Review

Calcium uptake mechanisms of mitochondria

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ABSTRACT

The ability of mitochondria to capture Ca^{2+} ions has important functional implications for cells, because mitochondria shape cellular Ca^{2+} signals by acting as a Ca^{2+} buffer and respond to Ca^{2+} elevations either by increasing the cell energy supply or by triggering the cell death program of apoptosis. A mitochondrial Ca^{2+} channel known as the uniporter drives the rapid and massive entry of Ca^{2+} ions into mitochondria. The uniporter operates at high, micromolar cytosolic Ca^{2+} concentrations that are only reached transiently in cells, near Ca^{2+} release channels. Mitochondria can also take up Ca^{2+} at low, nanomolar concentrations, but this high affinity mode of Ca^{2+} uptake is not well characterized. Recently, leucine-zipper-EF hand-containing transmembrane region (Letm1) was proposed to be an electrogenic 1:1 mitochondrial Ca^{2+}/H^+ antiporter that drives the uptake of Ca^{2+} into mitochondria at nanomolar cytosolic Ca^{2+} concentrations. In this article, we will review the properties of the Ca^{2+} import systems of mitochondria and discuss how Ca^{2+} uptake via an electrogenic 1:1 Ca^{2+}/H^+ antiport challenges our current thinking of the mitochondrial Ca^{2+} uptake mechanism.

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1. Introduction

Calcium (Ca^{2+}) is a highly versatile second messenger that control critical cellular responses in all eukaryotic organisms [1]. Ca^{2+} signals controls both short-term biological processes that occur in milliseconds, such as muscle contraction and neurotransmission, as well as long-term processes that require several days, such as cell proliferation and organ development [2]. The specificity of cellular Ca^{2+} signals is controlled by a sophisticated "toolkit" comprising numerous ion channels, pumps, and exchangers that drive the fluxes of Ca^{2+} ions across the plasma membrane and across the membrane of intracellular organelles. The development of genetically encoded Ca^{2+} probes targeted to specific intracellular compartments has illuminated the central role played by two organelles in cellular Ca^{2+} homeostasis: the endoplasmic reticulum (ER) and mitochondria. The ER is the major intracellular Ca^{2+} stores of cells, whereas mitochondria shape and decode cellular Ca^{2+} signals by taking up and then releasing Ca^{2+} ions. The Ca^{2+} uptake mechanisms of mitochondria have attracted much attention recently, due to the central role of mitochondria in cell metabolism and cell death.

The ability of mitochondria to act as Ca^{2+} buffers has important consequences on the pattern of the cytosolic Ca^{2+} signals. In excitable cells, mitochondria localized in the vicinity of voltage operated Ca^{2+} channels (VOCs) at the plasma membrane buffer entering Ca^{2+} ions. This Ca^{2+} buffering decreases the magnitude of the local Ca^{2+} microdomains generated around the open channels, and therefore the magnitude of exocytosis [3]. Mitochondrial Ca^{2+} buffering has opposite consequences on the activity of store-operated Ca^{2+} channels (SOCE). In this case, mitochondria located close to SOCE channels sustain their activity by reducing the negative feedback exerted by Ca^{2+} ions on the channel [4,5]. At the cellular level, mitochondrial Ca^{2+} buffering can also have different consequences depending on the arrangement of mitochondria inside cells. In pancreatic acinar cells, mitochondria act as a "firewall" that prevent the propagation of the cytosolic Ca^{2+} waves generated in the apical area of the cell, therefore splitting the cell in two functional compartments able to generate distinct cytosolic Ca^{2+} signals [6]. In HeLa cells however, mitochondria act as a "calcium relay" and, by returning the captured Ca^{2+} ions, are able to prevent the Ca^{2+} depletion of the ER [7] and to transport Ca^{2+} from the plasma membrane to the ER, bypassing the cytosol [8].

In addition to shaping cellular Ca^{2+} signals, Ca^{2+} uptake by mitochondria alters the activity of mitochondria in multiple ways. An increase in the free Ca^{2+} concentration within the mitochondrial matrix, $[Ca^{2+}]_{mit}$, activates several dehydrogenases (pyruvate dehydrogenase, isocitrate dehydrogense, oxoglutarate dehydrogenase) and carriers (citrin and aralar). The net result of the increase in $[Ca^{2+}]_{mit}$ is to increase the respiratory rate, H^+ extrusion and ATP production. The $[Ca^{2+}]_{mit}$ dependency of mitochondrial bioenergetics enables mitochondria to decode Ca^{2+} signals, and thus to tune ATP synthesis to the energetic requirements of the cell [9–11]. However, prolonged increases in $[Ca^{2+}]_{mit}$ can induce the opening of the mitochondrial permeability transition pore (PTP) leading to mitochondrial swelling, cytochrome C release, and cell death by apoptosis [12,13]. Here, we

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will review the main transporters involved in the uptake of Ca^{2+} into mitochondria, and discuss the possible role of the recently cloned mitochondrial Ca^{2+}/H^{+} exchanger Letm1 in mitochondrial Ca^{2+} transport.

2. The mitochondrial Calcium Uniporter: placing mitochondria in Ca^{2+} microdomains

The mechanism that is widely accepted to be responsible for the uptake of Ca^{2+} by mitochondria is the mitochondrial Calcium Uniporter (mCU). The activity of the mCU has been recorded for more than 50 years, using a variety of techniques (luminescence, fluorescence, isotopes, Ca^{2+} electrodes, patch-clamp) and experimental conditions (isolated mitochondria, intact and permeabilized cells, mitoplasts). This body of work generated a wealth of knowledge about the physiological and pharmacological properties of this transport mechanism. Early studies in isolated mitochondria revealed that the mCU catalyzes the passive uptake of Ca^{2+} across the inner membrane of mitochondria, driven by the negative mitochondrial potential ($\Delta\psi_{m}$) generated by the respiratory chain, and that the Ca^{2+} transport activity is inhibited by ruthenium red [14]. Subsequent electrophysiological studies showed the mCU to be an inward rectifying, highly Ca^{2+} selective ion channel located in the inner mitochondrial membrane [15]. These properties imply that the main driving force that allows Ca^{2+} accumulation into the mitochondrial matrix through the mCU channel is the negative $\Delta\psi_{m}$. Taking into account the Nernst equation and assuming a $\Delta\psi_{m}$ of -180 mV, thermodynamic equilibrium would require a concentration gradient of 10^{6} across the mitochondrial membrane. This implies that for a $[Ca^{2+}]_{cyt}$ of ~100 nM, $[Ca^{2+}]_{mit}$ would have to reach ~100 mM to achieve equilibrium. This concentration is never reached however due to the activity of mitochondrial Ca^{2+} extrusion systems. Therefore the mCU, as its names implies, is designed to work in a unidirectional fashion and import Ca^{2+} from the cytosol to the mitochondrial matrix. This mechanism endows energized mitochondria with the ability to potentially capture all the surrounding Ca^{2+} ions.

Fortunately for cells, uptake of Ca^{2+} across the mCU occurs only when energized mitochondria are exposed to high Ca^{2+} concentrations, above the micromolar level. Such concentrations occur only transiently during the peak of global cytosolic Ca^{2+} elevations, or in restricted cellular subcompartments near the mouth of Ca^{2+} entry of Ca^{2+} release channels. The binding of Ca^{2+} ions to the cytosolic side of the mCU channel is thus the main triggers for the activation of the mCU, probably via allosteric modulation of the channel [16]. Kinetic studies of mitochondrial Ca^{2+} uptake as a function of cytosolic $[Ca^{2+}]$ display a sigmoid relationship with a Hill coefficient of 2, indicating that two Ca^{2+} binding sites cooperatively activate the uniporter [16]. Calmodulin antagonists inhibit mitochondrial Ca^{2+} uptake in permeabilized cells perfused with 20 µM Ca^{2+}, suggesting that Ca^{2+} might also modulate the mCU via a Ca^{2+}-calmodulin interaction process [17]. Inhibition of the uniporter at higher $[Ca^{2+}]_{cyt}$ has been reported, a mechanism reminiscent of the bell-shaped Ca^{2+}-dependency of the IP3R and RyR [17]. The biphasic effects of Ca^{2+} on the mCU might avoid excessive Ca^{2+} accumulation in mitochondria.

Despite strong efforts to identify the channel protein(s), the molecules that compose the mCU channel are still unknown. Graier's group proposed that uncoupling proteins 2 and 3 (UCPs) are fundamental for Ca^{2+} uptake [18], but these findings are disputed because normal mitochondrial Ca^{2+} uptake was reported by several groups in 4 different tissues from UCP2 and UCP3 knock-out mice [19]. Given the multiplicity of mitochondria Ca^{2+} uptake mechanisms however, the normal phenotype of UCP2 and UCP3 knock-out mice does not rule out a role for these molecules in mitochondrial Ca^{2+} uptake, because the down regulation of one transport protein might have been compensated by the up-regulation of others to preserve function, masking the phenotype. Thus, new evidence must be

Table 1
Ca^{2+} uptake mechanisms of mitochondria.

Mechanism	Inhibitors	Activators	Ca^{2+} affinity
Uniporter	RuRed [69]	Polyamines [26]	Low
	Ru360 [70]	SB202190 [29]	
	Mg^{2+} [23]	Flavonoids [27]	
	KB-R7943 [25]	PPT [28]	
RaM	RuRed [66,67]	Spermine [66,67]	High
RyR	RuRed [20]	Imperatoxine A [22]	High
	Ryanodine [20–22]		
	Dantrolene [20,22]		
mNCX	CGP37157 [64,65]		No data
Letm1 (CHX)	RuRed [40]		High

provided to confirm or infirm that UCPs are involved in the uptake of Ca^{2+} by mitochondria. In rat cardiac cells a RyR type-1 was identified in the inner mitochondrial membrane of isolated mitochondria using electron microscopy and Western blotting [20,21]. Subsequently experiments in isolated mitochondria from rat heart showed that ryanodine blocked mitochondrial Ca^{2+} uptake and prevented Ca^{2+}-induced mitochondrial swelling and respiration [22], but whether RyR isoforms participate in mitochondrial Ca^{2+} uptake in other cell types remains to be established.

Several molecules have been reported to alter the activity of the mCU (Table 1). Magnesium (Mg^{2+}) is a well-established antagonist of mitochondrial Ca^{2+} uptake [23], via a putative Mg^{2+} binding site located on the cytosolic side of the inner mitochondrial membrane. Nucleotides inhibit Ca^{2+} uptake, in the sequence: ATP>CTP=UTP>GTP [24]. Ruthenium Red or its derivate Ru360 have been widely used as mCU inhibitors in isolated mitochondria and in permeabilized cells, but these compounds poorly permeate into cells, a drawback that has limited their use. The thiourea derivate KB-R7943, an inhibitor of the plasma membrane Na^{+}/Ca^{2+} exchanger, has been recently reported to inhibit the mCU in intact cells [25]. Polyamines [26], natural plant flavonoids, and certain estrogens receptor agonists activate mCU uptake [27–29]. Mitochondrial Ca^{2+} uptake is modulated by the activity of protein kinases. Overexpression and pharmacological inhibition of different PKC isoforms differently impact on the amplitude of the mitochondrial Ca^{2+} elevations evoked by agonists [30], with PKCβ activity decreasing and PKCζ enhancing the $[Ca^{2+}]_{mit}$ elevations. The role of p38 MAP-Kinase is more controversial. The p38 MAPK inhibitor SB202190 activates mitochondrial Ca^{2+} uptake, but the effect persists in permeabilized cells depleted of ATP, suggesting that the compound might directly activate the mCU, without involving kinases [29]. Recent papers showed that p38 MAPK knock-down increases mitochondrial Ca^{2+} uptake however, [31,32], suggesting that p38 MAPK might inhibit the mCU.

A characteristic feature of the uniporter is its low affinity for Ca^{2+}, with a Kd around 10 µM in permeabilized cells [14]. This early observation led to the dismissal of mitochondria as physiological Ca^{2+} stores. However, subsequent $[Ca^{2+}]_{mit}$ recordings in intact cells revealed that mitochondria are in fact perfectly able to take up large amounts of Ca^{2+} during physiological Ca^{2+} elevations. This paradox implied that mitochondria are located at privileged location inside cells, very close to Ca^{2+} release or Ca^{2+} entry channels. Functional and morphological evidence indicate that mitochondria are in close contacts with the endoplasmic reticulum (ER) and with plasma membrane channels [33]. The close contacts between the ER and mitochondria have received much attention, and several proteins have been proposed to link mitochondria to the ER [34,35]. This connection appears to be cell specific: in HeLa 60% of mitochondria are located close to the endoplasmic reticulum but this percentage drops to 4% in endothelial cells [36]. The requirement for microdomains has important functional consequences, as it implies that the spatial distribution of mitochondria determines both the pattern of cellular Ca^{2+} signals and the metabolic response of cells.

3. Calcium uptake at nanomolar Ca^{2+} levels

Despite the consensus that the mCU only takes up Ca^{2+} in the micromolar range, evidence exists that mitochondria also take up Ca^{2+} at nanomolar concentrations. In HeLa cells, mitochondria begin to accumulate Ca^{2+} whenever the global $[Ca^{2+}]_{cyt}$ measured with fluorescent indicators exceeds 100 nM [37]. In rat luteal cells, $[Ca^{2+}]_{cyt}$ increases that do not exceed 200 nM stimulate NADPH synthesis, indicating that Ca^{2+} has been transmitted to the mitochondrial matrix [38]. The caveat of such spatially-averaged $[Ca^{2+}]_{cyt}$ measurements is that they do not reflect the Ca^{2+} levels reached in Ca^{2+} microdomains. However, in luteal cells permeabilized with digitonin, mitochondrial Ca^{2+} uptake was evoked by an even smaller increase in Ca^{2+} from 50 nm to 180 nM [38], suggesting that mitochondrial Ca^{2+} uptake can indeed occur at nanomolar $[Ca^{2+}]_{cyt}$ levels. Similar findings were reported in other cell types including adrenal glomerulosa cells, INS-1 cells, and osteosarcoma cells, using different mitochondrial Ca^{2+} probes [39]. Different activation thresholds where observed among cell types, but Ca^{2+} uptake at nanomolar $[Ca^{2+}]_{cyt}$ levels was consistently inhibited by RuRed, suggesting the involvement of the uniporter [39]. An example of mitochondrial Ca^{2+} uptake in cells permeabilized and perfused with internal buffers containing nanomolar Ca^{2+} levels is shown in Fig. 1. The recent discovery of a high affinity mitochondrial Ca^{2+}/H^+ exchanger able to import Ca^{2+} in

energized mitochondria at nanomolar concentrations [40] has revived the interest for this high affinity mode of mitochondrial Ca^{2+} uptake (see below).

A possible confounding factor in comparing studies in intact cells with studies in permeabilized cells or isolated mitochondria stems from the simplified composition of the solutions used for Ca^{2+} measurements in the later configurations. As indicated previously, Mg^{2+} and nucleotides are effective modulators of the mitochondrial Ca^{2+} uptake. These compounds are often omitted from the "cytosolic-like" solutions used to perfuse permeabilized cells. In fact, Szanda et al. recently showed that Mg^{2+} is released from internal stores during activation of cells by physiological agonists, and that this divalent cation inhibits very effectively mitochondrial Ca^{2+} uptake at nanomolar Ca^{2+} levels, but not at micromolar Ca^{2+} levels [41]. By inhibiting the mCU, the released Mg^{2+} would inhibit mitochondrial Ca^{2+} uptake when Ca^{2+} is mobilized from ER Ca^{2+} stores. This could explain why microdomains appear to be required for ER-mitochondria Ca^{2+} transfer, but not for PM-mitochondria Ca^{2+} transfer, a process that can occur in the nanomolar range [42]. Together, these studies suggest that mitochondrial Ca^{2+} uptake can occur both at micromolar and nanomolar Ca^{2+} levels.

The possibility that mitochondria can import Ca^{2+} at nanomolar concentrations has important functional consequences for cell signalling. From a morphological standpoint, it implies that mitochondria do not need to be located close to Ca^{2+} sources, because mitochondria located far away from Ca^{2+} channels could capture significant amounts of Ca^{2+}. Close contacts between mitochondria and the ER as well as the generation of Ca^{2+} microdomains near plasma membrane Ca^{2+} channels might thus not be absolutely required for mitochondrial Ca^{2+} uptake. The co-existence of high- and low-affinity modes of Ca^{2+} uptake would allow different mitochondrial populations to take up different amounts of Ca^{2+} during cell activation, depending on their location relative to Ca^{2+} stores and channels (Fig. 2). The two modes also enable mitochondria to decode different patterns of cytosolic Ca^{2+} signals. The high affinity mode enables mitochondria to sense slow and sustained increases in $[Ca^{2+}]_{cyt}$, while the low-affinity mode enables mitochondria to decode high-amplitude, frequency-modulated oscillations in $[Ca^{2+}]_{cyt}$, as shown in seminal studies with hepatocytes [43]. The ability of mitochondria to sense slow and steady $[Ca^{2+}]_{cyt}$ elevations has important implications for cell fate, because mitochondrial Ca^{2+} overload can trigger apoptosis [13]. The high affinity mode of Ca^{2+} uptake can potentially transform small increases in $[Ca^{2+}]_{cyt}$ into sustained increases in $[Ca^{2+}]_{mit}$ and initiate the signalling cascade of apoptosis.

4. The Letm1 Ca^{2+}/H^+ antiporter: removing mitochondria from Ca^{2+} microdomains

A recent paper challenged several of the current concepts on mitochondria Ca^{2+} uptake by reporting that Letm1 (leucine-zipper-EF hand-containing transmembrane region), a protein located in the inner mitochondrial membrane, catalyzes the 1:1 electrogenic exchange of Ca^{2+} for H^+ [40]. The antiporter was identified in a genome-wide, unbiased siRNA screen using the strategy that led to the identification of the molecules involved in store-operated calcium entry [44,45]. Letm1 was previously identify as a gene located in the short arm of the chromosome 4 deleted in a subset of patients with Wolf–Hirschhorn syndrome (WHS), a disease characterized by growth and mental retardation, impaired muscular tone, and epileptic seizures. The predicted structure of the protein comprises a C-terminal domain bearing two EF hands domains and located in the intermembrane space, a single transmembrane domain, and an N-terminal domain bearing a PKC phosphorylation site predicted to reside in the mitochondrial matrix [46]. The presence of two putative Ca^{2+} binding sites on Letm1 suggested that impaired mitochondrial Ca^{2+} homeostasis due to lack of Letm1 could explain the seizures observed in some
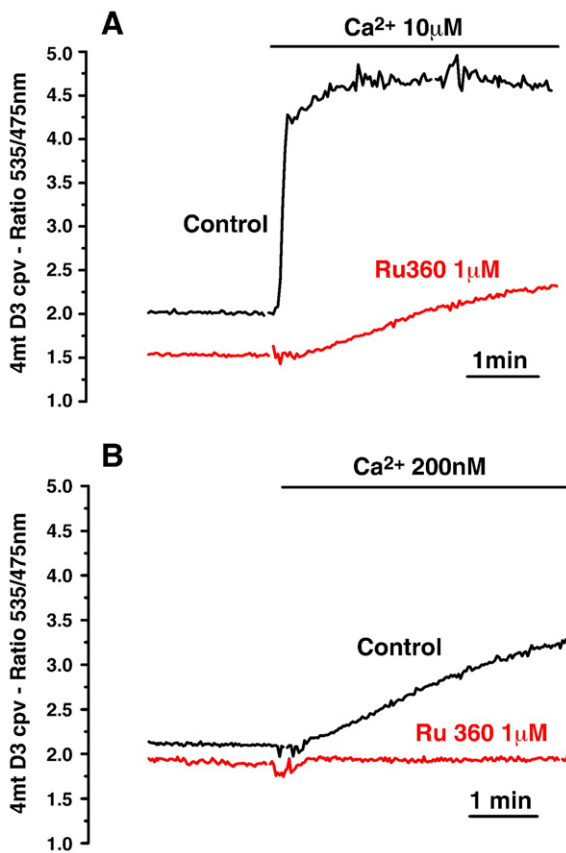

Fig. 1. Mitochondrial Ca^{2+} uptake in permeabilized cells. HeLa cells were transfected with the "cameleon" Ca^{2+} sensitive indicator 4mt-D3-cpv. Measurements were performed 48 h after transfection. Cells where permeabilized for 1 min with an internal solution containing 110 mM KCl, 10 mM NaCl, 0.5 mM H2KPO4, 0.1 M Hepes, 10 mM Succinate, 1 mM EGTA and 100 μM digitonin, and then maintained for 8 min in the same solution without digitonin before the addition of Ca^{2+} (A) $[Ca^{2+}]_{mit}$ responses evoked by the addition of 10 μM free Ca^{2+}, buffered with HEDTA, recorded in the presence or absence of Ru360, and added 3 min before the addition of Ca^{2+}. (B) $[Ca^{2+}]_{mit}$ responses evoked by the addition of 200 nM free Ca^{2+} buffered with EGTA. Traces in panel A show the mean of 12 cells (control) and 9 cells (Ru 360), in panel B 11 cells (control) and 7 cells (Ru360).
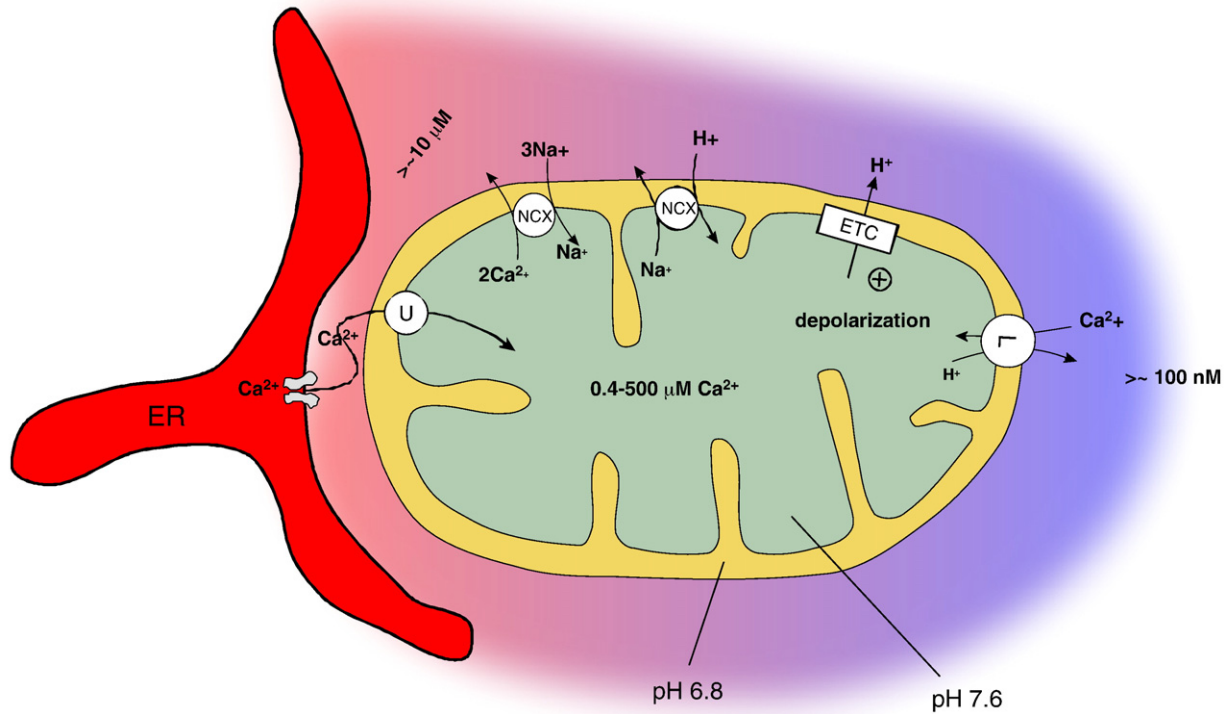
Fig. 2. Calcium uptake and extrusion mechanisms of mitochondria. The mitochondrial Ca^{2+} channel known as the uniporter (U) drives rapid and massive calcium entry, but only at high cytosolic Ca^{2+} concentrations (>10 μM) that are reached in microdomains near Ca^{2+} release channels on the endoplasmic reticulum (ER). The Letm1 Ca^{2+}/H^+ antiporter (L) drives the slow entry of calcium into mitochondria in exchange for protons. Letm1 operates at low cytosolic Ca^{2+} concentrations (>~100 nM) and is limited by the pH gradient generated by the mitochondria electron transport chain (ETC). Letm1 enables mitochondria to decode small cytosolic Ca^{2+} elevations without risking Ca^{2+} overload. The uniporter conveys rapid Ca^{2+} transients from the cytosol to the matrix but exposes mitochondria to Ca^{2+} overload and alterations in ER Ca^{2+} handling. Letm1 is bidirectional and can extrude Ca^{2+} along with the sodium–calcium exchanger (NCX) during large Ca^{2+} loads, but Ca^{2+} uptake dominates under physiological conditions.

WHS patients [47]. However, subsequent studies did not support a role for Letm1 in mitochondrial Ca^{2+} homeostasis, but rather indicated that Letm1 functions as a mitochondrial K^+/H^+ exchanger. Mutants in the yeast Letm1 homolog mdm38, which lack the two Ca^{2+} binding sites presents in Letm1, failed to swell when exposed to potassium acetate [48]. In this assay, lack of K^+/H^+ exchange activity causes K^+ accumulation inside the mitochondrial matrix, followed by water influx and osmotic swelling. The swelling defect was partially recovered by the expression of Letm1. Subsequent measurements in isolated mitochondria labelled with K^+ and H^+ fluorescent dyes confirmed the presence of a K^+/H^+ exchanger in the inner mitochondrial membrane of yeast. K^+/H^+ exchange activity was abolished in the Mdm38 mutant and complemented by the human homolog Letm1 [49], suggesting that Letm1 also function as a K^+/H^+ exchanger. Importantly, the growth defect of mdm38 mutants was rescued by the K^+/H^+ ionophore nigericin [50], suggesting that the physiological role of Mdm38 (and, by analogy, of Letm1) is to catalyze K^+/H^+ exchange. Defective K^+/H^+ exchange activity was thus proposed to account for the fragmentation and swelling of mitochondria reported in cells lacking Letm1 [47,51,52]. However, Mdm38 has also been reported to interact with ribosomes and proposed to be a component of the mitochondrial export machinery [53]. This could explain the reduced levels of respiratory chain proteins as well as the altered ψΔmit, respiration and ATP synthesis reported by some authors in cells lacking Mdm38 [52,54,55]. Moreover, Letm1 was also proposed to modulate the activity of OPA1, a protein that control cristae remodelling and the fusion of inner mitochondrial membranes [54].

In their screen, Jiang et al. used a targeted mitochondrial fluorescent probe dubbed "pericam" to monitor both the changes in $[Ca^{2+}]_{mit}$ and in the mitochondrial matrix pH (pH_{mit}) simultaneously. The functional assays were performed in Drosophila S2 cell and mammalian HEK-293 cells that were permeabilized, a configuration that enables to control the amounts of energetic substrates and ions in the cytosol. Importantly, Mg^{2+} was absent from their recording solutions, a condition that, as discussed above, favours the detection of the high affinity mode of mitochondrial Ca^{2+} uptake. Concomitant, but opposite, changes in $[Ca^{2+}]_{mit}$ and pH_{mit} were observed when the cytosolic $[Ca^{2+}]$ or pH were varied, with mitochondrial Ca^{2+} uptake coupled to a mitochondrial alkalinization, i.e. with protons leaving mitochondria. Only one gene fulfilled the screen criteria that included the ability to: 1) provide a template for siRNA able to suppress both $[Ca^{2+}]_{mit}$ and pH_{mit} changes, 2) encode for a mitochondrial membrane protein, and 3) have a human ortholog. Knock-down and overexpression of Letm1 in cells altered $[Ca^{2+}]_{mit}$ and pH_{mit} responses in a pattern consistent with Ca^{2+}/H^+ exchange. Reconstitution of the purified protein in liposomes confirmed that Letm1 mediates Ca^{2+}/H^+ exchange and revealed that the transport is electrogenic and blocked by ruthenium red. The stoichiometry was estimated to be 1:1, i.e. one calcium ion transported in exchange for one hydrogen ion. The charge imbalance implies that the transport is voltage dependent, a property confirmed in liposomes equilibrated at different potential with valinomycin and varying concentrations of K^+ [40]. Thus, Letm1 shares two of the properties of the mCU: the ability to catalyze Ca^{2+} uptake into mitochondria, and the inhibition by Ruthenium red. Unlike the mCU however, Letm1 catalyzes the electrogenic uptake of Ca^{2+} into mitochondria in exchange for H^+. This implies that proton efflux from mitochondria can drive Letm1-dependent Ca^{2+} entry into mitochondria. Indeed, $[Ca^{2+}]_{mit}$ increased upon cytosolic alkalinisation in permeabilized HEK cells overexpressing Letm1 [40]. Another important difference with the uniporter is the ability of Letm1 to take up Ca^{2+} at nanomolar concentrations. This implies that Letm1 should have less

reliance than the uniporter on microdomains for effective mitochondrial Ca^{2+} uptake. Instead, Letm1-mediated uptake of Ca^{2+} is driven by the negative potential of mitochondria, and by proton leaving the mitochondrial matrix (Fig. 2). This dual dependency favours Ca^{2+} entry into hyperpolarized mitochondria but limit Ca^{2+} entry into alkaline mitochondria. Because the very negative mitochondrial potential of -180 mV largely exceeds the opposing force generated by the mitochondrial pH gradient of ~1 pH unit, the 1:1 stoichiometry predicts that Letm1 should mediate Ca^{2+} entry under physiological conditions. Unlike the mCU however, thermodynamic equilibrium of an electrogenic 1:1 Ca^{2+}/H^+ antiporter only require combined Ca^{2+} and H^+ concentrations gradients of 10^3 to counterbalance a $\Delta\psi_m$ of -180 mV. For a pH gradient of ~1 pH unit (alkaline inside) and a $[Ca^{2+}]_{cyt}$ of ~100 nM, this means that equilibrium would be achieved at a $[Ca^{2+}]_{mit}$ of ~10 μM. Thus, unlike the mCU, the antiporter does not have the built-in ability to cause massive mitochondrial Ca^{2+} overload. Functional data in intact HeLa cells depleted of Letm1 indicate that the new antiporter can mediate both Ca^{2+} uptake and Ca^{2+} extrusion from mitochondria [40], but these observations remain to be confirmed by simultaneous Ca^{2+} and pH measurements during physiological stimulations. The role of Mg^{2+} and of nucleotides on the activity of the Letm1 antiporter should also be clarified, because most of the functional Letm1 data were obtained in solutions devoid of Mg^{2+} that favour the high affinity mode of mitochondrial Ca^{2+} uptake. As long as the ability of Letm1 to transport significant amounts of Ca^{2+} in physiological conditions containing millimolar $[Mg^{2+}]$ is not established, the physiological relevance of the new transporter will remain speculative. Finally, the unexpected sensitivity of Letm1 to RuRed highlights the limitations of the pharmacological approaches that were used to define the mitochondrial Ca^{2+} transport pathways. The mCU was largely defined by its sensitivity to RuRed and assumed to be the sole Ca^{2+} uptake mechanism of mitochondria. However, as mentioned earlier Ca^{2+} uptake by mitochondria exhibits several kinetics components, consistent with the presence of several Ca^{2+} uptake molecules. Moreover, several reports indicate that RuRed is not a specific inhibitor. RuRed has been reported to inhibit several types of ion channels: RyRs [56], VDAC [57], TRPs [58], TASK [59], K2P [60], and Kv [61] and to interfere with the binding of Ca^{2+} to calmodulin [62]. It is thus not unexpected that RuRed inhibits more than one mitochondrial Ca^{2+} transport pathway.

5. Other mechanisms: reverse Na/Ca exchange and rapid mode of uptake (RaM)

The idea that mitochondria are able to import Ca^{2+} via a sodium–calcium exchanger (NCX) operating in the "reverse" mode was first proposed by Jung et al. [63]. Using isolated heart mitochondria totally depolarized by FCCP and in presence of RuRed to inhibit the uniporter, they showed that mitochondria could accumulate significant amounts of Ca^{2+} and that Ca^{2+} uptake was inhibited by removing Na^+ from the internal solution. This is consistent with the reverse activity of the NCX, a transporter that is widely assumed to be the main Ca^{2+} extruder of mitochondria. In intact rat cardiomyocytes and MCDK cells maintained under hypoxic conditions that totally or partially depolarize mitochondria, the accumulation of Ca^{2+} was significantly lower in Na^+ depleted cells, consistent with reverse NCX activity [64,65]. These data have medical relevance since mitochondrial Ca^{2+} overload has been identified as a key step in the cell injury caused by the ischemia.

A kinetically distinct mode of mitochondrial Ca^{2+} uptake was reported in isolated liver mitochondria exposed to physiological Ca^{2+} pulses lasting 1–10 s: a rapid mode of Ca^{2+} uptake (RaM), hundred of times faster than the Ca^{2+} uptake by the mCU [66]. The RaM activated only transiently at the beginning of the Ca^{2+} pulses and rapidly recovered between pulses, enabling mitochondria to respond to repetitive Ca^{2+} transients. The rapid mode was detectable already when $[Ca^{2+}]_{cyt}$ increased above 200 nM and thus did not require

mitochondria to be in proximity to high Ca^{2+} microdomains. Subsequent experiments in isolated heart mitochondria also reported features consistent with RaM uptake [67], but rapid Ca^{2+} uptake at nanomolar $[Ca^{2+}]_{cyt}$ has not been reported in other cellular systems. Like the mCU, the RaM was inhibited by RuRed, activated by spermine, and driven by the electrochemical gradient, suggesting that this peculiar mode of transport could reflect an altered, high Ca^{2+} affinity mode of the mCU [68]. Whether the RaM is associated with mitochondrial H^+ transport or with the presence of Letm1 is not known.

6. Conclusion

The last few years have seen interesting advances in the molecular and functional characterization of the Ca^{2+} uptake mechanisms of mitochondria. *In situ* measurements in intact cells and tissues with ion-sensitive indicators have revealed the importance of mitochondrial Ca^{2+} uptake for cellular function. These studies have also showed that the knowledge derived from experiments in permeabilized cells or isolated mitochondria cannot be readily transposed to the more complex situation of intact cells. Gene silencing and overexpression studies in cells have led to disputed claims about the identity of the mCU, because the functional results in cells could not be reproduced in isolated mitochondria. The recent identification of Letm1 as a Ca^{2+}/H^+ antiporter that mediate the RuRed-sensitive, high affinity uptake of Ca^{2+} by mitochondria highlights the gap between prior assumptions and new findings from genome-wide screens. The only Ca^{2+} uptake mechanism of mitochondria was thought to be the mCU, a low-affinity uniporter operating at micromolar $[Ca^{2+}]_{cyt}$ and specifically inhibited by Ruthenium Red. This definition ignored reports showing that RuRed is a non-specific inhibitor and that high affinity mitochondrial Ca^{2+} uptake can occur at nanomolar $[Ca^{2+}]_{cyt}$. To date, Letm1 is the only *bona fide* mitochondrial Ca^{2+} transport protein validated by the functional reconstitution of its transport activity in purified lipids. Future experiments should use similar, unbiased screens to identify the elusive uniporter and to establish how many molecules are involved in mitochondrial Ca^{2+} uptake.

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