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Abstract

Mitochondria modulate Ca(2+) signals by taking up, buffering, and releasing Ca(2+) at key locations near Ca(2+) release or influx channels. The role of such local interactions between channels and organelles is difficult to establish in living cells because mitochondria form an interconnected network constantly remodeled by coordinated fusion and fission reactions. To study the effect of a controlled disruption of the mitochondrial network on Ca(2+) homeostasis, we took advantage of hFis1, a protein that promotes mitochondrial fission by recruiting the dynamin-related protein, Drp1. hFis1 expression in HeLa cells induced a rapid and complete fragmentation of mitochondria, which redistributed away from the plasma membrane and clustered around the nucleus. Despite the dramatic morphological alteration, hFis1-fragmented mitochondria maintained a normal transmembrane potential and pH and took up normally the Ca(2+) released from intracellular stores upon agonist stimulation, as measured with a targeted ratiometric pericam probe. In contrast, hFis1-fragmented mitochondria took up more slowly the Ca(2+) entering across plasma […]

Reference

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Ca\textsuperscript{2+} Homeostasis during Mitochondrial Fragmentation and Perinuclear Clustering Induced by hFis1\textsuperscript{*}\textsuperscript{S}

Maud Frieden\textsuperscript{‡}, Dominic James\textsuperscript{§}, Cyril Castelbou\textsuperscript{‡}, Anne Danckaert\textsuperscript{‡}, Jean-Claude Martinou\textsuperscript{§}, and Nicolas Demaurex\textsuperscript{‡}

From the \textsuperscript{1}Department of Physiology and the \textsuperscript{2}Bioimaging Core Facility, University of Geneva Medical Center, 1 Michel-Servet, CH-1211 Geneva 4, Switzerland and the \textsuperscript{3}Department of Cell Biology, University of Geneva, Quai Ernest-Ansermet 30, 1211 Geneva, Switzerland

Mitochondria modulate Ca\textsuperscript{2+} signals by taking up, buffering, and releasing Ca\textsuperscript{2+} at key locations near Ca\textsuperscript{2+} release or influx channels. The role of such local interactions between channels and organelles is difficult to establish in living cells because mitochondria form an interconnected network constantly remodelled by coordinated fusion and fission reactions. To study the effect of a controlled disruption of the mitochondrial network on Ca\textsuperscript{2+} homeostasis, we took advantage of hFis1, a protein that promotes mitochondrial fission by recruiting the dynamin-related protein, Drp1. hFis1 expression in HeLa cells induced a rapid and complete fragmentation of mitochondria, which redistributed around the nucleus. Despite the dramatic morphological alteration, hFis1-fragmented mitochondria maintained a normal transmembrane potential and pH and took up normally the Ca\textsuperscript{2+} released from intracellular stores upon agonist stimulation, as measured with a targeted ratiometric pericam probe. In contrast, hFis1-fragmented mitochondria took up more slowly the Ca\textsuperscript{2+} entering across plasma membrane channels, because the Ca\textsuperscript{2+} ions reaching mitochondria propagated faster and in a more coordinated manner in interconnected than in fragmented mitochondria. In parallel cytosolic fura-2 measurements, the capacitative Ca\textsuperscript{2+} entry (CCE) elicited by store depletion was only marginally reduced by hFis1 expression. Regardless of mitochondrial shape and location, disruption of mitochondrial potential with uncouplers or oligomycin/rotenone reduced CCE by ~35%. These observations indicate that close contact to Ca\textsuperscript{2+} influx channels is not required for CCE modulation and that the formation of a mitochondrial network facilitates Ca\textsuperscript{2+} propagation within interconnected mitochondria.

Mitochondria actively participate to the cellular Ca\textsuperscript{2+} homeostasis and modulate the pattern of agonist-induced Ca\textsuperscript{2+} signals by their ability to sequester and release Ca\textsuperscript{2+} (1). Because of the low Ca\textsuperscript{2+} affinity of the unipporter that constitutes the main mechanism of Ca\textsuperscript{2+} entry into mitochondria, it was proposed that the ability of these organelles to accumulate Ca\textsuperscript{2+} relies on their close location to Ca\textsuperscript{2+} release channels on the endoplasmic reticulum (ER)\textsuperscript{1} (2, 3). Mitochondria also interact with plasma membrane channels and thereby modulate the so-called capacitative Ca\textsuperscript{2+} entry (CCE) pathway, the ubiquitous Ca\textsuperscript{2+} entry mechanism triggered by emptying of the ER Ca\textsuperscript{2+} store (4, 5). Although the molecular identity of the channel(s) responsible for CCE as well as its mechanism of activation are still debated, recent evidence indicates that mitochondria represent a key organelle in CCE activity and/or activation. Indeed, CCE is inhibited by intracellular Ca\textsuperscript{2+} elevations, and mitochondria were shown to act as local buffers to prevent Ca\textsuperscript{2+}-mediated inhibition of the CCE pathway (6–9).

Local interactions between mitochondria and other subcellular structures are difficult to establish in living cells because mitochondria display a complex architecture that varies considerably between cell types. This ranges from a largely interconnected tubular network in COS-\textit{T}, endothelial, or HeLa cells to round punctuated structures in hepatocytes (10). Moreover, mitochondria are highly dynamic organelles that move in the cytosol and that constantly undergo fission and fusion. Both processes are under the control of certain GTPases and their associated proteins (11). hFis1, the human orthologue of the yeast Fis1p (12), is a 17-kDa transmembrane protein located in the outer membrane of the mitochondria that is involved in the machinery of mitochondria fission, and overexpression of this protein enhances the fission process in HeLa cells (13). In this study, we overexpressed the protein hFis1 in HeLa cells to induce a controlled fragmentation of mitochondria and measured the impact of these structural changes on cytosolic and mitochondrial Ca\textsuperscript{2+} signals with fura-2 and with a targeted ratiometric pericam probe, respectively. This approach allowed us to investigate the role of mitochondria on cytosolic and mitochondrial Ca\textsuperscript{2+} homeostasis and to distinguish the local and global effects of mitochondria on the Ca\textsuperscript{2+} entry process.

EXPERIMENTAL PROCEDURES

Materials—Minimum essential medium, fetal calf serum, penicillin, and streptomycin were obtained from Sigma. Acetoxyethyl ester form of fura-2 (fura-2/AM) and Mitotracker Red were obtained from Molecular Probes Europe (Leiden, Netherlands). Carboxyfluorescein diacetate, succinimimidyl ester (CMX-SS-AM) was obtained from Molecular Probes Europe (Leiden, Netherlands). Cathepsin B was obtained from Boehringer Mannheim. ATPase; RP3.1mit, ratiometric pericam targeted to the mitochondrial matrix; SERCA, sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPase; I\textsubscript{ATPase}, Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} current; I\textsubscript{Ca2+}, mitochondrial membrane potential; TMRR, tetramethylrhodamine methyl ester; GFP, green fluorescent protein.

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\textsuperscript{1} To whom correspondence should be addressed. Tel.: 41-22-379-5399; Fax: 41-22-379-5402; E-mail: Nicolas.Demaurex@medicine.unige.ch.
Mitochondrial Fragmentation and Ca$^{2+}$ Signaling

Fluka (Buchs, Switzerland). Transfet transfection reagent was purchased from Promega.

Cell Culture and Transfection—HeLa cells were grown in minimum essential medium containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin and were maintained at 37 °C under 5% CO$_2$. For experiments, cells were plated on 25-mm diameter glass coverslips 2–3 days before use. After reaching 40–60% of confluence, cells were transiently transfected with the different plasmids using the Transfectant reagent according to the protocol supplied by the manufacturer. For measurements of cytotoxic Ca$^{2+}$ concentration, [Ca$^{2+}$]$_{cyt}$, hFis1 was co-transfected with a GFP targeted to the nucleus to identify cells expressing hFis1. All experiments were performed 16–20 h after transfection with hFis1. To measure mitochondrial Ca$^{2+}$, cells were transfected with the same reagent, with the ratio of transfection efficiencies determined on 25-mm diameter glass coverslips 2–3 days before use. Prior to the experiment, cells were washed twice, and equilibrated for 15 min in the dark, washed twice, and equilibrated for 15 min in the same buffer. Cells were excited at 545 nm and emission collected through an LP 590 long pass filter. Changes in Δψ$_{m}$ were expressed as R$_{R}$, where R is the ratio of the fluorescence in the mitochondria divided by the cytosolic fluorescence at a given time and R$_{0}$ is the initial ratio of the mitochondrial over cytosolic fluorescence.

Mitochondrial Membrane Potential (Δψ$_{m}$) Measurements—To monitor changes in Δψ$_{m}$, cells were loaded for 20 min with 50 nM tetramethylrhodamine methyl ester (TMRM) in Hepes-buffered solution, and then were excited at 560 nm and emission at 577 nm recorded on a cooled, 16-bit CCD camera (CoolSnap HQ, Roper Scientific, Trenton, NJ) operated by the Meta morph 5.0 software (Universal Imaging, West Chester, PA). Images shown in Figs. 1A and 3 were deconvolved with the Huygens algorithm (Scientific Volume Imaging, Leiden, The Netherlands) using the Imaris software (Bitplane AG, Zurich, Switzerland). To determine the mitochondrial network into punctuate organelles that clustered around the nucleus. The fragmentation process occurred immediately upon hFis1 expression and, once initiated, was complete within 4 h as documented by time-lapse video microscopy of cells co-transfected with a nuclear-targeted GFP (See supplementary movie S1). Within 1 h, all the cells expressing the hFis1 cDNA had a punctiform mitochondria phenotype, consistent with a previous study (13). To quantify the extent of mitochondrial redistribution, we took several confocal optical sections of mitochondria (labeled with Mitotracker Red) and of the cell cytosol (labeled with the cytosolic protein ratiometric pericam). Using the mitochondrial image as a mask, we determined the surface of the cell occupied by mitochondria on the cytosolic images (the nucleus was included in the cell surface, see “Experimental Procedures”). As shown in Fig. 1B, mitochondria spread out to the periphery and covered a larger area of the cytosol in control cells. On average, the percentage of the cell surface “lacking” mitochondria was ~2-fold larger in hFis1-expressing cells compared with control (Fig. 1C, n = 15 and 32 cells, respectively). The same images were used to measure the number of contact points between mitochondria and the cell membrane, defined as the outline of the cytosolic staining. As shown in Fig. 1D, ~18% of the cell membrane was apposed to mitochondria in control cells (at the resolution of our confocal system of ~250 nm), a proportion that was reduced by ~3 times upon hFis1 expression. Thus, hFis1 not only induces mitochondrial fragmentation but also redistributes mitochondria away from the plasma membrane, leaving large regions of the cell periphery devoid of mitochondria and fewer contacts between mitochondria and the cell surface.

Effects of hFis1 Expression on Mitochondrial Membrane Potential, pH, and Ca$^{2+}$ Homeostasis—To assess the effects of
hFis1 expression on mitochondrial function, we first tested whether the mitochondrial membrane potential ($\Delta \Psi_m$) was altered after fragmentation. For this purpose, cells were loaded with TMRM and challenged sequentially with 5 g/ml oligomycin (to prevent ATP consumption) and 25 M rotenone (to block the complex I of the respiratory chain). As shown in Fig. 2, the drugs elicited similar changes in $\Delta \Psi_m$ in hFis1-overexpressing and control cells. In both cases, neither the application of oligomycin nor rotenone alone had a significant effect on $\Delta \Psi_m$, whereas their combined application dissipated $\Delta \Psi_m$ to a similar extent (Fig. 2B). The subsequent addition of the protonophore CCCP induced a rapid and complete dissipation of the membrane potential. The lack of depolarization in the presence of oligomycin indicates that the respiratory chain was functional in hFis1 cells and that the mitochondrial membrane potential was not maintained by the mitochondrial $\text{H}^+$ ATPase.
In concurrent Ca\textsuperscript{2+} measurements at 410 nm, addition of 50 μM histamine evoked identical [Ca\textsuperscript{2+}]_{mit} transients regardless of the induction of hFis1 (Fig. 3D). In the absence of extracellular Ca\textsuperscript{2+}, neither the maximal [Ca\textsuperscript{2+}]_{mit} elevation nor the duration of the response was different in control and hFis1-overexpressing cells (Fig. 3F). The [Ca\textsuperscript{2+}]_{mit} transients measured in the presence of extracellular Ca\textsuperscript{2+} were also not significantly different; the maximal amplitude of the signal averaged 0.167 ± 0.015 (n = 33) in untransfected cells and 0.141 ± 0.015 (n = 13) in hFis1 overexpressors. Taken together, these data indicate that mitochondrial pH and Ca\textsuperscript{2+} homeostasis is not affected by hFis1 expression despite the complete fragmentation of the mitochondrial network.

Effects of hFis1 Expression on ER Structure—The presence of normal [Ca\textsuperscript{2+}]_{mit} transients in HeLa cells with fragmented and clustered mitochondria is surprising, because the proximity of mitochondria to the Ca\textsuperscript{2+} source (i.e. the inositol 1,4,5-trisphosphate Ca\textsuperscript{2+} release channels) was shown to be crucial for a proper mitochondrial Ca\textsuperscript{2+} uptake. Mitochondria have been proposed to form stable, long-term interactions with ER Ca\textsuperscript{2+} release channels to account for the efficient transfer of Ca\textsuperscript{2+} between the two organelles (15). Because hFis1 induced dramatic alterations in mitochondrial architecture, we investigated whether the ER was also affected. As shown in Fig. 4, the staining pattern of the ER-targeted yellow cameleon probe (YC4.1.KR) was not grossly altered upon hFis1 expression, indicating that the mitochondrial remodeling was not accompanied by visible changes in ER architecture.

Effects of hFis1 on Ca\textsuperscript{2+} Transfer from Plasma Membrane Channels to Mitochondria—hFis1-fragmented mitochondria appear to handle normally the Ca\textsuperscript{2+} released from the ER, as indicated by the normal [Ca\textsuperscript{2+}]_{mit} transient elicited by histamine. Because the main effect of hFis1, apart from fragmentation, is to move mitochondria away from the plasma membrane (Fig. 1), we assessed whether hFis1 altered the ability of mitochondria to take up Ca\textsuperscript{2+} originating from the plasma membrane. For this purpose, Ca\textsuperscript{2+} was readmitted to cells previously stimulated with 50 μM histamine in the nominal absence of Ca\textsuperscript{2+}. As shown in Fig. 5A, the amplitudes of the [Ca\textsuperscript{2+}]_{mit} elevations were similar in control (0.109 ± 0.019; n = 12) and in hFis1-overexpressing cells (0.110 ± 0.014; n = 15). Interestingly however, the time needed to reach this level was significantly prolonged by hFis1 expression. The Ca\textsuperscript{2+} entering across the plasma membrane took, on average, 31 s longer to cause a maximal response in hFis1-fragmented mitochondria. Similar results were obtained in cells stimulated with the SERCA pump inhibitor, thapsigargin (1 μM), instead of histamine (Fig. 5, C and D), indicating that ER Ca\textsuperscript{2+} pumps were not involved in the transfer of Ca\textsuperscript{2+} from the plasma membrane to mitochondria.

To understand the structural basis of this slower [Ca\textsuperscript{2+}]_{mit} increase, we analyzed the spatio-temporal pattern of the [Ca\textsuperscript{2+}]_{mit} signal during Ca\textsuperscript{2+} readmittance to control and hFis1 cells. As shown in Fig. 6, [Ca\textsuperscript{2+}]_{mit} increased rapidly in large, contiguous regions of control tubular mitochondria. In contrast, [Ca\textsuperscript{2+}]_{mit} increased sequentially in discrete regions of hFis1-fragmented mitochondria. The slower response of hFis1-fragmented mitochondria was not because of a delay in the transfer of Ca\textsuperscript{2+} from the plasma membrane to mitochondria, because the [Ca\textsuperscript{2+}]_{mit} signal initiated at the same time or even earlier in individual mitochondria from hFis1-transfected cells (Fig. 6A). Rather, Ca\textsuperscript{2+} spread faster and in a more coordinated manner within tubular mitochondria (Fig. 6B), indicating that the propagation of the [Ca\textsuperscript{2+}]_{mit} signal was impaired by the fragmentation of the mitochondrial network.

The delayed [Ca\textsuperscript{2+}]_{mit} increase in hFis1 cells might possibly

mitochondrial matrix of live HeLa cells. For these measurements, we took advantage of the dual sensitivity to both Ca\textsuperscript{2+} and pH of a ratiometric pericam probe targeted to mitochondria, RP3.1mit (kindly provided by Dr. A. Miyawaki, Tokyo). RP3.1mit fluorescence is highly sensitive to pH at an excitation of 480 nm, but not at 410 nm (14, 15). Conversely, RP3.1mit fluorescence decreases with increasing concentrations of Ca\textsuperscript{2+} at 410 nm but is largely insensitive to Ca\textsuperscript{2+} at 480 nm. We could verify this dual sensitivity by exposing RP3.1mit-labeled HeLa cells to the mitochondrial uncoupler CCCP or to the calcium-mobilizing agonist histamine. As expected, CCCP caused a selective drop in RP3.1mit fluorescence at 480 nm as the mitochondria acidified to equilibrate its pH with the pH of the cytosol (Fig. 3A). In contrast, addition of histamine produced a transient drop only at 410 nm (Fig. 3B), confirming that changes in mitochondrial Ca\textsuperscript{2+} concentration, [Ca\textsuperscript{2+}]_{mit}, could be monitored selectively at this wavelength. We used this approach to evaluate the effect of hFis1 expression on mitochondrial Ca\textsuperscript{2+} and pH homeostasis. As shown in Fig. 3, C and E, addition of 1 μM CCCP elicited a drop in fluorescence at 480 nm that was of similar magnitude in control and in hFis1-expressing cells. The drop in fluorescence corresponded to a similar ΔpH, because the RP3.1mit calibration curves were similar in hFis1-overexpressing and -untransfected cells in the pH range 7.4–8.4 (see Supplementary Fig. 2). These data confirm the TMRM measurements and indicate that the pH of the mitochondrial matrix was not altered by hFis1 expression.
reflect a reduced or slower influx of Ca$^{2+}$ across the plasma membrane. To test this possibility, we measured Ca$^{2+}$ influx with fura-2. As shown in Fig. 7, the cytosolic Ca$^{2+}$ changes upon Ca$^{2+}$ readdition to cells stimulated with thapsigargin were of similar amplitude and kinetics in control and hFis1-overexpressing cells. To confirm this observation, CCE activity was measured by following the rate of Mn$^{2+}$ influx (Fig. 7C). The rates of Mn$^{2+}$ quench were not significantly different in control and hFis1-expressing cells (Fig. 7D), indicating that CCE was largely unaffected by the fragmentation and subcellular redistribution of the mitochondrial network.

**Effects of hFis1 on CCE Modulation by Mitochondria**—Functional mitochondria are required to sustain CCE, but it is not clear whether mitochondria act locally, i.e., as Ca$^{2+}$ buffers that remove Ca$^{2+}$-dependent channel inhibition, or globally, i.e., by modulating the filling state of the ER or by releasing a diffusible messenger. Because mitochondria in hFis1 cells were clearly located farther away from the plasma membrane than in untransfected cells, they provided a convenient model to separate the local and global effects of mitochondria on CCE. For this purpose, cells were stimulated with thapsigargin to activate CCE and mitochondria function was inhibited by either 1 μM CCCP or by a combination of 25 μM rotenone and 5 μg/ml oligomycin. The effects of the mitochondria inhibitors on CCE were then assessed by the Ca$^{2+}$ readdition protocol or by the Mn$^{2+}$ quench technique. As shown in Fig. 8, Ca$^{2+}$ entry...
was reduced by about one third in the presence of CCCP or of oligomycin/rotenone, regardless of hFis1 expression. Mn$^{2+}$/H$_{100}$entry was reduced to a similar extent in the presence of 1 $\mu$M CCCP, both in control (from 8.503 ± 1.442, n = 25; p < 0.05) and in hFis1-overexpressing cells (from 6.876 ± 1.196, n = 14 to 3.276 ± 0.225, n = 10; p < 0.05). These results indicate that functional mitochondria are required for optimal activation of CCE in HeLa cells, although the modulation of CCE by mitochondria (30–40%) is less pronounced than in other cell types.

**DISCUSSION**

In this study we investigated the effect of a controlled disruption of the mitochondrial network on the Ca$^{2+}$/H$_{110}$homeostasis of mitochondria. For this purpose, we expressed the protein hFis1 in HeLa cells to induce a rapid fragmentation and perinuclear clustering of their mitochondria. Surprisingly, these dramatic morphological alterations had little impact on the organelle function because mitochondria were still able to maintain a normal membrane potential and pH and to take up and release Ca$^{2+}$. This experimental paradigm allowed us to study Ca$^{2+}$/H$_{110}$handling by mitochondria located close or far from the plasma membrane, to define the role of mitochondria interconnection in the propagation of Ca$^{2+}$/H$_{110}$ signals, and to assess the local and global effects of mitochondria on plasma membrane Ca$^{2+}$/H$_{110}$ channels.

**hFis1 Initiates Mitochondria Fragmentation without Altering the Function of the Organelle**—Mitochondria are dynamic organelles that often form an extensive tubular network reflecting the balance of ongoing fusion and fission processes. Among the proteins regulating the fusion and fission processes, hFis1 was recently shown to induce mitochondrial fission in mammalian cells by recruiting the dynamin-related GTPases Drp1 from the cytosol to the outer mitochondrial membrane (13, 16). We could confirm that expression of hFis1 in HeLa cells induces a complete fragmentation of mitochondria within 16–20 h. This effect was selective for mitochondria because expression of hFis1 did not modify the ER architecture. Upon fragmentation, the mitochondria clustered around the nucleus, leaving large parts of the cytosol devoid of these organelles. Morphometric analysis revealed that 45% of the cellular area was lacking mitochondria in cells expressing hFis1 compared with 25% in control cells and that the fragmented mitochondria...
Mitochondrial Fragmentation and \( \text{Ca}^{2+} \) Signaling

were located farther away from the plasma membrane. We could not precisely evaluate the location of mitochondria relative to the upper portion of the plasma membrane (i.e., the “roof” of the cell) given the limited optical resolution of our confocal system in the \( z \)-axis. Nevertheless, functional data (see below) strongly suggest that upon hFis1 expression mitochondria moved toward deeper regions of the cytosol.

hFis1 overexpression did not affect the mitochondrial membrane potential (\( \Delta \psi \)) as measured \textit{in situ} with low concentrations of the rhodamine probe TMRM. Furthermore, oligomycin and rotenone, alone or in combination, had similar effects on \( \Delta \psi \). This latter experiment rules out the possibility that fragmented mitochondria maintained a normal membrane potential by the hydrolysis of glycolytic ATP, because the ATP synthase inhibitor oligomycin did not dissipate \( \Delta \psi \). Consistent with the maintenance of a normal \( \text{H}^+ \) electrochemical gradient, the \( \text{pH} \) of the mitochondrial matrix was not altered 16 h after transfection of hFis1 (Fig. 3). We did not try later time points, because expression of hFis1 for >48 h has been shown to induce cytochrome \( c \) release and apoptosis (13). Thus, during acute fragmentation mitochondria maintained a normal membrane potential and \( \text{pH} \), in agreement with a recent report on hippocampal neurons showing that mitochondrial redistribution and aggregation did not modify their energy status (17).

Mitochondrial Fragmentation and \( \text{Ca}^{2+} \) Handling—Given the preserved function but altered shape and location of mitochondria, the question arises as to whether they were still able to accumulate \( \text{Ca}^{2+} \) during cell stimulation. This question is of particular interest because the mitochondrial \( \text{Ca}^{2+} \) uniporter, which drives the entry of \( \text{Ca}^{2+} \) into mitochondria, has a low affinity for \( \text{Ca}^{2+} \) (18). It was thus postulated that mitochondria must be located in close vicinity to \( \text{Ca}^{2+} \) release sites on the ER (i.e., near inositol 1,4,5-trisphosphate-gated \( \text{Ca}^{2+} \) release channels) to rapidly and efficiently accumulate \( \text{Ca}^{2+} \) (2, 3). This “high microdomain” model is widely accepted to account for the rapid \([\text{Ca}^{2+}]_{\text{mit}}\) increase that occurs during cell stimulation. As an extension of this postulate, it was proposed recently that the contact points between the ER and mitochondria are highly stable over time, suggesting that specific structural interactions exist between the two organelles (15). This conclusion was based on 1) the similar distribution of highly responsive mitochondria inside cells during successive histamine stimulations, and 2) the larger than expected reduction in aequorin responses to repetitive histamine challenges, which indicates

**Fig. 5.** hFis1 overexpression delays the transfer of \( \text{Ca}^{2+} \) from the plasma membrane to mitochondria. A and C, after stimulation with 50 \( \mu \text{M} \) histamine (A) or 1 \( \mu \text{M} \) thapsigargin (C) in \( \text{Ca}^{2+} \)-free medium, 2 \( \text{mM} \) \( \text{Ca}^{2+} \) was readded and the \( \text{Ca}^{2+} \) uptake by mitochondria was evaluated. The fragmented mitochondria needed significantly more time to accumulate \( \text{Ca}^{2+} \) maximally. B and D, statistical evaluation of the delayed mitochondrial \( \text{Ca}^{2+} \) uptake. Bars are mean \pm S.E. (\( n = 11 \) for untransfected cells and \( n = 15 \) for hFis1-overexpressing cells in panel B; \( n = 18 \) for untransfected cells and \( n = 10 \) for hFis1-overexpressing cells in panel D). * \( p < 0.05 \).
**FIG. 6. Disruption of the mitochondrial network increases the heterogeneity of the \([\text{Ca}^{2+}]_{\text{mit}}\) signal.** Cells were transiently transfected with RP3.1 and \([\text{Ca}^{2+}]_{\text{mit}}\) measured during \(\text{Ca}^{2+}\) readdition by ratio imaging. A threshold corresponding to 80% of the spatially averaged \([\text{Ca}^{2+}]_{\text{mit}}\) response was applied to delineate regions of high \([\text{Ca}^{2+}]_{\text{mit}}\). A, fluorescence images taken every 15 s during the \(\text{Ca}^{2+}\) readdition are shown with pixels exceeding the 80% threshold highlighted in red. **Bar**, 10 \(\mu\)m, applies to all images. **B**, the percentage of the mitochondrial area exceeding the threshold is plotted over time. **Arrow** indicates start of the \(\text{Ca}^{2+}\) readdition.
that mitochondria that capture large amounts of Ca$^{2+}$ (and thus consume aequorin) are not replaced by other mitochondria from the remaining population. In our imaging measurements, we did not detect any significant differences in the ability of fragmented or tubular mitochondria to take up Ca$^{2+}$ during histamine stimulation, neither in the amplitude nor in the kinetic of the response. Although this result is not contradictory to the concept of a close vicinity between certain parts of the ER and mitochondria, it is hard to reconcile with the existence of permanent physical contacts. Our data showed that the ER structure was not affected by the fragmentation of the mitochondrial network, indicating that hFis1 specifically altered the structure and location of one organelle (the mitochondria) while leaving the other intact (the ER). Although it is conceivable that mitochondria can move and reform specific interactions with other ER Ca$^{2+}$ channels at a new location, it is difficult to envisage that stable ER-mitochondria complexes can move inside cells without altering the ER structure. Thus, to account for the rapid uptake of Ca$^{2+}$ into fragmented mitochondria, the most likely hypothesis is that close contacts between the ER and mitochondria occur stochastically but at a relatively high frequency given the density of the two organelles in perinuclear regions.

Although fragmented mitochondria captured normally the Ca$^{2+}$ released from the ER, they accumulated Ca$^{2+}$ with a significant delay compared with tubular mitochondria when the Ca$^{2+}$ source was the extracellular space. The kinetic was about two times slower, whereas the maximal Ca$^{2+}$ increase was not affected. Because morphometric analysis indicated that fragmented mitochondria are located deeper in the cytosol, Ca$^{2+}$ ions must, on average, travel a longer distance before reaching a fragmented than a tubular mitochondria. However, this longer distance is unlikely to account for the delayed transfer of Ca$^{2+}$ to fragmented mitochondria, because during fura-2 measurements Ca$^{2+}$ equilibrated within seconds in the cytosol. The increased distance from the plasma membrane might, however, translate into a slightly reduced Ca$^{2+}$ concentration around perinuclear mitochondria. In this case, Ca$^{2+}$ would enter at a lower rate through the mitochondrial Ca$^{2+}$ uniporter without altering its capacity to accumulate Ca$^{2+}$. Re-
regardless of the mechanism, the ability of perinuclear mitochondria to take up Ca\(^{2+}\), albeit at slower rates, indicated that close contacts to plasma membrane channels were not required for Ca\(^{2+}\) uptake by mitochondria during capacitative Ca\(^{2+}\) entry. Moreover, our observations indicated that the transfer of Ca\(^{2+}\) from the extracellular space to mitochondria did not involve the ER, because mitochondria located deep in the cell were still able to take up Ca\(^{2+}\) when ER SERCA ATPases were inhibited by thapsigargin (Fig. 5). These data indicate that Ca\(^{2+}\) does not transit through the ER to reach mitochondria and that high Ca\(^{2+}\) microdomains are not required for the slow uptake of Ca\(^{2+}\) that occurs during CCE.

Our observations also indicated that the formation of a tubular network facilitates the propagation of Ca\(^{2+}\) along mitochondria. As shown in Fig. 6, Ca\(^{2+}\) uptake was not only slower during Ca\(^{2+}\) readdition but also more heterogeneous in fragmented than in tubular mitochondria. In fragmented mitochondria, Ca\(^{2+}\) increased sequentially in small regions that accumulated Ca\(^{2+}\) in an uncoordinated manner. In contrast, Ca\(^{2+}\) increased in an explosive manner within large, contiguous regions of the tubular network. This indicates that the Ca\(^{2+}\) entering mitochondria can tunnel within the matrix and equilibrate rapidly along tubular, but not fragmented, mitochondria. Lumenal connectivity between mitochondria has been tested in HeLa cells using the fluorescence recovery after photobleaching (FRAP) technique (10). The fluorescence of DsRed recovered within 90 s after bleaching when a long (\(-25 \mu m\)) filamentous mitochondrial cluster was irradiated but failed to recover for up to 1 h when larger regions of the cell were bleached (10). This indicates that small molecules such as Ca\(^{2+}\) can tunnel within the matrix of fused mitochondria but not between mitochondrial clusters that are not interconnected. Our observations are consistent with these findings, because during Ca\(^{2+}\) readdition [Ca\(^{2+}\)]\(_{\text{mit}}\) did not increase at the same time in all mitochondria, even in cells with an extensive tubular network. The response was relatively homogenous, however, because the [Ca\(^{2+}\)]\(_{\text{mit}}\) signal initiated simultaneously at several sites at the cell periphery and then rapidly propagated toward deeper cellular regions in a synchronous manner. Such a coordinated [Ca\(^{2+}\)]\(_{\text{mit}}\) increase was not observed in cells with fragmented mitochondria, indicating that the tubular organization of mitochondria facilitates Ca\(^{2+}\) propagation between individual organelles.

**Local and Global Effects of Mitochondria on Plasma Membrane Ca\(^{2+}\) Channels**—Because mitochondria remained largely functional upon hFis1 expression, we investigated whether these fragmented mitochondria located far from the plasma membrane were still able to modulate CCE. Depletion of the ER Ca\(^{2+}\) stores triggers an influx of extracellular Ca\(^{2+}\) called CCE (4). Several studies have shown that mitochondria are involved in the maintenance and/or activation of CCE in different cells, but their exact contribution as well as the mech-

![Figure 8](https://example.com/figure8.png)

**Fig. 8. Functional mitochondria are required for optimal Ca\(^{2+}\) entry regardless of hFis1 expression.** A and B, effect of mitochondrial depolarization due to 1 \(\mu\)M CCCP on the Ca\(^{2+}\) entry following thapsigargin treatment in untransfected cells (A) and hFis1-overexpressing cells (B). C, statistical evaluation of the effect of CCCP on Ca\(^{2+}\) entry. Bars are mean \(\pm\) S.E. (n = 13 for untransfected cells and 6 for hFis1-overexpressing cells). *, p < 0.05 versus control. D, similar experiments were performed with a mixture of 25 \(\mu\)M rotenone and 5 \(\mu\)g/ml oligomycin to poison mitochondria. Bars are mean \(\pm\) S.E. (n = 25 for untransfected cells and 20 for hFis1-overexpressing cells). *, p < 0.05 versus control.
anism of action are still debated. In electrophysiological studies, the best characterized current supporting CCE is $I_{\text{URAC}}$, a current that is highly selective for $\text{Ca}^{2+}$ and carried by channels of small unitary conductance (19–21). This current is inhibited at high intracellular $\text{Ca}^{2+}$ concentrations, and mitochondria were shown to prevent $\text{Ca}^{2+}$-dependent channel inactivation by their ability to accumulate $\text{Ca}^{2+}$ near the cytosolic mouth of the channel (6). In agreement with this hypothesis, a recent cell-attached patch-clamp study on endothelial cells showed that mitochondria can maintain low $\text{Ca}^{2+}$ concentrations under single plasma membrane channels (9). In addition to their buffering effects, mitochondria were recently proposed to release an as yet unidentified diffusible factor(s) that regulates the activity of $I_{\text{URAC}}$ (22).

Our experiments using either thapsigargin or histamine to deplete ER $\text{Ca}^{2+}$ stores showed that upon $\text{Ca}^{2+}$ readdition the bulk cytosolic $\text{Ca}^{2+}$ elevation was not significantly different in control or hFis1-overexpressing cells. This was also confirmed by $\text{Mn}^{2+}$ quench experiments, although with both assays a small but not significant reduction in $\text{Ca}^{2+}$ entry was observed in hFis1-overexpressing cells. Because in hFis1 cells large parts of the plasma membrane are devoid of underlying mitochondria, the presence of mitochondria near membrane channels is clearly not essential for CCE. This does not imply that mitochondria do not exert local effects on CCE, because reduced subplasmalemmal $\text{Ca}^{2+}$ buffering could have opposite effects on membrane channels. $\text{Ca}^{2+}$-dependent K⁺ channels are more active when located far from underlying mitochondria (9), leading to a larger hyperpolarization and enhanced driving force for $\text{Ca}^{2+}$ entry. Because $\text{Ca}^{2+}$-dependent K⁺ channels are also present in HeLa cells, (23), lack of subplasmalemmal mitochondria might elicit opposite mechanisms, the reduction in local $\text{Ca}^{2+}$ buffering enhancing the $\text{Ca}^{2+}$ feedback inhibition on $\text{Ca}^{2+}$ entry channels while increasing the driving force for $\text{Ca}^{2+}$ entry. Thus, a local role of mitochondria on $\text{Ca}^{2+}$ entry channels cannot be formally excluded but is apparently not the dominant mechanism by which mitochondria modulate CCE in HeLa cells.

Regardless of their location, functional mitochondria were required for optimal CCE, because poisoning mitochondria either with CCCP or oligomycin/rotenone significantly reduced CCE both in control and hFis1-overexpressing cells. It should be noted, however, that in other cellular systems such as RBL-1 (22), T lymphocytes (7), or endothelial cells (9) such inhibition of mitochondrial function resulted in a more pronounced reduction of CCE (−80–90%). Thus, the proportion of CCE under the influence of mitochondria is relatively modest in HeLa cells (−30–40%). Nonetheless, the observation that optimal CCE activity requires functional mitochondria even if the organelles are located far from the plasma membrane suggests that CCE modulation is not a local effect but rather a global effect that might involve a diffusible factor, as proposed recently (22). Thus, function, but not location, of mitochondria is critical for CCE modulation.

In conclusion, our data show that mitochondrial fragmentation and perinuclear clustering did not alter the ability of mitochondria to take up $\text{Ca}^{2+}$ ions released by the ER or to modulate CCE but significantly decreased the speed of $\text{Ca}^{2+}$ propagation between these organelles during $\text{Ca}^{2+}$ influx. Thus, mitochondria remain functional and able to modulate CCE regardless of their shape and location, although the formation of a mitochondrial network might facilitate the propagation of specific $\text{Ca}^{2+}$ signals within cells.

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