Cytoplasmic and Mitochondrial Calcium Signaling: A Two-Way Relationship

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Intracellular Ca^{2+} signals are well organized in all cell types, and trigger a variety of vital physiological processes. The temporal and spatial characteristics of cytosolic Ca^{2+} increases are mainly governed by the fluxes of this ion across the membrane of the endoplasmic/sarcoplasmic reticulum and the plasma membrane. However, various Ca^{2+} transporters also allow for Ca^{2+} exchanges between the cytoplasm and mitochondria. Increases in mitochondrial Ca^{2+} stimulate the production of ATP, which allows the cells to cope with the increased energy demand created by the stimulus. Less widely appreciated is the fact that Ca^{2+} handling by mitochondria also shapes cytosolic Ca^{2+} signals. Indeed, the frequency, amplitude, and duration of cytosolic Ca^{2+} increases can be altered by modifying the rates of Ca^{2+} transport into, or from, mitochondria. In this review, we focus on the interplay between mitochondria and Ca^{2+} signaling, highlighting not only the consequences of cytosolic Ca^{2+} changes on mitochondrial Ca^{2+}, but also how cytosolic Ca^{2+} dynamics is controlled by modifications of the Ca^{2+}-handling properties and the metabolism of mitochondria.

Mitochondria are organelles found in nearly all cell types of eukaryotic organisms, with the notable exception of erythrocytes (Repsold and Joubert 2018). They are characterized by two membranes, the inner mitochondrial membrane (IMM) that encloses the mitochondrial matrix and the outer mitochondrial membrane (OMM). These two membranes are a few nanometers apart and delimit the so-called intermembrane space (IMS). The OMM is smooth and highly permeable to most solutes, whereas the IMM is impermeable. Besides, the IMM is folded into cristae, allowing to increase the surface of this membrane (El-Hattab et al. 2018). Mitochondria form unconnected, functionally distinct entities (Collins et al. 2002) and their number varies from tens (in lymphocytes [Potter and Ward 1942]) to thousands (in hepatocytes) per cell (Weibel et al. 1969). They are usually considered as 1-μm wide spherocylinders, varying in length from 1 to 10 μm. These organelles of widely different sizes also display heterogenous cellular distribution, with a denser aggregation of mitochondria around the nucleus than in the cell periphery (Collins et al. 2002). Heterogeneity is further amplified by the

dynamic behavior of these organelles. Indeed, mitochondria continuously move along the cytoskeleton. They are able to grow, divide, or fuse with each other (Wang et al. 2012). Moreover, when submitted to stressful conditions, they can connect and form a network that allows a better adaptation to maintain cellular homeostasis (Zemirli et al. 2018).

The roles of mitochondria are numerous and notably include cell cycle regulation, apoptosis, bioenergetics, and also Ca^{2+} homeostasis (Murphy and Hartley 2018). Indeed, although Ca^{2+} signaling in nonexcitable cells mainly involves fluxes between the cytosol, the plasma membrane (PM) and the endoplasmic reticulum ([ER], the main Ca^{2+} storage of the cell), mitochondria, as well as other organelles such as lysosomes, are able to alter cytosolic Ca^{2+} signals (Dupont et al. 2011; Patel and Muallem 2011). At rest, the Ca^{2+} concentration inside mitochondria is of the same order as that in the cytosol (∼0.1 µM). The close apposition between mitochondria and ER membranes favors Ca^{2+} exchanges between these organelles (Rizzuto et al. 1998; Csordás et al. 1999, 2010). Cytosolic Ca^{2+} increases, mostly as a result of the release of ER Ca^{2+} through the inositol 1,4,5-trisphosphate receptor (IP_3R), can indeed be transmitted into mitochondria via the different transporters located in the mitochondrial membranes. As mitochondria can take up large amounts of cytosolic Ca^{2+}, this sequestration modifies the quantitative and dynamic characteristics of Ca^{2+} signaling in the cytosol (Nicholls and Chalmers 2004).

Ca^{2+} is an essential activator of mitochondrial metabolism and stimulates the synthesis of ATP molecules (Fig. 1A). Notably, Ca^{2+} activates three dehydrogenases involved in the Krebs cycle (Rizzuto et al. 2012). This cycle oxidizes acetyl-coenzyme A, typically issued from pyruvate generated by glycolysis. This oxidation is coupled with the reduction of NAD^+ into NADH. NADH molecules in turn transfer their electrons to the electron transport chain (ETC). The four first complexes of the ETC (I–IV) use the energy provided by electrons to export protons from the matrix into the IMS, hence generating an important transmembrane potential difference between the matrix and the IMM

($\Delta\Psi$). The resting mitochondrial $\Delta\Psi$ is around 150–160 mV, negative inside. Complex V, which is the F_1F_0-ATP synthase, consumes the energy provided by the electrochemical gradient to synthesize ATP (Murphy and Hartley 2018). The $\Delta\Psi$ is crucial not only for energetic production, but also for Ca^{2+} fluxes in and out of mitochondria. Indeed, the Ca^{2+} transporters through the IMM are electrogenic and dependent on the electrochemical gradient of Ca^{2+} between the cytosol and the mitochondrial matrix. Thus, changes in $\Delta\Psi$ affect the balance between influx and efflux of mitochondrial Ca^{2+}. Interestingly, this is not the case for transporters in the ER where fluxes of protons and other monovalent cations compensate the potential variations due to Ca^{2+} transport (Meissner 1981). Perturbations in mitochondrial Ca^{2+} dynamics can be associated with pathological conditions: disturbances in Ca^{2+} fluxes, in molecular mitochondrial components, or in the distance between mitochondria and ER can lead to impaired bioenergetics of the cell or cellular death (Contreras et al. 2010; Naon and Scorrano 2014; Mishra 2016; Wang et al. 2016). Ca^{2+} thus constitutes a signal of life and death that is finely tuned by molecular and geometric parameters (Berridge et al. 1998).

In this review, we focus on the interplay between mitochondria and Ca^{2+} signaling, highlighting not only the consequences of cytosolic Ca^{2+} changes on mitochondrial Ca^{2+}, but also how cytosolic Ca^{2+} dynamics can be altered by modifications of the Ca^{2+}-handling properties and the metabolism of mitochondria. We first list the main mitochondrial Ca^{2+} transporters and report evidence of the impact of their activity on mitochondrial and cytosolic Ca^{2+} dynamics. We next focus on the mitochondrial permeability transition pore (mPTP) and on its less well-established participation in mitochondrial Ca^{2+} dynamics. The bidirectional relation between Ca^{2+} signaling and metabolism, the main physiological target of mitochondrial Ca^{2+} increases, is then discussed. In the last section, we report observations highlighting the importance of the spatial arrangement of the Ca^{2+} transporters located in the ER and in the mitochondrial membranes.

MITOCHONDRIAL Ca²⁺ TRANSPORTERS

On cell stimulation, cytoplasmic Ca^{2+} increases are transmitted to mitochondria. Ca^{2+} ions need to cross two membranes to pass from the cytosol into the mitochondrial matrix (Fig. 1D). Ca^{2+} first passes through the OMM via a highly expressed voltage-dependent anion-selective channel (VDAC). This channel behaves as a dif-

fusion pore that is permeable to ions and small hydrophilic metabolites, thus accounting for the high permeability of the OMM. VDAC exists in three isoforms (1,2,3) and displays multiple conductance states (Shoshan-Barmatz et al. 2018). The so-called "closed" states (i.e., the states with the lower permeability to metabolites) have the highest permeability to Ca^{2+} (Tan and Colombini 2007). In HeLa cells stim-
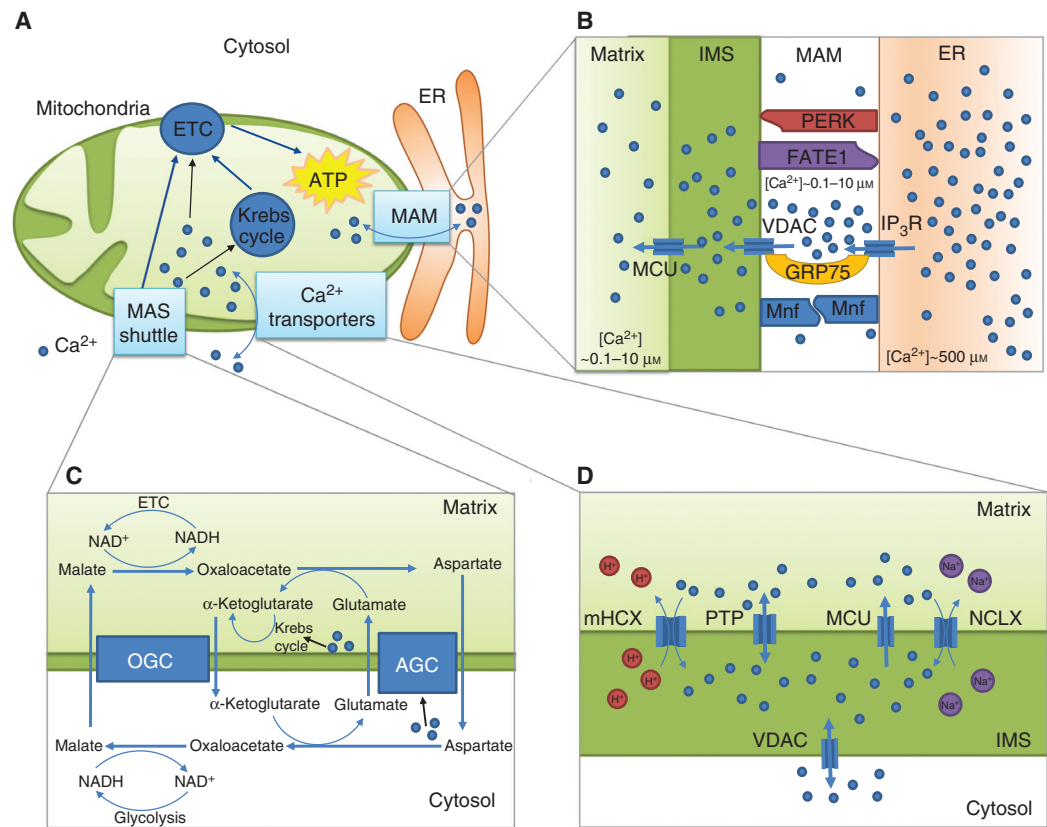

Figure 1. Schematic representation of some key aspects of mitochondrial Ca^{2+} dynamics and of the interplay between Ca^{2+} and mitochondrial metabolism (*A*). Ca^{2+} exchanges between the cytosol and mitochondria allow the activation of mitochondrial metabolism, by boosting the Krebs cycle and the electron transport chain (ETC). (*B*) Membranes of the ER and the mitochondria are linked via tethering proteins (PERK, FATE, GRP75, Mfn, and others not indicated in this simplified scheme) to form mitochondria-associated membranes (MAMs). These membranes delimit areas where Ca^{2+} can reach high concentrations. (*C*) The electrons of the NADH produced in the cytosol by glycolysis are transferred into mitochondria by the malate-aspartate shuttle (MAS). The aspartate-glutamate carrier (AGC) of the MAS is activated by cytosolic Ca^{2+}. At high mitochondrial Ca^{2+} concentration, the activation of the Krebs cycle consumes α-ketoglutarate and limits the impact of the MAS. OGC stands for oxoglutarate carrier (see text for details). (*D*) Ca^{2+} has to cross two membranes to enter inside a mitochondrion: the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). The first one is passed via a voltage-dependent anion-selective channel (VDAC), a nonselective channel. More specific Ca^{2+} transporters are involved in the crossing of the IMM (see text for details).

ulated by histamine, overexpression of the three VDAC isoforms induces an increase in the mitochondrial Ca^{2+} levels, whereas silencing their expression tends to decrease mitochondrial Ca^{2+} uptake (de Stefani et al. 2012). Similarly, in mouse embryonic fibroblasts, the knocking out of VDAC1 limits agonist-induced mitochondrial Ca^{2+} uptake (Monaco et al. 2015). In heart cells, VDAC2-dependent mitochondrial Ca^{2+} uptake plays a critical modulatory role in the regulation of cardiac rhythmicity, most probably by controlling the spatial and temporal extent of the Ca^{2+} increases in the cytosol (Shimizu et al. 2015). However, it is most often assumed that in physiological conditions the Ca^{2+} transport via VDAC across the OMM is kinetically not limiting.

The transfer of Ca^{2+} from the IMS into the mitochondrial matrix is much more complex. Various transporters are indeed expressed in the IMM (Fig. 1D). Ca^{2+} ions enter into mitochondria through a channel known as the mitochondrial Ca^{2+} uniporter (MCU). The MCU is located in the IMM, is highly selective to Ca^{2+}, and is inhibited by ruthenium red. The opening of the uniporter has a sigmoidal dependence on cytosolic Ca^{2+}, and is characterized by a rather low affinity for Ca^{2+} ($K_d \sim 2-10$ μM) (Mallilankaraman et al. 2012; Csordás et al. 2013; Paillard et al. 2017). However, the effective dependence of the MCU on Ca^{2+} is intricate, as when Ca^{2+} enters into the mitochondrial matrix, it provokes a decrease in $\Delta\Psi$ that diminishes the driving force for Ca^{2+} entry. Although the existence of a Ca^{2+} uniporter was postulated for a few decades thanks to functional studies of mitochondrial Ca^{2+} uptake, its molecular identity was identified much later as the 40-kDa MCU (Baughman et al. 2011; de Stefani et al. 2011). MCU oligomers form a highly selective Ca^{2+} pore that is part of a multiprotein complex composed of a large number of regulators, notably the mitochondria Ca^{2+} uptake proteins 1,2,3 (MICU 1,2,3) and the essential MCU regulator (EMRE). These regulators allow a fine tuning of the opening of the MCU pore (Mammucari et al. 2018; Nemani et al. 2018; Penna et al. 2018) and modulate the sigmoidal dependence on Ca^{2+} of the MCU (Csordás et al. 2013; Patron et al.

2014). Specific stoichiometries between the MCU and its regulators may be responsible for the observed tissue-specific differences of activities of the uniporter, and explains, for example, the variation in Ca^{2+} dynamics observed in mitochondria of heart and liver (Paillard et al. 2017; Wacquier et al. 2017). Indeed, as MICU1 is less expressed in cardiomyocytes, the Ca^{2+} uptake rate is higher at low cytosolic Ca^{2+} concentrations, but smaller at high cytosolic Ca^{2+} levels, as compared with hepatocytes that express much more MICU1. This tissue-specific MICU1:MCU expression ratio is highly relevant as heart cells characterized by a liver-like MICU1:MCU expression ratio display contractile dysfunction (Paillard et al. 2017). Interestingly, in modeling approaches, it is possible to provide a unifying description of mitochondrial Ca^{2+} dynamics in different cell types by taking into account the level of expression of the MCU (which is lower in heart cells [Fieni et al. 2012]) and the MICU1:MCU expression ratio (Wacquier et al. 2017). As a matter of fact, the regulation of the MCU is highly sophisticated and is covered in detail in recent reviews (Giorgi et al. 2018; Nemani et al. 2018; Wang et al. 2018). Although the MCU is the predominant Ca^{2+}-import mechanism, other transport systems have been reported to enable mitochondrial Ca^{2+} uptake. Such systems include mitochondrial ryanodine receptors in cardiomyocytes and neurons (Jakob et al. 2014), mitochondrial connexin 43 in cardiac cells (Gadicherla et al. 2017), and the rapid mode of Ca^{2+} uptake (RaM) in cardiomyocytes and hepatocytes (Buntinas et al. 2001). However, the exact roles of these channels remain elusive.

Ca^{2+} is extruded from mitochondria via exchangers. The IMM has two main types of Ca^{2+} exchangers: a H^+/Ca^{2+} exchanger (mHCX, expressed mainly in nonexcitable cells) and a Na^+/Ca^{2+} exchanger (NCX, expressed mainly in excitable cells). The mNCX activity is attributed to the protein NCLX (Palty et al. 2010). This transporter is generally thought to import three Na^+ in exchange for one Ca^{2+}, although the exact stoichiometry of this transporter is still controversial (Kim et al. 2013). NCLX is electrogenic and its activity depends on $\Delta\Psi$. It is noncoop-

eratively activated by mitochondrial Ca^{2+}, whereas it is cooperatively regulated by Na^+, with a Hill coefficient of 2 (Wingrove and Gunter 1986). Interestingly, it has been shown that the NCLX displays a reverse transport mode. This happens in depolarized mitochondria, under the control of the protein mitofusin 2. This reverse mode could be relevant in pathological conditions when $\Delta\Psi$ is transiently or chronically collapsed, as Ca^{2+} uptake via the MCU is dependent on $\Delta\Psi$ (Samanta et al. 2018).

The nature of the mHCX is still an ongoing and controversial issue. Some reports suggest that the leucine zipper EF hand-containing transmembrane (LETM1) protein is responsible for this activity. Indeed, in vitro, LETM1 favors an electrogenic exchange of H^+ and Ca^{2+} (Tsai et al. 2014). Additionally, HeLa cells that do not express LETM1 show higher mitochondrial Ca^{2+} levels, highlighting the importance of LETM1 in Ca^{2+} efflux (Shao et al. 2016). However, other studies indicate that mitochondrial Ca^{2+} efflux is mediated by the NCLX and not by LETM1 in HeLa cells. Indeed, in cells stimulated by histamine to generate cytosolic and mitochondrial Ca^{2+} signals, an overexpression of LETM1 does not change the rate of Ca^{2+} efflux from mitochondria (de Marchi et al. 2014b). Other groups also suggest that LETM1 is a K^+/H^+ exchanger. In this case, LETM1 would alter NCLX activity, maybe by changing the osmotic balance (Austin et al. 2017).

Through the action of uniporter and the exchangers, cytosolic Ca^{2+} signals are transmitted to mitochondria. Ca^{2+} oscillations can thus be observed in these organelles (Ishii et al. 2006). In some cell types, mitochondria are able to integrate the cytosolic Ca^{2+} oscillations. For example, in cat ventricular myocytes, the cytosolic oscillations induce a progressive elevation of the mitochondrial Ca^{2+} level (Sedova et al. 2006). In hepatocytes, the mitochondrial Ca^{2+} signals themselves are integrated into different levels of activation of metabolism (Hajnóczky et al. 1995). The physiological consequences of these increases in mitochondrial Ca^{2+} have been much described (Jouaville et al. 1999; Denton 2009; Griffiths and Rutter 2009; Williams et al. 2015). Less emphasis has been put on the fact

that mitochondria alter Ca^{2+} signaling in the cytosol, both by sequestering and releasing Ca^{2+}. Experiments and mathematical models highlight well-defined differences in the phases of the Ca^{2+} oscillations in different compartments (ER, cytosol, and mitochondria). Interestingly, it appears that between two successive cytosolic spikes of the oscillations, mitochondria are slowly releasing Ca^{2+} while the ER is still replenishing. It suggests that mitochondria are able to gradually release the Ca^{2+} accumulated during a single spike, allowing the reactivation of the IP_3R, and the triggering of a new spike (Ishii et al. 2006; Wacquier et al. 2016). Thus, Ca^{2+} release from mitochondria is in part responsible for the pacemaker Ca^{2+} increase in the cytosol that progressively activates IP_3R and stimulates Ca^{2+} release from the ER, before the onset of each spike of the oscillations. This is supported by data obtained in different cell types when using inhibitors of the MCU or of the NCLX. For example, in HeLa cells stimulated by histamine, siRNA-mediated reduction of MCU expression tends to slow down the cytosolic oscillations (Fig. 2A,B). This is explained by a lower accumulation of Ca^{2+} in mitochondria, and thus a weaker subsequent reactivation of IP_3R (Wacquier et al. 2016). In the same cell type, the addition of CGP37157, an NCLX inhibitor, favors fast oscillations (Hernández-SanMiguel et al. 2006). A more drastic impact of altering MCU expression is observed in mast cells, in which the proinflammatory leukotriene C4 signal induces damped Ca^{2+} oscillations in the cytosol. If the MCU is knocked down in these cells, the damping is hastened considerably (Samanta et al. 2014). Mitochondria also have a significant impact on the electric activity of heart cells. This activity is attributable to Ca^{2+} release from the sarcoplasmic reticulum (SR) and induces spontaneous heartbeats. Such a disturbance increases the risk of arrhythmia. In cardiomyocytes, this spontaneous electrical activity is sensitive to perturbations of the mitochondrial Ca^{2+} fluxes. Inhibition of the MCU by Ru360 indeed decreases the frequency of these beats. In a similar way, inhibiting the NCLX by CGP37157 completely inhibits spontaneous Ca^{2+} release, and the associated beats (Xie et al. 2018).
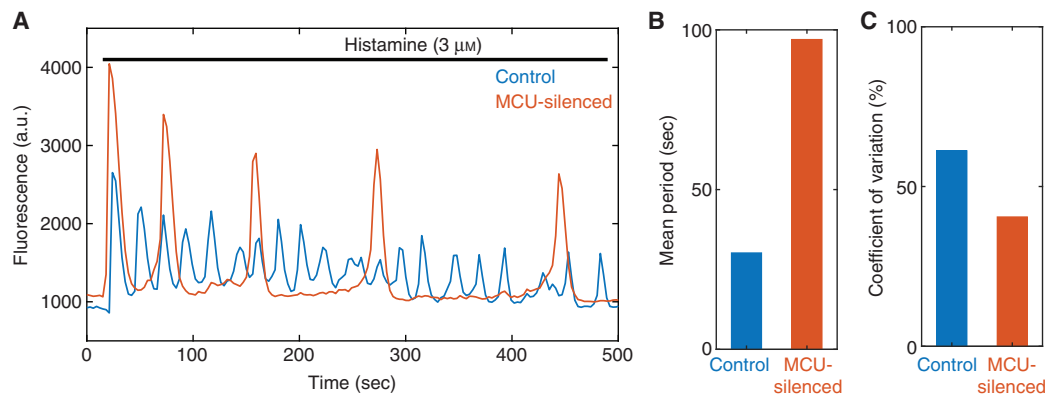
Figure 2. Impact of the mitochondrial Ca^{2+} uniporter (MCU)-silencing on cytosolic Ca^{2+} oscillations. (*A*) Cytosolic Ca^{2+}-probe fluorescence in HeLa cells stimulated with 3 µM histamine. The Ca^{2+} oscillations induced by histamine are drastically different in control cells (blue) compared with those in MCU-silenced cells (red), thus showing how Ca^{2+} handling by mitochondria can affect cytosolic Ca^{2+} signals. (*B*) Mean period of the Ca^{2+} oscillations shown in panel *A*. In MCU-silenced cells, the period of the cytosolic Ca^{2+} oscillations is much increased, as compared with control cells. (*C*) Coefficient of variation (CV) (standard deviation/mean) of the time interval between two successive peaks of these oscillations. The MCU-silenced cells display a lower CV, which is associated with more regular spikes. (The data are taken, with permission, from Wacquier et al. 2016.)

In HeLa cells, individual mitochondria of one single cell display heterogeneities in their Ca^{2+} profiles following a stimulation by histamine (Suzuki et al. 2014). It remains to be established whether such differences are the result of variations in the levels of expression of Ca^{2+} transporters, in mitochondrial Ca^{2+} buffering strengths, or in ER-mitochondria coupling. However, modeling approaches predict that changes in the mitochondrial properties (notably Ca^{2+} buffering or MCU level) greatly alter the amplitude of mitochondrial Ca^{2+} responses. In addition to these heterogeneities, mitochondria are likely to be the source of molecular noise because of their small volume. This is visible experimentally and in stochastic models (Lu et al. 2016; Gonze et al. 2018). Fluctuations in mitochondrial Ca^{2+} concentrations can be transmitted to rates of Ca^{2+} transport, and thus to cytosolic Ca^{2+} signals. In agreement with this hypothesis, considering mitochondria in stochastic models for Ca^{2+} oscillations increases their coefficient of variation ([CV] defined as the ratio between the standard deviation and the mean of the interspikes intervals) of ∼20% (Gonze et al. 2018). MCU-silenced HeLa cells display Ca^{2+} oscillations characterized by a lower

CV than those of a control case (Fig. 2C; Wacquier et al. 2016). This effect is attributable both to an increase of the period, and to stochastic disturbances in Ca^{2+} exchanges. In other words, the coupling between cytosolic and mitochondrial Ca^{2+} signaling has a cost in terms of the regularity of the cytosolic Ca^{2+} spikes.

MITOCHONDRIAL PERMEABILITY TRANSITION PORE

Under some circumstances such as mitochondrial Ca^{2+} overload or metabolic stress, a sudden increase in the permeability of the IMM can be observed. This phenomenon, called permeability transition (PT), is imputed to the opening of a nonselective channel, the mitochondrial permeability transition pore (mPTP). A sustained opening of this pore leads to the leakage of ions, metabolic molecules, and proapoptotic agents, resulting in mitochondrial swelling, dissipation of the electrochemical gradient, impaired ATP production, and, finally, cell death (Giorgio et al. 2018). The properties, and the regulation, of the mPTP have been highlighted in the late 1970s by the famous works of Hunter et al. (1976) and Hunter and Haworth (1979a,b,c). The opening

of the mPTP is favored by Ca^{2+}, extramitochondrial pH, fatty acids, cyclophilin D protein (CyPD), reactive oxygen species (ROS), or inorganic phosphates. It is inhibited by other divalent ions (Mg^{2+}, Sr^{2+}, Mn^{2+}), nucleotides, matrix acidification, $\Delta\Psi$, or cyclosporin A (CsA), a drug widely used to study the mPTP (Szabo and Zoratti 2014). Interestingly, mitochondrial Ca^{2+} is necessary for mPTP opening: in a suspension of mitochondria, the opening of the PTP (assessed by the swelling of mitochondria) can be triggered by drugs such as phenylarsine oxide (PhAsO) or p-hydroxyphenylglyoxal (OH-PGO). In the presence of the membrane permeant BAPTA-AM, responsible for mitochondrial Ca^{2+} chelation, the PhAsO- or OH-PGO-induced opening of the pore is blocked. This pinpoints the crucial role of mitochondrial Ca^{2+}, even in residual amounts. However, matrix Ca^{2+} is by itself not always sufficient to elicit PTP opening, as no swelling is noticed in the same experiments without PhAsO or OH-PGO addition (Giorgio et al. 2018).

However, despite its early discovery and characterization, the mPTP is still subject to intensive research as its molecular nature was unsolved until recently. It was long considered that the mPTP forms at a contact site between the IMMs and the OMMs, as a complex between the VDAC and the adenine nucleotide transporter (ANT). However, this idea was rejected on the basis of experiments involving genetic deletion of these components: mitochondria lacking ANT or VDAC are still able to undergo a PT inhibited by CsA (Kokoszka et al. 2004; Baines et al. 2007). The involvement of the F_1F_0-ATP synthase in PT was then proposed, because CyPD, an inducer of the mPTP, was found to bind this complex and to promote its partial deactivation, an effect that can be removed by CsA (Giorgio et al. 2009). This has been validated by experiments using purified dimers of ATP synthase reconstituted in lipid bilayers. In such a system, it is possible to trigger the opening of a channel, the electrophysiological properties of which are similar to those of the mPTP (Giorgio et al. 2013).

The molecular identification of the PTP allowed a better understanding of the interplay between the inducers of PT and the pore itself. For example, the reversible protonation of a histidine residue, located in the oligomycin sensitivity-conferring protein (OSCP) unit of the ATP synthase, has been shown to inhibit the opening of the pore (Antoniel et al. 2018). The Ca^{2+}-binding site of ATP synthase, which allows the opening of the pore, has recently been identified. Binding changes the conformation of the complex, leading to the opening of the pore (Giorgio et al. 2017).

Despite its recent molecular identification, the exact location of the pore in the ATP synthase still constitutes an ongoing issue. Two hypotheses emerge on this topic: the pore forms itself either between two monomers of ATP synthase or in the C subunit ring of the enzyme (the main transmembrane subunit forming the F_0 rotor). In both cases, it seems that a dimerization step is necessary: ATP synthases purified in a lipid bilayer do not display any conductivity in their monomeric form, but are permeable when assembled in dimers (Giorgio et al. 2013, 2017). It has been further suggested that the opening of the pore is associated with the dissociation of the dimers, as the opening of the mPTP leads to an increase of the monomer/dimer ratio. As C subunits appear to play a role in this dissociation, the opening of the ATP synthase dimers would be a result of a reorganization of the C ring (Bonora et al. 2013, 2017). Nevertheless, it has also been reported that the opening of the PTP can occur even in the absence of C subunits (He et al. 2017).

As mentioned above, the opening of the mPTP is favored by ROS (Bernardi et al. 2006), which can be generated as a consequence of ATP production by mitochondria and are important for cellular signaling, although they also contribute to oxidative stress and cellular damage (Feissner et al. 2009). Mitochondrial Ca^{2+} overload increases ROS production through complex II disintegration. These Ca^{2+}-induced ROS subsequently activate mPTP opening and cellular death (Hwang et al. 2014). On the other hand, depending on the targeted protein, the type and concentration of ROS, and the duration of exposure, ROS can stimulate or inhibit Ca^{2+} signaling (Csordás and Hajnóczky 2009; Tadic

et al. 2014). For example, at the ER-mitochondria interface, H_2O_2 generated by Ca^{2+} overload modulates agonist-induced Ca^{2+} release and oscillations (Booth et al. 2016). This effect may be because of changes in the redox environment and thus in the redox state of IP_3R1 (Joseph et al. 2018). There is thus an intricate cross talk between Ca^{2+} signals, ROS production, and mPTP opening, which is frequently dysregulated in severe human pathologies such as cardiovascular diseases, cancer, and neurodegeneration (Feissner et al. 2009; Marchi et al. 2017).

The physiological role of the mPTP depends on its conductance state. Indeed, the mPTP can open in two states: low and high conductance modes (Fig. 3A). The latter one is well known and occurs in stressful or pathological conditions. In this case, the pore becomes permeable to large molecules (<1.5 kDa). This opening is physiologically irreversible, leads to the dissipation of $\Delta\Psi$, and triggers cell apoptosis (Brenner and Moulin 2012). Pathological consequences linked to the mPTP are usually associated with increased Ca^{2+} uptake and overload, which tend to favor the opening of the pore. For example, the mPTP has a role in the ischemia/reperfusion injury (I/RI): when the blood flow in a vessel is occluded and then restored, an infarct, the size of
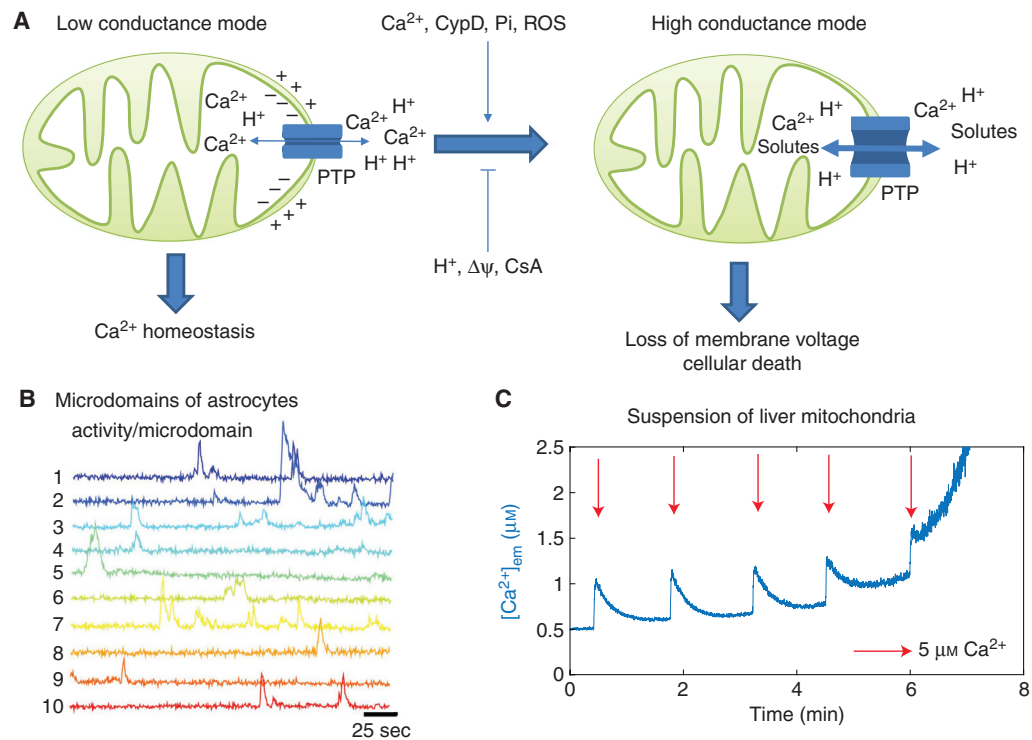

Figure 3. The mitochondrial permeability transition pore (mPTP) can open in two conductance modes. (*A*) Schematic representations of the mPTP opening in the low (*left*) and in the high (*right*) conductance modes. (*B*) Experimental observation of transient openings of the mPTP in the low conductance mode. Each trace shows the local Ca^{2+} concentration in a well-defined microdomain of an astrocyte. These transient elevations are attributed to the mPTP as adding CsA decreases the probability of occurrence of these events. The data are from Agarwal et al. (2017) with permission. (*C*) Ca^{2+} measured in the extramitochondrial medium of a suspension of mitochondria extracted from hepatocytes. After several Ca^{2+} additions in the medium (arrows), the mPTP opens in its high conductance mode. This is visible thanks to the increase in Ca^{2+} in the medium, as a result of the leakage of mitochondrial Ca^{2+} through the mPTP in its high conductance state. See Wacquier et al. (2017) for experimental methods.

which is reduced by inhibiting the mPTP, appears (Bulluck et al. 2016). This effect is linked to mitochondrial Ca^{2+} influx as the inhibition of the MCU protects neurocytes from I/RI (Yu et al. 2016). As the C subunit of the ATP synthase seems to be a core component of the mPTP, it is likely to play an important role in I/RI (Morciano et al. 2015). Drugs targeting the C subunits have recently been developed. By administrating these compounds in the reperfusion phase, the apoptotic rate of the cells is indeed attenuated (Morciano et al. 2018). Hypersensitivity of the mPTP is also encountered in neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, or Parkinson's disease (Pérez and Quintanilla 2017). In contrast, a decrease in the propensity of the pore to switch to an open state can lead to cancers (Rasol et al. 2010).

However, it seems than the PTP is not only associated with pathological conditions, in particular in its low conductance mode. This mode occurs in a transient and flickering way. In this state, the mPTP is only permeable to small ions (Ca^{2+}, H^+, K^+). Although the exact mechanism of pore opening and closing in the low conductance mode remains to be fully elucidated, this conductance state seems to be involved in various physiological responses. For example, in neural progenitor cells, repetitive mPTP openings are associated with neuronal differentiation, whereas CsA (which blocks mPTP flickering) induces the proliferation of the cell (Hou et al. 2012). In mouse embryonic fibroblasts, the reprogramming into a pluripotent state seems to be favored by transient openings of the mPTP. Indeed, these openings activate the demethylation of promoter regions of pluripotency genes (Ying et al. 2018). In contrast to the high conductance mode that irreversibly leads to the dissipation of the $\Delta\Psi$, the transient opening of the mPTP in the low conductance mode could participate to Ca^{2+} homeostasis by allowing the release of Ca^{2+} from mitochondria. The low conductance mode would thereby avoid mitochondrial Ca^{2+} overload without impairing mitochondrial bioenergetics associated with the release of metabolic substrates. In myocyte mitochondria, these opening events are indeed as-

sociated to synchronized and CsA-dependent drops of matrix Ca^{2+} and mitochondrial membrane potential. In this system, these events are rare and remain localized in single mitochondria (Lu et al. 2016). These transient openings have also been indirectly observed in resting astrocytes, via the detailed observation of the local and stochastic increases of cytosolic Ca^{2+}, which are inhibited by CsA (Fig. 3B; Agarwal et al. 2017).

As the mPTP in its low conductance mode is permeable to Ca^{2+}, it is likely to alter cytosolic Ca^{2+} signaling. This is supported by experiments performed in HeLa cells: when the mPTP is inhibited by CsA, the Ca^{2+} oscillations in the cytosol are slightly slowed down (Wacquier et al. 2016). However, when mPTP activity is modulated by changing the expression level of the C subunit of the F_1F_0-ATP synthase in HeLa cells, the mitochondrial Ca^{2+} fluxes are not significantly altered (De Marchi et al. 2014a). A plausible explanation for these apparently contradictory results is that the frequency of the oscillations is very sensitive to small Ca^{2+} changes, which may not be visible when measuring global fluxes at supramaximal histamine concentrations.

The dynamics of mPTP opening in its high conductance mode has been much studied in suspensions of mitochondria submitted to additions of exogenous Ca^{2+} (Fig. 3C). In these conditions, most of the Ca^{2+} added in the extra-mitochondrial medium is rapidly taken up by mitochondria, via the MCU. Successive Ca^{2+} additions finally lead to the rapid release of the sequestered Ca^{2+}, probably when the level of mitochondrial Ca^{2+} reaches some threshold concentration leading to the opening of the mPTP in its high conductance mode. Interestingly, before this massive Ca^{2+} release, the basal level of Ca^{2+} slowly increases in the extramitochondrial medium. This increase may reflect the progressively increasing opening of the PTP in its low conductance mode and/or a decrease in $\Delta\Psi$. Interestingly, Ichas et al. (1997) even observed traveling waves of Ca^{2+} in preparations of mitochondria suspended in a gel. These waves were ascribed to mitochondrial CICR relying on the low conductance mode of the PTP (Oster

et al. 2011). How the regulations of the same pore in the two modes are related remains largely unknown. In a mathematical model, Oster et al. (2011) postulated two independent mechanisms of regulation for the two modes: an activation by matrix pH and by mitochondrial Ca^{2+} for the low and the high conductance states, respectively. Alternatively, to describe mitochondrial Ca^{2+} swelling in cardiac cells, it was hypothesized that the pore switches from the low to the high conductance mode if the extramitochondrial Ca^{2+} level increases (Chapa-Dubocq et al. 2018), which remains to be established experimentally.

MITOCHONDRIAL METABOLISM

Mitochondrial Ca^{2+} regulates bioenergetics in various ways. First, by increasing the NADH pool in mitochondria, which is necessary to establish the proton gradient. Ca^{2+} indeed activates several key enzymes of the Krebs cycle. By binding to the isocitrate and α-ketoglutarate dehydrogenases, Ca^{2+} increases their affinities for their substrates without altering their maximal velocities. This increases the rates of reaction of these enzymes (Williams et al. 2015). Ca^{2+} also indirectly activates the pyruvate dehydrogenase. This enzyme is regulated by reversible phosphorylation, catalyzed by the pyruvate dehydrogenase phosphatase (PDP). When Ca^{2+} binds to the PDP the activity of this enzyme is increased, leading to the activation of pyruvate dehydrogenase (Denton 2009).

Ca^{2+} is also an activator of the malate-aspartate shuttle (MAS), which effectively allows translocation across the IMM of electrons from the NADH molecules produced in the cytosol during glycolysis (Fig. 1C). As the mitochondrial membrane is impermeable to NADH, this coenzyme is first metabolized into malate that is conveyed into mitochondria by a α-ketoglutarate-malate transporter (OGC). There, malate is converted into aspartate, which occurs together with the reduction of NAD^+ into NADH. Ca^{2+} regulates the activity of the aspartate-glutamate carrier (AGC), which is necessary for the replenishment of cytosolic malate. Ca^{2+} activates the two main AGCs with a high affinity (100 to 350

nM, depending on the tissue): citrin (in heart or liver) and aralar (in brain, skeletal muscles, or heart). Thus, MAS activation is theoretically another pathway through which Ca^{2+} increases in the cytosol could stimulate mitochondrial metabolism. However, this pathway is only effective near basal Ca^{2+} levels. Indeed, at higher mitochondrial Ca^{2+}, the activation of the Krebs cycle triggers a decrease in α-ketoglutarate, which is a crucial metabolite of the MAS as it must be exported to allow the influx of malate (Contreras et al. 2007; Satrústegui et al. 2007).

Components of the ETC are also activated by Ca^{2+}: the activity of complexes I, III, and IV, which extrude protons out of the matrix, is increased twofold by Ca^{2+} (Territo et al. 2000; Glancy et al. 2013; Williams et al. 2015). However, it is not known if this regulation is performed by Ca^{2+} in the matrix or in the IMS (Territo et al. 2000; Glancy et al. 2013). Furthermore, Ca^{2+} is able to bind and activate the F_1F_0-ATP synthase (Territo et al. 2000). As Ca^{2+} is a main activator of metabolism, it is not surprising to see that altering mitochondrial Ca^{2+} transporters impairs bioenergetics. LETM1 silencing induces lower mitochondrial Ca^{2+} levels and an impaired oxygen consumption (Doonan et al. 2014). In MICU1 mutant fibroblasts, the level of mitochondrial Ca^{2+} and dephosphorylated PDH are increased, although the level of ATP and the membrane potential remain unaffected (Bhosale et al. 2017).

Besides the direct activation of mitochondrial metabolism by Ca^{2+}, the close interplay between Ca^{2+} signaling and metabolism is further complicated by the modulation of the rates of the Ca^{2+} transporters by ΔΨ. Strikingly, a large peak in cytosolic Ca^{2+} triggers a transient decrease of metabolic intermediates: a drop in mitochondrial/cytosolic ATP concentrations and in ΔΨ is indeed observed in HeLa cells stimulated by histamine (Jouaville et al. 1999; Griffiths and Rutter 2009). This is probably because of a transient depolarization following the Ca^{2+} influx into mitochondria. This effect is rapidly counterbalanced by the activation of the Krebs cycle by Ca^{2+} (Wacquier et al. 2016). A similar transient decrease in ATP is observed in mouse eggs when the increase in cytosolic Ca^{2+} is in-

duced by the addition of the SERCA inhibitor thapsigargin (Campbell and Swann 2006). Conversely, in *Xenopus* oocytes, the injection of metabolic substrates induces drastic changes in cytosolic Ca^{2+} responses induced by IP_3. These substrates allow the synchronization of the Ca^{2+} waves propagating in different regions of the egg. Synchronized waves display a higher amplitude and a larger velocity of propagation (Jouaville et al. 1995).

SPATIAL ASPECTS

The spatial intracellular distribution of mitochondria has a significant impact on Ca^{2+} signaling. For example, in mouse pancreatic acinar cells, the Ca^{2+} responses induced by an agonist at the apical pole of the cell remain localized in this area. This confinement is ascribed to a barrier of mitochondria, acting as a firewall by buffering Ca^{2+} (Tinel et al. 1999). In atrial cardiomyocytes, Ca^{2+} signals are generated at the periphery of the cell, but are not fully transmitted toward the cell center. If mitochondria are depolarized and thus unable to load Ca^{2+}, the peripheral Ca^{2+} signal propagates in the cell and becomes global (Mackenzie et al. 2004).

The spatial arrangement of Ca^{2+} channels also needs to be considered: to transport Ca^{2+} efficiently, the MCU needs to be confronted with high Ca^{2+} concentrations, because of its affinity for this ion is low ($K_D \sim 2$–$10\ \mu M$) (Mallilankaraman et al. 2012; Csordás et al. 2013; Paillard et al. 2017). However, the global (i.e., spatially averaged) concentration in the cytosol varies between $\sim 0.1\ \mu M$ at rest and $\sim 1\ \mu M$ at the peak of a Ca^{2+} oscillation. The low affinity of the MCU for Ca^{2+} prevents overloading mitochondria with Ca^{2+}, but physiological Ca^{2+} sequestration requires specific mechanisms to reach sufficiently high Ca^{2+} concentrations to promote significant MCU activity. Hotspots of high Ca^{2+} concentration that promote Ca^{2+} uptake via the MCU can be achieved locally in regions known as mitochondria-associated membranes (MAMs). These are confined areas delimited by the membranes of the ER and the mitochondria, which are associated thanks to tethering proteins (Fig. 1B; Csordás et al. 2010). These zones contain Ca^{2+} channels, notably IP_3R, VDAC1 (Szabadkai et al. 2006) or MCU (de la Fuente et al. 2016). Following a Ca^{2+} release by IP_3R, the Ca^{2+} signal first stays localized in the MAMs as this ion diffuses quite slowly in the cytoplasm. Thus, the Ca^{2+} concentrations are transiently higher in MAMs than in the rest of the cytoplasm, by a factor ~ 5–10 (Csordás et al. 2010; Giacomello et al. 2010; Wacquier et al. 2017). These spatially restricted areas with high Ca^{2+} concentrations allow a better Ca^{2+} uptake by the MCU.

Accumulating evidence highlights the importance of these MAMs for physiological responses inside mitochondria but also on Ca^{2+} signaling in the cytosol. Indeed, mathematical modeling indicates that the drastically different kinetics of mitochondrial Ca^{2+} increases in intact cells and in suspensions of mitochondria can be ascribed to the absence of MAMs in the latter experimental conditions (Wacquier et al. 2017). At the MAMs, VDAC1 forms a complex with IP_3R via the glucose-regulated protein 75 (GRP75) (Szabadkai et al. 2006). The formation of such a complex is favored by apoptotic stimuli, which pinpoints the importance of direct routes between the ER and mitochondria on the establishment of specific pathophysiological responses. It has been shown that overexpression of the three isoforms of VDAC increases physiological mitochondrial Ca^{2+} uptake, but that only the overexpression of VDAC1 enhances proapoptotic Ca^{2+} transfer (de Stefani et al. 2012; Monaco et al. 2015). Interestingly, the Ca^{2+} concentration and thus the rate of Ca^{2+} transport is also tuned by the size of the MAMs, in particular by the distance between the membranes of the ER and those of mitochondria (Qi et al. 2015). This space is tightly controlled by tethering proteins, such as the fetal and adult testis expressed 1 (FATE1), the protein kinase RNA-like ER kinase (PERK), or mitofusin 2 (Mfn2) (Naon and Scorrano 2014; Kerkhofs et al. 2017). For example, increasing FATE1 expression will enhance the distance between ER and mitochondria, and thereby decrease Ca^{2+} uptake, thus making cells more resistant against apoptotic stimuli. Such a mechanism is exploited in testicular cancer (Doghman-Bouguerra et al. 2016). On the other hand, knocking down

FATE1 sensitizes cells to death stimuli (Doghman-Bouguerra et al. 2016). Mfn2 has long been reported as a tethering protein, strengthening ER-mitochondria interactions. Mfn2 ablation leads to a lower Ca^{2+} uptake and to impaired energetic response (de Brito and Scorrano 2008; Chen et al. 2012). However, other studies pinpoint an increase in the number of close contacts between mitochondria and ER in Mfn2 knocked-out cells, compared with wild-type cells. This is associated with an improved Ca^{2+} transfer at the ER-mitochondria interface. The low Ca^{2+} uptake observed in the first studies would occur because of a lower MCU expression and not because of any structural reason (Filadi et al. 2015, 2018). MAMs are also enriched in PERK proteins. The ablation of PERK induces weaker ER-mitochondria interactions and disturbs Ca^{2+} signaling (Verfaillie et al. 2012). Considering this link between MAMs and Ca^{2+} uptake, oncogenes and tumor suppressors may promote/prevent the survival of cancer cells by modulating MAMs size and hence the Ca^{2+} exchanges that act as life and death signals (Bittremieux et al. 2016; Danese et al. 2017). Tethering proteins thus constitute a target of choice for chemotherapeutics (Kerkhofs et al. 2018). In a general way, alterations of the spatial characteristics of the MAMs, and thus of Ca^{2+} dynamics in these microdomains, potentially lead to various pathological disorders, such as obesity, neurodegenerative diseases, or cancers (Marchi et al. 2017; Pinton 2018).

Intramitochondrial spatial aspects also play a key role in Ca^{2+} sequestration. In heart cells, the MCU, which is much less expressed than the NCLX, is mainly found in areas close to the junctional SR. Strikingly, the NCLX is excluded of these areas. There is thus a spatial separation of Ca^{2+} uptake and extrusion spots. This segregation could allow an effective mitochondrial response. Indeed, in a "mixed" situation in which the NCLX is also expressed near the junctional SR, the NCLX would extrude mitochondrial Ca^{2+} too fast. This limits the cost in terms of depolarization of the IMM, as the NCLX is electrogenic and induces depolarization of the membrane (de la Fuente et al. 2016, 2018).

CONCLUDING REMARKS

Ca^{2+} signaling and its physiological consequences have been intensively studied for decades. Ca^{2+} exchanges between the cytoplasm and the ER/SR play a primary role in this pathway. Although mitochondria are not constitutively loaded with Ca^{2+} in the absence of cell stimulation, various Ca^{2+} transporters allow for Ca^{2+} exchanges between the cytoplasm and these organelles (Csordás et al. 2013; Samanta et al. 2018; Shoshan-Barmatz et al. 2018). Thus, Ca^{2+} handling by mitochondria plays an important role in Ca^{2+} signaling of stimulated cells. Importantly, changes in the mitochondrial Ca^{2+} fluxes modify the frequency of cytosolic Ca^{2+} oscillations by reshaping the pacemaker-like Ca^{2+} increase that progressively sensitizes the IP_3R (Hernández-SanMiguel et al. 2006; Wacquier et al. 2016). Ca^{2+} dynamics are also important for bioenergetics as mitochondrial Ca^{2+} uptake stimulates the production of ATP, which allows cells to cope with the increased energy demand created by stimuli. Reciprocally, mitochondrial metabolism can to some extent control Ca^{2+} signaling. In this review, we have mainly focused on the less studied impact of mitochondrial Ca^{2+} dynamics and metabolism on cytosolic Ca^{2+} signaling.

The regulation and molecular nature of the mitochondrial Ca^{2+} transporters have been well characterized, but some important questions are still open. For example, the nature of the mHCX and its participation in mitochondrial Ca^{2+} efflux in different cell types remain to be established (Austin et al. 2017). Similarly, the involvement of the mPTP in its low conductance mode for cellular Ca^{2+} homeostasis has been suggested (Lu et al. 2016; Wacquier et al. 2016; Agarwal et al. 2017), but the dynamical and quantitative aspects of this flux have still to be characterized. Such progress could also help deciphering the mechanisms that regulate the transition of this pore from the low- to the high-conductance mode.

Mitochondrial Ca^{2+} impacts on metabolism by activating the MAS shuttle, the Krebs cycle, and the ETC (Denton 2009; Williams et al. 2015). Computational modeling predicts that

in electrically nonexcitable cells such as HeLa cells or hepatocytes, the activation of the MAS shuttle by Ca^{2+} is only significant at low cytosolic Ca^{2+}. At higher Ca^{2+} concentrations the activation of the glutamate-aspartate transporter is counterbalanced by the decrease in α-ketoglutarate brought about by the activation of the Krebs cycle. The generality of this prediction remains to be tested experimentally. Other parameters affect the relation between Ca^{2+} increases and mitochondrial metabolism. First, changes in the ratio between cytosolic and mitochondrial Ca^{2+} concentrations modify $\Delta\Psi$, which controls all mitochondrial Ca^{2+} fluxes and reaction rates in the Krebs cycle and in the ETC. The intracellular spatial arrangement of mitochondria is also important. Various studies have indeed highlighted that mitochondria and ER are tightly coupled by microdomains (Csordás et al. 2010). The size of these areas is a crucial parameter because it can modulate Ca^{2+} uptake and induce either impaired bioenergetics or cellular death (Chen et al. 2012; Doghman-Bouguerra et al. 2016). The number, the size, and the dynamics of individual mitochondria are other characteristics of these organelles that require further investigation to fully understand the interplay between mitochondria and Ca^{2+} signaling in physiological and pathological conditions.

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