanodine. A: images taken 15 min after incubation with 100 μM ryanodine.
The first image in the series is a composite image; ensuing images are single planes. Arrows indicate a mitochondrion in which changes in FI were observed. The mitochondrion is more clearly resolved in a single plane than a composite image. Scale bar, 5 μm. Trace at right shows changes in $\Delta V_m$ (mV). B: effect of ryanodine on the percentage of mitochondria that flickered, the no.
of flickers per flickering mitochondrion, and the flicker amplitude ($n = 55$ mitochondria). Bar at top indicates duration of ryanodine application. Bar graphs indicate results from a series of image sequences. From left to right: 2 control sequences separated by 5 min, and sequences beginning 0.5, 5, and 15 min after ryanodine application.
Fig. 3. Mitochondrial flickering in the presence of xestospongin C (XeC). A: images taken 10 min after incubation with 0.5 μM xestospongin C. The first image in the series is a composite image; ensuing images are single planes. Arrows indicate a mitochondrion in which changes in FI were observed. Scale bar, 5 μm. Trace at right shows changes in ΔΨm (mV). B: effect of xestospongin C on the percentage of mitochondria that flickered, the no. of flickers per flickering mitochondrion, and flicker amplitude (n = 66 mitochondria). Bar at top indicates duration of xestospongin C application. Bar graphs indicate results from a series of image sequences. From left to right: 2 control sequences separated by 5 min, and sequences beginning 10 s, 5 min, and 10 min after xestospongin C application.
ing that neither depletion of the SR nor ryanodine decreased flicker frequency or magnitude in the present study. One explanation for this discrepancy may be differences in intracellular organization between cardiomyocytes and smooth muscle cells. A much higher density of mitochondria is observed in images of cardiomyocytes loaded with TMRE or its analog, tetramethylrhodamine methyl ester (TMRM) than in images of the smooth muscle cells used here (17, 36, 49). Thus the likelihood of mitochondria and SR being in sufficiently close contact for mitochondria to respond to microdomains of high Ca\(^{2+}\) close to the RyR release sites is much higher in cardiomyocytes than smooth muscle cells. Alternatively, the microdomain theory may prove to be applicable only to regions of ER containing IP3Rs and not to SR and RyR. Finally, it is possible that sparks can result in

\[\text{Fig. 4. Mitochondrial flickering after sarcoplasmic reticulum (SR) Ca}^{2+}\ \text{depletion. A: images taken 10 min after store depletion.}
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First image is a composite image; subsequent images are single planes. Arrows indicate a depolarizing mitochondrion. Scale bar, 5 \(\mu\)m. Trace at right shows changes in \(\Delta\Psi_m\) (mV). A: arrows point to 2 mitochondria of similar FI. The first mitochondrion (horizontal arrow, filled square) is stable throughout the first sequence but depolarizes during the second sequence. The second mitochondrion (vertical arrow, open square) depolarizes and recovers during the first sequence but remains stable during the second sequence.

\[\text{Fig. 5. Flickers are independent of exposure to light. Single planes extracted from image stacks taken during 2 consecutive sequences (i and ii). Scale bars, 5 \(\mu\)m. The traces (iii) show changes in FI. Scale bars at right in A and B show change in } \Delta\Psi_m \text{ (mV). A: arrows point to 2 mitochondria of similar FI. The first mitochondrion (horizontal arrow, filled square) is stable throughout the first sequence but depolarizes during the second sequence. The second mitochondrion (vertical arrow, open square) depolarizes and recovers during the first sequence but remains stable during the second sequence.}
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mitochondrial Ca\(^{2+}\) uptake but that insufficient Ca\(^{2+}\) enters the mitochondria to affect $\Delta \Psi_m$.

**Mitochondrial flickers and the PTP.** In all studies in which flickers were attributed to ROS generation or PTP activation, flickers preceded an irreversible global mitochondrial depolarization. Inhibition of the PTP by cyclosporin A prevents mitochondrial depolarization in astrocytes (16, 28) and cardiomyocytes (3). In the present study cyclosporin A had no effect on mitochondrial flickers. It has been reported that in some cell preparations cyclosporin A fails to inhibit the PTP (25, 49). However, the patch pipette solution contained Mg\(^{2+}\) and ATP, which inhibit Ca\(^{2+}\)-mediated activation of the PTP (22, 24). Mg\(^{2+}\) also facilitates PTP inhibition by cyclosporin A (2, 33). The ROS scavenger trolox inhibits the PTP in cardiomyocytes and Cos-7 cells (12, 49). In contrast, trolox did not inhibit mitochondrial flickering in the cells studied here (data not shown). We conclude that flickers do not reflect progressive failure of mitochondrial function due to phototoxic activation of the PTP. In neurons, irreversible depolarization of the mitochondria does not occur during imaging, and consequently mitochondrial flickering is not attributed to phototoxicity (4). Additionally, Jacobson and Duchen (28) report that with low illumination, flickers are smaller, more infrequent, and do not result in cell death. Therefore, it appears that flickers that are independent of phototoxic events can occur in several cell types.

The PTP can operate in a low-conductance state, which does not impair mitochondrial function. It has been proposed that
Fig. 6. Mitochondrial flickering during exposure to cyclosporin A (CsA). A: images taken 10 min after incubation with 10 μM cyclosporin A. The first image is a composite image; the following images are single planes. Arrows indicate a mitochondrion in which depolarization and repolarization are observed. Scale bar, 5 μm. Trace at right shows changes in ∆Ψm (mV). B: effect of caffeine (Cfn) and cyclosporin A on the percentage of mitochondria that flickered, the no. of flickers per flickering mitochondrion, and the flicker amplitude (n = 41 mitochondria). Bar at top indicates duration of caffeine and cyclosporin A application. From left to right, histograms show results from 2 control sequences separated by 5 min, sequences beginning 0.5, 5, and 10 min after caffeine application, and 0.5, 5, and 10 min after cyclosporin A application.
rapid uptake of Ca$^{2+}$ into the mitochondria after localized release from stores activates the low-conductance PTP (27). However, in light of the lack of inhibition of flickers by manipulations designed to prevent Ca$^{2+}$ signaling, it is unlikely that Ca$^{2+}$-dependent low-conductance PTP activity underlies flickers. Furthermore, reversal of PTP activation occurs rapidly after chelation of free Ca$^{2+}$ (11, 21, 24). Here, buffering of cytosolic Ca$^{2+}$ with BAPTA-AM failed to abolish the flickers. It is known that the PTP can switch from low to high conductance after an increase in [Ca$^{2+}$]$_i$ or a slight mitochondrial depolarization (2, 27). Such an increase in [Ca$^{2+}$]$_i$ is expected after caffeine application in the presence of thapsigargin, i.e., in the immediate aftermath of store depletion. If flickers were caused by low-conductance PTP activity, we might expect that store depletion would result in global mitochondrial depolarization due to Ca$^{2+}$-dependent activation of high-conductance PTP. This was not observed after store depletion. The accumulated evidence indicates that flickering is not caused by induction of PTP in these cells.

**Molecular basis of mitochondrial flickers.** Many ion transporters have been identified in the inner mitochondrial membrane, several of which might cause flickers (37). Entry of H$^+$ into the matrix via the F$_1$F$_0$ATPase during ATP generation (4) or through uncoupling proteins, recently identified in intestinal smooth muscle (42), may result in mitochondrial depolarization. Opening of mitochondrial K$^+$ channels may also result in depolarization. In isolated mitochondria, activation of ATP-sensitive K$^+$ (K$_{ATP}$) channels in the inner mitochondrial membrane has been shown to result in $\Delta \Psi_m$ depolarization, although the extent of depolarization is currently debated (23, 30). K$_{ATP}$ channel opening is unlikely in the present study as the patch pipette included ATP. However, Ca$^{2+}$-activated K$^+$ channels were recently identified in mitochondria of glioma and cardiac cells (43, 45), and K$^+$ entry into the mitochondrial matrix via these channels may also elicit $\Delta \Psi_m$ depolarization. Finally, anion channels in the inner mitochondrial membrane have been linked to PTP-independent oscillations in $\Delta \Psi_m$ in substrate-deprived myocytes (37).

Their reversibility and independence from spontaneous intracellular Ca$^{2+}$ release events and PTP activity indicate that flickers represent a previously unknown process in myocyte mitochondria. The quantitative analysis of flickers used here and in our earlier study (36) should be useful in the further elucidation of the mechanism and role of mitochondrial flickers in smooth muscle and other cell types.

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