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# Mitochondria and Ca$^{2+}$ signaling: old guests, new functions

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## Abstract

Mitochondria are ancient endosymbiotic guests that joined the cells in the evolution of complex life. While the unique ability of mitochondria to produce adenosine triphosphate (ATP) and their contribution to cellular nutrition metabolism received condign attention, our understanding of the organelle's contribution to Ca$^{2+}$ homeostasis was restricted to serve as passive Ca$^{2+}$ sinks that accumulate Ca$^{2+}$ along the organelle's negative membrane potential. This paradigm has changed radically. Nowadays, mitochondria are known to respond to environmental Ca$^{2+}$ and to contribute actively to the regulation of spatial and temporal patterns of intracellular Ca$^{2+}$ signaling. Accordingly, mitochondria contribute to many signal transduction pathways and are actively involved in the maintenance of capacitative Ca$^{2+}$ entry, the accomplishment of Ca$^{2+}$ refilling of the endoplasmic reticulum and Ca$^{2+}$-dependent protein folding. Mitochondrial Ca$^{2+}$ homeostasis is complex and regulated by numerous, so far, genetically unidentified Ca$^{2+}$ channels, pumps and exchangers that concertedly accomplish the organelle's Ca$^{2+}$ demand. Notably, mitochondrial Ca$^{2+}$ homeostasis and functions are crucially influenced by the organelle's structural organization and motility that, in turn, is controlled by matrix/cytosolic Ca$^{2+}$. This review intends to provide a condensed overview on the molecular mechanisms of mitochondrial Ca$^{2+}$ homeostasis (uptake, buffering and storage, extrusion), its modulation by other ions, kinases and small molecules, and its contribution to cellular processes as fundamental basis for the organelle's contribution to signaling pathways. Hence, emphasis is given to the structure-to-function and mobility-to-function relationship of the mitochondria and, thereby, bridging our most recent knowledge on mitochondria with the best-established mitochondrial function: metabolism and ATP production.

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**Keywords**

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## Introduction

Right from its beginning until our days, the history of mitochondria has been a story of visionary suggestions, wrong assumptions, and reconsiderations. Following the visionary endosymbiotic theory, proposed by Andreas Schimper in 1883 and Konstantin Mereschkowsky in 1905, Ivan Wallin proposed in the 1920s that mitochondria are bacteria that have joined preeukaryotic cells. However, based on the false assumption that mitochondria do not contain DNA, this hypothesis was discontinued but became reestablished after mitochondrial DNA was identified in the 1960s. Today, the endosymbiotic theory is widely accepted, and based on similarities in their genomes, rickettsias are thought to be the closest relatives to the ancestors of mitochondria. However, mitochondrial DNA only encodes for 13 proteins of the respiratory chain, while all other mitochondria-localized proteins are encoded by the nuclear DNA and get imported into the organelle by the very sophisticated TIM/TOM machinery (for review, see [146]).

Beyond any doubt, the key role of mitochondria is to provide energy in the form of adenosine triphosphate (ATP), which is continuously necessary in the struggle against entropy to abide life. Additionally, in recent years, evidence has accumulated that mitochondria are much more than efficient suppliers of energy but are elementarily involved in virtually every signaling cascade and metabolic process that has been described to date. Therefore, it is not surprising that mitochondrial dysfunctions have been found to be involved in many different diseases, which, in turn, brings this organelle into the focus of (patho-)physiologists as well as pharmaceutical industry.

Accordingly, mitochondria play a crucial role in the initiation of apoptosis. Therein, proteins of the BCL-2 family interfere with mitochondrial carriers and poreforming proteins of the inner and outer mitochondrial membranes [59, 195]. The interrelation between mitochondrial $Ca^{2+}$ and apoptosis has been convincingly reported [48, 196] and was very recently excellently reviewed by Hajnoczky et al. [80], Armstrong [4], or Chan [32]. Consequently, we decided to exclude the apoptotic branch of the mitochondria from this review and refer on the intriguing work of others.

Strikingly, the property of mitochondria to contribute to manifold physiological processes other than apoptosis is also tightly linked to the organelle's $Ca^{2+}$ homeostasis. The positive charged calcium ions are of crucial importance in many cellular physiological processes as they specifically govern versatile processes within almost all different cells to induce intrinsic functions. $Ca^{2+}$ signal transduction is accomplished by a reversible binding of $Ca^{2+}$ to specific different $Ca^{2+}$ binding sites, such as EF-hands, C2-domains, or annexin-domains. Such $Ca^{2+}$ binding sites have been found in numerous signaling-proteins, which alter their enzyme activity, cellular distribution, binding affinity to other proteins or heterologous biomolecules upon a $Ca^{2+}$ induced steric rearrangement.

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The versatility of $Ca^{2+}$ as a second messenger is accomplished by a sophisticated machinery of an impressive number of $Ca^{2+}$ shuttling proteins that include ion channels, transporters, pumps, exchangers, and buffer proteins that concertedly control the spatiotemporal $Ca^{2+}$ signaling [16]. Notably, mitochondria play an ambiguous role in the regulation of $Ca^{2+}$ homeostasis as they house targets for signaling $Ca^{2+}$ but contribute to local and global $Ca^{2+}$ signaling also. Accordingly, mitochondria not only act as simple $Ca^{2+}$ sinks, but also specifically respond to this universal messenger.

In this review, we attempt to give a condensed overview on the molecular aspects of mitochondrial $Ca^{2+}$ homeostasis and its regulatory potential as fundamental basis for the organelle's contribution to signaling pathways. This review does not claim to be complete but represents the authors' subjective assortment on mitochondrial $Ca^{2+}$ signaling and $Ca^{2+}$ function in these old guests that joined the cells in the evolution of complex life.

# Mitochondrial $Ca^{2+}$ handling

## Mitochondrial $Ca^{2+}$ uptake

The phenomenon of mitochondrial $Ca^{2+}$ uptake basically builds on two landmark observations that lay 30 years apart from each other. In the late 1960s, Naranjan S. Dhalla described for the first time strong $Ca^{2+}$ accumulation in isolated respiring mitochondria [49]. However, due to the low sensitivity of the mitochondrial $Ca^{2+}$ uptake machinery, it was assumed that $Ca^{2+}$ sequestration by mitochondria in living cells is physiologically irrelevant. This false paradigm lasted until reliable measurements of mitochondrial $Ca^{2+}$ uptake with targeted protein based $Ca^{2+}$ sensors clearly demonstrated that mitochondria in intact cells promptly sequester $Ca^{2+}$ upon cell stimulation under physiological condition [184]. The apparent discrepancy between the striking mitochondrial $Ca^{2+}$ response in intact cells and the low affinity of the mitochondrial $Ca^{2+}$ uptake system of isolated mitochondria has not been solved entirely, but is most likely due to the exposure of the mitochondria to microdomains of high $Ca^{2+}$ that meet the low $Ca^{2+}$ affinity of the mitochondrial $Ca^{2+}$ uptake system [187]. Such microdomains of high $Ca^{2+}$ are thought to be achieved by a close proximity of sites of mitochondrial $Ca^{2+}$ uptake with those of $Ca^{2+}$ release at the endoplasmic reticulum (ER) and/or by functional coupling with $Ca^{2+}$ entry channels at the plasma membrane [44, 126, 186]. Another aspect, which has been barely considered so far, but may also explain the obvious discrepancy between the prompt mitochondrial $Ca^{2+}$ response in intact cells and the rather ponderous $Ca^{2+}$ sequestration of isolated mitochondria, is that mitochondrial $Ca^{2+}$ uptake in intact cells might be modulated by soluble proteins, which trail away upon cell permeabilization and/or isolation of the organelles. In line with this suggestion, mitochondrial $Ca^{2+}$ homeostasis has been linked to protein kinase C family members [171], although the final proof is still missing.

Regardless of whether the organelles are isolated or reside within a cell, $Ca^{2+}$ uptake into mitochondria critically depends on the electrical driving force for $Ca^{2+}$ influx that is established by a huge negative membrane potential across the inner mitochondrial membrane (IMM). In respiring, energized mitochondria, a membrane potential of approximately −180 mV is generated by a large $H^+$ gradient at the IMM that is established by continuous translocation of $H^+$ from the matrix into the intermembrane space, which is accompanied by electron fluxes between the complexes of the respiratory chain [140]. According to Nernst equation, equilibrium would be reached only if $Ca^{2+}$ inside the mitochondria reaches values $10^6$ higher than in the extramitochondrial area. Dissipation of the proton gradient across the IMM by chemical uncouplers such as carbonyl cyanide 4-(trifluoromethoxyl)s-phenyl-hydrazone (FCCP), which immediately causes a depolarization of the IMM, reduces the driving force for $Ca^{2+}$ entry and thus strongly diminishes mitochondrial $Ca^{2+}$ uptake (Fig. 1).

At this point it should be mentioned that protonophores like FCCP rapidly provoke a severe morphological alteration of the structural organization of the mitochondria in living cells. Notably, under physiological conditions mitochondria form a tubular highly interconnected network while mitochondrial structure turns toward disconnected, spherical, singular mitochondria upon FCCP (Fig. 2). Notably, such structural changes of mitochondria are accompanied with alterations in mitochondrial $Ca^{2+}$ homeostasis independently of the initiator of such structural reorganization of this organelle [160].

**$Ca^{2+}$ transit through the outer mitochondrial membrane—**The access of cytosolic $Ca^{2+}$ to the mitochondrial matrix is restricted by the outer mitochondrial membrane (OMM) and by the IMM. Notably, for a long time, the OMM that contains various large pored porines like the voltage-dependent anion-selective channel (VDAC) [198] was thought to be freely permeable for $Ca^{2+}$. However, this assumption has changed by a report in which the overexpression of VDAC1 was found to facilitate the transfer of $Ca^{2+}$ from the ER into mitochondria upon cell stimulation, thus indicating that the number of porines in the OMM correlates with mitochondrial $Ca^{2+}$ sequestration [177]. Complement findings were obtained with tcBid, a proapoptotic protein, that increases the permeability of the OMM [42]. Very recently, the group of György Hajnóczky demonstrated an enhanced $Ca^{2+}$ conductance through the OMM by $Ca^{2+}$ when the cytosolic $Ca^{2+}$ concentration was raised from ~0 to 2 mM or above. While this effect may have significance during $IP_3$-induced $Ca^{2+}$ release in the microdomain between ER and mitochondria however, these data do not indicate whether $Ca^{2+}$ concentrations out of these microdomains may have any similar effect [12]. Further to this limited permeability to cytosolic $Ca^{2+}$, the OMM, by housing certain adaptor/scaffold proteins, essentially contributes to the establishment of junction with other organelles or the plasma membrane (see: Endoplasmic reticulum—mitochondria $Ca^{2+}$ crosstalk) and organelle motility (see: Effect of $Ca^{2+}$ on mitochondrial motility and morphology).

**$Ca^{2+}$ transport through the inner mitochondrial membrane—**In contrast to the OMM, the IMM was very early expected to be impermeable for ions to maintain the membrane potential and to run the endoergonic generation of ATP. Interestingly, despite the molecular nature of all $Ca^{2+}$ shuttling proteins in the IMM are unknown or a matter of intensive debate, various phenomena of the $Ca^{2+}$ transport through the IMM have been carefully characterized, and pharmacological tools were developed. In the following, the most frequent $Ca^{2+}$ shuttling phenomena across the IMM are listed:

**The mitochondrial $Ca^{2+}$ uniporter:** Most of $Ca^{2+}$ flux across the IMM is postulated to be accomplished by the so-called mitochondrial $Ca^{2+}$ uniporter (MCU), which represents a gated, highly selective ion channel [106, 192] and allows $Ca^{2+}$ influx along the electrochemical gradient without any accompanying other ion or the need for ATP hydrolysis [77]. Based on carefully elaborated experiments using the patch clamp technique on so-called mitoblasts (isolated swollen mitochondria lacking the OMM), David Clapham's group emphasized that the MCU is a highly selective $Ca^{2+}$ channel, which shows a second-order kinetics with both an activation domain as well as a transport site [106]. Notably, these electrophysiological data revealed that the MCU is hardly saturable by $Ca^{2+}$ and exhibits a half-activation constant, $K_{0.5}$ of 19 mM $Ca^{2+}$. However, previous data obtained in suspended isolated mitochondria reported a $K_{0.5}$ of 10 μM $Ca^{2+}$ [76, 78]. Since in whole-cell recordings the membrane potential is imposed, whereas in mitochondria suspension, the membrane potential rapidly depolarized upon $Ca^{2+}$ entry, and thus, the MCU gets saturated at lower $Ca^{2+}$ concentration, this difference was suggested to be due to the different techniques used [106].

**Pharmacology of the MCU:** While $Ca^{2+}$ itself is thought to activate MCU, other ions like the lanthanides, ruthenium red [178], its derivate Ru360 [131], diamino-pentane pentamic acid [40] and the plasma membrane $Na^+/Ca^{2+}$ exchange inhibitor KB-R7943 [191] inhibit MCU. However, none of these inhibitors is known to act selectively on MCU and many exhibit no or very limited membranepermeability. Furthermore, $Mg^{2+}$ as well as nucleotides were shown to either inhibit or stimulate the MCU [115, 120]. In the 1980s, Nicchitta and Williamson reported that spermine activates mitochondrial $Ca^{2+}$ uniport, suggesting that polyamines may have an important physiological role in intracellular $Ca^{2+}$ handling [147]. A similar effect of activation on the transport of $Ca^{2+}$ by isolated mitochondria from rat liver was described for 2-aminoethanesulfonic acid (taurine) [157]. Very recently, Montero et al. [143] found out that the p38 MAPK inhibitor SB202190, estrogen receptor agonists and antagonists [121] as well as several plant flavonoides [142] accelerate mitochondrial $Ca^{2+}$ sequestration in intact as well as permeabilized cells, suggesting that the MCU can be activated selectively by certain pharmacological compounds. Whether or not pharmacological modulation of MCU activity can have some therapeutic potential is currently unknown. However, in view of the overwhelming reports that point to the crucial involvement of mitochondria in the development of numerous diseases (for review, see [55, 56]), it seems imperative that respective tests are performed in the near future.

**Identification of components of the MCU:** Although the phenomenon of mitochondrial $Ca^{2+}$ sequestration has been convincingly reported and explicitly characterized by numerous sophisticated approaches, all efforts to genetically identify the protein(s) that actually account for the MCU have been unsuccessful until now [193].

We have recently demonstrated that UCP2 and UCP3 are essentially involved in MCU [215]. *Prima facie*, these findings seem to be misleading as one would expect UCPs to depolarize mitochondria and, thus, to attenuate the electrical driving force for mitochondrial $Ca^{2+}$ uptake. However, a closer examination reveals that the physiological function of UCP2 and UCP3, which are embedded in the inner mitochondrial membrane and belong to the superfamily of mitochondrial ion transporters [181], is still a matter of debate. In a recent review, Michael Duchen wrote “the uncoupling proteins, or UCPs, are at present a rather mysterious group of proteins waiting for a role” [55]. Indeed, the many reported functions such as modulation of free radical formation, apoptosis, regulation of hormone secretion and glucose and fatty acid metabolism that have been suggested for UCP2/3 [26] can hardly be explained by a smooth uncoupling function of these proteins [102]. In contrast, in view of the versatility of $Ca^{2+}$ as a multifactorial second messenger, UCP2/3 facilitated $Ca^{2+}$ fluxes across the IMM might represent an attractive explanation of the molecular mechanisms behind the diverse biological processes that were described to be affected by UCP2 and UCP3. Notably, there is a wide consensus that these orthologs do not exhibit a thermogenetic function like UCP1. UCP2 and UCP3 have been identified in many tissues [181] and promote $H^+$ influx and smooth uncoupling in isolated mitochondria only under specific conditions [25], while their involvement in heat production could not be confirmed so far (for review, see [109]). Moreover, since UCP2 and UCP3 related proteins also exist in ectothermic fish and plants that do not require thermogenesis, further/alternative functions of these UCPs were emphasized. A contribution of either/both proteins was described in mitochondrial free radical production [25], apoptosis [46], the regulation of hormone secretion (UCP2) [110], and glucose and fatty acid metabolism (UCP3) [86].

Our assumption that UCP2 and UCP3 are essentially involved in the MCU is based on experiments using overexpression and knock-down (siRNA) experiments in single endothelial, HeLa, HEK293, and AtT20 cells [215]. In all cells tested, overexpression of UCP2 or UCP3 resulted in enhanced $Ca^{2+}$ sequestration into mitochondria upon cell stimulation with an $IP_3$-generating agonist. Notably, mitochondrial $Ca^{2+}$ extrusion remained unchanged, and no significant differences in mitochondrial pH compared to wild type cells were obtained. Notably, UCP2- and UCP3-mediated enhanced mitochondrial $Ca^{2+}$ elevation occurred independently of the source of $Ca^{2+}$ (i.e., $IP_3$-initiated intracellular $Ca^{2+}$ release and entering $Ca^{2+}$). In line with these findings, mitochondrial $Ca^{2+}$ uptake was strongly reduced in cells treated with siRNA against either UCP2 or UCP3 and was almost prevented if both siRNAs were combined. Convincingly, in HeLa cells that only express UCP3, expression of UCP2 could rescue the abolished mitochondrial $Ca^{2+}$ sequestration in cells treated with siRNA against UCP3. Supportingly, single isolated liver mitochondria of $UCP2^{-/-}$ mice lacked MCU, while a ruthenium red-insensitive $Ca^{2+}$ uptake, which was responsible for approximately 50% of mitochondrial $Ca^{2+}$ accumulation in liver mitochondria of wild type animals, remained. In line with our data, a role of UCP2/3 as carriers of ions other than $H^+$ has been recently postulated in CHO cells in which UCP3 did not cause uncoupling, but controls mitochondrial metabolic activity [145]. Additional analogies of UCP2/3 and the MCU concern their sensitivity to fatty acids and nucleotides. UCP2 and UCP3 were described to be activated by fatty acids and blocked by nucleotides [26, 99, 100], a sensitivity that was also recently reported for ruthenium red-sensitive MCU [120, 229]. These data and reports conclusively point to an elementary contribution of UCP2 and UCP3 to MCU in response to cell stimulation under physiological conditions and may explain the molecular mechanism beyond the reported effects of UCP2 and UCP3 [26, 101, 109].

In recent experiments, MCU could not be detected in isolated single yeast mitochondria, despite there was a significant, ruthenium red-insensitive $Ca^{2+}$ accumulation. Since there is no UCP-homolog in yeast, these data may indicate that the MCU phenomenon is causally linked to the expression of UCP2 or 3. However, a heterologous expression of human UCP2

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or UCP3 in this system failed to establish ruthenium red-sensitive MCU in yeast. Accordingly, and in view of the MCU rescue by UCP2 in siRNA-treated HeLa cells mentioned above, we speculate that UCP2/3 alone are not sufficient to establish MCU in yeast due to the lack of so far unknown mammalian constituent/s (e.g., VDAC) that is/are essential to assemble an active MCU [215] (Fig. 3).

**Alternative routes of mitochondrial $Ca^{2+}$ uptake:** Along with our recent findings on the involvement of UCP2 and UCP3 in the MCU phenomenon, it remains important to elucidate the involvement of the two more recently described UCP homologous [60] UCP4 and UCP5 in mitochondrial ion homeostasis. Notably, UCP5 is the closest family member to UCP1/2/3. Considering our findings that UCP2 and UCP3 can substitute each other for MCU, an assessment of the involvement of UCP5 in mitochondrial $Ca^{2+}$/ion homeostasis remains mandatory. In regard to UCP4, recent data in which expression of UCP4 attenuated store-operated $Ca^{2+}$ influx in PC12 cells tend to support an uncoupling function of this homolog even in intact cells [33]. Although it has not been measured in this study, mitochondrial depolarization as a result of a still-to-be-approved UCP4 uncoupling activity in intact cells would reduce the mitochondrial capacity to buffer entering $Ca^{2+}$. Consequently to the lack of subplasmalemmal $Ca^{2+}$ buffering by the mitochondria, one can expect $Ca^{2+}$-inhibitable entry pathway to be reduced (see: $Ca^{2+}$ entry pathways).

In heart as well as in liver, an alternative mitochondrial $Ca^{2+}$ uptake pathway has been described that shows distinct properties that do not apply for the MCU described above. This pathway has the feature to transfer $Ca^{2+}$ very rapidly into mitochondria during brief $Ca^{2+}$ pulses and is therefore termed the rapid uptake mode (RaM, 77). So far, the physiological function of RaM is unknown, and whether RaM is molecularly distinct from the MCU or exhibits a certain state of the MCU awaits further investigation. However, the sensitivity of RaM to ruthenium red fuels the latter concept. In chicken neurons, a ruthenium red-insensitive, electroneutral $Na^+$- and $H^+$-independent $Ca^{2+}$ antiporter has been described that accounts for spatially organized mitochondrial $Ca^{2+}$ uptake [37], thus indicating that depending on species and/or tissue, there might be various mitochondrial $Ca^{2+}$ uptake pathways.

In line with these assumptions, we have recently reported that in liver mitochondria isolated from wild type as well as $UCP2^{-/-}$ mice, a ruthenium red-insensitive $Ca^{2+}$ sequestration mechanism exists besides the classical MCU [215]. These data are in agreement with our findings in yeast in which mitochondrial $Ca^{2+}$ sequestration was found to be rather insensitive to this blocker of MCU [215]. Alternatively, since higher concentrations of ruthenium red (>1 μM) diminished these non-MCU typed $Ca^{2+}$ uptake into mammalian mitochondria, it remains possible that either ruthenium red exhibits additional inhibitory properties on various mitochondrial $Ca^{2+}$ uptake pathways or the sensitivity of MCU to ruthenium red depends on the protein composition of a multiprotein signalplex that accomplishes mitochondrial $Ca^{2+}$ sequestration (Fig. 3).

However, there are some additional candidates of putative ion carriers described in mitochondria that are most likely not involved in MCU and may be responsible for ruthenium red-insensitive $Ca^{2+}$ uptake into the mitochondria. The formation of the

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mitochondrial permeability transition pore has been described to be involved in mitochondrial $Ca^{2+}$ sequestration upon agonist-induced $Ca^{2+}$ mobilization from the ER [222]. Despite these convincing data, the circumstances that lead to such transient formation of the mitochondrial permeability transition pore upon physiological cell stimulation and the modulation of its gating remains unclear. Moreover, a mitochondrial $Ca^{2+}$-ATPase, that belongs to the SERCA family, has been identified in brown adipose tissue and described to account for heat/ATP production even under conditions in which mitochondria were depolarized [45]. In endothelial cells, a long-lasting incubation of the SERCA inhibitor, BHQ, results in slow accumulation of $Ca^{2+}$ in the mitochondria (Fig. 4). Since this effect occurs far later than ER depletion by BHQ [127, 154], it is unlikely that this increase in mitochondrial $Ca^{2+}$ by BHQ is due to ER depletion. Among other possible explanations of this slow mitochondrial $Ca^{2+}$ accumulation induced by SERCA inhibitors, the existence of mitochondria-localized $Ca^{2+}$-ATPase needs to be evaluated.

Beside the UCPs, another interesting protein of the solute carrier family 25 (SLC25) that may be involved in mitochondrial ion homeostasis is the human mitochondrial carrier CGI-69. This protein has two predicted transmembrane domains and shares high homology in the mitochondrial energy-transfer signature motifs found in UCPs [228]. However, just like UCP2 and UCP3 [26, 215], overexpression of CGI-69 did not initiate depolarization of mitochondria in HEK293 cells [228]. Like most other members of the SLC25 family that do not contain an EF-hand motif for $Ca^{2+}$ binding, the physiological function of CGI-69 is unknown. Accordingly, an assessment of the involvement of these SLC25 family members in mitochondrial $Ca^{2+}$ signaling is necessary for a better understanding of the molecular nature of mitochondrial $Ca^{2+}$ transporters.

While there is overwhelming evidence that the mitochondrial $Na^+/Ca^{2+}$ exchanger ($NCX_{mito}$) accounts for mitochondrial $Ca^{2+}$ extrusion in most cells (see: Mitochondrial $Ca^{2+}$ extrusion processes), under certain conditions, this exchanger might work in the opposite direction resulting in $Ca^{2+}$ uptake and $Na^+$ extrusion [200]. This feature, which $NCX_{mito}$ shares with $NCX_{pm}$ and other antiporters, may gain particular importance under conditions in which the cellular $Na^+$ homeostasis is disturbed. Accordingly, it remains to be resolved whether or not $NCX_{mito}$ is functionally linked with other, nonmitochondrial ion carriers/channels (e.g., TRPC3 [58]), and if so, what might be the contribution of such arrangement to mitochondrial $Ca^{2+}$ homeostasis and organelle function. Importantly, the switch between the modes of the $NCX_{mito}$ critically depends on its electrogenity and $Na^+$:$Ca^{2+}$ stoichiometry that is not entirely clear so far. Nevertheless, even a 2 $Na^+$:1 $Ca^{2+}$ stoichiometry like the $NCX_{pm}$ would allow switching this antiporter to the reversed mode under conditions where microdomains of $Na^+$ and or $Ca^{2+}$ are established.

In heart mitochondria, besides the ruthenium red-sensitive MCU and RaM, ryanodine receptors (mRyR) that are localized in the IMM contribute to mitochondrial $Ca^{2+}$ uptake [18]. Pharmacologically, mRyR have been characterized as type 1 RyR and their contribution to mitochondrial $Ca^{2+}$ sequestration during excitation–contraction coupling to stimulate oxidative phosphorylation for ATP production to meet metabolic demands has been described [19].

## Mitochondrial $Ca^{2+}$ buffering and $Ca^{2+}$ storage

The ability of isolated mitochondria to buffer and store large amounts of $Ca^{2+}$ depends on phosphate and physiological concentrations of adenine nucleotides [148, 150], and the level of the free matrix $Ca^{2+}$ concentration is controlled by the formation of an instantly reversible $Ca^{2+}$-phosphate complex in the mitochondrial matrix [232]. In the presence of sufficient phosphate concentrations in the buffer, the free matrix $Ca^{2+}$ concentration of isolated mitochondria is surprisingly invariant from $Ca^{2+}$ load and was found to change less than 50% when total matrix $Ca^{2+}$ was increased 50-fold [31]. While there is considerable uncertainty on the actual structure of the $Ca^{2+}$-phosphate complex formed, the importance of matrix pH in dissolving this complex is known. Notably, matrix acidification induced by protonophores counteract the formation of $Ca^{2+}$-phosphate complexes within mitochondria and thus reduces the mitochondrial $Ca^{2+}$ buffer capacity while initiating large mitochondrial $Ca^{2+}$ release [15, 149]. The formation of $Ca^{2+}$-phosphate complexes as a high capacity mechanism of strong mitochondrial $Ca^{2+}$ buffering could also be observed in intact bovine adrenal chromaffin cells [53].

On the contrary, in intact human endothelial cells, no large mitochondrial $Ca^{2+}$ release is observed upon mitochondrial acidification, and free matrix $Ca^{2+}$ concentration is more likely under the control of the balance between the amount of $Ca^{2+}$ uptake and the capacity of its extrusion [125–128, 215]. These data may suggest that the formation of $Ca^{2+}$-phosphate complexes, at least in endothelial cells , is marginally involved in mitochondrial $Ca^{2+}$ homeostasis under physiological conditions. In view of the signaling function of matrix $Ca^{2+}$ to stimulate various metabolic and housekeeping enzymes and to facilitate a fast regulator and tunable regulatory function of mitochondrial $Ca^{2+}$, initial mitochondrial $Ca^{2+}$ elevations up to 1 to 2 μM free $Ca^{2+}$ might not be buffered by the formation of $Ca^{2+}$-phosphate. Nevertheless, it is reasonable to assume that under conditions of altered mitochondrial/cellular $Ca^{2+}$ homeostasis, the formation of $Ca^{2+}$-phosphate complex occurs and may counteract massive accumulation of free matrix $Ca^{2+}$ that would initiate apoptosis. Notably, such mitochondrial $Ca^{2+}$ buffer capacity via formation of $Ca^{2+}$-phosphate critically depends on the availability of phosphate that enters the mitochondria as $H_3PO_4$ ($H_2PO_4^-/OH^-$ antiport) via the electroneutral phosphate transporter [149]. Once the $Ca^{2+}$-phosphate complex is formed, the $H^+$ release needs to be compensated by the respiratory chain. A limited disposal of phosphate under pathological conditions of mitochondrial $Ca^{2+}$ overload might explain the brake of mitochondrial $Ca^{2+}$ buffer capacity and initiation of $Ca^{2+}$-triggered apoptosis. In line with this assumption, the environmental buffer phosphate concentration was described to enhance $Ca^{2+}$ buffer capacity of isolated mitochondria [232].

Whether the appearance of $H^+$ in the matrix that is associated with the deprotonation of $H_3PO_4$ to form $PO_4^{3-}$ prior complexing with $Ca^{2+}$ exhibits a beneficial smooth uncoupling that might decrease excessive ROS production is unknown but would be a further possibility how UCP2 and UCP3, which facilitate $Ca^{2+}$ entry into the mitochondria, trigger smooth uncoupling and attenuation of mitochondrial ROS production [25, 26].

## Mitochondrial $Ca^{2+}$ efflux and functional coupling with other organelles

**Mitochondrial $Ca^{2+}$ extrusion processes—**In most cells, the main mechanism of $Ca^{2+}$ extrusion from the mitochondria is the $NCX_{mito}$. The postulation of the existence of a $Na^+/Ca^{2+}$ carrier in mammalian mitochondria is based on findings of a $Na^+$-dependent $Ca^{2+}$ flux across the inner mitochondrial membrane [41] and on pharmacological characterization using the quite selective inhibitor of $NCX_{mito}$, chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP 37157 [11]). There is a broad consensus that $NCX_{mito}$ is responsible for mitochondrial $Ca^{2+}$ extrusion in most cells [77] and thus represents the physiological counterpart to the MCU. In line with these reports, in endothelial cells, mitochondrial $Ca^{2+}$ extrusion after agonist-induced elevation in $[Ca^{2+}]_{mito}$ highly depends on cytosolic $Na^+$ [127, 197] and is prevented by CGP 37157 [127, 128]. Although the existence of a $Na^+$-dependent $Ca^{2+}$ efflux through the IMM has been experimentally confirmed, the actual protein(s) that account(s) for $NCX_{mito}$ is/are still unknown and await(s) further investigation.

In liver mitochondria a $Ca^{2+}/H^+$ exchanger was described to be responsible for mitochondrial $Ca^{2+}$ extrusion [79, 170]. So far, it is not clear whether this phenomenon is specific for liver mitochondria, but the large acidification of mitochondria in intact endothelial cells, Hek293 cells, or HeLa cells upon $Ca^{2+}$ sequestration in the presence of the $NCX_{mito}$ inhibitor CGP37157 (Fig. 5) may point to its ubiquitous existence. In addition to the ion exchanger, a transient formation of the mitochondrial permeability transition pore may be an alternative mechanism of mitochondrial $Ca^{2+}$ extrusion [61, 139].

**Endoplasmic reticulum—mitochondria $Ca^{2+}$ crosstalk—**In living cells, the mitochondria were found to be in close proximity (~10–100 nM) to the ER [44, 183, 187]. Although mitochondria exhibit high motility in living cells, in cultured human endothelial cells, which expressed organelle-targeted fluorescent proteins, the proportion of mitochondria that colocalizes with the ER and vice versa is remarkably constant at approximately 50–55 and 22–27%, respectively [215]. This tight vicinity is actually the basis to explain how mitochondria are able to accumulate $Ca^{2+}$ efficiently despite the low affinity of the uniporter for $Ca^{2+}$ [185]. In line with this report, mitochondrial $Ca^{2+}$ uptake was found to depend on the establishment of high $Ca^{2+}$ microdomains by ER $Ca^{2+}$ release sites close to mitochondria [183, 205]. Notably, the maintenance of such high $Ca^{2+}$ gradients essentially rely on a continuous $Ca^{2+}$ cycling through the ER and is maintained by $Ca^{2+}$ influx from the extracellular space [205].

Filippin et al. [63] also proposed that the interaction between the mitochondria and the ER is stable over time, suggesting a specific interaction between both organelles that might include a physical linkage between the ER and the mitochondria. Morphological evidence was reported by Mannella et al. [129, 130], and recently, the group of Hajnoczky [43] showed, using electron tomography, a visible linker between mitochondria and the ER. In line with this, they construct a synthetic linker and reported an increased mitochondrial $Ca^{2+}$ uptake, which eventually led to $Ca^{2+}$ overload. In line with these findings, a chaperone-mediated coupling of endoplasmic reticulum and mitochondrial $Ca^{2+}$ channels has been described [203] and strongly point to a possibly reversible organelle junction that allows rapid and very efficient $Ca^{2+}$ transfer from the ER to the mitochondria and *vice versa* [5, 125, 127, 128].

However, in view of the high mobility of the mitochondria and their constant fusion/fission processes [14, 64], the kinetics and duration of the establishment of a physical linker that attach mitochondria to the ER as well as the molecular initiators of organelle linkage need further attention. Most reports indicate that mitochondria are more efficient in accumulating $Ca^{2+}$ when the $Ca^{2+}$ concentration is locally high like in hotspots and thus support the concept of the formation of a physical organelle junction. Besides the importance of such $Ca^{2+}$ hotspots for the $Ca^{2+}$ transport via a low $Ca^{2+}$-sensitive uptake machinery, spatial $Ca^{2+}$ elevation might also be responsible for initiating the linkage between mitochondria and the ER [220]. However, it was also shown that mitochondria are able to take up $Ca^{2+}$ at low $Ca^{2+}$ concentration range [39, 105, 172, 207], and thus such physical linkage might not be prerequisite for all mitochondrial $Ca^{2+}$ uptake processes.

*ER $Ca^{2+}$ refilling* A reverse $Ca^{2+}$ flux from mitochondrial $Ca^{2+}$ toward the ER fuels SERCA-mediated ER-refilling [128]. Since in the presence of the agonist $Ca^{2+}$ refilling of the ER essentially depends on $Ca^{2+}$ that enters the cell through CCE and is rapidly sequestered by subplasmalemmal mitochondria [126], a coupling, at least functional, between the mitochondria and the plasma membrane can be assumed (see: Plasma membrane—mitochondria $Ca^{2+}$ connection).

Such reverse $Ca^{2+}$ flux from the mitochondria toward the ER was found to be important for the ER $Ca^{2+}$ refilling process. In HeLa cells, depolarizing mitochondria lead to a more profound $Ca^{2+}$ store depletion during cell stimulation, showing that part of the $Ca^{2+}$ accumulated by mitochondria during the release phase is returned back to the ER, thus limiting the level of depletion [5]. In endothelial cells, the same effect was observed and, in addition, mitochondria were shown to relay $Ca^{2+}$ entering the cells through the plasma membrane toward the ER [128]. This effect gained even more importance under conditions of reduced $Ca^{2+}$ entry by plasma membrane depolarization [125]. In agreement with these reports that point to an important contribution of mitochondria to ER $Ca^{2+}$ refilling, the refilling process of the ER following bradykinin stimulation is slowed down after mitochondria were uncoupled by FCCP in BHK-21 cells [112].

Another reflection of the contribution of mitochondria to ER $Ca^{2+}$ refilling has been described under conditions of mitochondrial fragmentation by exposure to elevated D-glucose in which fragmentation of mitochondria yielded delayed mitochondrial $Ca^{2+}$ extrusion [160] and diminished ER $Ca^{2+}$ refilling (Graier and Malli, unpublished data).

*Maintenance of protein folding processes in the ER* Causally linked to the mitochondrial contribution to ER $Ca^{2+}$ refilling, the $Ca^{2+}$ transfer from the mitochondria toward the ER should be considered of particular importance for $Ca^{2+}$-dependent protein folding in the lumen of the ER [136–138]. Accordingly, mitochondrial $Ca^{2+}$ homeostasis and especially the $Ca^{2+}$ transfer from the mitochondria toward the ER via the $NCX_{mito}$ have been found to be prerequisite to maintain calreticulin/calnexin-mediated protein folding in the ER [153]. Additionally, ER protein folding is attenuated by mitochondrial ROS production that evokes




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sustained ER $Ca^{2+}$ depletion in leukemia cells [230] and thus induces activation-enhanced cell death [201]. Thus, convincing evidence on the contribution of a pathological, altered mitochondrial ion homeostasis to protein dysfunction/ER stress exist [72] and further point to a considerable new function of mitochondrial $Ca^{2+}$ homeostasis in cellular metabolism.

**Plasma membrane—mitochondria $Ca^{2+}$ connection—**In view of the mitochondrial contribution to subplasmalemmal $Ca^{2+}$ buffering to maintain the activity of $Ca^{2+}$-sensitive store-depletion activated $Ca^{2+}$ entry pathways [69, 70, 93, 94, 126], a functional interplay that might build on actual junctions between the mitochondrial $Ca^{2+}$ uptake machinery and plasma membrane $Ca^{2+}$ channels seems possible. Moreover, in view of the recent data that indicate $Ca^{2+}$-triggered mitochondrial moving arrest [43], such junction might get established on demand in a $Ca^{2+}$ dependent manner. In line with such expectations, the molecular mechanisms of the development of $Ca^{2+}$ current in response to $Ca^{2+}$ store depletion represents a dynamic assembly of various TRPC family members, STIM1, and Orai1 [117, 152] and might be extended to a so far unknown linkage protein between the OMM and the TRPC/STIM1/Orai complex or a protein of the OMM that couples to the store-operated $Ca^{2+}$ channel complex and allows mitochondrial $Ca^{2+}$ buffering of entering $Ca^{2+}$. Additionally, in view of the strict dependency of the main mitochondrial $Ca^{2+}$ extrusion pathway from environmental/cytosolic $Na^+$, one might speculate that $Na^+$ permeable plasma membrane ion channels (e.g., TRPC3, [141]) and/or the plasma membrane $Na^+/Ca^{2+}$ exchanger ($NCX_{pm}$) are, at least functionally, linked to mitochondrial $Na^+$ carriers.

While a physical linkage between mitochondria and the plasma membrane can be conceptionally anticipated, further studies are essential to identify proteins in the OMM that serve as anchors for such junction.

## Tuning mitochondrial $Ca^{2+}$ signaling

**Mutual regulation of mitochondrial ions—**Mitochondrial protonophores like FCCP, which equalize $H^+$ concentration in the matrix and the intermembrane space/cytosol, strongly diminish mitochondrial $Ca^{2+}$ uptake due to their mitochondria-depolarizing property [215]. Besides this electrophysical importance of $H^+$ to establish the negative membrane potential, the homeostasis of $Ca^{2+}$ is closely linked to that of $H^+$. Notably, $Ca^{2+}$ indirectly affects $H^+$ homeostasis by its effect on metabolic enzymes, which, in turn, fuel the respiratory chain resulting in enhanced $H^+$ flux [134] (see: Effect of $Ca^{2+}$ on mitochondrial metabolism).

Besides $H^+$, there are at least three other ions that need to be considered as to be involved in mitochondrial $Ca^{2+}$ homeostasis: $Na^+$, $K^+$, and $Mg^{2+}$. For all three cations, mitochondrial carriers/channels exist [53] and preliminary data point to a mutual regulation of these ions with mitochondrial $Ca^{2+}$. In case of $Na^+$, the importance of cytosolic/mitochondrial $Na^+$ for mitochondrial $Ca^{2+}$ homeostasis has been already indicated above (see: Mitochondrial $Ca^{2+}$ extrusion processes). However, in regard of the role of $Na^+$ for mitochondrial $Ca^{2+}$ homeostasis, one needs to consider the possibility of functional interaction between mitochondrial $Na^+$ carriers and plasma membrane $Na^+$ fluxes. In endothelial cells, the occurrence of subplasmalemmal $Na^+$ gradients, which are generated by activation of $Na^+$-permeable ion channels, most likely TRPC3 [58], affect $NCX_{pm}$ [159, 212]. Whether or not mitochondrial $Ca^{2+}$ homeostasis is regulated by plasma membrane $Na^+$ currents is unclear and deserves more attention particularly considering the pathological consequences of altered $Na^+$ signaling on mitochondrial ion homeostasis.

While the molecular nature of mitochondrial transporters for $Na^+$ is unknown, putative carriers for $K^+$ and $Mg^{2+}$ in this organelle have already been identified. Three classes of mitochondrial $K^+$ channels have been pharmacologically and functionally characterized and were found to be similar to some of the $K^+$ channels present in the plasma membrane [208]: the ATP-regulated $K^+$ channel (mito$K_{ATP}$; apparently Kir6.1 and Kir6.2 [111]), the large conductance $Ca^{2+}$-activated $K^+$ channel (mito$BK_{Ca}$; apparently Slo1 [223]) and the voltage-dependent $K^+$ channel (mitoKv1.3 [206]). Whereas the physiological function of the latter one has not been well characterized so far, mito$K_{ATP}$ and mito$BK_{Ca}$ are described to be involved in the regulation of mitochondrial membrane potential, ROS production, mitochondrial transition pore and oxidative phosphorylation [208]. Moreover, the $K_{ATP}$ channel opener diazoxide was found to be cardioprotective presumably due to its mitochondrial depolarizing effect [67]. However, although the molecular structure of the channel is often described as Kir6.1/2 and SUR2, this assumption has been challenged [30, 217] and the respective current has also been attributed to the function of a complex of five proteins not involving Kir6.1/2 or SUR2 [3]. In line with these reports, this drug has been described to act directly on mitochondrial $K^+$ influx [28, 51, 83, 84, 90, 155].

Besides mitochondrial $K^+$ channels, a mitochondrial $K^+/H^+$ carrier has been described [66] of which the contribution to mitochondrial $Ca^{2+}$ is unknown but may influence mitochondrial $Ca^{2+}$ homeostasis via its impact on matrix $H^+$ concentration.

For mitochondrial $Mg^{2+}$, the human mitochondrial Mrs2 protein [233] was recently described to substitute functionally its yeast homologue for the mitochondrial $Mg^{2+}$ carrier [108]. The contribution of mitochondrial $Mg^{2+}$ and its carrier for mitochondrial $Ca^{2+}$/ion homeostasis has not been evaluated so far.

Additionally, to $H^+$, $Na^+$, $K^+$, and $Mg^{2+}$, cytosolic $Ca^{2+}$ itself may regulate mitochondrial $Ca^{2+}$ homeostasis by its regulatory effect on proteins in the outer or inner mitochondrial membranes. Recently, cellular $Ca^{2+}$ has been shown to modulate activity of VDAC, which may tune mitochondrial $Ca^{2+}$ sequestration and facilitate mitochondrial overload upon huge cytosolic $Ca^{2+}$ elevation [12]. Additionally, there are several members of the mitochondrial SLC25 family that contain one or more EF-hand motifs for $Ca^{2+}$ binding (e.g., Aralar, short $Ca^{2+}$-binding mitochondrial carriers) and thus may serve as transducers of outer $Ca^{2+}$ into the mitochondria and/or adapt ion carrier activities to the existing $Ca^{2+}$ concentration outside the organelle. The family of short $Ca^{2+}$-binding mitochondrial carriers (SCaMC) consists of three genes in the human genome that code for highly conserved proteins (70–80% homology) with a characteristic mitochondrial carrier domain and EF-hand $Ca^{2+}$-binding motifs at either the C-(SCaMC-1 & 2) or N-terminal side [47].

**Protein phosphorylation for regulation of mitochondrial $Ca^{2+}$ homeostasis—** Mitochondrial protein phosphorylation is a largely unresolved and complex phenomenon ($Ca^{2+}$ as regulator of mitochondrial phosphoproteom). A bidirectional modulation of mitochondrial $Ca^{2+}$ uptake was described for the protein kinase C family, of which protein kinase Cβ diminished and protein kinase Cζ enhanced mitochondrial $Ca^{2+}$ accumulation upon cell stimulation [171]. Considering the existence of many kinases and phosphatases in the mitochondrial matrix [92], an assessment whether or not reversible protein phosphorylation represents a regulatory mechanism in mitochondrial $Ca^{2+}$ homeostasis is mandatory.

**Second messenger-modulation of mitochondrial $Ca^{2+}$ signaling—**There is already strong evidence of a modulation of mitochondrial $Ca^{2+}$/ion carrier by intracellular messengers/small molecules. Among them, NO• has been described to diminish mitochondrial $Ca^{2+}$ accumulation [213], whereas peroxynitrite and other reactive oxygen species ($O_2^-$;$H_2O_2$) were found to yield accumulation of $Ca^{2+}$ in the mitochondria [75]. Additionally, nucleotides regulate mitochondrial $Ca^{2+}$ transport [120] by $P2Y_1$- and $P2Y_2$-like mitochondrial receptors [13]. On the other hand, polyunsaturated fatty acids cause $Ca^{2+}$ release from mitochondria [229].

Overall, there is strong evidence that mitochondrial $Ca^{2+}$ homeostasis represents a target of multiple modulator effects of intracellular messengers/small molecules. The actual molecular mechanisms and proteins involved in these regulatory processes are so far unknown and deserve a detailed assessment.

## Contribution of mitochondria to cytosolic $Ca^{2+}$ homeostasis

### Shaping cytosolic $Ca^{2+}$ elevation

$Ca^{2+}$ mobilization from the internal $Ca^{2+}$ stores occurs primarily from the ER upon activation of the $IP_3$ receptor ($IP_3R$) or the RyR. $IP_3R$ is activated following cell stimulation by extracellular agonists, activation of the phospholipase C and production of $IP_3$. On the other hand, the RyR can be activated by a conformational change of the plasma membrane voltage-operated $Ca^{2+}$ channels in skeletal muscle, by $Ca^{2+}$ itself leading to $Ca^{2+}$-induced $Ca^{2+}$-release or by cADP ribose or NAADP [16]. The latter two mechanisms are less well documented at present. Once $Ca^{2+}$ is elevated in the cytosol as a result of $Ca^{2+}$ mobilization, it is immediately taken up by several pumps or exchangers to counteract the $Ca^{2+}$ rise.

In excitable cells, in which cytosolic $Ca^{2+}$ elevation is due to $Ca^{2+}$ entry through voltage-gated $Ca^{2+}$channels, the impact of mitochondria is rather clear and consistent. For instance, in chromaffin cells, mitochondria rapidly accumulate $Ca^{2+}$ in an initial phase and thus delivers $Ca^{2+}$ back to the cytosol, thus prolonging the low plateau phase of the $Ca^{2+}$ elevation [8, 9, 54, 89]. However, if the ER represents the source of $Ca^{2+}$, the way how mitochondria shape the cytosolic $Ca^{2+}$ is less clear. In that case, the release of $Ca^{2+}$ is accompanied by v entry through store-operated $Ca^{2+}$ channels, which are also modulated by mitochondria (see: $Ca^{2+}$ entry pathways). Thus, to precisely assess the role of the mitochondria, experiments should be performed in the absence of extracellular $Ca^{2+}$. In such circumstances, preventing mitochondrial $Ca^{2+}$ uptake results in an increased cytosolic $Ca^{2+}$

signal [5]. In case of intracellular $Ca^{2+}$ wave propagation, mitochondria were shown to accelerate or slow down the wave, depending most likely on the $IP_3R$ subtype [54], which exhibit isoform specific $Ca^{2+}$ dependence. Thus, in case of a linear $Ca^{2+}$ activation of $IP_3R$, mitochondria slowed down the wave propagation [20], whereas in case of a bell shape $Ca^{2+}$ activation, mitochondria accelerated the process by preventing $Ca^{2+}$-dependent inhibition of the $IP_3R$ [103].

The inhibition of local $Ca^{2+}$ elevation by mitochondrial $Ca^{2+}$ uptake that yields suppression of $Ca^{2+}$-triggered feedback to $IP_3R$ has also been found to contribute to cytosolic $Ca^{2+}$ oscillation [8, 62, 81, 103, 112, 182, 187]. Recent data show that $Ca^{2+}$ concentrations in the mitochondria and the ER oscillate concomitantly with cytosolic $Ca^{2+}$ oscillations and that the $Ca^{2+}$ shuttling between these organelles exhibits a pacemaker role in cytosolic $Ca^{2+}$ oscillation [96].

Another important aspect in modulating cytosolic $Ca^{2+}$ signaling by mitochondria is their capacity to exhibit spatial $Ca^{2+}$ buffering in distinct area of the cytosol. Notably, the property of mitochondria to act as an intracellular $Ca^{2+}$ buffering mechanism in contractile tissue with regard to the organelle's intracellular location was already described in 1980 [135]. Subsequently, numerous reports approved these early findings in many excitable cells, such like smooth muscle cells, in which mitochondria were found to modulate $Ca^{2+}$ sparks and $Ca^{2+}$-activated $K^+$ currents [35]. In chromaffin cells mitochondria were found to modulate $Ca^{2+}$ inhibition of various $Ca^{2+}$ channels [88]. Moreover, mitochondria may also be responsible for the difference in $Ca^{2+}$ signaling pattern in the perinuclear and subplasmalemmal cytosol [207]. In cardiac myocytes, mitochondrial $Ca^{2+}$ buffering was found to be responsible for the delay between the peak of the $Ca^{2+}$ transient and the peak shortening in myocytes [209]. Intriguingly, in myocytes as well as smooth muscle cells, mitochondrial $Ca^{2+}$ buffering was found to be linked to the cellular $Na^+$ homeostasis [123, 173], thus pointing to the strong inter-dependence of mitochondrial $Ca^{2+}$ homeostasis with that of other ions.

In respect of spatial mitochondrial $Ca^{2+}$ buffering, the group of Ole Petersen has conducted pioneer work in pancreatic acinar cells in which the mitochondria are distinctively located around the granular pole and form a belt that retains the $Ca^{2+}$ signal in the granular pole [162, 166–168, 214]. This landmark observation was complemented by their findings on the focal $Ca^{2+}$ delivery function of the ER in this cell type [22, 68, 162, 166, 167, 169].

This overview on the aspect of spatial mitochondrial $Ca^{2+}$ buffering only merely touches the role of mitochondria as a local and/or global regulator of cytosolic $Ca^{2+}$ signal, but indicates that the quality of the modification of the cytosolic $Ca^{2+}$ signal by mitochondria is complex and highly depends on the cellular system investigated.

## $Ca^{2+}$ entry pathways

The role of mitochondria as a regulator of $Ca^{2+}$ entry has been mainly studied in the particular context of the activation of store-operated $Ca^{2+}$ entry. This mechanism, originally described by Putney [175], is now evident in virtually every cell type. Upon store depletion, $Ca^{2+}$ permeable channels open at the plasma membrane and allow $Ca^{2+}$ entry that eventually refilled the store. The best characterized current supporting this influx was described in blood cells and termed CRAC for $Ca^{2+}$ release activated $Ca^{2+}$ current. One of the characteristics of this current is a feedback inhibition due to rise in intracellular $Ca^{2+}$ concentration [161]. Mitochondria, by their ability to take up $Ca^{2+}$, were shown to prevent $Ca^{2+}$-dependent inhibition of the current, and thus allow a sustained $Ca^{2+}$ entry through CRAC [94]. This mechanism of SOCE was also reported in other cell types, and mitochondria were shown to have the same effect. In endothelial cells, it was clearly shown that mitochondria are able to maintain low level of $Ca^{2+}$ concentration in their vicinity, thus preventing $Ca^{2+}$-depedent inhibition of SOCE [126].

In the case of $Ca^{2+}$ entry, it is also postulated that mitochondria have to be located close to the membrane to efficiently buffer entering $Ca^{2+}$. This was indeed confirmed by experiments where mitochondria were relocalized more distantly from the membrane after overexpression of dynamitin [216]. Intriguingly, a sustained activity of CRAC requires translocation of mitochondria to the plasma membrane [176]. In that particular case, the SOCE was significantly reduced. However, overexpression of hFis1 that also relocated mitochondria away from the plasma membrane, did not significantly impact on SOCE [65]. In this condition, mitochondria were still able to accumulate entering $Ca^{2+}$ (even with a slower time course), and depolarizing mitochondria reduced the SOCE. Thus, mitochondria prevent $Ca^{2+}$-dependent inhibition of SOCE by local $Ca^{2+}$ buffering, but they may also release a diffusible compound that activates $Ca^{2+}$ entry as shown in RBL cells [71], as well as in immune cells [6].

Until recently, neither the mechanism of SOCE activation nor the molecular identity of the channel was known. During the last 2 years, major advances in the field have been obtained with the identification of STIM 1 as a key regulator of SOCE [119, 189] and Orai1 as the channel supporting the CRAC current [218, 226]. With this in hand, the role of mitochondria in SOCE regulation will probably be reassessed and some remaining controversies clarified.

$Ca^{2+}$ entry does not exclusively rely on SOCE. The arachidonic-activated $Ca^{2+}$ entry pathway is a well-documented route of $Ca^{2+}$ influx that is distinct from SOCE [199, 221]. In addition, the large family of TRP cation channels also constitute an important way of $Ca^{2+}$ influx [141]. However, the role of mitochondria in these pathways has not been investigated so far. An interesting exception is the TRPM2 channel. This nonselective cation channel is $Ca^{2+}$-permeable and activated by different compounds, among them oxidative stress and ADP-ribose. Recently, it was shown that oxidative stress stimulates mitochondria to produce ADP-ribose that subsequently activates TRPM2 [165]. Whether mitochondria are also involved in modulating the activation of other TRP channels is currently unknown.

## Contribution of $Ca^{2+}$ to mitochondrial function

### Effect of $Ca^{2+}$ on mitochondrial metabolism

In most cells, mitochondria provide most of the cell’s energy as ATP of which the synthesis from ADP is linked to a series of electron transport through the IMM. This complex endergonic reaction is powered by the oxidation of reduced cofactors ($NADH^{+}$+$H^{+}$, $FADH_2$), which are generated during nutrition catabolism via, e.g., the Krebs cycle, and deliver electrons to complex I and complex II of the respiratory chain in the IMM. The subsequent electron transfer to complex III and further to complex IV of the electron transport chain supplies energy to establish a proton gradient across the IMM, which is finally converted into the synthesis of ATP molecules at the ATP synthase complex (complex V). This vital process leading to mitochondrial ATP production has to be up- or downregulated according to the cellular needs of energy and the fluctuating supply of nutrition as well [21].

Mitochondrial $Ca^{2+}$ signaling appears to be fundamental in the control of mitochondrial metabolism. Forty years ago, Hansford and Chappell reported that the oxidation of glycerol phosphate is activated by $Ca^{2+}$ [85] and later on, McCormack and Denton precisely described that pyruvate dehydrogenase is activated by $Ca^{2+}$-dependent dephosphorylation, while the $NAD^+$-isocitrate deyhydrogenase and the 2-oxoglutarate dehydrogenase are directly activated by $Ca^{2+}$ [133]. Thus, an increase in matrix $Ca^{2+}$ concentration in the mitochondria accelerates the enzymatic activities of these three dehydrogenases leading to increased NADH levels [52, 82, 174], and subsequently, this $Ca^{2+}$-triggered activation of Krebs cycle dehydrogenases actually culminates in an augmentation of the mitochondrial ATP production in intact living cells. That was shown in elegant experiments using the luciferin luciferase reaction [104]. Moreover, they could also describe a so far not further characterized mitochondrial memory that allows a long-term activation of mitochondrial ATP production up to 60 min after agonist-induced $Ca^{2+}$ signal elevation. In line with these reports, we could demonstrate that an augmentation of mitochondrial $Ca^{2+}$ uptake capacity upon cell stimulation with an IP3 generating agonist by overexpression of UCP2 or UCP3 resulted in a significant enhancement of the agonist-induced mitochondrial ATP generation in endothelial cells [215]. In addition, there are several reports indicating that the $Ca^{2+}$-sensitive dehydrogenases are not the only targets of the mitochondrial metabolic pathways for $Ca^{2+}$-dependent activation of mitochondrial ATP production. $Ca^{2+}$-sensitivity was also reported for the $F_0/F_1$-ATPase (heart, [87, 211]), the adenine nucleotide translocase (liver, [144]), complexes of the respiratory chain [188], and EF-hand containing substrate carriers of the IMM [113, 158].

All these reports on the stimulatory effect of $Ca^{2+}$ for mitochondrial ATP production illustrate the functional significance of mitochondrial $Ca^{2+}$ sequestration and explain how a cell can accommodate the increasing demand of energy during stimulated states. However, the effects of matrix $Ca^{2+}$ that exceed its regulatory function on oxidative phosphorylation might be multiple and await further investigation.

Interestingly, there is a limited number of reports that are contrary to the dogma that mitochondrial $Ca^{2+}$ accumulation accelerates mitochondrial metabolism [17, 122]. In these reports, conditions are described in which the depolarizing effect of $Ca^{2+}$ on the IMM exceeds its stimulatory effect on the respiratory chain/dehydrogenases, and consequently leads to a $Ca^{2+}$-induced decrease in NADH levels. Such a scenario seems conceivable under conditions of excessive activation of respiration by substrate overload and/or under conditions of pathologically increased mitochondrial $Ca^{2+}$ sequestration as elaborated in a mathematical model by Bertram et al. [17]. Notably, under conditions of substrate or $Ca^{2+}$

overload of the mitochondria the formation of reactive oxygen species (ROS) is assumed to play an important role in mitochondrial degeneration/dysfunction.

## Impact of mitochondrial $Ca^{2+}$ on ROS generation and ROS defense

In most cells mitochondria represent the main source of the physiological production of ROS. Basically, mitochondrial-generated ROS come from an interaction between oxygen ($O_2$) with unpaired electrons, which occur along the respiration. In average 1 to 2% of the total electrons transported through the respiratory chain leak to produce superoxide anions $\left(\bullet O_2^-\right)$ [24], which can be rapidly converted into the more reactive $H_2O_2$ and its aggressive derivative the hydroxyl radicals ($\bullet OH^-$). Among the actual sites of ROS generation in mitochondria complex I and complex III have attracted most attention [10, 114]. However, very recently, matrix enzymes such as the alpha-ketoglutarate dehydrogenase and other components of dehydrogenase complexes of the Krebs cycle have been also considered as important sources of ROS within mitochondria [36]. To balance the permanent production of ROS, mitochondria also house a sophisticated defense network of enzymatic and nonenzymatic antioxidants against ROS [2]. However, although ROS have been accused to exhibit numerous pathological effects in mitochondria and can lead to lipid peroxidation, which subsequently causes oxidative damage of mitochondrial DNA, RNA and enzymes, mitochondrial ROS generation might constitute an important signaling molecule to modulate cellular signal transduction in health and disease.

The impact of mitochondrial $Ca^{2+}$ signaling on mitochondrial ROS homeostasis is in most instances ambiguous. There are various reports implying that an excessive mitochondrial $Ca^{2+}$ sequestration facilitate the generation of ROS within mitochondria [57, 179, 180]. However, the underlying mechanisms for $Ca^{2+}$-induced mitochondrial ROS production has not been clarified entirely but might include activation of the electron transport chain as well as $Ca^{2+}$-induced opening of the mitochondrial permeability transition pore (PTP).

A different conclusion of the consequence of mitochondrial $Ca^{2+}$ uptake for mitochondrial ROS generation can be obtained if the electrical effect of $Ca^{2+}$ on the potential of the IMM ($\Delta\psi_{mito}$) is considered. $Ca^{2+}$ uptake into mitochondria reduces $\Delta\psi_{mito}$, which has been shown to counteract generation of $\bullet O_2^-$ during oxidative phosphorylation [202, 219]. Moreover, $Ca^{2+}$ directly or via calmodulin modulates the activity of enzymes of the antioxidant defense systems (e,g, MnSOD), indicating that $Ca^{2+}$ exhibits dual contribution in the regulation and control of the mitochondrial ROS homeostasis [91, 225]. Additionally, mitochondrial $Ca^{2+}$ inhibits the removal of hydrogen peroxide and its succinate-fueled production, thus indicating that mitochondria function as intracellular $Ca^{2+}$-modulated peroxide sinks [231].

Our understanding on mitochondrial ROS production is controversial and rather confused. This is not surprising as the methods to measure ROS production lack specificity and mitochondria from different tissue origin might metabolically not be comparable. Notably, UCP2 and UCP3 have been shown to suppress mitochondrial ROS production under various experimental conditions presumably via their uncoupling function (for a recent review see Dlaskova et al. [50]). We have recently demonstrated in intact as well as isolated mitochondria that UCP2 and UCP3 are fundamental for mitochondrial $Ca^{2+}$ uniport while no obvious evidence for $H^+$ conductance was found [215]. Consequently, in addition to the dogma of smooth uncoupling via UCP2/UCP3-mediated $H^+$ flux, the discovered $Ca^{2+}$ function of these proteins might be, at least partly, responsible for the reported reduction in mitochondrial ROS production by these UCP proteins. Since the correlation of UCP2/3-dependent mitochondrial $Ca^{2+}$ fluxes with mitochondrial ROS production has not been elucidated so far, the molecular mechanisms beyond UCP2/UCP3-associated attenuation of mitochondrial ROS production awaits further investigation.

On the other hand, extramitochondrial-produced ROS might affect mitochondria as well. In recent studies, exposure of mitochondria to ROS have been shown to affect the structural organization and $Ca^{2+}$ homeostasis [160] and trigger $IP_3$-linked apoptotic cascade [124], thus indicating that this organelle can be affected by cytosolic-/plasma membrane-originated ROS also.

## $Ca^{2+}$ as regulator of mitochondrial phosphoproteom

Reversible phosphorylation of enzymes, receptors, ion channels, transcription factors and cytoskeletal elements is a hallmark of cell signaling (Greengard, Science, 1976, 146–152). It has recently emerged that the role of phosphorylated proteins as physiological effectors is not restricted to the cytosol, but is also fundamentally involved in the homeostasis of mitochondrial functions. With the development of improved phosphoproteomic screens, the existence of multiple phosphoproteins in mitochondria could be confirmed [91]. Importantly, there are protein kinases and phosphatases that are exclusively located within the mitochondrial matrix [132], in the IMM [107], the intermembrane space [194], and at the cytoplasmic surface of mitochondria [34]. Moreover, cytosolic protein kinases are known to be imported into mitochondria by a yet not fully understood mechanisms [97, 156].

Although the $Ca^{2+}$ dependent dephosphorylation of the pyruvate dehydrogenase complex within mitochondria was already described in the late 1960s [118], only limited knowledge is available about the impact of $Ca^{2+}$ on posttranslational modification of mitochondrial proteins. Very recently, a dynamic change in the phosphorylation state of multiple mitochondrial proteins has been shown in porcine heart mitochondria in response to $Ca^{2+}$ elevation [91]. The mitochondrial proteins that got phosphorylated in response to matrix $Ca^{2+}$ elevation included components of the electron transport chain and the Krebs cycle, indicating that $Ca^{2+}$ impacts mitochondrial bioenergetics on various levels. Moreover, a $Ca^{2+}$-induced dephosphorylation of the MnSOD was also described, which was associated with an increase in its enzymatic activity, while neither the kinase(s) nor the phosphatase(s) involved in this process has been identified so far. Similar findings were obtained in rat brain mitochondria, in which several, so far unidentified, low molecular mass proteins were found to be phosphorylated in a $Ca^{2+}$-dependent manner [7]. Interestingly, this $Ca^{2+}$-induced protein phosphorylation depended on the opening of the mitochondrial PTP while its particular function in $Ca^{2+}$-activated phosphorylation of mitochondrial proteins remained unresolved.

In summary, convincing evidence points to an importance of mitochondrial $Ca^{2+}$ fluxes for protein phosphorylation and dephosphorylation as a crucial mechanism in the dynamic




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regulation of various mitochondrial functions. However, this remains an emerging field, which requires further investigation to be fully integrated into a complex understanding of the molecular mechanisms of the regulation of mitochondrial functions.

## Effect of $Ca^{2+}$ on mitochondrial motility and morphology

Mitochondria display a very complex architectural organization that varies continuously over time depending on their metabolic activity, the level of cell activation [224], the cell cycle status [14, 64], and the impact of physical or chemical environmental signals. In most cells, mitochondria are largely interconnected tubular structures [190] (Fig. 6a), whereas mitochondria with round punctuated morphology are less frequently described [38] and may represent an early indication of mitochondrial stress initiated by either metabolic overload [160], chemical depolarization (Fig. 2), uncontrolled fission processes [1, 163, 164], or physical disturbance (R. Malli and W.F. Graier, unpublished observations). In addition, in certain cell types, mitochondrial structure reflects their function, such like spatial $Ca^{2+}$ buffering in pancreas acinar cells [22, 68, 162, 166, 167, 169], cardiac muscle [135, 209], chromaffin cells [88], or nerves [29, 32, 151].

Besides the complexity of their architectural organization, mitochondria exhibit complex dynamics (Fig. 6b) that have been shown to depend on cytoskeleton-based transport mainly along microtubule via motor proteins such as the mitochondria linked kinesin Kif1B [210]. Hence, mitochondrial structure continuously changes by processes of fusion, branching, or fission [14, 224] that results in a permanent rearrangement of the mitochondria (Fig. 6b). In contrast to the molecular mechanisms responsible for mitochondrial motility that are poorly understood so far, major proteins that participate in the regulation of the organelles fission, fusion, and transport along cytoskeleton elements have already been identified (for review, see [190, 224]). The processes of fission and fusion are mainly under the control of guanosine triphosphatases (GTPases) such Drp1, Mitofusins (Mfn) and OPA-1 [23], and their associated adaptor proteins like hFis1 [98] as well as Rho family proteins [95, 204].

Both, mitochondrial motility and morphology are significantly affected by $Ca^{2+}$. Particularly, mitochondrial motility is regulated by $Ca^{2+}$ in the physiological range with maximal movement at low resting cytosolic $Ca^{2+}$ levels and a complete arrest at 1–2 μM free $Ca^{2+}$ in the cytosol [227]. The molecular mechanism behind this $Ca^{2+}$-triggered arresting of mitochondria is unknown, but indicates that mitochondria might be recruited in a $Ca^{2+}$-dependent manner to enhance local $Ca^{2+}$ buffering and/or ATP supply on distinct cellular demands. Hence, the elevation of intracellular $Ca^{2+}$ concentration initiates the translocation of Drp1 to the mitochondria and thus initiation of fission processes that consequently results in mitochondrial fragmentation [27]. In this regard, a reversible $Ca^{2+}$-dependent fragmentation of interconnected tubular mitochondria upon stimulation with an $IP_3$-generating agonist was described in HeLa cells [204]. Whether this $Ca^{2+}$ induced mitochondrial fission is due to alterations of the potential of the inner mitochondrial membrane and alteration of the matrix pH or depends on an activation of GTPases most likely of Drp-1 [116] remains however elusive.

In summary, mitochondria are very mobile organelles, which move, fuse, branch, and divide to create a flexible and highly dynamic tubular network, whose morphology, motility, and intracellular distribution is evidently affected by $Ca^{2+}$. Although the molecular processes, pathways of regulation as well as the physiological function of this striking aspect of mitochondrial $Ca^{2+}$ signaling are not fully understood yet, one can expect that along their exploration we will learn further exciting and so far unknown functions of these old guests.

## Conclusion

During recent years, mitochondria have been discovered to represent much more than the cell's power plants that are crucially involved in nutrition metabolism and energy production. Utilizing newly developed techniques and instrumentations we started to explore the contribution of this fascinating organelle to signal transduction pathways, regulation, and tuning of multiple cell functions and ion homeostasis. Due to the versatile signaling properties of $Ca^{2+}$, mitochondrial $Ca^{2+}$ homeostasis, which was proved to represent an active and complexly regulated process, obviously plays a crucial role in most already established as well as newly discovered mitochondrial functions. In spite of many outstanding contributions regarding the regulation and function of mitochondrial $Ca^{2+}$ most of the molecular contributors to mitochondrial $Ca^{2+}$ homeostasis urgently await identification. Complementing this task will be prerequisite for further exploration of new $Ca^{2+}$-dependent functions of these old guests.

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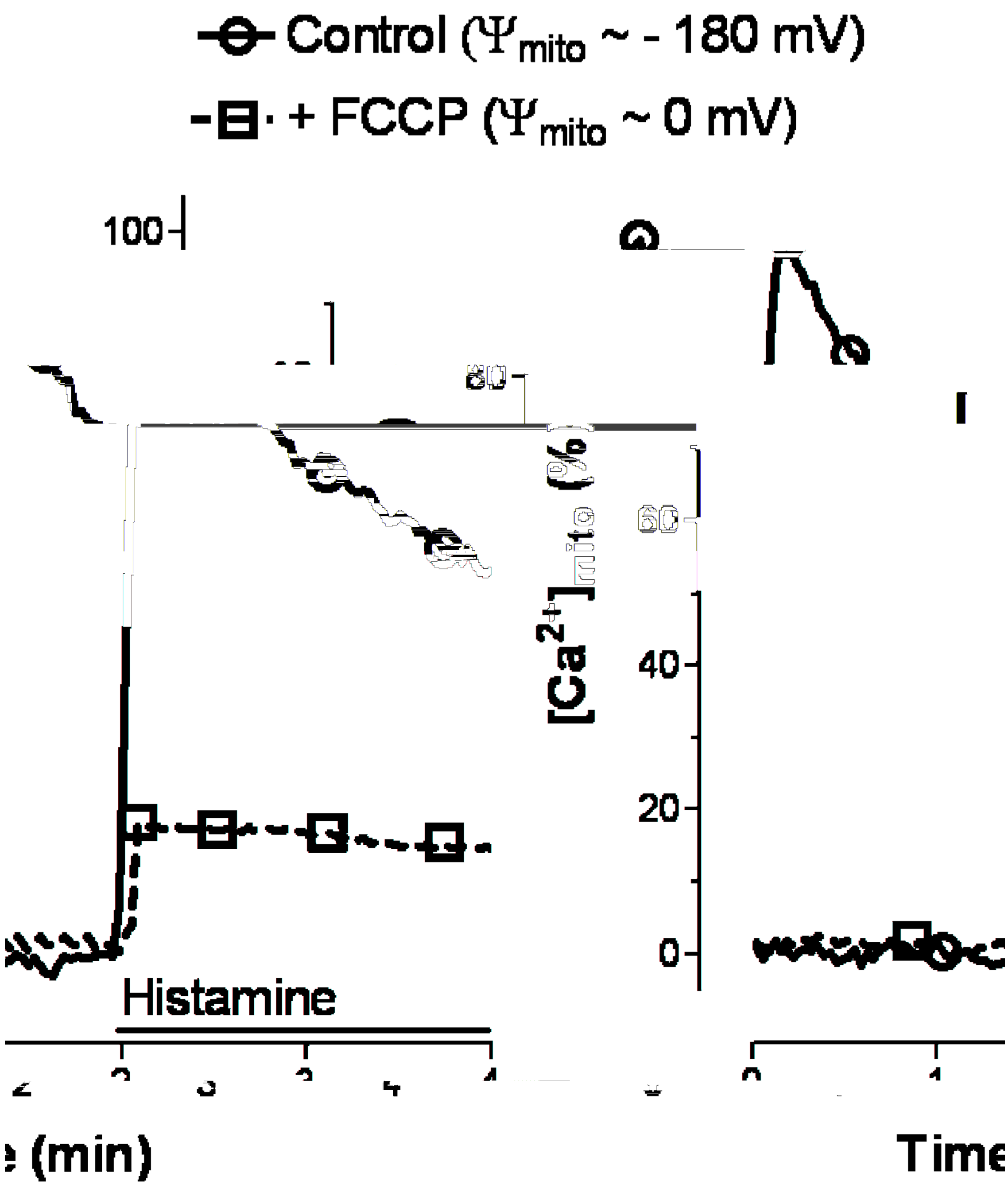


**Fig. 1.**

Effect of membrane depolarization of the mitochondria on the organelle's $Ca^{2+}$ sequestration upon cell stimulation with the $IP_3$-generating agonist histamine. Mitochondrial $Ca^{2+}$ signaling was measured in single endothelial cells that expressed mitochondrial targeted ratiometric pericam using a high resolution fluorescence microscope as described previously [127, 128]. In $Ca^{2+}$ containing solution, mitochondria were depolarized by 2 μM FCCP. Changes of the fluorescence intensity at 430 nm excitation and 535 nm emission are shown in percent of the maximal effect of histamine under control conditions (i.e., in the absence of the chemical uncoupler)

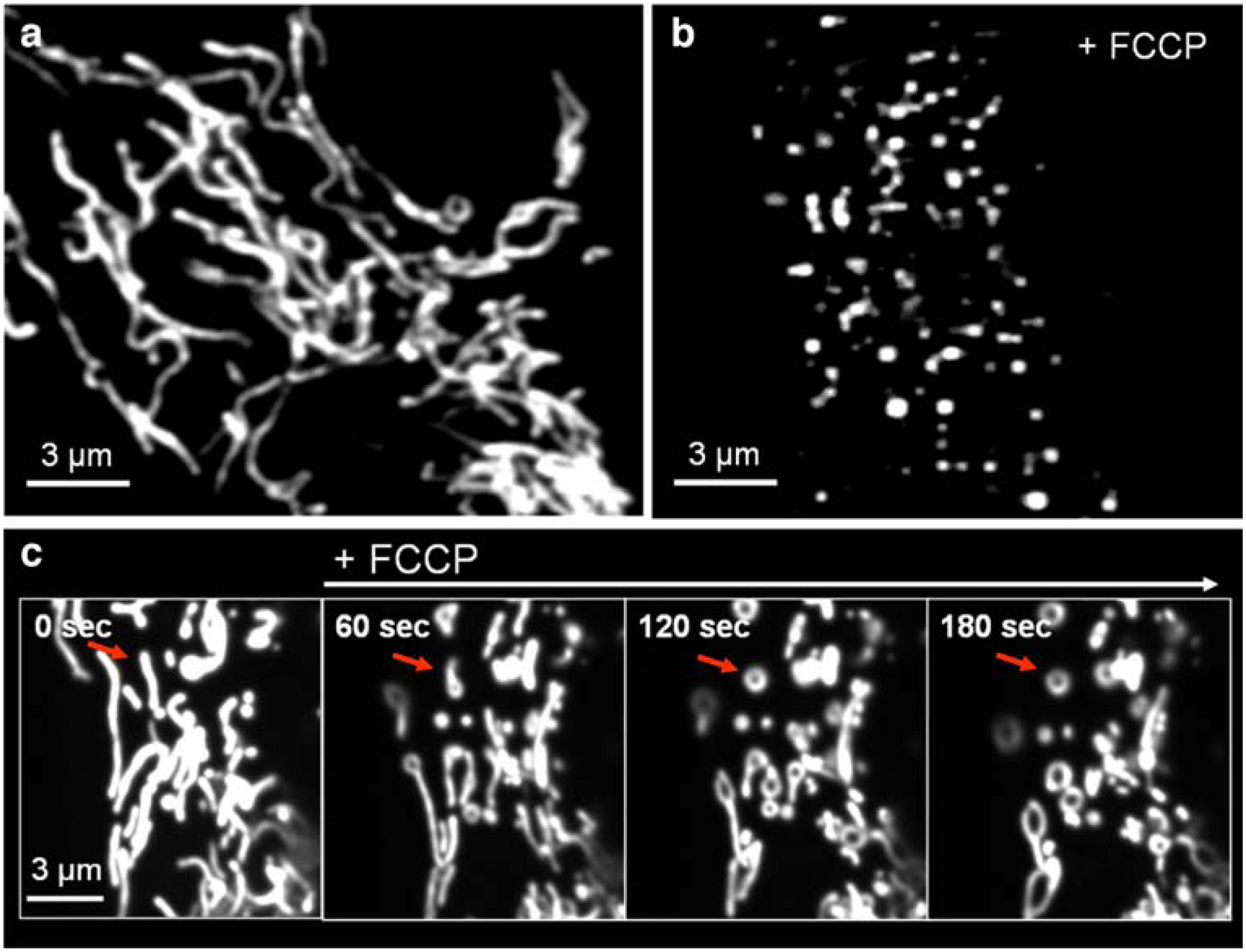


**Fig. 2.**

Effect of membrane depolarization of the mitochondria on the organelle’s morphology and structural integrity. The architectural organization of mitochondria was visualized in human endothelial cells, which transiently expressed mitochondrial-targeted DsRed using an array confocal laser scanning microscope as described previously [160, 215]. **a** Under basal conditions, mitochondria consist as tubular, highly interconnected network. **b** After treatment with 2 μM FCCP for 10 min, mitochondria fragment and form singular round mitochondria. **c** Time course of FCCP induced fragmentation of tubular mitochondria. Upon membrane depolarization by the chemical uncoupler, mitochondrial fragmentation is preluded by the formation of ring-like structures

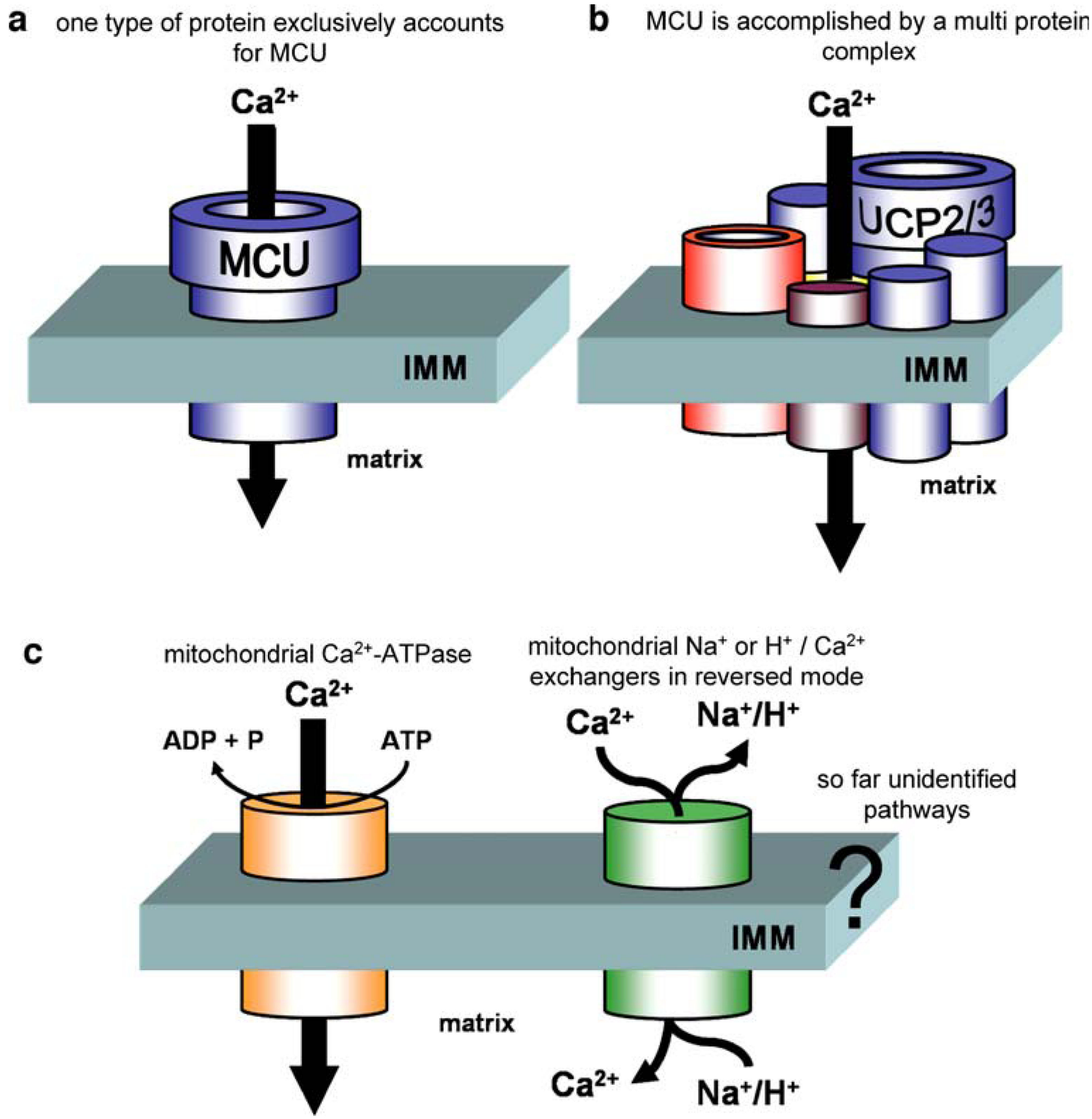


**Fig. 3.**

Schematic illustration of putative pathways for mitochondrial $Ca^{2+}$ fluxes across the inner mitochondrial membrane (IMM; **a–b** ruthenium red-sensitive MCU). **a** The ruthenium red-sensitive MCU might consist of just one type of protein (s). Probably UCP2/UCP3 or some other, so far unidentified, protein works alone or forms homomultimeres to establish MCU. However, because of our recent work, this possibility seems unlikely at least for UCP2/UCP3 [215]. **b** Alternatively, MCU is accomplished by the assembly of a multi protein complex that may form a $Ca^{2+}$ permeable channel similar to what has been described for the mitochondrial permeability transition pore. Our recent work favors this possibility and points to a fundamental contribution of UCP2/UCP3 in this process [215]. **c** The ruthenium red-insensitive mitochondrial $Ca^{2+}$ uptake pathways that either depend on ATP or other ions ($Na^+$, $H^+$) might also contribute to mitochondrial $Ca^{2+}$ signaling under certain conditions and tissues


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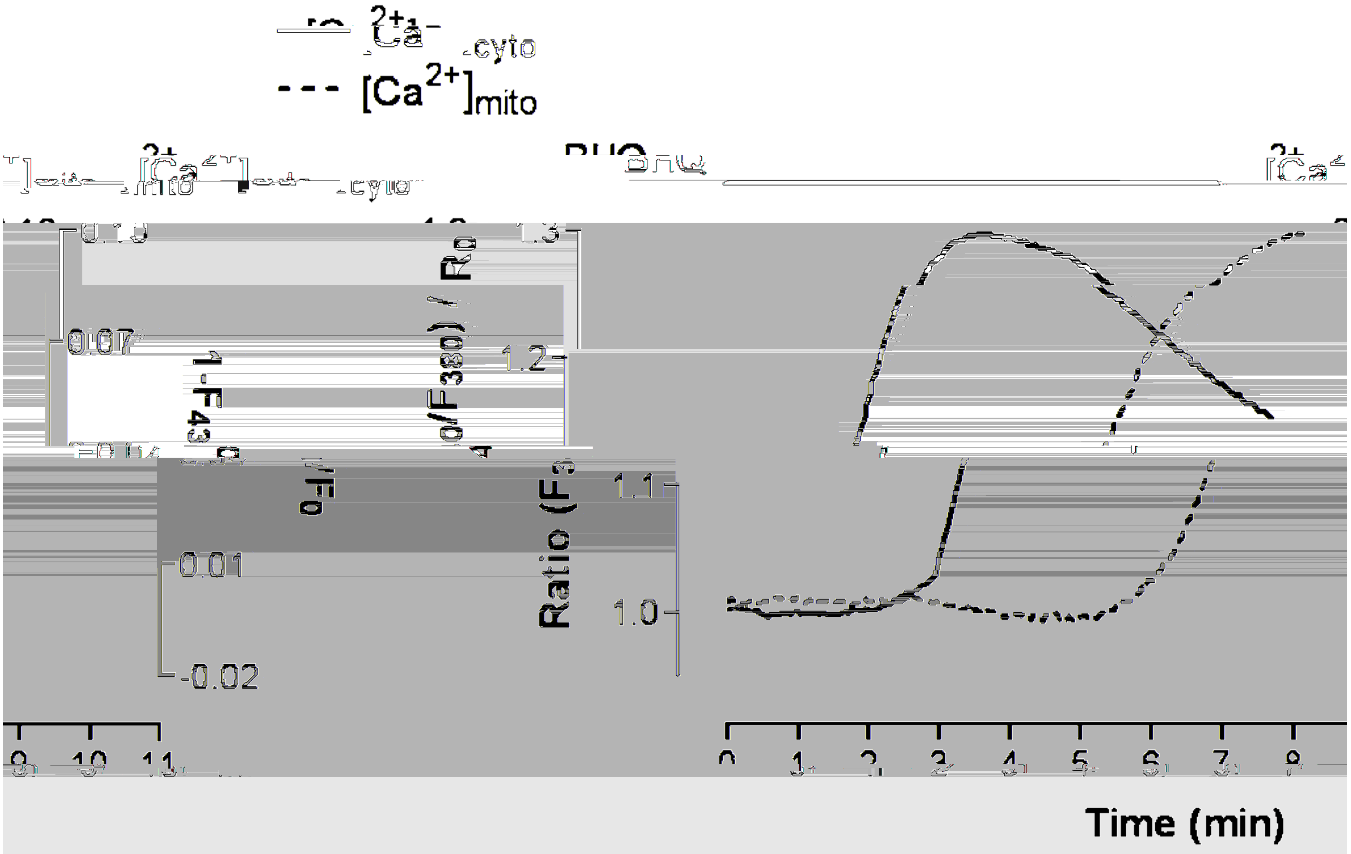


**Fig. 4.**

Correlation between the effect of an inhibition of SERCA on the cytosolic and mitochondrial $Ca^{2+}$ concentration. Cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_{cyto}$) was measured in single human endothelial cells using fura-2 in a high resolution fluorescence microscope as described previously [73, 74, 125]. Analog experiments were performed with cells, which stably expressed mitochondrial targeted ratiometric pericam, to monitor mitochondrial free $Ca^{2+}$ content ($[Ca^{2+}]_{mito}$). Changes in $[Ca^{2+}]_{mito}$ are expressed as 1-$F_{430}/F_0$, as at 430 nm excitation the fluorescence intensity reflects the $Ca^{2+}$ sensitivity of this sensor [128]. As indicated, SERCA was inhibited by the addition of 2,5-di-*tert*-butylhydroquinone (BHQ, 15 μM)

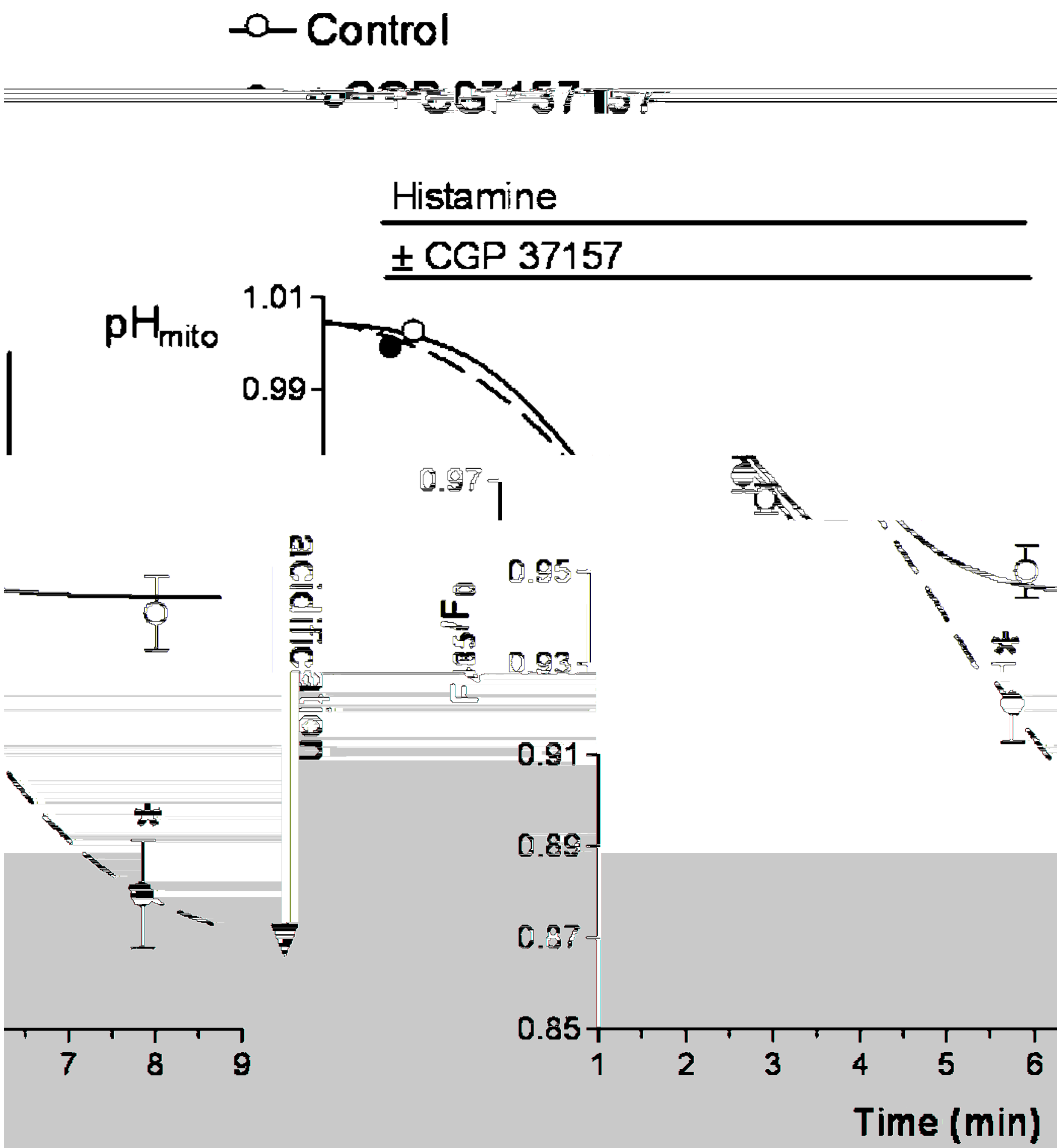


**Fig. 5.**

Effect of an inhibition of the mitochondrial $Na^+/Ca^{2+}$ exchanger on histamine-induced changes in the pH of the mitochondrial matrix. Endothelial cells, which stably express mitochondrial targeted ratiometric pericam were used to monitor changes of the matrix pH by following the pH sensitive wavelength from the sensor. Cells were illuminated at 480 nm and emission was collected at 535 nm on a high-resolution fluorescence microscope [127, 128]. As indicated cells were stimulated with 100 µM histamine in the absence (*continous line*, *open circles*) or in the presence of 20 µM CGP 37157 (*dotted line*, *filled circles*)

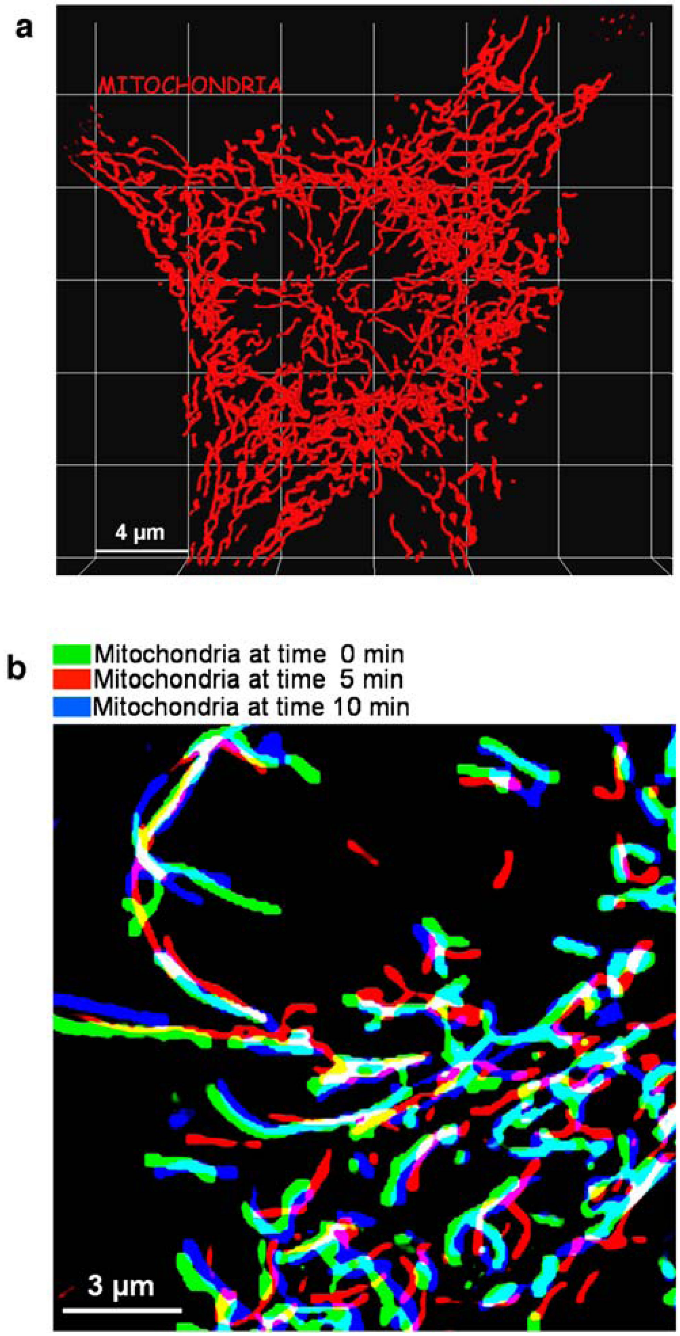


**Fig. 6.**

Mitochondrial morphology and motility. **a** Highly interconnected mitochondria in a single living HeLa cell that transiently expressed mitochondria-targeted DsRed. Z-Scans were performed on an array confocal laser scanning microscope applying 514 nm excitation and measuring 570 nm emission [160]. 3-D reconstruction of the mitochondrial network was performed using Imaris 4.2 software. **b** Overlay of mitochondrial structures in a single endothelial cell expressing mitochondria-targeted DsRed at 0, 5 and 10 min




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