Biochimica et Biophysica Acta 1787 (2009) 1383–1394

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Review

Regulation of plasma membrane calcium fluxes by mitochondria

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ARTICLE INFO

Article history:
Received 17 November 2008
Received in revised form 21 December 2008
Accepted 29 December 2008
Available online 6 January 2009

Keywords:
Calcium
Mitochondria
Ion channel
Endoplasmic reticulum
Cell signaling

ABSTRACT

The role of mitochondria in cell signaling is becoming increasingly apparent, to an extent that the signaling role of mitochondria appears to have stolen the spotlight from their primary function as energy producers. In this chapter, we will review the ionic basis of calcium handling by mitochondria and discuss the mechanisms that these organelles use to regulate the activity of plasma membrane calcium channels and transporters.

Mitochondria are multifunctional organelles that control a large number of cellular processes. Mitochondria are the cell energy factories that burn oxygen during oxidative phosphorylation to produce the cellular ATP used for biochemical reactions. This process is so central for life that mitochondria consume most of the biological oxygen on earth to supply energy in aerobic organisms. Besides performing oxidative phosphorylation, mitochondria play a central role in the generation of reducing equivalents by the tricarboxylic acid (TCA) cycle, in fatty acid oxidation, amino acid metabolism, and ketone body synthesis. Mitochondria are also a critical component of the cell signaling machinery, via their ability to sense and shape Ca^{2+} signals that control fundamental cellular functions. This later aspect has attracted much interest in the last decade, as the results from studies in isolated mitochondria were transposed into the more physiological context of intact cells and tissues.

The ability of mitochondria to move Ca^{2+} ions has important implications, firstly because Ca^{2+} controls numerous functions of mitochondria, and secondly because Ca^{2+} handling by mitochondria impacts on cytosolic Ca^{2+} signals and on the activity of Ca^{2+}-dependent proteins. Mitochondria both encode and decode Ca^{2+} signals and these two interrelated functions directly impact on the cell metabolism and signaling. Mitochondria take up and release Ca^{2+}, and this filtering of Ca^{2+} ions has numerous effects on cell Ca^{2+} homeostasis. By acting as a Ca^{2+} buffer, mitochondria can activate or deactivate plasma membrane channels that are regulated by changes in Ca^{2+} concentrations, or restrict Ca^{2+} signals to specific cellular domains. By acting as a "Ca^{2+} relay", mitochondria can propagate Ca^{2+} signals and funnel Ca^{2+} ions inside cells to reload intracellular Ca^{2+} stores. Mitochondria also decode Ca^{2+} signals as either life or death signals. Physiological increases in the cytosolic free Ca^{2+} concentration are transmitted to the mitochondrial matrix by the mitochondrial Ca^{2+} uniporter, and stimulate α-ketoglutarate, isocitrate, and pyruvate dehydrogenases to boost mitochondrial metabolism. Several mitochondrial metabolite transporters are also indirectly modulated by $[Ca^{2+}]_{mit}$, and two exchanger families (aspartate-glutamate carriers and ATP·Mg/phosphate exchangers) are regulated by physiological elevations of cytosolic Ca^{2+} [1]. Excessive $[Ca^{2+}]_{mit}$ elevations cause the release of cytochrome *C* from the intermembrane space of mitochondria and trigger apoptosis [2,3]. Mitochondria thus shape Ca^{2+} signals and translate them as metabolic or apoptotic responses. The regulation of mitochondrial functions by Ca^{2+} has been extensively covered and will not be discussed in this chapter. Instead, we will focus on the modulation by mitochondria of the activity of membrane transporters.

Mitochondria contain two membranes, an inner membrane folded into cristae that harbor the respiratory chain complexes, and an outer membrane permeable to solutes. The control of ion gradients across the inner membrane of mitochondria is central to bioenergetics, and mitochondria have evolved a sophisticated array of carriers, ion channels, and transporters to move ions and metabolites across their inner membrane (Fig. 1). Mitochondria move electrons and protons to produce energy, and rely on ionic gradients to exchange metabolic substrates and products between the intermembrane space and the mitochondrial matrix. At the same time, mitochondria, like other organelles, must maintain their ionic homeostasis and respond to changes in their surrounding cytosolic environment. These functions are accomplished by a large number of transport proteins involved in energy production (light green and brown in Fig. 1), metabolite transport (blue), and ion transport (green). A common property of

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doi:10.1016/j.bbabio.2008.12.012

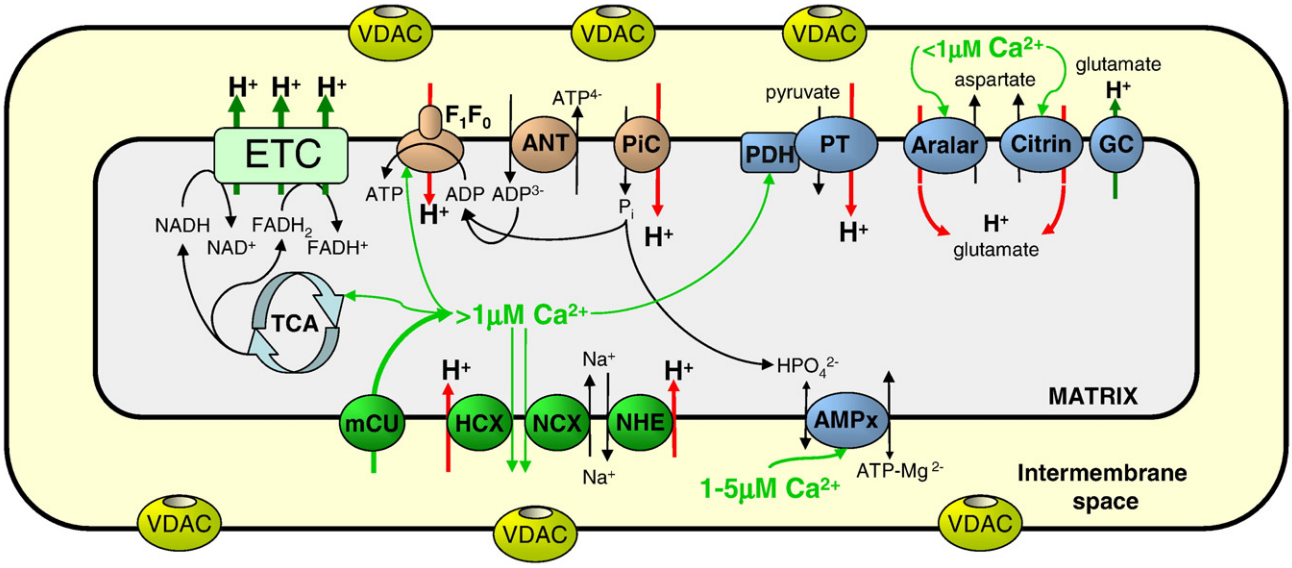

Fig. 1. Energy production and ion transport by mitochondria. The scheme shows proteins that move electrons and ATP (light green and brown), metabolic substrates (blue), and ions (green) across the inner and outer membrane of mitochondria.

these transport molecules is their reliance on the proton gradient, as highlighted in Fig. 1. This should not come as a surprise, as the cycling of protons across the mitochondrial inner membrane is the basis of the chemioosmotic theory that won Peter Mitchell a Nobel Prize in 1978. According to this theory, protons are pumped out of mitochondria during oxidative phosphorylation as electrons flow from reduced substrates in the matrix to oxygen in the intermembrane space. The potential energy stored in the proton gradient is then used to synthesize ATP by the F_1F_o-ATP synthase as protons are drawn back into the matrix [4]. The proton gradient is also necessary to import metabolic substrates and export ATP, the product of oxidative phosphorylation, as the entry of pyruvate and phosphate are coupled to the entry of protons into the mitochondrial matrix. Finally, the pumping of electrons across the inner membrane generates a negative transmembrane potential that provides a huge driving force for the entry of cations into the mitochondrial matrix. This property allows Ca^{2+} transporters within the inner membrane to move Ca^{2+} ions across mitochondria, thus altering the cytosolic concentration of an ion that is central to cell signaling. The fundamentals of mitochondrial Ca^{2+} handling have been discussed extensively in recent reviews [5,6], and we will only highlight some important features of the Ca^{2+} transporters of mitochondria that underlie the ability of these organelles to shape cellular Ca^{2+} signals.

1. The unique but so elusive mitochondrial calcium uniporter

The Ca^{2+} uptake capacity of mitochondria was recognized early in studies from purified isolated mitochondria (for a historical review, see [7]), and attributed to the presence of a unidirectional transporter that catalyzes the passive entry of Ca^{2+} into mitochondria, driven by the large negative membrane potential of mitochondria. These studies determined the fundamental properties of the mitochondrial Ca^{2+} uniporter (mCU), i.e. high transport rate, low temperature dependency, block by the ruthenium red, and sensitivity to membrane potential. The activity of the mCU was subsequently measured by recording changes in the free Ca^{2+} concentration within the mitochondrial matrix, $[Ca^{2+}]_{mit}$, in intact cells, confirming that the basic features of the mitochondrial Ca^{2+} uptake system are conserved in living cells [8–10]. Then, in a "tour de force" study, the mCU was characterized electrophysiologically by patch-clamp recordings from mitoplasts, isolated vesicles made of inner mitochondrial membranes. These recordings revealed the presence of a voltage-dependent, inwardly-rectifying, highly Ca^{2+}-selective channel (MiCa) that retained several key properties of the uniporter such as the RuR sensitivity [11]. Unfortunately, despite a long quest to unravel the protein responsible for mitochondrial Ca^{2+} uptake, the molecular identity of the Ca^{2+} uniporter is still hotly debated. The uncoupling proteins UCP2 and UCP3 have been proposed to be an essential component of the mitochondrial Ca^{2+} uniporter [12], because overexpression of these proteins increased mitochondrial Ca^{2+} responses in intact cells while their silencing had the opposite effect. These "novel" UCP proteins were previously reported to conduct protons when activated by fatty acids or ROS [13], but their uncoupling function is still debated [14]. Although strong evidence was provided that UCP2 and UCP3 participate in Ca^{2+}uptake, the claim that the novel UCPs transport Ca^{2+} ions has then been vigorously challenged [15], because several groups found that mitochondria from knock-out mice lacking UCP2 or UCP3 were able to take up Ca^{2+} normally. In this contradicting study, the activity of the uniporter was assessed in purified mitochondria preparations, by measuring the changes in extra-mitochondrial Ca^{2+} with a Ca^{2+} electrode. Using mitochondria isolated with a different procedure, the group of Wolfgang Graier reported a slightly reduced Ca^{2+} uptake with this assay in mitochondria prepared from UCP2 null mice [16]. The most striking difference however, was the diverging results obtained in intact cells and in isolated mitochondria. In intact cells, the amplitude and kinetics of the mitochondrial Ca^{2+} signals were clearly altered by the invalidation and overexpression of the two novel UCPs [12], an effect that was partially or entirely lost when the activity was measured in isolated mitochondria. These two opposite datasets are most easily reconciled by postulating that the two novel UCPs have a modulatory role in cells that is not observed in purified mitochondria.

2. Following the path of calcium in and out of mitochondria

Ca^{2+} entry into mitochondria is only the beginning of the story, firstly because mitochondria have a finite capacity to store Ca^{2+} ions and secondly because the fluxes of Ca^{2+} influence the fluxes of other ions. Fig. 2 shows the coupling between the fluxes of Ca^{2+}, Na^+, and H^+ across the inner membrane of mitochondria, according to the model developed by Chalmers and Nicholls ([17], reviewed in [18]). In this chain of events, calcium enters via the uniporter (mCU) and leaves mitochondria in exchange for sodium ions, a process catalyzed by the Na^+/Ca^{2+} exchanger (mNCX). Sodium ions are then extruded

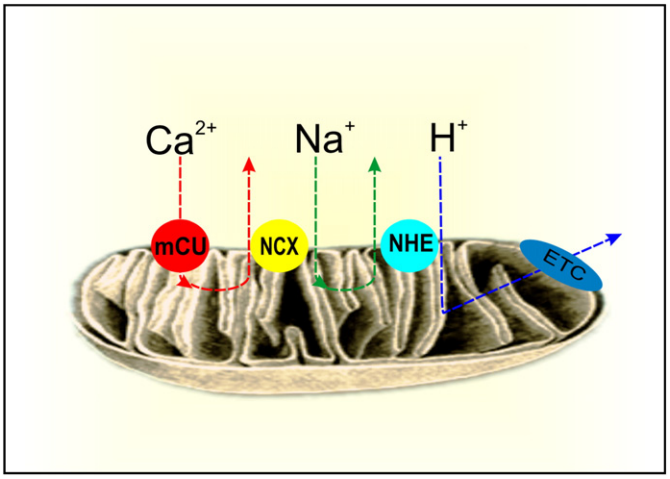

Fig. 2. Ion transporters driving Ca^{2+} fluxes across the inner mitochondrial membrane. Ca^{2+} enters mitochondria via the uniporter (mCU), and exits mitochondria in exchange for Na^+ via the Na^+/Ca^{2+} exchanger (mNCX). Na^+ leaves mitochondria in exchange for H^+ via the Na^+/H^+ exchanger (mNHE), and H^+ ions are ultimately extruded by the electron transport chain (ETC).

in exchange for protons by the Na^+/H^+ exchanger (mNHE). Note that protons must ultimately leave the mitochondria to negate the disturbances in ionic homeostasis caused by the entry of calcium into mitochondria, and that the cost of the entry of one calcium ion is the extrusion of three protons (assuming that the electrogenic mNCX imports 3 Na^+ ions for each Ca^{2+} ion extruded, Na^+ extrusion requires the entry of 3 protons that must then be extruded from mitochondria: $Ca^{2+} \rightarrow 1\ Ca^{2+}/3Na^+ \rightarrow 3Na^+/3H^+$). Because proton extrusion is normally performed by the respiratory chain, Ca^{2+} fluxes across mitochondria divert protons away from the ATP synthase and thus uncouple respiration from ATP production. The energy cost of Ca^{2+} ions transiting across mitochondria is thus akin to the activation of uncoupling proteins in the mitochondrial membrane. However, Ca^{2+} fluxes are not strictly coupled to respiration, but only require the extrusion of protons to compensate for charge and to maintain a sufficient driving force for Ca^{2+} entry into mitochondria. Proton extrusion can occur in the absence of oxidative phosphorylation by the reversal of the F_1F_0-ATPase, which can consume ATP to pump protons, and even in cells lacking mitochondrial DNA and thus both a functional respiratory chain and the proton channel component (F_0) of the ATPase [19,20]. These "ρ0" cells use ATP from glycolysis to drive the catalytic activity of their remaining F_1-ATPase, the inverted activity of the adenine nucleotide translocator (ANT) driving proton extrusion by the phosphate transporter and promoting hyperpolarization (Fig. 1, brown transporters working in inverted mode). These oxidation-defective mitochondria are able to mount normal Ca^{2+} signals [20], indicating that the ability of mitochondria to take up and release Ca^{2+} can be separated from their oxygen consumption. Thus, although respiring mitochondria have a more negative membrane potential and can take up Ca^{2+} more effectively than resting mitochondria, Ca^{2+} handling by mitochondria are not strictly coupled to the energetic state of the organelle. In fact, Ca^{2+} uptake can occur even in fully depolarized mitochondria, provided that the electrochemical gradient for Ca^{2+} favors Ca^{2+} entry, i.e. that the concentration difference between the cytosol and the mitochondrial matrix, ($[Ca^{2+}]_{cyt} - [Ca^{2+}]_{mit}$) outbalances the loss of the negative mitochondrial membrane [12].

3. Coupling of ion fluxes across mitochondria

Several points are also remarkable in the mitochondrial $Ca^{2+}/Na^+/H^+$ ion exchange system described in Fig. 2: 1) the activation of one process (as described here for Ca^{2+} entry) causes the successive activation of two other transporters, altering the fluxes of two other ions and disturbing the local ionic microenvironment. 2) Except the uniporter, all the ion transporters in this chain are exchangers that can work both in forward or reverse mode, depending on the ion gradients of Na^+, Ca^{2+}, and H^+. Thus, although the mNCX is generally assumed to function as a Ca^{2+} extruder, it can also catalyze the entry of Ca^{2+} into mitochondria in exchange for sodium leaving mitochondria. The mitochondrial Na^+ concentration can raise significantly [21], suggesting that $[Na^+]_{mit}$ might momentarily exceed $[Na^+]_{cyt}$, and evidence that Ca^{2+} enters mitochondria by mNCX reversal has been provided [22]. Similarly, the NHE can drive the entry of Na^+ into mitochondria, if the gradient for sodium entry exceeds the gradient for proton entry into mitochondria. NHE reversal is predicted to occur when the cytosolic Na^+ concentration increases sharply, as is the case under the plasma membrane during the activation of Na^+-permeable channels. The increased mitochondrial Na^+ can, in turn, drive the entry of Ca^{2+} into mitochondria by reversing the activity of the mNCX when the cytosolic Na^+ returns to pre-stimulatory values. In this scenario, the whole chain is inverted and the opening of Na^+ channels drives Ca^{2+} uptake by mitochondria. 3) Two transport processes are electrogenic: the uniporter and the mNCX. Their activity thus both depends and alters the membrane potential of mitochondria. In contrast, the NHE is electroneutral and its activity does not depend on the mitochondrial membrane potential. The electrogenic nature of the mCU was recognized long ago and was predicted from the unidirectional fluxes across an ion channel [7]. mNCX-operated Na^+/Ca^{2+} exchange was initially thought to be electroneutral [23], but the stoichiometry of the mNCX has been reassessed recently and evidence of an electrogenic 3 $Na^+/1\ Ca^{2+}$ exchange is now compelling [22,24], reviewed in [25]. Thus, both the mCU and the mNCX are voltage dependent and their activity depends on the membrane potential of mitochondria. A negative mitochondrial membrane potential will therefore not only favor Ca^{2+} entry across the uniporter, but also Ca^{2+} extrusion across the exchanger, favoring its forward mode by driving the entry of 3 Na^+ ions into mitochondria in exchange for one Ca^{2+} ion leaving mitochondria. The net result of the voltage sensitivity of the two Ca^{2+} transporters is to increase Ca^{2+} turnover across mitochondria at negative potentials. 4) The mCU and mNCX have different kinetics, affinity, and capacity. The mCU is an ion channel that allows the fluxes of millions of ions per second, while the transport rate of the mNCX is more limited due to the slower reaction kinetics of ion exchangers. The capacity of the uniporter to import Ca^{2+} ions thus greatly exceeds the capacity of the exchanger to extrude Ca^{2+} ions, but the explosive entry of Ca^{2+} by the uniporter is significantly dampened before Ca^{2+} is slowly extruded by the mNCX, due to the efficient buffering of Ca^{2+} by phosphate in the mitochondrial matrix [14].

Studies of the mitochondrial involvement in intracellular Ca^{2+} signaling often rely on the pharmacological inhibition of mitochondrial Ca^{2+} uptake or extrusion. This warrants a brief consideration of the mitochondrial inhibitors (Table 1) most commonly used to study the role of mitochondria in Ca^{2+} handling. Inhibition of the mitochondrial uniporter represents the most direct approach to study the effects of mitochondrial Ca^{2+} uptake without grossly altering mitochondrial energetic or metabolism. Ruthenium red and its purified active compound RU360 are the prototypical uniporter inhibitors [26,27]. Both compounds are potent and effective on isolated mitochondria, but their usefulness for in situ studies is limited by poor membrane permeability (reviewed in [28]) and by the overlapping affinities of ruthenium red for the uniporter and ryanodine receptors [28,29]. For these reasons, mitochondrial Ca^{2+} uptake is often inhibited indirectly by depolarizing mitochondria to remove the driving force for Ca^{2+} uptake. The mitochondrial membrane potential is created by the proton pumping of the electron transport chain complexes I, III and IV, while complex II supplies

Table 1
Common mitochondrial inhibitors used to study Ca^{2+} signaling.

Class	Drug (IC_{50})	Comments	Reference
Protonophores	FCCP (~1 μM)	Depolarize PM at ~10 μM	[125]
	CCCP (~1 μM)		
	DNP (3 mM)		
Complex I inhibitors	Rotenone (1 nM)	Depolarize $\Delta\psi_m$, ↑ ROS production	[126]
	Amytal (0.2 mM)	Better controlled titration	[127]
	DPI (23 nmol/mg protein)	Inhibits PM-bound oxidases	[128]
Complex III inhibitors	Antimycin A (3 pM)	Complex IIIb/c(1)-Qi site	[129]
	Myxothiazol	Complex IIIb/c(1)-Qo site	
	HQNO (37 μM))	Complex IIIb(562)	[130]
ATPase synthase (complex V) inhibitors	Oligomycin A and B (0.1 μM) apoptolidin (4–5 μM)	F_0 subunit	[131]
	Aurovertin B	F_1 subunit	
Uniporter blockers	ruthenium red (~0.2 nM)	Inhibits RyR at higher concentration [29]	[27]
	RU360 (0.2 nM)		[26]
mNCX inhibitors	CGP-37157 (0.4 μM)	Selective over PM NCX	[30]
	Diltiazem (7 μM)		[132]

Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; DNP, dinitrophenol; DPI, diphenyleneiodonium; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; HQNO, 2-heptyl-4-hydroxyquinoline-N-oxide; TTFA, theonyltrifluoroacetate.

complex III with reducing equivalents in parallel with complex I. Since proton pumping at complex IV requires reducing equivalents from complex III, via cytochrome *C*, complete inhibition of proton pumping can be achieved by inhibiting both complexes I and III, typically with rotenone and antimycin, respectively. Inhibition of complex I or III alone causes a variable and lesser degree of depolarization that is often accompanied by increased ROS production, complicating the interpretation of the effects of the given treatment on Ca^{2+} handling. Mitochondrial depolarization can cause reversal of the mitochondrial ATP synthase, which will generate a small membrane potential through the hydrolysis of cytosolic ATP. For this reason, oligomycin A, an inhibitor of the ATP synthase, is often added to mitochondrial depolarizing cocktails. This combination is quite effective in intact cells, because rotenone, antimycin A and oligomycin are readily membrane permeable. Alternatively, mitochondria can be depolarized by collapsing the proton gradient across the inner membrane with protonophores, lipid soluble H^+ shuttles, like FCCP or dinitrophenol. However, these compounds can also collapse protons gradients across the plasma membrane and other membranous organelles at concentrations similar to those used to inhibit mitochondria, producing confounding effects on membrane potential and intracellular pH. Another way to study the impact of mitochondrial Ca^{2+} fluxes on cell signaling is to inhibit Ca^{2+} extrusion from mitochondria. Mitochondrial Ca^{2+} extrusion in most cells is primarily mediated by the mitochondrial NCX, which is readily inhibited by the benzothiazepine compound CGP-37157 [30] and somewhat less potently and selectively by diltiazem [26]. While CGP-37157 effectively blocks the mitochondrial NCX, it is also reported to inhibit several other targets such as voltage-dependent Ca^{2+} channels, which necessitates accompanying control experiments [31,32]. Addition of CGP-37157 to cells effectively traps Ca^{2+} inside the mitochondrial matrix whenever mitochondria accumulate Ca^{2+}, preventing trans-mitochondrial Ca^{2+} fluxes and the propagation of Ca^{2+} ions by mitochondria inside cells [33,34].

4. Shaping of cytosolic Ca^{2+} signals by mitochondria

The mismatch between rapid Ca^{2+} uptake by the uniporter and slow Ca^{2+} release by the exchanger, combined with the presence of a high concentration of phosphate inside the mitochondrial matrix, endows mitochondria with the capacity to act as a temporary storage compartment for Ca^{2+}. The shuffling of Ca^{2+} by mitochondria alters the amplitude of Ca^{2+} signals as well as their spatial and temporal dimensions. By diverting Ca^{2+} ions away from the cytosol, mitochondria blunt the peak of the cytosolic Ca^{2+} elevations, while the delayed release of the Ca^{2+} that has been buffered by mitochondria creates a shoulder that prolongs the Ca^{2+} signal. Mitochondria can thus turn brief, high-amplitude Ca^{2+} transients into biphasic, low amplitude Ca^{2+} elevations, a mechanism that plays a significant role in neurons by shaping the plateau phase of the Ca^{2+} response in presynaptic terminals ([35,36], reviewed in [6]). In the spatial domain, mitochondria can act either as propagating devices or as Ca^{2+} confining devices, depending of the cellular context. This dichotomy was already apparent in early studies, as energized mitochondria were shown to facilitate the propagation of Ca^{2+} waves in oocytes [37] but to limit the propagation of Ca^{2+} waves in astrocytes [38]. Whether mitochondria act as Ca^{2+} propagating or Ca^{2+} confining devices depend on their spatial arrangement. In pancreatic acinar cells, mitochondria are arranged in a belt that separates the apical and the basolateral pole of these polarized secretory cells. By their ability to buffer Ca^{2+}, mitochondria effectively prevent the propagation of Ca^{2+} signals from the apical pole to the basolateral pole, restricting the diffusion of the Ca^{2+} signal to the site of secretion [39]. However, the same mechanism of mitochondrial Ca^{2+} buffering can be used in a totally opposite manner to funnel Ca^{2+} ions inside cells and thus to propagate Ca^{2+} signals. In Hela cells, mitochondria recycle Ca^{2+} back to the endoplasmic reticulum during Ca^{2+} release [40], and transfer Ca^{2+} ions from plasma membrane channels to intracellular stores during Ca^{2+} influx [41]. By acting as a temporary Ca^{2+} store, mitochondria transfer Ca^{2+} ions from discrete sites of capture to a wide region surrounding the active mitochondria (Fig. 3). This process plays a significant role in the refilling of intracellular stores, because the slow kinetics of the exchanger better matches the Ca^{2+} pumping kinetics of SERCA pumps than the explosive increase achieved with the opening of an ion channel. By facilitating the transmission of Ca^{2+} ions inside cells, mitochondria thus enable a few ion channels to sustain the efficient refilling of large intracellular Ca^{2+} stores. This Ca^{2+} funnelling process is so efficient that mitochondria enable store refilling to take place even without detectable changes in $[Ca^{2+}]_{cyto}$, as discussed below.

Although the imbalance between Ca^{2+} uptake and Ca^{2+}extrusion from mitochondria causes detectable changes in $[Ca^{2+}]_{mito}$ during bouts of Ca^{2+} signaling, such high cytosolic concentrations are only

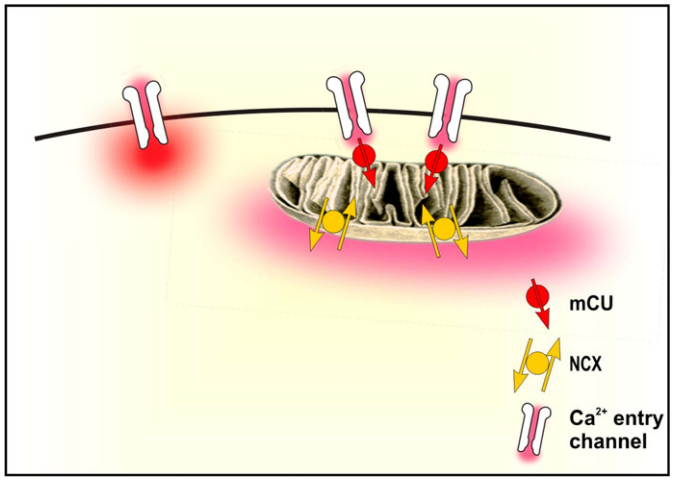

Fig. 3. Ca^{2+} propagation by mitochondria. The opening of plasma membrane channels produces spatially restricted area of elevated Ca^{2+} (left). The presence of mitochondria beneath the active channels enable the diffusion of Ca^{2+} ions to a larger cellular domain as the Ca^{2+} taken up by mitochondria is subsequently extruded by the organelle (right). The main Ca^{2+} transporters are represented.

reached transiently and locally. Most physiological Ca^{2+} signals remain within a range of intermediate Ca^{2+} concentrations, and do not translate into visible increases in $[Ca^{2+}]_{mito}$ as the Ca^{2+} ions entering mitochondria via the mCU are immediately buffered by phosphate within the mitochondrial matrix and extruded by the mNCX. The Ca^{2+} value at which the Ca^{2+} uptake rate exceeds the rate of Ca^{2+} extrusion is known as the "setpoint" [42], and has been estimated to range between ~500 and 800 nM in studies from isolated mitochondria [18]. Below the setpoint, Ca^{2+} entry across the uniporter is balanced by Ca^{2+} extrusion by the mNCX, and no net changes in the mitochondrial free Ca^{2+} occurs. This does not mean, however, that Ca^{2+} ions do not enter mitochondria during submicromolar Ca^{2+} increases in $[Ca^{2+}]_{cyto}$. Instead, in these conditions, the model by Chalmers and Nicholls would predict that Ca^{2+} cycle across the inner membrane of mitochondria without altering the free Ca^{2+} concentration within the mitochondrial matrix. These "silent" fluxes of Ca^{2+} can only be detected in cells by pharmacological means, i.e. either by blocking the uniporter with ruthenium red or by inhibiting the exchanger with CGP 37157. This strategy was used to show that silent transfer of Ca^{2+} ions across mitochondria indeed occur during stimulation with agonists. In endothelial cells, preventing mNCX caused a large $[Ca^{2+}]_{mit}$ elevation and a corresponding decrease in $[Ca^{2+}]_{ER}$, revealing that mitochondria were silently funnelling Ca^{2+} ions from plasma membrane channels to the ER [43]. The efficient refilling of the ER in this cell type thus required the presence of a trans-mitochondrial Ca^{2+} flux that did not translate into detectable changes in the free $[Ca^{2+}]_{mit}$, highlighting the capacity of mitochondria to function as a stealthy Ca^{2+} propagating device within cells.

5. Dynamics of mitochondria

One of the most interesting aspects of mitochondria now being unraveled is that cells have the ability to regulate the shape, mobility and connectivity of their mitochondria. Mitochondria are dynamic organelles that actively move, fuse, and divide within cells [44]. In mammalian cells, the fusion of mitochondria is regulated by a family of mitochondrial proteins, the large dynamin-related GTPases Mitofusin (Mfn) 1 and 2 and Optic Atrophy 1 (Opa1) [45,46], while fission is regulated by the cytosolic dynamin-related protein 1 (Drp1) and its receptor on the outer mitochondrial membrane Fis1 [47,48]. Proper mitochondrial fusion is essential for embryonic development [45] and for neuronal function, as highlighted by human diseases linked to mutations in mitochondrial fusion proteins. Mutations in Mitofusin 2 cause Charcot-Marie-Tooth disease type 2A [49], a neuropathy characterized by defects in the longest motor and sensory nerves, while mutations in Opa1 are associated with autosomal dominant optical atrophy, a loss of visual acuity due to the degeneration of retinal ganglion cells [50]. Mitochondrial motility is of particular importance for neurons, because mitochondria must cover a great distance to travel from the cell body to the periphery of long motor or sensory neurons. Neurons are thus highly sensitive to disturbances in mitochondrial movement and distribution. Mitochondria in multicellular eukaryotes move along microtubule tracks and their motility is controlled by a set of motor and adaptor proteins [51], including the outer mitochondrial membrane Miro GTPases that contain two calcium-binding motifs [52]. In cardiac myocytes, the motility of mitochondria was reduced at high Ca^{2+} levels, an effect likely mediated by the Miro 1 and Miro2 proteins [53]. By this mechanism, mitochondria can be immobilized at sites of Ca^{2+} elevations, near the mouth of Ca^{2+} entry or Ca^{2+} release channels. This provides a built-in mechanism for the stabilization and amplification of Ca^{2+} signals, as Ca^{2+} buffering by mitochondria prevents the inactivation of the channels and enable the entry of more Ca^{2+} ions that can be used to immobilize additional mitochondria in the vicinity. In neurons, the anchoring of axonal mitochondria requires the protein syntaphilin, and altered recruitment of mitochondria in axons lacking syntaphilin was associated with abnormal Ca^{2+} signals at presynaptic boutons [54]. Anchoring of stationary mitochondria below the plasma membrane is particularly important for Ca^{2+} signaling, because a plethora of plasma membrane Ca^{2+} transporters are regulated by Ca^{2+} or ATP and are thus sensitive to mitochondria. In Hela cells, removal of subplasmalemmal mitochondria by overexpressing hFis1 impaired the refilling of ER Ca^{2+} stores, rendering the cells more prone to ER Ca^{2+} depletion. As a result of the ER depletion, the activity of store-operated Ca^{2+} channels was increased, leading to a compensatory increase in the activity of plasma membrane Ca^{2+} pumps [55]. In smooth muscle, a similar hFis-1-mediated relocalization of mitochondria away from the plasma membrane also had a profound effect on the refilling of the sarcoplasmic reticulum during agonist stimulation [56]. In this case, reversal of plasmalemmal Na^+/Ca^{2+}-exchange was the primary Ca^{2+} entry mechanism, and loss of sub-plasmalemmal mitochondria was associated with increased cytosolic $[Ca^{2+}]$ elevations. Increase in $[Ca^{2+}]_{cyto}$ fragment mitochondria by activating the Ca^{2+}-dependent phosphatase calcineurin, which activates Drp1 to drive hFis1-mediated mitochondria fission [57], and alterations in Ca^{2+} handling associated with a fragmentation of the mitochondrial network are observed in hypertensive vasculopathies [58]. The loss of subplasmalemmal mitochondria might be a critical factor in this cascade, because the resulting amplification of the Ca^{2+} signals might activate calcineurin and favor further fragmentation of mitochondria.

6. Mechanisms of the modulation by mitochondria

In essence, the mechanisms that mitochondria use to modulate intracellular targets can be separated in two categories, ionic and metabolic. Mitochondria move ions while consuming and producing metabolites, and the resulting changes in the chemical microenvironment alters several cellular functions. The ionic effects primarily involve changes in the local concentration of Ca^{2+} ions, as already mentioned and discussed in more detail in the following section. Mitochondria also alter the Na^+ and H^+ concentrations in their vicinity and thus might modulate pH- or Na^+-dependent cellular processes, but examples of such modulations have not yet been reported. The metabolic effects comprise either a delivery of metabolic products, primarily ATP, or the consumption of metabolic substrates, primarily pyruvate. Supply of ATP directly drives the activity of energy-dependent processes, fueling the activity of membrane ATPases. ATP also indirectly regulates the activity of several channels and exchangers that are allosterically regulated by nucleotides. Interestingly, ATP is in fact a dual agent that has both ionic and metabolic effects, because ATP is also a potent Ca^{2+} buffer, a fact that is often overlooked [59]. Delivery of ATP at the right time and place can thus not only energize and allosterically regulate transporters, but also directly regulate Ca^{2+}-dependent processes. Note that while mitochondria are usually viewed as a source of ATP, when oxidative phosphorylation is defective mitochondria consume ATP via the reverse activity of the F_1F_0 ATPase. In this inverted mode, mitochondria can, in theory, modulate transporters in their vicinity by decreasing the local ATP concentration. An increased concentration of pyruvate, on the other hand, has been shown to increase the activity of the CRAC channel in mast cells [60], suggesting that mitochondria can regulate transporters in their vicinity by altering the concentration of metabolic substrates. Finally, mitochondria can also deliver other metabolic products, such a glutamate, which has been involved in the regulation of exocytosis in insulin-secreting cells [61]. Although complex and very diverse effects can stem from the integration of these metabolic and ionic changes, a few common rules emerge: 1) all processes require a close proximity between mitochondria and their target protein(s), because the concentration of ions and metabolites is tightly controlled within cells. The location of mitochondria is thus critical for their ability to regulate signaling processes. 2) The ionic

and metabolic pathways are usually engaged simultaneously, because Ca^{2+} uptake by mitochondria increases the metabolic activity of the organelle. Ca^{2+} regulates the activity of three rate-limiting enzymes of the tricarboxylic cycle, causing Ca^{2+} signals to be translated into metabolic responses by mitochondria [9]. This implies that the local ATP concentration increases while the local pyruvate concentration decreases when mitochondria take up Ca^{2+}. 3) Both additive and opposite modulations can occur during concurrent ionic and metabolic changes. For instance, the supply of ATP together with the slow release of Ca^{2+} by the mitochondrial mNCX provides both substrate and energy for the activity of Ca^{2+} pumps located on the ER (SERCA) or plasma membrane (PMCA). Ca^{2+} uptake combined with ATP released by mitochondria synergize to buffer local $[Ca^{2+}]_{cyt}$ increases, preventing the Ca^{2+}-dependent inactivation of Ca^{2+} entry and of Ca^{2+} release channels. By relieving their Ca^{2+}-dependent inhibition, mitochondria drastically change the temporal pattern of activation of these channels, which can remain active for several minutes in the presence of nearby mitochondria. At the same time, ATP can positively or negatively regulate membrane channels and exchangers, closing K_{ATP} channels [62] or enhancing the activity of the plasmalemmal NHEs [63]. The next sections discuss the plasma membrane channels and transporters that are modulated by mitochondria.

7. Regulation of Ca^{2+} entry channels by mitochondria

7.1. Store-operated Ca^{2+} channels

Store-operated Ca^{2+} entry (SOCE) is a process whereby the depletion of the ER Ca^{2+} store leads to the opening of plasma membrane Ca^{2+} channels. Originally described by Putney in parotid acinar cells [64], SOCE turned out to be ubiquitous and to control numerous cellular functions such as lymphocyte proliferation [65]. The fundamental reason for store-operated Ca^{2+} entry is that cells inevitably loose Ca^{2+} during signaling, as Ca^{2+} ions released into the cytoplasm by the opening of Ca^{2+} release channels on the ER/SR are pumped out of the cell by plasma membrane Ca^{2+} ATPases (PMCA). To ensure sustained signaling, the Ca^{2+} ions that have been extruded must be returned to the cytosol and ultimately re-accumulated into intracellular stores by SERCA. SOCE has been best characterized electrophysiologically as the prototypical Ca^{2+} release-activated Ca^{2+} (CRAC) current of blood cells [66]. Other cell types have SOCE with different characteristics than CRAC, often called CRAC-like [66]. The CRAC current (I_{CRAC}) is characterized by a very low single channel conductance, a high Ca^{2+} selectivity, an inward rectification and a complex regulation by both intra- and extracellular Ca^{2+} (reviewed in [66]). The regulation of CRAC channels by intracellular Ca^{2+} comprises a fast and a slow Ca^{2+}-dependent inactivation processes. The slow Ca^{2+}-dependant inactivation develops over tens of seconds following CRAC activation [67,68], and is prevented by intracellular Ca^{2+} buffers like EGTA. Slow inactivation by Ca^{2+} is a well-described feature of the I_{CRAC}, but its precise mechanism remains unknown.

Mitochondria have been shown to modulate several steps in the store-operated Ca^{2+} entry process, by buffering Ca^{2+} ions or by supplying ATP and other soluble factors. Mitochondria modulate the activity of CRAC channels by buffering the incoming Ca^{2+} ions, thus relieving the feed-back inhibition of the channels by Ca^{2+}. Most studies describing this mode of regulation were performed on blood cells, by analyzing the effect of mitochondrial inhibitors on the I_{CRAC} current. It was reported for the first time in 1997 that functional mitochondria are required to allow a sustained SOCE in T lymphocytes [69]. Preventing mitochondrial Ca^{2+} uptake with CCCP or antimycin A led to a transient cytosolic Ca^{2+} elevation following store depletion, without affecting the activation phase. Similar effects were reproduced in Jurkat T cells, in which mitochondria were required to sustain the CRAC currents, but were not necessary for CRAC activation [70]. The importance of mitochondria for sustaining I_{CRAC} was further extensively studied by the group of A. Parekh on RBL-1 cells and Jurkat T lymphocytes [71,72]. By introducing in the patch pipette an "energizing cocktail" that sustains the mitochondrial respiration, they showed that I_{CRAC} could develop under physiological conditions. Until then, to record I_{CRAC} in the whole-cell configuration, SERCA pumps had to be inhibited and a high concentration of Ca^{2+} buffer included inside the patch pipette (typically 10 mM EGTA or BAPTA) to prevent any kind of Ca^{2+}-dependent inactivation of the current. The ability of energized mitochondria to substitute for artificial Ca^{2+} buffers highlighted their Ca^{2+} buffering capacity. The role of mitochondria was then studied on other cell types by Ca^{2+} imaging. These studies gave a slightly different outcome, as preventing mitochondrial function reduced both the activation and the sustained phase of SOCE in endothelial cells [43], Hela cells [73] and myotubes [74]. Thus, in these "non-blood" cells, inhibiting mitochondrial function affected not only the sustained phase of the Ca^{2+} influx, but also the initial phase of the Ca^{2+} entry process. This subtle difference between non-blood cells and blood cells in the sensitivity of the activation phase to mitochondria might well reflect a difference in the nature of the channel supporting SOCE, which might not necessarily be the CRAC channel, or a distinct sensitivity of SOCE to intracellular Ca^{2+} in different cell types.

In order for mitochondria to buffer efficiently Ca^{2+} and to prevent the slow Ca^{2+}-dependent inactivation, it was proposed that the organelles should be located close to the Ca^{2+} entry channel [7]. Confirming this assumption, Quintana et al. [75] showed in T-lymphocytes that mitochondria translocate to the plasma membrane during Ca^{2+} entry and take up large amounts of entering Ca^{2+}. Preventing this movement with nocodazole to de-polymerize microtubules led to a less sustained Ca^{2+} entry phase. These observations point to a double positive feedback whereby Ca^{2+} entry "attracts" mitochondria close to the PM, while the recruited mitochondria in turn allow more sustained Ca^{2+} entry. The necessity for mitochondria to be close to the PM was further supported by data showing that the relocation of the mitochondria far from the PM in Hela cells overexpressing dynamitin reduced SOCE significantly [76]. Interestingly, in the same cell type, the relocation of mitochondria away from the PM by overexpressing hFis1 did not decrease SOCE [73], although functional mitochondria were still required for full SOCE activation. The reason for such discrepancy is not known but might give credit to another aspect of mitochondrial regulation of SOCE. Indeed, in parallel to the previously cited works, it was proposed that mitochondria may release compounds that act positively on CRAC [77]. Recently, it was shown in Jurkat T-cells that ATP produced by mitochondria efficiently buffers Ca^{2+} and thus prevents the slow Ca^{2+}-dependent inactivation of the CRAC [59]. Unlike mitochondria, ATP is a small mobile buffer that can diffuse in restricted compartments and might exert a buffering action at sites that mitochondria cannot reach. This later point needs to be experimentally confirmed but it opens a new perspective regarding the role of the mitochondria, especially in the context of the recent discoveries on the mechanism of SOCE activation. During the last 3 years, two key players were discovered that play a central role in the machinery leading to SOCE; STIM1 and Orai1. STIM1 is a single spanning transmembrane protein located mainly on the ER membrane, which possesses an EF hand motif on its ER-luminal domain [78–80]. Orai1 is a plasma membrane protein with four predicted transmembrane domains that form a critical component of the CRAC channel, if not the channel itself [81,82]. The current view is that the ER Ca^{2+} sensor STIM1 re-localizes and forms puncta at the junction of the ER and the PM upon store depletion. By a mechanism that remains to be fully elucidated, STIM1 interacts with Orai1, leading to its activation. Such a spatial arrangement implies a very close association between the superficial part of the ER and the PM, estimated to be around 10–25 nM [83]. Previous EM studies in pancreatic acinar cells indicated that both the ER and mitochondria can be extremely close to the plasma membrane, and that the two

organelles exclude each other, with spots of ER-PM appositions and others of mitochondrial-PM apposition [84], reviewed in [85]. In this context, it becomes difficult to envisage a physical space for mitochondria between the superficial part of the ER and the PM (Fig. 4). It should be noted that since the discovery of STIM1 and Orai1 as the major players in SOCE activation, the role of mitochondria was not re-evaluated. The role assigned to mitochondria in previous studies would translate in a regulation of the Orai1 channel, but whether mitochondria prevented the slow Ca^{2+}-dependent inactivation of the Orai1 current by buffering Ca^{2+}, by releasing ATP, or by other means has not been investigated.

Another regulatory role of intracellular Ca^{2+} on SOCE is the fast Ca^{2+}-dependent inactivation, which results from a negative feedback of Ca^{2+} entering the SOCE on the channel itself. This process occurs within milliseconds and can be partially prevented by the use of fast Ca^{2+} chelators like BAPTA [86]. Unlike the slow Ca^{2+}-dependent inactivation, mitochondria were not reported to be involved in the prevention of the fast inactivation of the CRAC current [71,77]. However, Bakowski and Parekh [60] showed recently that the intermediate metabolite pyruvate reduced the extent of the fast Ca^{2+}-dependent inactivation. Several questions remain to be answered regarding the physiological relevance of such a regulatory mechanism, but as pyruvate is metabolized within the mitochondria, it is tempting to speculate that the metabolic status of the organelle can influence the concentration of surrounding pyruvate and thus the extent of fast Ca^{2+}-dependent inactivation of SOCE.

The third regulation of SOCE by mitochondria is linked to the level of store depletion achieved during cell stimulation. In order for I_{CRAC} to be activated, a substantial level of store depletion has to be reached, while at the end of the process store refilling deactivates SOCE. Parekh's group showed that the amount of IP_3 required to activate I_{CRAC} is reduced if mitochondria are maintained in an energized state [72]. The proposed explanation is that the Ca^{2+} release from the ER is taken up by mitochondria, causing a more pronounced ER Ca^{2+} depletion that facilitates I_{CRAC} activation. In other words, for the same level of IP_3, and by extension of cell stimulation, the ER is more depleted and thus promotes a larger I_{CRAC} when mitochondria are fully functional. This assumption appears to be in contradiction with studies using genetically encoded Ca^{2+} indicators targeted to the ER, which showed that mitochondria promote store refilling by recycling Ca^{2+} ions back to the ER or by funneling Ca^{2+} ions from SOCE channels to the ER [40,43,56]. Several reports pointed to a role of mitochondria as organelles that minimize the level of store depletion during physiological stimulation, reducing SOCE activation rather than favoring it. The discrepancy with earlier data obtained with electrophysiological recordings might reflect the different methodologies used, and Parekh recently proposed a unifying theory in which mitochondria promote store depletion initially and subsequently favor ER refilling, thus playing a role at each end of the store-operated Ca^{2+} entry process [87]. In this framework, the role of mitochondria in simply supplying ATP should not be underestimated. Ca^{2+} pumping by both SERCA and PMCA is an ATP-dependent process that is favored by the presence of respiring mitochondria. By supplying ATP to the PMCA, mitochondria favor the shift of Ca^{2+} ions from the lumen of the ER to the extracellular solution during periods of signaling, and thus favor SOCE activation. In contrast, by energizing SERCA, mitochondria favor store refilling and prevent SOCE activation. Whether mitochondria enhance or reduce the level of ER depletion thus depends on the

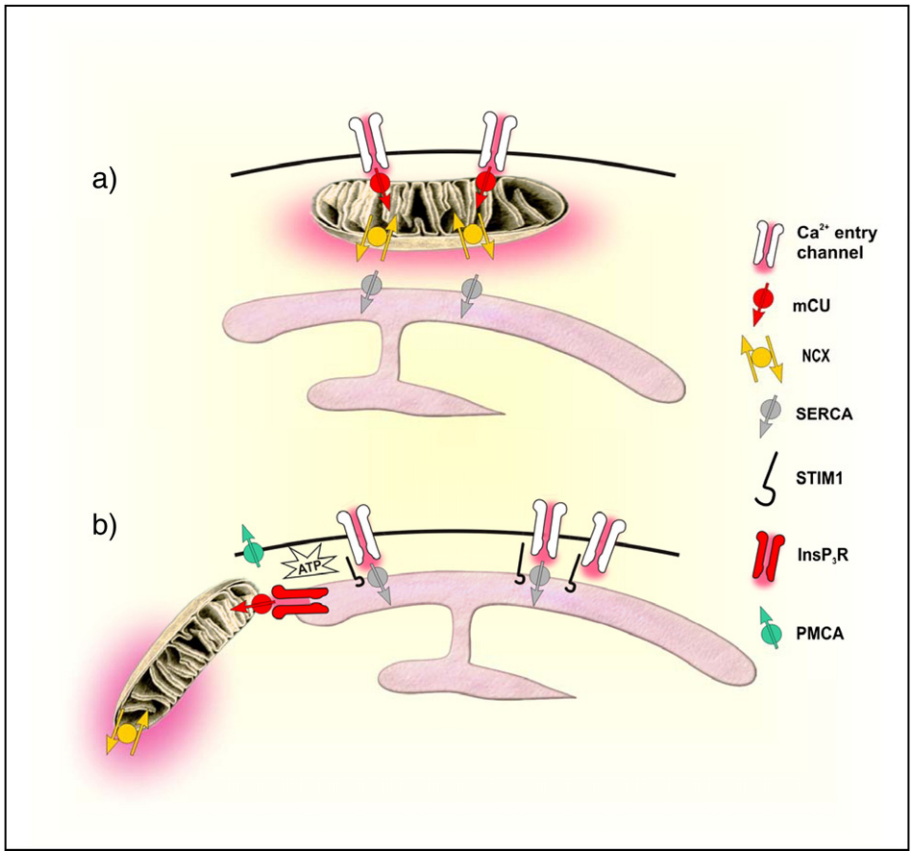

Fig. 4. Modulation of store-operated Ca^{2+} entry by mitochondria. (a) Schematic view of the spatial arrangement enabling mitochondria to funnel Ca^{2+} ions from store-operated membrane Ca^{2+} channels to the endoplasmic reticulum Ca^{2+} stores. (b) Current model of the STIM1 and Orai1 interactions that regulate store-operated entry. The tight junctional space between the endoplasmic reticulum and the plasma membrane precludes direct contact between Ca^{2+} channels and mitochondria. How mitochondria modulate SOCE in this context remains to be established, but ATP produced locally by mitochondria can act as a Ca^{2+} buffer system and provides the energy necessary to fuel the Ca^{2+} pumps on the ER (SERCA) and plasma membrane (PMCA). Both effects will favor the maintenance of a low Ca^{2+} concentration below the plasma membrane.

spatial organization of the organelles and on the relative contribution of mitochondria in buffering Ca^{2+} near Ca^{2+} release and Ca^{2+} entry channels and in supplying ATP for SERCA and PMCA.

7.2. Non-Store operated channels

In non-excitable cells, Ca^{2+} entry does not exclusively take place through the SOCE pathway, but also through other non store-operated Ca^{2+}/cation channels. Among these Ca^{2+} entry pathways, the arachidonic acid activated Ca^{2+} channel (ARC) is the best characterized [88]. To the best of our knowledge, the possible impact of mitochondria on ARC channels is so far unfortunately not assessed. We showed recently that on endothelial cells, agonist-induced Ca^{2+} entry is due in large part to receptor-activated Ca^{2+} entry (RACE) channels [89] and not to SOCE channels. During physiological stimulation by histamine, preventing the mitochondrial function only modestly affected the Ca^{2+} entry phase. In contrast, SOCE activation by store depletion with thapsigargin was highly sensitive to mitochondrial inhibition. Thus, the RACE pathway in endothelial cells appears to be, at least acutely, independent from the mitochondrial function.

7.3. TRP channels

The vast majority of Ca^{2+} entry channels belong to the TRP family of cation channels, which comprises 6 mammalian subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPA (ankyrin) and TRPML (mucolipin) [90]. The Ca^{2+} selectivity of TRP channels varies greatly from a very high Ca^{2+} selectivity for TRPV5 and TRPV6 to Ca^{2+}-impermeable channels for TRPM4-5 [91]. Whether or not mitochondria may modulate the activity of TRP channels is largely unknown except for three members, TRPM2, TRPM7 and TRPV1. TRPM2 is found mainly in the brain, but also in pancreatic beta-cells, intestinal cells and various immune cells. Multiple factors can modulate its activity such as intracellular Ca^{2+}, oxidative stress, ADPR, NAD^+ or cADPR [92]. In addition, TRPM2 has a peculiar characteristic as it possess an ADP-ribose hydrolase activity on its C-terminal part, designated NUDT9-H (nucleoside diphosphate linked moiety X-type motif 9 homology domain). Even though the enzymatic activity, transforming ADPribose to AMP and ribose 5′ phosphate, is likely not required for the channel gating, it has been shown that ADPR binds to the NUDT9-H domain to induce the channel opening [93]. Indeed, inclusion of ADPR in the patch pipette induced large whole-cell currents, and in inside-out configuration ADPR stimulates single channel activity in TRPM2 overexpressing cells [94,95]. Physiologically, the source of ADPR is not fully elucidated, but mitochondria were reported as being potentially involved in ADPR production. Interestingly, Perraud et al. [96] showed that oxidative stress stimulates a mitochondrial metabolic pathway leading to the formation of ADPR. However, mitochondria are not the only source of ADRP and thus their role in the activation of TRPM2 requires further experimental evidences. TRPM7 is a Mg^{2+} and Ca^{2+} permeable channel, ubiquitously expressed and constitutively activated at low level in resting cells. Like TRPM2, it has an enzymatic activity on the C-terminal part demonstrated to be a kinase. Free cytosolic Mg^{2+} and Mg^{2+}-nucleotide are inhibitors of TRPM7 that might explain its low level of activity at rest. Recently, Kim et al. [97] showed that CCCP and oligomycin have inhibitory effect on the whole-cell current supported by TRPM7. The authors suggest that the mitochondrial membrane potential and the F_1/F_0 ATP synthase might be important regulators of TRPM7. Whether the mechanism is linked to the ATP production and thus acts on the kinase domain of the channel is for the moment not known. In taste cells, mitochondria were recently reported to be involved in keeping the resting Ca^{2+} concentration at low levels, as poisoning mitochondria led to a considerable increase in cytosolic Ca^{2+} due to Ca^{2+} entry [98]. The authors proposed that TRPV1 is responsible, probably with other channels, for the significant basal entry of Ca^{2+} ions that are taken up by mitochondria and subsequently extruded from cells to maintain a low cytosolic Ca^{2+} concentration. It is not known, however, whether mitochondria in turn modulate the activity of TRPV1 in this system. As evident from the above-cited studies, the involvement of mitochondria as possible modulators of TRP channel function starts only to be explored, but considering the multiple regulation pathways acting on TRPs, it is likely that the field will bring new exciting findings.

7.4. Voltage-gated Ca^{2+} channels

The functional roles of voltage-gated Ca^{2+} channels (VGCC) are at least as diverse as the cells in which they are expressed. In many of these cells mitochondria functionally interact with the VGCC. As a comprehensive review of this topic is beyond the scope of this chapter, we only provide representative examples of known interactions between VGCC and mitochondria. As described above for other Ca^{2+} sources, mitochondria can buffer Ca^{2+} entry mediated by VGCC**.** This buffering reduces the diffusion of Ca^{2+} entry to limit downstream effector activity and to restrict spatially the range of Ca^{2+} signals. In vascular smooth muscle, mitochondrial buffering of VGCC-mediated Ca^{2+} entry reduces the activation of Ca^{2+}-sensitive Cl^- and K^+ channels, thus moderating positive feedback between Ca^{2+} influx and depolarization [99]. The moderation of Ca^{2+}-dependent vesicle fusion, as in adrenal chromaffin cells [100], is another common effect of mitochondria buffering the Ca^{2+} entering though VGGC. Similarly, in neurons mitochondria buffer and slowly release VGCC Ca^{2+} influx, which prevents asynchronous vesicle release at motor nerve terminals [101] and is essential for short term potentiation of glutamatergic synapses in the supraoptic nucleus [102]. In addition, Ca^{2+} buffering by mitochondria reduces Ca^{2+}-dependent inactivation of VGCC, as is the case for SOCE channels. This is particularly important in heart and neurons, when stimulation frequencies increase to levels where passive diffusion alone becomes insufficient to counteract the accumulation of Ca^{2+} near the mouth of the channel [103]. The interactions between mitochondria and VGCC can also have important pathological consequences. Mutations in the neuronal P/Q-type channels increase channel opening and underlie familial hemiplegic migraine and episodic ataxia. These mutations cause excess cerebellar granule cell death as early as 10 days post natal, and mitochondrial Ca^{2+} overload in response to this Ca^{2+} influx appears to contribute to cell death [104]. Beyond responding to VGCC Ca^{2+} entry, mitochondria can also indirectly regulate VGCC through modulation of K^+ channels. In pancreatic beta cells, increases in cytosolic glucose stimulate mitochondrial ATP production. The increase in ATP:ADP ratio inactivates K_{ATP} channels, depolarizes the beta cells and opens VGCC resulting in Ca^{2+} entry and insulin secretion (reviewed in [62]). In a variation on this theme, mitochondria function as O_2-sensors for vasoconstriction. Mitochondria in the ductus artereosus respond to increased O_2 concentrations at birth by increasing H_2O_2 production, which inhibits K_V channels, depolarizes the smooth muscle, and activates VGCC [105]. In contrast, this mechanism is activated by decreases in O_2 concentration in pulmonary resistance arteries, which is thought to be caused by reduced expression of ETC complexes I and III in this tissue relative to systemic blood vessels [106]. These few examples illustrate the diversity of the interactions of mitochondria with VGCC in health and disease.

8. Regulation of Ca^{2+}-activated K^+ channels by mitochondria

On endothelial cells, the stimulation by an IP_3 generated agonist leads to a transient Ca^{2+} release from the ER followed by a Ca^{2+} entry plateau phase. The Ca^{2+} elevation activates Ca^{2+}-dependent K^+ channel resulting in a membrane hyperpolarization, which in turns increase the driving force for Ca^{2+} entry. Using single channel recordings of the activity of large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) on EA.hy926 cells (an endothelial cell line derived

from human umbilical vein) has allowed to estimate the Ca^{2+} concentration under the PM. Combining this approach with the visualization of mitochondria expressing a fluorescent protein highlighted the ability of mitochondria to maintain low level of Ca^{2+} in their close environment during cell stimulation [107]. Indeed, when a functional mitochondria was localized under the patch pipette, cell stimulation with histamine minimally activated the BK_{Ca} channel, reflecting the buffering of Ca^{2+} by the mitochondria. On the contrary, when mitochondria were not visible under the pipette or when mitochondria were poisoned with FCCP, the channel was strongly activated by histamine, indicating a higher Ca^{2+} concentration in its close proximity. On smooth muscle cells also, poisoning mitochondria with CCCP or rotenone increased the basal single channel activity of K_{Ca} channel as a result of elevating Ca^{2+} concentration [108]. Thus, in the same cell, a Ca^{2+}-activated channel would have a low level of activity if localized close to mitochondria, while a Ca^{2+}-inhibited channel, like the SOCE channel, would have a strong activity. In non-excitable cells, the membrane hyperpolarization favors the entry of Ca^{2+}. By locally buffering Ca^{2+}, mitochondria might exert an opposite role of favoring Ca^{2+} entry by activating SOCE, while at the same time diminishing the membrane hyperpolarization and thus reducing the driving force for Ca^{2+} entry. One can speculate that this dual function serves as a fine-tuning of the Ca^{2+} signal generated during cell stimulation.

9. Interactions of mitochondria with the Na^+/Ca^{2+}-exchanger (NCX)

The Na^+/Ca^{2+}-exchanger (NCX) is a well known Ca^{2+} extrusion mechanism, but there is a growing appreciation that NCX reversal can mediate Ca^{2+} entry in physiological and pathological contexts, both in response to receptor stimulation and store-depletion [109–115]. Indeed, the interaction of mitochondria with the NCX is best characterized for the "reverse" Ca^{2+}-entry mode of NCX transport, and mitochondrial uptake of NCX-mediated Ca^{2+} entry can be readily generated by reducing extracellular Na^+ [116,117]. Physiological reversal of the NCX, however, requires significant increases in cytosolic $[Na^+]$ that only occur in restricted compartments, because cytosolic diffusion of Na^+ is very fast. In smooth muscle, the NCX clusters with TRPC channels and with the low-Na^+ affinity $\alpha 2$ Na^+/K^+-ATPase at diffusionally restricted junctions of the PM and sarco/endoplasmic reticulum (summarized in [118,119]). This molecular and structural organization permits the generation of local $[Na^+]_{cyto}$ elevations (LNaTs, localized Na^+ transients) that are sufficient to drive NCX reversal [21]. NCX-mediated Ca^{2+} entry is particularly important for Ca^{2+} store refilling during agonist stimulation in vascular smooth muscle [56], and mitochondria enhance the efficiency of NCX-mediated SR refilling by catching Ca^{2+} ions spilling from PM-SR/ER junctions and transferring them to the SR [56]. Thus, as in endothelial cells [43], mitochondria silently funnel Ca^{2+} ions to the SR and prevent elevations of bulk cytosolic $[Ca^{2+}]$ during NCX reversal [117]. In addition, mitochondria also buffer Na^+ spilling from the junctions in vascular smooth muscle, and the increased Na^+ delivery likely supports the increased activity of the mNCX that favors SR refilling while preventing mitochondrial Ca^{2+} overload. By these sequential actions, mitochondria effectively contain the local Ca^{2+} and Na^+ elevations to the sub-plasmalemmal cytosol. Mitochondria appear to play a less important role during the normal "forward" mode of NCX-mediated Ca^{2+} extrusion. NCX-mediated Na^+ entry into cells can in theory support the activity of the mNCX and thus couple Ca^{2+} efflux from mitochondria to Ca^{2+} extrusion from cells, but this aspect of the NCX-mitochondria interaction remains to be examined. Mitochondrial Ca^{2+} uptake can inhibit reversal of cardiac NCX over-expressed in CHO cells, suggesting that mitochondria may provide feedback regulation of the NCX [116], but the physiological relevance of these findings is not clear. The coordinated interactions between mitochondria, TRPC channels, and reverse NCX at PM-SR/ER junctions, on the other hand, may be particularly important in pathological conditions. In conditions of vascular injury and hypertension, TRPC channels take on a more substantial role in Ca^{2+} entry that is usually mediated by voltage-gated Ca^{2+} channels [120–122], and the contribution of the NCX to agonist-stimulated Ca^{2+} entry is increased [123]. During reperfusion following an ischemic event, NCX reversal is driven by the over-activity of Na^+/H^+ exchangers that clear the ischemic acid load, and mitochondria take-up the NCX-mediated Ca^{2+} entry. Furthermore, the hypoxic ischemia conditions inhibit respiration and depolarize mitochondria, favoring Ca^{2+} uptake rather than extrusion by the electrogenic mNCX. This renders mitochondria especially susceptible to Ca^{2+} overload during short ischemic events, and their demise can be prevented by inhibitors of reverse-mode NCX [124].

10. Conclusion

In summary, it is difficult to name a plasma membrane transport process that is not modulated by mitochondria. Although incomplete, our review hopefully covers most of the studies that established our current understanding of the mechanisms used by mitochondria to regulate Ca^{2+} transporters, exemplified by the complex interactions between Ca^{2+} entry channels and mitochondria. Among the arsenal of tools that mitochondria use to positively or negatively modulate plasma membrane transporters, regulation by Ca^{2+} comes first, followed closely by regulation by ATP. Current evidence suggests that the role of other ions, metabolites, or byproducts of the cellular respiration is marginal but these factors might gain importance as mitochondria are being studied in more physiological contexts. The location of mitochondria determines their ability to regulate the activity of membrane Ca^{2+} transporters, and a picture of mitochondria as silent propagators of Ca^{2+} inside cells is emerging that contrasts to the conventional view of mitochondria as fixed Ca^{2+} buffers

Acknowledgments

We thank Nadine Dupont for the artwork and the Swiss National Science Foundation for financial support (grants N° 31-118393/1 and N° 31-120186/1).

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