Subplasmalemmal mitochondria modulate the activity of plasma membrane Ca2+-ATPases

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Abstract
Mitochondria are dynamic organelles that modulate cellular Ca2+ signals by interacting with Ca2+ transporters on the plasma membrane or the endoplasmic reticulum (ER). To study how mitochondria dynamics affects cell Ca2+ homeostasis, we overexpressed two mitochondrial fission proteins, hFis1 and Drp1, and measured Ca2+ changes within the cytosol and the ER in HeLa cells. Both proteins fragmented mitochondria, decreased their total volume by 25-40%, and reduced the fraction of subplasmalemmal mitochondria by 4-fold. The cytosolic Ca2+ signals elicited by histamine were unaltered in cells lacking subplasmalemmal mitochondria as long as Ca2+ was present in the medium, but the signals were significantly blunted when Ca2+ was removed. Upon Ca2+ withdrawal, the free ER Ca2+ concentration decreased rapidly, and hFis1 cells were unable to respond to repetitive histamine stimulations. The loss of stored Ca2+ was due to an increased activity of plasma membrane Ca2+-ATPase (PMCA) pumps and was associated with an increased influx of Ca2+ and Mn2+ across store-operated Ca2+ channels. The increased Ca2+ influx compensated for the loss [...]
Mitochondria are dynamic organelles that modulate cellular Ca\(^{2+}\) signals by interacting with Ca\(^{2+}\) transporters on the plasma membrane or the endoplasmic reticulum (ER). To study how mitochondria dynamics affect cell Ca\(^{2+}\) homeostasis, we overexpressed two mitochondrial fission proteins, hFis1 and Drp1, and measured Ca\(^{2+}\) changes within the cytosol and the ER in HEK293 cells. Both proteins fragmented mitochondria, decreased their total volume by 25–40%, and reduced the fraction of subplasmalemmal mitochondria by 4-fold. The cytosolic Ca\(^{2+}\) signals elicited by histamine were unaltered in cells lacking subplasmalemmal mitochondria as long as Ca\(^{2+}\) was present in the medium, but the signals were significantly blunted when Ca\(^{2+}\) was removed. Upon Ca\(^{2+}\) withdrawal, the free ER Ca\(^{2+}\) concentration decreased rapidly, and hFis1 cells were unable to respond to repetitive histamine stimulations. The loss of stored Ca\(^{2+}\) was due to an increased activity of plasma membrane Ca\(^{2+}\)-ATPase (PMCA) pumps and was associated with an increased influx of Ca\(^{2+}\) and Mn\(^{2+}\) across store-operated Ca\(^{2+}\) channels. The increased Ca\(^{2+}\) influx compensated for the loss of stored Ca\(^{2+}\), and brief Ca\(^{2+}\) additions between successive agonist stimulations fully corrected subsequent histamine responses. We propose that the lack of subplasmalemmal mitochondria disrupts the transfer of Ca\(^{2+}\) from plasma membrane channels to the ER and that the resulting increase in subplasmalemmal [Ca\(^{2+}\)] up-regulates the activity of PMCA. The increased Ca\(^{2+}\) extrusion promotes ER depletion and the subsequent activation of store-operated Ca\(^{2+}\) channels. Cells thus adapt to the lack of subplasmalemmal mitochondria by relying on external rather than on internal Ca\(^{2+}\) for signaling.

Mitochondria are intracellular organelles that play a key role in Ca\(^{2+}\) signaling. Mitochondria take up and release Ca\(^{2+}\), and physiological increases in the cytosolic free Ca\(^{2+}\) concentration, [Ca\(^{2+}\)]\(_{cyt}\), are transmitted to the mitochondrial matrix as changes in [Ca\(^{2+}\)]\(_{mit}\), [Ca\(^{2+}\)]\(_{mit}\) signals during cell stimulation activate mitochondrial dehydrogenases and boost ATP production, but excessive [Ca\(^{2+}\)]\(_{mit}\) increases can damage mitochondria and trigger apoptosis (1, 2). On the other hand, Ca\(^{2+}\) handling by mitochondria has numerous effects on cell Ca\(^{2+}\) homeostasis. By taking up Ca\(^{2+}\), mitochondria generate microdomains of low Ca\(^{2+}\) that enable the full activation of Ca\(^{2+}\) channels that are normally inhibited at high Ca\(^{2+}\) concentrations (3, 4). In addition, mitochondria have been shown to recycle the Ca\(^{2+}\) released from the ER back to this intracellular Ca\(^{2+}\) store, preventing the Ca\(^{2+}\) depletion of the ER (5), and to relay the Ca\(^{2+}\) entering across plasma membrane Ca\(^{2+}\) channels directly to the ER, short circuiting the cytosol (6). Mitochondria thus not only integrate intracellular Ca\(^{2+}\) signals to produce the appropriate metabolic response but also control the fluxes of Ca\(^{2+}\) inside cells to shape Ca\(^{2+}\) signals.

Ca\(^{2+}\) enters mitochondria down its electrochemical gradient via a Ca\(^{2+}\) uniporter, using the negative mitochondrial membrane potential generated by the electron transport chain, and is released from mitochondria by a Na\(^{+}/\)Ca\(^{2+}\) exchanger or by the opening of the mitochondrial permeability transition pore. Because of the low Ca\(^{2+}\) affinity of the mitochondrial Ca\(^{2+}\) uniporter, micromolar Ca\(^{2+}\) concentrations are required for efficient Ca\(^{2+}\) uptake into mitochondria. Such high Ca\(^{2+}\) concentrations occur only transiently in cells at the mouth of Ca\(^{2+}\) channels, implying that mitochondria are in close proximity to plasma membrane Ca\(^{2+}\) entry channels or to intracellular Ca\(^{2+}\) release channels (7). Accordingly, morphological and functional evidence indicate that mitochondria are very close and are possibly physically connected to Ca\(^{2+}\) release channels on the endoplasmic reticulum (8, 9).

Mitochondria are structurally complex organelles that move, fuse, and divide within cells (10–12). This constant remodeling regulates the morphology, number, and function of mitochondria and ensures the proper transmission of mitochondria to daughter cells during cell division. In most cells, mitochondria fusion and fission are usually balanced, leading to the formation of a tubular network of interconnected mitochondria. This cable-like architecture facilitates the transfer of the mitochondrial membrane potential (ΔΨ\(_{mit}\)) from oxygen-rich to oxygen-poor cellular regions (13) and allows the rapid transmission of Ca\(^{2+}\) signals along the interconnected mitochondria (14). Disruption of the mitochondrial network by excessive mitochondrial fission or impaired fusion is a common feature of apoptosis, and the breakdown of the mitochondrial network is associated with a decrease in mitochondrial respiration, an increase in the production of reactive oxygen species, and the loss of the mitochondrial DNA (10).

Mitochondrial fusion and fission reactions are regulated by a highly evolutionary conserved set of dynamin-related proteins. Mitochondrial fission is operated by Drp1, a GTPase located in the cytosol that is recruited to mitochondria by hFis1, a small adapter protein located on the outer mitochondrial membrane (15, 16). Mitochondrial fusion is regulated by the transmembrane GTPases mitofusin and OPA-1, located on the outer and inner mitochondrial membrane, respectively (reviewed in Ref. 17). By overexpressing or ablating the genes coding for these mitochondrial fission and fusion proteins, it is now possible to selectively manipulate the mitochondrial network toward fusion or fission. Using this strategy, we previously showed that the overexpression of hFis1 induced a complete fragmentation of the mitochondrial net-
work in HeLa cells (18). The fragmented mitochondria clustered around the nucleus but retained a normal membrane potential and pH and normally took up the Ca\(^{2+}\) released from the ER upon stimulation with agonists.

In the present study, we investigated the impact of a hFis1 or Drp1-induced mitochondrial fragmentation on the cytosolic and ER Ca\(^{2+}\) homeostasis. Our data show that the lack of subplasmalemmal mitochondria in cells overexpressing hFis1 or Drp1 is associated with an increased activity of plasma membrane Ca\(^{2+}\) pumps, which, in turn, induces a faster Ca\(^{2+}\) depletion of the ER and a subsequent activation of store-operated Ca\(^{2+}\) influx. These defects are balanced as long as Ca\(^{2+}\) is present in the extracellular medium and become apparent only when Ca\(^{2+}\) is removed. The lack of mitochondria below the plasma membrane thus renders cells more dependent on extracellular Ca\(^{2+}\) for signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—HeLa cells were grown on minimum essential medium supplemented with 2 mM L-glutamine, 50 μg/ml streptomycin, and 50 units/ml penicillin along with 10% heat-inactivated fetal calf serum 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 TransFast reagent according to the protocol supplied by the manufacturer (Promega). For measurements of [Ca\(^{2+}\)]\(_{\text{cyt}}\), hFis1 or Drp1 were co-transfected with a GFP targeted to the nucleus in order to identify cells expressing the mitochondrial fission proteins. All experiments were performed between 16 and 20 h after transfection with hFis1, and after 48 h with Drp1. To measure endoplasmic reticulum Ca\(^{2+}\), [Ca\(^{2+}\)]\(_{\text{ER}}\) cells were transfected with the cameleon YC4.1 targeted to the endoplasmic reticulum (YC4.1, a gift from Dr. R. Y. Tsien, University of California at San Diego) and 24 h later with hFis1.

**Materials**—Minimum essential medium, fetal calf serum, penicillin, and streptomycin were obtained from Invitrogen. Histamine and thapsigargin were obtained from Sigma. The acetoxymethyl ester form of Fura-2 (Fura-2/AM) and MitoTracker Red were obtained from Molecular Probes Europe. TransFast transfection reagent was purchased from Promega (Catalysis AG).

**Cytosolic Ca\(^{2+}\) Measurements**—Experiments were performed in Heps-buffered solution containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 20 mM Heps, and 10 mM glucose, pH 7.4, with NaOH. The Ca\(^{2+}\)-free solution contained 1 mM EGTA instead of CaCl\(_2\) except in the Mn\(^{2+}\) quench experiments, where no EGTA was added. The low Na\(^{+}\) solution contained 121 mM N-methyl-D-glucamine, 19 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 20 mM Heps, and 10 mM glucose, pH 7.4, with HCl. Glass coverslips were mounted in a thermostatic chamber (Harvard Apparatus, Holliston, MA) equipped with gravity feed inlets and a vacuum outlet for solution changes. Cells were imaged on an Axiovert S100 TV using a 100×, 1.3 numerical aperture, oil immersion objective (Carl Zeiss AG, Feldbach, Switzerland). Cells were loaded for 30 min with 2 μM Fura-2/AM at room temperature in the dark, washed twice, and equilibrated for 15–20 min to allow de-esterification. To monitor [Ca\(^{2+}\)]\(_{\text{cyt}}\), cells were alternatively excited at 340 and 380 nm with a monochromator (DeltaRam, Photon Technology International Inc., Monmouth Junction, NJ) through a 430 DCLP dichroic mirror, and emission was monitored through a 510/40 filter (Omega Optical, Brattleboro, VT). Transfected cells were recognized by the fluorescence of the nuclear-targeted GFP (480-nm excitation, 535-nm emission), and the characteristic morphology of mitochondria (globular versus tubular) was verified by imaging the MitoTracker labeling (577-nm excitation, 590-nm emission). For this purpose, cells were loaded prior the experiments with 500 nM MitoTracker Red for 90 s and washed 2–3 times with experimental buffer. To minimize cross-talk of the nuclear GFP fluorescence into the 380 nm Fura-2 signal, we only selected cells with a moderate nuclear fluorescence, and the region of the cytosol used to estimate [Ca\(^{2+}\)]\(_{\text{cyt}}\) did not include the nucleus. Fluorescence emission was imaged using a cooled, 16-bit, charge-coupled device, back-illuminated, frame transfer MicroMax camera (Princeton Instruments, Roper Scientific, Trenton, NJ). Image acquisition and analysis were performed with the MetaFluor 6.2 software (Universal Imaging, West Chester, PA).

**ER Ca\(^{2+}\) Measurements**—HeLa cells transfected with the YC4.1ER were excited at 430 nm through a 455 DRLP dichroic mirror (Omega Optical), and emissions were collected alternatively at 475 and 535 nm (475/515 and 535/525, Omega Optical) through emission filters mounted on a filter wheel (Ludl Electronic Products, Hawthorn, NY). To recognize the fragmented aspect of the mitochondria due to hFis1 overexpression, HeLa cells were loaded with 500 nM MitoTracker Red prior to the experiments. A low intensity threshold was applied to define the fluorescent signal associated with the ER. The total fluorescent area was taken for measurement.

**Measurements of Mitochondria Morphology.**—Cells were imaged on an Axiovert 200M microscope equipped with an array laser confocal spinning disk (QLC100; VisiTech, Sunderland, UK) using a 63×, 1.4 numerical aperture, oil-immersion objective (Carl Zeiss AG). Cells were alternately excited at 488 and 568 nm to image the green emission (525/50 nm) from the nuclear targeted enhanced GFP (EGFP) or the membrane-targeted yellow cameleon YC3\(_{\text{em}}\) (red and 615/60 nm) emission from MitoTracker Red or RFP\(_{\text{mit}}\). These fluorescent images were acquired using a cooled, 12-bit, charge-coupled device camera (CoolSnap HQ; Roper Scientific, Trenton, NJ) operated by the Metamorph 6.2 software (Universal Imaging). Optical slices of 300-nm step size z sections were acquired, and the green and red channels were further deconvoluted with the Huygens algorithm (Scientific Volume Imaging BV, Hilversum, The Netherlands). Three-dimensional rendering of the deconvoluted z stacks (shadow projections or cross-sections) was done with the Imaris 4.2 software (Bitplane AG, Zurich, Switzerland). For measurements of mitochondrial volume, complete series of the deconvoluted z stacks were processed with the "measure volume" routine from the Metamorph software after application of a low intensity threshold on the MitoTracker Red or RFP\(_{\text{mit}}\) fluorescence. The percentage of membrane volume colocalized with mitochondria was calculated in the whole cell volume using the colocalization option from the Imaris software after restriction of each fluorescence signal by a low intensity threshold to define the respective structures.

**Measurements of Ca\(^{2+}\) Entry—Mn\(^{2+}\) (100 μM) was substituted for Ca\(^{2+}\) to estimate the ion flux through Ca\(^{2+}\) entry channels according to the Mn\(^{2+}\)-quench technique (19). Cells were excited at 356–358 nm, which corresponded to the isosbestic point of Fura-2. The rate of fluorescence decrease reflects the rate at which Mn\(^{2+}\) enters the cells, and the slope during the first 1–2 min was used as an indicator for Ca\(^{2+}\) entry.

**Statistics**—Unless indicated, statistical analysis was performed using the unpaired Student’s t test.

**RESULTS**

**Effects on hFis1 and Drp1 on Mitochondria Distribution**—To study the impact of hFis1 or Drp1 overexpression on mitochondria morphology, we analyzed the structure of mitochondria by confocal imaging.
HeLa cells were co-transfected with a nuclear targeted GFP together with the fission protein, labeled with MitoTracker Red, and imaged on a spinning wheel confocal microscope. As shown in Fig. 1, overexpression of either hFis1 or Drp1 induced the fragmentation of mitochondria, but the organelles redistributed very differently inside the cells. hFis1-fragmented mitochondria clustered around the nucleus, whereas Drp1-fragmented mitochondria remained scattered throughout the cell. Interestingly, the expression of the fission proteins was associated with a reduction in the mitochondrial volume (Fig. 1, bottom right panel). The total volume labeled with MitoTracker in the confocal stacks was reduced by 39% upon the expression of hFis1 and by 25% upon the expression of Drp1. A 34% reduction in mitochondrial volume was also observed upon the expression of hFis1 using RFPmit as a mitochondrial marker. The reduction in mitochondrial volume was not associated with a change in cellular volume (data not shown). Thus, the two fission proteins not only caused the fragmentation of mitochondria but also reduced the total mitochondrial content. In addition, the two proteins redistributed mitochondria differently within cells, with hFis1 inducing a collapse of mitochondria around the nucleus, whereas Drp1-fragmented mitochondria remained distributed throughout the cell.

We showed previously that hFis1 expression removed mitochondria from the cell periphery (18), but this analysis has not been performed with Drp1. Because of the different fragmentation pattern obtained with Drp1, mitochondria could still be close to the plasma membrane in the z axis in our fairly thin HeLa cells. To establish the location of mitochondria relative to the plasma membrane, cells were transfected with a fluorescent protein bearing the membrane anchor sequence CAAX of ki-Ras (YC3.6pm) (20). As shown in Fig. 2 (green channel), the membrane-targeted probe was expressed at the cell periphery and in filopodial and lamellipodial structures. In con-
control cells, mitochondria decorated the inner leaflet of the plasma membrane, as seen in the xz and yz cross-section images. In contrast, in cells expressing hFis1 or Drp1, mitochondria were not distributed evenly below the plasma membrane. As a result, large contiguous regions of the plasma membrane were devoid of neighboring mitochondria. Using the complete series of the z stacks, we performed a quantitative analysis of the interactions between mitochondria and the plasma membrane (Fig. 2, bottom right panel). The fraction of the plasma membrane bearing mitochondria decreased from 10.1 ± 2.5 to 2.5 ± 0.3 and 2.9 ± 0.4% upon hFis1 and Drp1 expression, respectively (p < 0.01). Thus, both fission proteins reduced the fraction of the plasma membrane bearing mitochondria by 4-fold.

Effects of hFis1 on Histamine-induced Cytosolic Ca\(^{2+}\) Elevations—To evaluate the impact of the mitochondrial redistribution on the cytosolic Ca\(^{2+}\) signals, HeLa cells were challenged with the Ca\(^{2+}\)-mobilizing agonist histamine or with the SERCA inhibitor thapsigargin, and changes in the cytosolic Ca\(^{2+}\) concentration were measured with Fura-2. The effects of hFis1, which induced the most dramatic mitochondrial redistribution, were analyzed in detail. The resting Ca\(^{2+}\) concentrations were not different between control and hFis1-overexpressing cells, but the responses elicited by histamine differed markedly depending on the presence of external Ca\(^{2+}\) (Fig. 3). When Ca\(^{2+}\) was present in the extracellular medium the application of a low dose of histamine (1 μM) elicited Ca\(^{2+}\) elevations of similar amplitude and duration in control and hFis1-overexpressing cells (Δratio was 0.77 ± 0.08 and 0.75 ± 0.10 for control and overexpressing cells, n = 18 and n = 14, respectively; Fig. 3A). In contrast, in the absence of external Ca\(^{2+}\) the histamine-induced Ca\(^{2+}\) elevation was reduced by 40% in hFis1-expressing cells (Δratio was 0.53 ± 0.04 versus 0.95 ± 0.04, n = 32 and n = 77, p < 0.0001; Fig. 3B). An even more severe reduction was observed when cells were kept for a longer duration in Ca\(^{2+}\)-free medium before the application of histamine (15 min; Fig. 3C). The integrated Ca\(^{2+}\) responses measured in control and hFis1-expressing cells, expressed as the area under the curve, are illustrated in Fig. 3E for the different protocols. The Ca\(^{2+}\) responses were similar in the presence of Ca\(^{2+}\) but were reduced by 60% when Ca\(^{2+}\) was omitted for 2 min and by 80% when Ca\(^{2+}\) was omitted for 15 min. Interestingly, a normal response was observed in Ca\(^{2+}\)-free medium when hFis1-expressing cells were stimulated with supramaximal doses of histamine (50 μM; data not shown). However, in all conditions tested (1 and 50 μM histamine in Ca\(^{2+}\)-free medium), the percentage of cells displaying an oscillatory response was lower in hFis1 overexpressers, suggesting that these cells had a defect in intracellular Ca\(^{2+}\) handling.

To verify that the reduced Ca\(^{2+}\) response was due to a decreased release of Ca\(^{2+}\) from intracellular stores, cells were stimulated with 1 μM thapsigargin in the absence of extracellular Ca\(^{2+}\) to indirectly assess the ER Ca\(^{2+}\) content. As shown in Fig. 3D, the thapsigargin-induced response of hFis1-expressing cells was reduced by 58%, suggesting that the Ca\(^{2+}\) content of the ER was reduced by an equivalent amount. ER Ca\(^{2+}\) content was also assessed at low extracellular Na\(^{+}\) concentration.
FIGURE 3. Effect of hFis1 overexpression on cytosolic Ca^{2+} responses. HeLa cells were loaded with 2 μM Fura-2 and stimulated with histamine or thapsigargin. A, responses elicited by the application of 1 μM histamine to cells maintained in a medium containing 2 mM Ca^{2+}. Traces are averages of 18 and 14 recordings for control and hFis1, respectively. B and C, responses elicited by the application of 1 μM histamine to cells maintained in Ca^{2+}-free medium for 2 min (B, n = 77 and 32 for control and hFis1, respectively) or for 15 min (C, n = 21 and 15 for control and hFis1, respectively). D, cells maintained in Ca^{2+}-free medium for 2 min were stimulated with 1 μM thapsigargin (TG). Traces are the average of 17 and 12 recordings for control and hFis1, respectively. E, statistical evaluation of the Ca^{2+} increase induced by histamine, expressed as the area under the curve (A.U.C.). Bars are mean ± S.E.; *, p < 0.05. F, statistical evaluation of the Ca^{2+} increase induced by 1 μM thapsigargin done under normal (145 mM) and low (19 mM) extracellular Na^{+} conditions, expressed as the area under the curve (A.U.C.). Bars are mean ± S.E.; *, p < 0.05.
Calcium Fluxes in Cells Lacking Subplasmalemmal Mitochondria

Effects of hFis1 on ER Ca\(^{2+}\) Homeostasis—Because both the cytosolic and the ER [Ca\(^{2+}\)] were decreasing faster in hFis1 cells kept in Ca\(^{2+}\)-free medium even without agonist stimulation, we assessed whether the fluxes of Ca\(^{2+}\) at the plasma membrane were altered. To test the basal activity of the Ca\(^{2+}\) extrusion and Ca\(^{2+}\) influx machinery, we used a simple Ca\(^{2+}\) switch protocol, alternating the bath solution from Ca\(^{2+}\)-containing to Ca\(^{2+}\)-free media. As shown in Fig. 5, A and B, Ca\(^{2+}\) removal caused a more pronounced [Ca\(^{2+}\)]\(_{\text{cyt}}\) decrease in hFis1 overexpressers, whereas the readquisition of Ca\(^{2+}\) produced a faster and larger [Ca\(^{2+}\)]\(_{\text{cyt}}\) increase in these cells. The increased Ca\(^{2+}\) entry was confirmed by using the Mn\(^{2+}\) quench technique. The addition of 100 \(\mu\)M Mn\(^{2+}\) to cells kept for 3 min in nominal Ca\(^{2+}\)-free medium caused a significantly faster decrease in Fura-2 fluorescence in hFis1 overexpressers (Fig. 5C). Interestingly, this faster decrease was only observed when Mn\(^{2+}\) was added to cells in the absence of Ca\(^{2+}\). When the experiment was performed in the presence of extracellular Ca\(^{2+}\), the rate of Mn\(^{2+}\) entry was not altered by hFis1 overexpression (Fig. 5D). Next, we tested the activity of plasma membrane Ca\(^{2+}\) extruders at high [Ca\(^{2+}\)]\(_{\text{cyt}}\) levels. For this purpose, a high [Ca\(^{2+}\)]\(_{\text{cyt}}\) was imposed by incubating cells sequentially with 1 \(\mu\)M thapsigargin and 2 \(\mu\)M Ca\(^{2+}\), and Ca\(^{2+}\) was removed from the extracellular medium 3 min later to assess the rates of [Ca\(^{2+}\)]\(_{\text{cyt}}\) recovery (Fig. 5E, inset). As shown in Fig. 5E, [Ca\(^{2+}\)]\(_{\text{cyt}}\) decreased faster in hFis1-overexpressing cells, indicating that the Ca\(^{2+}\) extrusion mechanisms are more active in these cells regardless of the [Ca\(^{2+}\)]\(_{\text{cyt}}\) levels. An exponential fit of the [Ca\(^{2+}\)]\(_{\text{cyt}}\) decay gave a time constant of \(\tau\) = 20.69 ± 1.78 s with \(n = 27\) and \(\tau = 14.85 ± 1.84\) s with \(n = 13\) for control and hFis1-overexpressing cells, respectively (Fig. 5F). These observations indicate that the activity of Ca\(^{2+}\) extruding transporters was increased in hFis1-overexpressing cells both at low and high [Ca\(^{2+}\)]\(_{\text{cyt}}\), whereas the activity of Ca\(^{2+}\) entry channels increased only when Ca\(^{2+}\) was removed from the medium.

Effects of Drp1 on Ca\(^{2+}\) Homeostasis—To test whether the effects observed with hFis1 could be reproduced with another fission protein, we tested the impact of the Drp1-induced mitochondrial fragmentation on Ca\(^{2+}\) homeostasis. Cells were stimulated with 1 \(\mu\)M thapsigargin in the absence of extracellular Ca\(^{2+}\) to assess the ER Ca\(^{2+}\) content. As shown in Fig. 6A, the thapsigargin-induced response of Drp1-overexpressing cells was reduced by 55% compared with that of control cells, indicating that the Ca\(^{2+}\) content of the ER was reduced. Next, we tested whether the Ca\(^{2+}\) fluxes at the plasma membrane were also increased in Drp1 overexpressers by using the Ca\(^{2+}\) switch protocol. As shown in Fig. 6B, C and D, [Ca\(^{2+}\)]\(_{\text{cyt}}\) decreased much more rapidly in Drp1 overexpressers than in non-transfected cells upon Ca\(^{2+}\) removal and recovered more rapidly upon Ca\(^{2+}\) readmission. Thus, despite the different fragmentation patterns, hFis1 and Drp1 induced very similar alterations in Ca\(^{2+}\) handling.

Effect of Extracellular Ca\(^{2+}\) Readmission on Repetitive Ca\(^{2+}\) Transients—To assess whether the increased Ca\(^{2+}\) influx could compensate for the loss of ER Ca\(^{2+}\) and explain the surprisingly normal Ca\(^{2+}\) responses observed in Ca\(^{2+}\)-containing medium, we tested whether brief additions of Ca\(^{2+}\) could rescue the Ca\(^{2+}\) signaling defect of hFis1 overexpressing cells. For this purpose, cells were stimulated twice with 1 \(\mu\)M histamine at a 12-min interval in Ca\(^{2+}\)-free medium, and Ca\(^{2+}\) was readmitted or not readmitted during the interval. Fig. 7A and D show typical recordings performed with and without Ca\(^{2+}\) readmission. To estimate the amount of Ca\(^{2+}\) that recycled back into the ER after stimulation, the amplitude of the second histamine response was

![Graph](image-url)

**FIGURE 4. Effect of hFis1 overexpression on ER Ca\(^{2+}\) homeostasis.** HeLa cells were transiently transfected with the ER-targeted cameleon probe YC4.1ER and 1 day later with hFis1. A, original recordings of ER Ca\(^{2+}\) following transition from Ca\(^{2+}\)- to Ca\(^{2+}\)-free medium and subsequent stimulation with 50 \(\mu\)M histamine and 1 \(\mu\)M thapsigargin. B, statistical evaluation of the ER Ca\(^{2+}\) decrease elicited by Ca\(^{2+}\) removal and by application of 50 \(\mu\)M histamine (n = 11 and 10 for control and hFis1, respectively). Bars are mean ± S.E.; *, p < 0.05.
As shown in Fig. 7B, the second response was significantly blunted in hFis1-overexpressing cells when Ca^{2+} was not readmitted, confirming that their ER Ca^{2+} stores were more depleted. The amplitude of the second histamine response averaged 40% of the first response in control cells but only 20% in hFis1-overexpressing cells. The difference was even more striking when the total Ca^{2+} response was integrated, with the second response averaging 22% in control cells and 10% in hFis1-overexpressing cells (Fig. 7C). In contrast, when Ca^{2+} was readmitted between the two histamine stimulations the responses became similar in control and hFis1-overexpressing cells, with the peak amplitude averaging 50% and the integrated Ca^{2+} responses averaging 35% of the first response in both control and hFis1-overexpressing cells (Fig. 7E and F). These observations indicate that the transient addition of extracellular Ca^{2+} can res-
Calcium Fluxes in Cells Lacking Subplasmalemmal Mitochondria

DISCUSSION

In this study, we show that HeLa cells overexpressing the two fission proteins hFis1 and Drp1 lack subplasmalemmal mitochondria and require external Ca$^{2+}$ for signaling. Ca$^{2+}$ signals were normal in cells expressing hFis1 or Drp1 when the medium contained Ca$^{2+}$ but were significantly blunted when Ca$^{2+}$ was removed from the medium. Upon Ca$^{2+}$ removal, both the [Ca$^{2+}$]$_{cyt}$ and [Ca$^{2+}$]$_{ER}$ levels decreased rapidly, indicating that Ca$^{2+}$ was leaving the ER and the cytosol. These changes occurred without concomitant changes in [Ca$^{2+}$]$_{mit}$ (data not shown), indicating that the missing Ca$^{2+}$ was not accumulating in mitochondria. Moreover, Ca$^{2+}$ was not sequestered in other organelles by SERCA, because increased [Ca$^{2+}$]$_{cyt}$ clearance was observed in the presence of thapsigargin (Fig. 5E). Thus, Ca$^{2+}$ was extruded from cells, either by plasma membrane Ca$^{2+}$-ATPase (PMCA) pumps or by Na$^{+}$/Ca$^{2+}$ exchange. Increased Ca$^{2+}$ efflux was observed both at resting and high [Ca$^{2+}$]$_{cyt}$ levels (Fig. 5), and ER Ca$^{2+}$ depletion was also observed at low [Na$^{+}$], a condition that prevent Na$^{+}$/Ca$^{2+}$ exchange (Fig. 3F). This observation indicates that the contribution of Na$^{+}$/Ca$^{2+}$ exchange is minimal and that the major Ca$^{2+}$ alteration occurring in cells with fragmented mitochondria is an increased activity of PMCA. Increased influx of Ca$^{2+}$ and Mn$^{2+}$ was also observed in hFis1 cells, but only under conditions associated with decreased [Ca$^{2+}$]$_{ER}$ levels and not in cells with normal or fully depleted ER Ca$^{2+}$ stores. This finding indicated that the activation of plasma membrane Ca$^{2+}$ channels was secondary to the decrease in [Ca$^{2+}$]$_{ER}$. Thus, the primary Ca$^{2+}$ signaling defect associated with the expression of the mitochondrial fission proteins is, surprisingly, an increased activity of plasma membrane Ca$^{2+}$ pumps. The increased PMCA activity, in turn, induces a rapid Ca$^{2+}$ depletion of the ER and a subsequent activation of store-operated Ca$^{2+}$ channels.

Surprisingly, Ca$^{2+}$ homeostasis was disturbed in a nearly identical way by overexpression of Drp1 and hFis1 (Fig. 6), although the pattern of mitochondrial fragmentation was very different (Fig. 1). In both cases, the major Ca$^{2+}$ defect was an increased PMCA activity, but with Drp1 the mitochondria remained dispersed within the cytosol and did not cluster around the nucleus. However, a detailed quantitative analysis of the confocal stacks revealed that Drp1 removed mitochondria from the plasma membrane as efficiently as hFis1 (Fig. 2). The fraction of the plasma membrane bearing mitochondria was reduced by 4-fold upon expression of the two fission proteins, indicating that the amount of subplasmalemmal mitochondria decreased to a similar extent. The similarity of the Ca$^{2+}$ phenotype obtained with Drp1 and hFis1 suggests that the Ca$^{2+}$ machinery is extremely sensitive to the presence of subplasmalemmal mitochondria.
The impact of mitochondria remodeling on Ca\(^{2+}\) handling in HeLa cells has been analyzed in three recent studies using overexpression of different proteins from the mitochondria fusion/fission machinery, namely hFis1 (18), dynamitin (21), and Drp1 (22). The studies focused on the transfer of Ca\(^{2+}\) between the ER and mitochondria and on the observed Ca\(^{2+}\) alterations that correlated with a specific shape and location of mitochondria. With dynamitin, mitochondria did not fragment but accumulated near the nucleus. The number of close contacts between the ER and mitochondria increased by 2.5-fold without detectable effects on the [Ca\(^{2+}\)]\(_{mit}\) responses (21). With Drp1, mitochondria fragmented extensively but remained in the cell periphery. The number of ER-mitochondria contacts was not changed, but the [Ca\(^{2+}\)]\(_{mit}\) responses were significantly reduced, and a substantial portion of mitochondria failed to display [Ca\(^{2+}\)]\(_{mit}\) elevations (22). With hFis1, mitochondria fragmented and accumulated near the nucleus, and [Ca\(^{2+}\)]\(_{mit}\) responses were normal (18). In the present study, we...
Calcium Fluxes in Cells Lacking Subplasmalemmal Mitochondria

attempts to quantify the number of ER-mitochondria contacts, but the density of the ER near the nucleus precluded precise measurements. Overall, mitochondria remained largely embedded in the ER, and no changes in the extent of colocalization between the two organelles could be demonstrated. Taken together, these studies suggest that the integrity of the mitochondrial network is as important as its connections to the Ca\(^{2+}\) sources in shaping [Ca\(^{2+}\)]\(_{\text{mit}}\) responses. When a substantial portion of mitochondria are located far from Ca\(^{2+}\) sources, as observed with Drp1, Ca\(^{2+}\) tunneling within interconnected organelles appears to determine the amplitude of the averaged [Ca\(^{2+}\)]\(_{\text{mit}}\) response (22). In this case, the connectivity of the mitochondrial network ensures that all mitochondria respond adequately to physiological Ca\(^{2+}\) signals. On the other hand, when mitochondria accumulate in ER-dense regions, as observed with dynamitin and hFis1, Ca\(^{2+}\) propagation within the network is no longer required, because nearly all mitochondria are located close to ER Ca\(^{2+}\) channels (18, 21). Our results extend these previous studies and show that the main effect of hFis1 on cell Ca\(^{2+}\) homeostasis are not due to alteration in Ca\(^{2+}\) transfer from the ER to mitochondria, but instead to an increased activity of PMCA initiated by the lack of subplasmalemmal mitochondria.

We propose the following mechanism to explain the increased activity of PMCA in cells lacking subplasmalemmal mitochondria (Fig. 8). Under normal conditions, subplasmalemmal mitochondria capture Ca\(^{2+}\) ions entering across plasma membrane channels and relay the captured Ca\(^{2+}\) to the ER, sustaining the activity of SERCA (Fig. 8A). In cells with long, tubular mitochondria such as HeLa cells, Ca\(^{2+}\) tunneling within interconnected mitochondria might allow propagation of the Ca\(^{2+}\) captured at the plasma membrane to ER regions located deep in the cells (18). The loss of peripheral mitochondria disrupts this Ca\(^{2+}\) relay mechanism and decreases the local Ca\(^{2+}\) buffering capacity near plasma membrane channels. As a result, nearby PMCA pumps are continuously exposed to an increased Ca\(^{2+}\) load because of the constant leak of Ca\(^{2+}\) across the plasma membrane and intracellular Ca\(^{2+}\) release channels (Fig. 8B). Cells adapt to this increased subplasmalemmal Ca\(^{2+}\) load by increasing the activity of PMCA, which eventually restores normal subplasmalemmal [Ca\(^{2+}\)] levels. As a result of this adaptation, cells become exquisitely sensitive to the presence of extracellular Ca\(^{2+}\). In the absence of Ca\(^{2+}\), ER Ca\(^{2+}\) stores become rapidly depleted, because Ca\(^{2+}\) ions are more efficiently pumped out of the cells by the overactive PMCA. The ER depletion might be compounded by the lack of subplasmalemmal mitochondria, as these organelles can recycle part of the released Ca\(^{2+}\) ions back to neighboring ER regions. In cells lacking subplasmalemmal mitochondria, a large part of the Ca\(^{2+}\) released from the ER during agonist stimulation is thus pumped outside of the cells instead of being recycled back to intracellular stores. The ensuing decrease in [Ca\(^{2+}\)]\(_{\text{ER}}\) then activates capacitative Ca\(^{2+}\) channels at the plasma membrane, allowing the entry of Ca\(^{2+}\) to substitute for the deficit in internally stored Ca\(^{2+}\) (Fig. 8C). The lack of subplasmalemmal mitochondria thus initiates a sequence of compensatory mechanisms that restores the ability of cells to generate Ca\(^{2+}\) signals by external rather than internal Ca\(^{2+}\).

The adaptation of Ca\(^{2+}\) transport proteins to cope with increased Ca\(^{2+}\) loads is expected to maintain cell Ca\(^{2+}\) homeostasis. PMCA pumps are good candidates for such Ca\(^{2+}\)-dependent modulation, as they are the first line of defense of cells against a Ca\(^{2+}\) overload. Indeed, Ca\(^{2+}\)-dependent increase in PMCA activity has been reported in human T lymphocytes following [Ca\(^{2+}\)]\(_{\text{EC}}\) elevations induced by the opening of Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels (23). Both the maximal pump rate and the Ca\(^{2+}\) sensitivity of the PMCA pumps were increased, resulting in a 5-fold increase in PMCA activity that matched the increased Ca\(^{2+}\) influx. In our cells overexpressing hFis1, the increase in PMCA activity was observed at normal resting [Ca\(^{2+}\)]\(_{\text{rest}}\) levels, suggesting that subplasmalemmal [Ca\(^{2+}\)] levels were increased. We attempted to measure subplasmalemmal Ca\(^{2+}\) levels with Fura-2 and YC3.6pm to demonstrate that the increased PMCA activity is linked to a
subplasmalemmal Ca\(^{2+}\) overload, but the spatio-temporal resolution of our imaging system was insufficient to resolve subcellular Ca\(^{2+}\) gradients. However, the increased PMCA activity was readily apparent in the Fura-2 recordings and produced specific alterations in Ca\(^{2+}\) handling that mimicked those reported previously for PMCA overexpression. In Chinese hamster ovary cells, overexpression of the four PMCA pump isoforms decreased the steady-state [Ca\(^{2+}\)]\(_{\text{ER}}\) levels and reduced the amplitude and duration of the cytosolic Ca\(^{2+}\) transients elicited by ATP (24). Larger effects were observed with the neuron-specific PMCA2 and PMCA3 than with the ubiquitously expressed isoforms 1 and 4 (25). As observed in our cells lacking subplasmalemmal mitochondria, the increased Ca\(^{2+}\) clearance had minimal impact on resting cytosolic Ca\(^{2+}\) levels, and the general pattern of Ca\(^{2+}\) signals was preserved as long as Ca\(^{2+}\) was present in the extracellular medium.

Our results indicate that subplasmalemmal mitochondria control cellular Ca\(^{2+}\) handling by modulating the activity of PMCA. Subplasmalemmal mitochondria thus appear to have multiple roles in Ca\(^{2+}\) signaling. Growing evidence suggests that subplasmalemmal mitochondria are involved in the transfer of Ca\(^{2+}\) from the extracellular medium to the ER. Ca\(^{2+}\) uptake by mitochondria located beneath plasma membrane channels generates microdomains of low Ca\(^{2+}\), enabling the activation of store-operated Ca\(^{2+}\) entry channels during agonist stimulation (3). During this process, Ca\(^{2+}\) continuously flows through mitochondria, and inhibition of mitochondrial Ca\(^{2+}\) efflux with CGP 37157 (an inhibitor of the mitochondrial Na\(^+/\)Ca\(^{2+}\) exchanger) abolishes the ability of mitochondria to buffer subplasmalemmal Ca\(^{2+}\) (4). Importantly, CGP 37157 also reversibly inhibits ER refilling, indicating that mitochondria delay Ca\(^{2+}\) from plasma membrane channels to the ER. In endothelial cells, ER Ca\(^{2+}\) refilling is prevented by CGP 37157 only in the presence of agonists, indicating that Ca\(^{2+}\) might transit through mitochondria only during cell activation (26). We now show that the lack of subplasmalemmal mitochondria increases the activity of PMCA and promotes ER Ca\(^{2+}\) depletion, highlighting the importance of these organelles in cell Ca\(^{2+}\) homeostasis. Furthermore, our colocalization measurements indicate that in HeLa cells only 10% of the plasma membrane is close to mitochondria at the resolution of our confocal microscope (0.7 \(\mu\)m in the z axis). These mitochondria play a critical role, because a decrease from 10 to 2.5% is sufficient to induce major alterations in cell Ca\(^{2+}\) homeostasis. By placing mitochondria at specific sites near the plasma membrane, cells can use mitochondria to modulate membrane Ca\(^{2+}\) channels, relay Ca\(^{2+}\) to the ER, and control the activity of PMCA.

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Calcium Fluxes in Cells Lacking Subplasmalemmal Mitochondria