Mitochondria: The Hub of Cellular Ca\textsuperscript{2+} Signaling

Mitochondria couple cellular metabolic state with Ca\textsuperscript{2+} transport processes. They therefore control not only their own intra-organelle [Ca\textsuperscript{2+}], but they also influence the entire cellular network of cellular Ca\textsuperscript{2+} signaling, including the endoplasmic reticulum, the plasma membrane, and the nucleus. Through the detailed study of mitochondrial roles in Ca\textsuperscript{2+} signaling, a remarkable picture of inter-organelle communication has emerged. We here review the ways in which this system provides integrity and flexibility for the cell to cope with the countless demands throughout its life cycle and discuss briefly the mechanisms through which it can also drive cell death.

The Principles of Mitochondrial Ca\textsuperscript{2+} Handling

Over recent years, mitochondria have taken center stage as remarkably autonomous and dynamic cellular organelles that are intimately involved in orchestrating a diverse range of cellular activities. Mitochondrial integrity is required for fertility and for early development. Throughout adult life mitochondria provide mechanisms to adapt to various stress conditions, and ultimately they have the power to determine cell death (36, 37, 95, 111). The emergence of this picture follows inevitably from the recognition of mitochondria as the “powerhouse of the cell,” a role intimately and flexibly coupled to ion transport processes across their double membrane barrier. Although generation of a mitochondrial transmembrane H\textsuperscript{+} gradient is the basis of coupling fuel oxidation to ATP production, it also provides a playground for the versatile handling of ions with signaling functions, the most notable of which is Ca\textsuperscript{2+} (9). Although general issues relating to mitochondrial Ca\textsuperscript{2+} handling have recently been widely reviewed (e.g., Refs. 10, 28, 40, 77, 89, 96), here we will concentrate on a particular aspect of mitochondrial Ca\textsuperscript{2+} handling, i.e., how it provides signals for the rest of the cell by shaping the cellular Ca\textsuperscript{2+} signaling network through direct and indirect interactions with other cellular organelles.

More than 50 years of studies have established a cohesive model of Ca\textsuperscript{2+} handling in isolated mitochondria based on the cooperation of three modules: Ca\textsuperscript{2+} uptake from the extramitochondrial space, Ca\textsuperscript{2+} buffering in the mitochondrial matrix, and Ca\textsuperscript{2+} extrusion into the surroundings (FIGURE 1). Importantly, all these processes are tightly coupled to the energetic state of the organelle (7, 80). Respiring mitochondria, supplied with oxygen and sources of carbon, maintain a membrane potential (\(\Delta\psi_{m}\)) and a pH gradient through the activity of electron transport chain (ETC). Ca\textsuperscript{2+} accumulation through the mitochondrial Ca\textsuperscript{2+} uniporter (MCU) depends on the electrochemical gradient for Ca\textsuperscript{2+} defined by the \(\Delta\psi_{m}\) and the [Ca\textsuperscript{2+}] gradient between the cytosol and the mitochondrial matrix (\([\text{Ca}\textsuperscript{2+}]_{c} - [\text{Ca}\textsuperscript{2+}]_{m}\)). Ca\textsuperscript{2+} extrusion through a membrane H\textsuperscript{+} gradient is the basis of coupling fuel oxidation to ATP production, it also provides a playground for the versatile handling of ions with signaling functions, the most notable of which is Ca\textsuperscript{2+} (9). Although general issues relating to mitochondrial Ca\textsuperscript{2+} handling have recently been widely reviewed (e.g., Refs. 10, 28, 40, 77, 89, 96), here we will concentrate on a particular aspect of mitochondrial Ca\textsuperscript{2+} handling, i.e., how it provides signals for the rest of the cell by shaping the cellular Ca\textsuperscript{2+} signaling network through direct and indirect interactions with other cellular organelles.

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molecular identity of this pathway remains obscure, but its properties are reasonably well characterized. Ca$^{2+}$ flux rates through the uniporter are equivalent to those measured for fast gated pores but rather slower than those seen for most channels (see Ref. 39 for review). The activity of the uniporter shows little sensitivity to changes in temperature, and it also shows a wide spectrum of cation selectivity, together suggesting that it is a channel rather than a carrier. Likewise, Ca$^{2+}$ uptake via the uniporter is inhibited by ruthenium red (RuR), a compound that inhibits a variety of cation channels, including L-type plasmalemmal Ca$^{2+}$ channels (27), ryanodine-sensitive ER Ca$^{2+}$ release channels (64), and vanilloid receptor-operated channels (113). Moreover, patch-clamp experiments in mitoplasts (isolated IMM vesicles) revealed expression of an inwardly rectifying, highly Ca$^{2+}$ selective, voltage-dependent Ca$^{2+}$ channel (MiCa) with properties consistent with a possible identity as the Ca$^{2+}$ uniporter (58).

One of the most important features of the uniporter is an apparent gating by extramitochondrial Ca$^{2+}$ ([Ca$^{2+}$]$_{e}$). This was identified in early studies of Ca$^{2+}$ influx on isolated mitochondria (14) and later through studies of the Ca$^{2+}$ sensitivity of RuR-sensitive mitochondrial Ca$^{2+}$ accumulation (53, 69). Montero et al. (69) elegantly showed that an increase in [Ca$^{2+}$]$_{i}$ promoted RuR-sensitive (and thus uniporter-mediated) Ca$^{2+}$ release from depolarized mitochondria preloaded with Ca$^{2+}$. This is consistent with suggestions that the uniporter is allosterically gated by [Ca$^{2+}$]$_{i}$ (53), an observation that may also explain why local [Ca$^{2+}$]$_{i}$ needs to be higher than one might expect from the behavior of a conducting Ca$^{2+}$ channel to see significant increases in [Ca$^{2+}$]$_{i}$.

As mentioned above, the Ca$^{2+}$ uniporter is surprisingly and somewhat worryingly resistant to attempts of identification. Some studies point to the existence of one or more glycoprotein (complexes) with regulated Ca$^{2+}$ binding and channelling properties, but none of them have been carefully proved to fulfil this role (for reviews, see Refs. 80, 96). Alternatively, it has been recently proposed that other mitochondrial carriers might represent the long sought IMM Ca$^{2+}$ uniporter. Graier’s group has recently provided compelling genetic evidence that the novel uncoupling proteins UCP-2 and -3 play an essential role in RuR-sensitive mitochondrial Ca$^{2+}$ accumulation (106). Overexpression of UCP-2 and -3 increased the peak [Ca$^{2+}$]$_{m}$ response to Ca$^{2+}$ mobilizing stimuli in different cell types, their silencing significantly reduced Ca$^{2+}$ uptake both in intact cells and isolated liver mitochondria from UCP-2 knockout animals. Nevertheless,
expression of human UCPs in the yeast *Saccharomyces cerevisiae* was not sufficient to establish RuR-sensitive Ca\(^{2+}\) uptake, suggesting that these proteins may not represent the Ca\(^{2+}\) conducting channel itself; rather, they may reside in a macromolecular complex with other membrane components to create or modulate the uptake pathway.

An uptake mechanism, named rapid uptake mode (RaM), with properties distinct from those of the uniporter has also been described (17, 97). This pathway has the capacity to transfer Ca\(^{2+}\) very rapidly into the mitochondria during the rising phase of a Ca\(^{2+}\) pulse. The properties of the pathway differ in different tissues (17), but in the heart the pathway saturates quickly and is slow to reset after activation. Again, the functional significance of this pathway remains to be established.

**Ca\(^{2+}\) extrusion routes**

The major route for Ca\(^{2+}\) efflux from mitochondria is through an exchange of Ca\(^{2+}\) for Na\(^{+}\). It has a discrete pharmacology distinct from the plasmalemmal exchanger, but again its molecular identity remains to be determined. Similarly, and remarkably, the stoichiometry of the exchanger seems still to be controversial. Initially, it was thought to be an electroneutral 2Na\(^{+}\)/Ca\(^{2+}\) exchanger (15), but this has been questioned (57), and a stoichiometry of 3Na\(^{+}\)/Ca\(^{2+}\) was suggested. In this case, the operation of the exchange will be dependent on \(\Delta \psi_{m}\), supported by the observation that mitochondrial depolarization leads to inhibition of mitochondrial Ca\(^{2+}\) efflux (8, 69). An electrogenic stoichiometry would predict mitochondrial depolarization during Ca\(^{2+}\) efflux. The lack of evidence for this process might reflect the necessity of a Na\(^{+}\) gradient for Ca\(^{2+}\) extrusion, built on the H\(^{+}\) gradient of polarized mitochondria through the Na\(^{+}\)/H\(^{+}\) exchange process.

Even if it was never studied or even considered, we cannot rule out that the OMM might play a role as a significant permeability barrier not only in Ca\(^{2+}\) uptake but also in Ca\(^{2+}\) efflux. VDAC is thought to be part of the mPTP (Refs. 7, 20; although see below), which is in turn regulated by [Ca\(^{2+}\)]\(_{m}\). mPTP opening provides a potential efflux pathway for Ca\(^{2+}\) (52), although the physiological relevance of this pathway is debated.

A “unified model” of mitochondrial Ca\(^{2+}\) handling

Altogether, we might depict the complex nature of mitochondrial Ca\(^{2+}\) transport between the extracellular and intramitochondrial space by dividing it into three phases (FIGURE 2).

1) [Ca\(^{2+}\)]\(_{m}\) equilibrates with small elevations of [Ca\(^{2+}\)]\(_{e}\) until the removal of Ca\(^{2+}\) from the matrix by the xNa\(^{+}\)/Ca\(^{2+}\) exchange keeps pace with the rate of Ca\(^{2+}\) uptake.

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**FIGURE 2.** Compartmentalized cellular Ca\(^{2+}\) signaling

A: a simplified three-compartment model of the cell indicates the principal organelles participating in cellular Ca\(^{2+}\) handling and their resting [Ca\(^{2+}\)]. The endoplasmic reticulum (ER) represents the main intracellular Ca\(^{2+}\) store, having a basal Ca\(^{2+}\) level almost as high as in the extracellular space, roughly 10\(^4\) times higher than the cytoplasm and mitochondria. Cell stimulation leads to the activation of at least two seminal pathways. First, the formation of IP\(_{3}\) leads to Ca\(^{2+}\) release from the ER, triggering a reciprocal [Ca\(^{2+}\)] increase in the cytosol and [Ca\(^{2+}\)] drop in the ER (as measured by the luminescent recombinant aequorin Ca\(^{2+}\) probes; top and middle in B, respectively). Second, extracellular signals or ER Ca\(^{2+}\) depletion activate Ca\(^{2+}\) influx routes, refilling the ER partially through mitochondria (see also **FIGURE 3A**). Note the almost 100 times greater response in mitochondria (bottom in B) compared with the cytosol, partly explained by the close physical association between the organelles.
2) As \([\text{Ca}^{2+}]_m\) rises above ~4–500 nM, the \(\text{Ca}^{2+}\)-dependent activation of the \(\text{Ca}^{2+}\) uptake rate exceeds the capacity of the exchanger, and mitochondria start to accumulate \(\text{Ca}^{2+}\). However, at this point, the \(P_{\text{ATP}}\)-dependent \(\text{Ca}^{2+}\) buffering mechanism counterbalances the increase of \([\text{Ca}^{2+}]_m\) as recently pointed out by Nicholls and Chalmers (79). Net \(\text{Ca}^{2+}\) flux into the mitochondrial matrix might result in loss of \(\Delta\psi_m\) and an increase of \(\Delta\phi\), if protons move out through the ETC to compensate for \(\text{Ca}^{2+}\) charge. This process would rapidly stop \(\text{Ca}^{2+}\) uptake, if charge compensating ions (e.g., \(\text{P}_i\) or acetate) are not transported along together with \(\text{Ca}^{2+}\) (94). Importantly, in the presence of \(P_{\text{ATP}}\), mitochondria are able to accumulate much more \(\text{Ca}^{2+}\) [in contrast to acetate (118)] due to the additional buffering role of phosphate in \(\text{Ca}^{2+}\) handling. Thus the role of phosphate uptake accompanying \(\text{Ca}^{2+}\) is to maintain \([\text{Ca}^{2+}]_m\) at a constant value through the formation of insoluble \(\text{xCa}^{2+}\cdot\text{xP}_i\cdot\text{xOH}\) complexes (18). The stoichiometry of these complexes is not defined unambiguously but appears to be close to that of hydroxyapatite \([\text{Ca}_4(\text{PO}_4)_6\cdot\text{OH}\]) There are various important consequences of this process. First, since the solubility of the \(\text{Ca}^{2+}/\text{P}_i\) complexes is steeply dependent on matrix pH, a rapid, vast increase of \([\text{Ca}^{2+}]_m\) will occur during acidification, e.g., as predicted following addition of a protonophore. Furthermore, slow \(\text{Ca}^{2+}\) addition to isolated mitochondria does not lead to a measurable \([\text{Ca}^{2+}]_m\) elevation due to immediate formation of \(\text{Ca}^{2+}/\text{P}_i\) precipitates, whereas bolus application of \(\text{Ca}^{2+}\) (or maximal cell stimulation) causes a \([\text{Ca}^{2+}]_m\) signal, detectable by (fluorescent/luminescent) mitochondrial \(\text{Ca}^{2+}\) sensors. Thus it is worth considering that \(\text{Ca}^{2+}\) flux into mitochondria is not necessarily synonymous with a net increase in \([\text{Ca}^{2+}]_m\). This is not purely semantic, since \(\text{Ca}^{2+}\) uptake by the uniporter is electrogenic and is therefore associated with small changes in \(\Delta\phi_m\). Experimentally, changes in \(\Delta\phi_m\) will reflect the rate of \(\text{Ca}^{2+}\) flux, and may therefore prove a more sensitive measurement of \(\text{Ca}^{2+}\) movement into mitochondria than measurement of \([\text{Ca}^{2+}]_m\) itself. Depolarization following \(\text{Ca}^{2+}\) uptake is a transient phenomenon (e.g., see Ref. 8). Thus two further aspects need to be considered in interpreting the relationship between mitochondrial \(\text{Ca}^{2+}\) uptake and changes in \(\Delta\phi_m\). 1) The kinetics of the response of the potentiometric probe used to make the measurements, which might lag behind the kinetics of the true change in \(\Delta\phi_m\) and 2) the activation of compensatory mechanisms (comprising \(\text{H}^+, \text{K}^+, \text{P}_i\) fluxes), which might affect not only the repolarization phase of the transient drop in \(\Delta\psi_m\) but also its extent. Finally, modification of the \(P_{\text{ATP}}\) transport machinery (e.g., by inhibiting or downregulating the \(P_{\text{ATP}}\) carrier (51, 85)) might exert profound changes on mitochondrial \(\text{Ca}^{2+}\) uptake, which, however, has never to our knowledge been tested experimentally. The \([\text{Ca}^{2+}]_m\) value at which the rate of \(\text{Ca}^{2+}\) uptake exceeds that of the extrusion was termed as "set point" (76, 78), which, however, has another interpretation, maybe reflecting better the impact of mitochondria on cellular \(\text{Ca}^{2+}\) homeostasis. It also represents a \([\text{Ca}^{2+}]_m\) value, maintained by the mitochondrial \(\text{Ca}^{2+}\) cycling described above, in the surrounding cytosol. Indeed, isolated liver mitochondria lower \([\text{Ca}^{2+}]_m\) to ~4–800 nM (depending on the amount of total \(\text{Ca}^{2+}\) added to the medium), whereas if \([\text{Ca}^{2+}]_m\) is experimentally lowered below this value by the addition of a \(\text{Ca}^{2+}\) chelator, matrix \(\text{Ca}^{2+}\) is released until this same value is attained (76). In a cellular context (see below), where several other mechanisms operate to maintain cytosolic \([\text{Ca}^{2+}]_m\) equilibrium, this observation may have a limited relevance, but it nicely illustrates the fundamental purpose of mitochondrial \(\text{Ca}^{2+}\) handling, i.e., to maintain and even dictate the \([\text{Ca}^{2+}]_m\) level in its mother cell, through its capacity to accumulate, buffer, and release \(\text{Ca}^{2+}\). Importantly, these capacities are the function of \(\Delta\psi_m\) and \(\Delta\phi\); thus we can interpret the set point as a signal of mitochondrial metabolism and integrity for the rest of cell, translated into a \([\text{Ca}^{2+}]\) value.

3) When mitochondrial \(\text{Ca}^{2+}\) load exceeds the buffering capacity of the matrix, \([\text{Ca}^{2+}]_m\) rises steeply, leading to irreversible swelling, uncoupling, and loss of soluble mitochondrial content, a process dubbed as mitochondrial permeability transition (MPT), mostly explained by the opening of a large nonselective pore encompassing both mitochondrial membranes (in turn called opening of the mPTP). The underlying molecular mechanisms again are poorly clarified (for details, see below), but there is no doubt about the profound pathophysiological significance of the process, which has been most convincingly demonstrated as a trigger to ischemic reperfusion injury in the heart (2).

**Mitochondrial \(\text{Ca}^{2+}\) Handling in the Cellular Context**

Early studies on the impact of \(\text{Ca}^{2+}\) accumulation on mitochondrial function led to the characterization of \(\text{Ca}^{2+}\)-dependent metabolic processes, which was further established in intact cells, through the development of imaging techniques to study mitochondrial function (29). Furthermore, the shift to more physiological systems also revealed that the interaction between mitochondria and cellular \(\text{Ca}^{2+}\) homeostasis is rigorously mutual, leading also to the acknowledgment that interaction between mitochondria and other cellular organelles has a large influence on cell function, doubtless even larger than regulating ATP production.

**Impact of \(\text{Ca}^{2+}\) uptake on mitochondrial function**

In teleological terms, it seems that the major functional significance of mitochondrial \(\text{Ca}^{2+}\) uptake is in the regulation of mitochondrial metabolism. In the late
1980s, it was shown that the three major rate-limiting enzymes of the citric acid cycle are all upregulated by Ca$^{2+}$ (for review, see Ref. 67). The first suggestions that Ca$^{2+}$ operates also in intact cells came from measurements of changes in mitochondrial redox state, reflected as changes in mitochondrial NADH and flavoprotein autofluorescence, in response to changes in [Ca$^{2+}$]$_{m}$ (27, 86). These observations showed clearly that 1) mitochondria must be taking up Ca$^{2+}$ during [Ca$^{2+}$]$_{c}$ signals and 2) this was sufficient to activate the TCA cycle, causing increased net reduction of the coenzymes. More recently, transfection of cells with firefly luciferase allowed a clear and unequivocal demonstration that mitochondrial Ca$^{2+}$ uptake increases mitochondrial ATP production (56). The relative importance of this mechanism in the regulation of mitochondrial oxidative phosphorylation over the more traditional model, in which the rate of ATP generation is regulated largely by the ATP-to-ADP:Pi ratio, is not clear. It is very attractive to suggest that the transfer of Ca$^{2+}$ from the cytosol to mitochondria during [Ca$^{2+}$]$_{c}$ signals represents a major mechanism to couple ATP supply with demand, since in almost all systems increases in work are associated with increases in [Ca$^{2+}$]$_{m}$. Nevertheless, direct evidence for a significant role in intact systems is limited, and there are conflicting data (see for example Refs. 49, 71).

The time course of the changes in [Ca$^{2+}$]$_{m}$ and in activation of the enzyme systems becomes crucial. [Ca$^{2+}$]$_{c}$ signals are typically brief, transient phenomena. Typically, it seems that the resultant mitochondrial activation is prolonged with respect to the change in [Ca$^{2+}$]$_{c}$ (27, 42, 92), and this in turn will be a function of the rate of mitochondrial Ca$^{2+}$ efflux and the half-life of the activated states of the enzymes. A further major question that is important in considering the impact of Ca$^{2+}$ on mitochondrial function is: How high does [Ca$^{2+}$]$_{m}$ rise during these signals? The Ca$^{2+}$ transport model depicted in the first part of this essay gives some clues to answer this question. In isolated mitochondria, two phases of [Ca$^{2+}$]$_{m}$ changes were observed following a rise in [Ca$^{2+}$]$_{c}$. At submicromolar [Ca$^{2+}$]$_{c}$, [Ca$^{2+}$]$_{m}$ increases in a range (0.2–3 μM) that allows the parallel activation of Ca$^{2+}$-dependent enzymes of the Krebs-cycle, leading to increased supply of reducing equivalents [NAD(P)H] (67, 84, 86). An increase of [Ca$^{2+}$]$_{m}$ into the range where the activity of Ca$^{2+}$-dependent dehydrogenases is controlled by Ca$^{2+}$ activates mitochondrial metabolism. At [Ca$^{2+}$]$_{m}$ above the micromolar level, [Ca$^{2+}$]$_{m}$ is relatively stable (see above), allowing mitochondria to accumulate as much as 700–1,000 nmol Ca$^{2+}$/mg mitochondrial protein, but one should not expect further activation of metabolism. In intact cells, a further increase of [Ca$^{2+}$]$_{m}$ was observed using low-affinity variants of targeted recombinant aequorin probes. This increase, as discussed below, seems to depend on interaction of mitochondria with other Ca$^{2+}$ handling organelles (ER and the plasmamembrane) and most probably plays a role in the regulation of cell death processes by mitochondria.

The capacity to study mitochondria in intact cells that has accompanied the improvements in digital and confocal microscopy has led to the (re-)discovery of their dynamic morphological properties, adding a new layer to the plasticity of mitochondrial signaling. Although it is out of the scope of this essay to describe in detail the mechanisms controlling the continuous fusion, fission, and movement of the mitochondrial population [forming a dynamic interconnected network in several cell types (for a recent review, see Ref. 48)], we would just like to emphasize that shaping mitochondria seems to be closely interconnected with cellular and mitochondrial Ca$^{2+}$ signals. The large mechanochemical GTPase enzyme dynamin-like protein-1 (Drp-1), driving the mitochondrial division apparatus, translocates to the OMM following a cytoplasmic [Ca$^{2+}$] rise in mammalian cells (16) and becomes activated by calcineurin-driven dephosphorylation (19). Similarly, intracellular distribution of mitochondria, as well as their fusion, is regulated by a newly discovered mitochondrial family of Rho GTPases (Miro 1 and 2), bearing two functionally important Ca$^{2+}$ binding EF-hand domains (31). A recently emerging outstanding question is: How do mitochondrial dynamics (and their Ca$^{2+}$ regulation) affect their metabolic properties and participation in the regulation of different cell death pathways?

In effect, mitochondrial Ca$^{2+}$ uptake may have profound consequences for mitochondrial function under pathological conditions. A combination of mitochondrial Ca$^{2+}$ loading and oxidative stress and/or ATP depletion may promote opening of the mPTP. This appears to reflect a pathological conformation of a group of mitochondrial membrane proteins, notably the adenine nucleotide translocase (ANT) and VDAC, with the association of cyclophilin D, a regulatory protein that confers sensitivity of the permeability transition (5, 62, 74), allowing as well an initial molecular definition of a Ca$^{2+}$-dependent
cell death pathway that is clearly distinct from apoptosis but plays a fundamental role in reperfusion injury in the heart and in glutamate neurotoxicity in the CNS. Still, the potential role of mPTP in other cell death models (e.g., cancer cell death induced by antitumor agents, or degenerative diseases of the CNS) now can only be reliably interpreted through findings regarding cyclophilin D, since the role of other proposed components (the ANT and VDAC) is now thrown into doubt by the generation of knockout mice for each set of proteins, which still seems able to display a MPT (3, 59). However, it is important to note that, similar to cyclophilin D−/− cells, ANTI/2 knockouts also display less sensitivity to Ca2+ than their wild-type counterparts (59, 117).

The reverse signaling: impact of mitochondria on cellular Ca2+ homeostasis

A vast number of studies identified the crucial importance of mitochondria in shaping/determining cellular Ca2+ responses in intact cells of virtually any type. Here we have to limit ourselves to mention just a few remarkable examples to summarize the principles underlying the mechanism of this interaction.

Mitochondria seem capable of generating local microdomains of ATP that may be crucial in supplying the ATP required for ER/SR Ca2+ accumulation. Thus ER Ca2+ refilling was slowed by mitochondrial uncouplers even in permeabilized cells supplied with ATP in the buffer (60). In the mammalian egg, a remarkable cell for many reasons, inhibition of oxidative phosphorylation (for example with oligomycin) caused an almost immediate loss of ER Ca2+, followed after a variable but prolonged delay by Ca2+ influx. These data were interpreted to suggest that the (demonstrable) close apposition between ER and mitochondria created local microdomains in which mitochondrial ATP was required for ER Ca2+ cycling, with a rapid rate of ATP consumption. Inhibition therefore caused a rapid local ATP depletion, allowing immediate efflux of ER Ca2+, whereas global [ATP] fell much later (30). These observations also belie the commonly held view that mitochondria in the mammalian oocyte are relatively dormant.

Mitochondrial Ca2+ handling may also dictate changes in local Ca2+ during cell signaling. Many exciting cells respond to depolarization with a rise in [Ca2+]c, which rises rapidly and recovers with an initial rapid phase and a slower second phase that can even form a plateau (1, 24, 104). It has been established in many preparations that the slow recovery phase reflects the redistribution of mitochondrial Ca2+ through the activity of the mitochondrial Na+/Ca2+ exchanger, reflecting the set point, typically initiated at a [Ca2+]c of ~500 nM. The operation of this system has functional consequences at presynaptic terminals, where the [Ca2+]c plateau that follows repetitive stimulation, maintained by the re-equilibration of mitochondrial Ca2+, provides an elevated [Ca2+]c baseline on which subsequent stimulation initiates an enhanced synaptic response, which is the basis for posttetanic potentiation of synaptic transmission (24, 103). It is also intriguing that the poststimulus plateau phase is not seen in nonexcitable cells following the transmission of [Ca2+]c signals from ER to mitochondria.

There has been some debate about the quantitative relationships between ambient [Ca2+]c and mitochondrial uptake. In HeLa cells transfected with mitochondrially targeted aequorin and then permeabilized, net mitochondrial Ca2+ accumulation was only detectable
if the added Ca\(^{2+}\) reached concentrations higher than 3 \(\mu\)M, whereas \([\text{Ca}^{2+}]_{\text{m}}\) signals evoked by IP\(_3\) mobilizing agonists were far more effective at raising \([\text{Ca}^{2+}]_{\text{m}}\), even though the mean \([\text{Ca}^{2+}]_{\text{m}}\) signal might rise to <1 \(\mu\)M (22). This led to the idea that a fundamental in situ feature of the mitochondrial Ca\(^{2+}\) uptake machinery stems from its strategic localization at different sources of the cellular \([\text{Ca}^{2+}]_{\text{c}}\), signal. Indeed, intimate contacts between the PM and ER membranes and the OMM was revealed by morphological and functional imaging studies (23, 61, 91), showing that immediate Ca\(^{2+}\) sequestration occurs at these sites into the organelle. Mitochondria, positioned at the cyttoplasmic face of the ER Ca\(^{2+}\) release channels [inositol 1,4,5-trisphosphate receptors (IP\(_3\)R) and ryanodine receptors (RyRs)] as well as to different PM Ca\(^{2+}\) influx channels [capacitative Ca\(^{2+}\) entry (CCE) or ionotropic glutamate receptors], thus are exposed to Ca\(^{2+}\) concentrations well above those measured in the bulk cytosol, now generally termed as Ca\(^{2+}\) microdomains (23, 70, 90).

The proximity of mitochondria to SR or ER Ca\(^{2+}\) release sites has been further emphasized through evidence that focal, nonpropagating ER/SR Ca\(^{2+}\) release can cause a transient increase in \([\text{Ca}^{2+}]_{\text{m}}\) in mitochondria close to the release site. This is reflected by spontaneous transient mitochondrial depolarizations (23), local transient increases in \([\text{Ca}^{2+}]_{\text{m}}\) (24), and the resistance of SR-mitochondrial Ca\(^{2+}\) transfer to cytosolic Ca\(^{2+}\) buffering by BAPTA in cardiomyocytes (25). Moreover, in primary endothelial and HeLa cells, high-resolution fast kinetic imaging of \([\text{Ca}^{2+}]_{\text{m}}\) showed that intramitochondrial Ca\(^{2+}\) signals, following IP\(_3\)-induced Ca\(^{2+}\) release, propagate in the matrix from distinct foci (termed “hot spots”), most probably representing ER-mitochondrial contacts (34, 99).

These observations provided the impetus to identify further molecular components in this microdomain, and an intriguingly complex picture emerges concerning the functional consequences of the interactions operating in this tiny space (FIGURE 3). First, biochemical characterization of the long-known mitochondria-associated membrane fraction [MAM; comprising OMM and ER segments, originally identified by the presence of specific lipid transfer enzymes (108)] revealed direct coupling between ER and mitochondrial Ca\(^{2+}\) permeating channels (the IP\(_3\)R and VDAC, respectively) (100), and an elegant molecular tool has also been developed to directly boost the physical coupling between the organelles (22). The functional consequences were partly predictable, since linking the ER and OMM membranes increased IP\(_3\)-mediated Ca\(^{2+}\) transfer into mitochondria (22), but surprisingly the IP\(_3\)R itself also appeared to augment the efficiency of this process by protein-protein interaction (100). Moreover, the ER-mitochondria linkage and consequent direct Ca\(^{2+}\) channelling between the lumen of the organelles appears to be a target of protein signaling pathways, indicated by the requirement of chaperones [grp75 (100) and a newly discovered family of sigma-1 receptors (44)]. Since it is now well established that the IP\(_3\)R integrates a wide range of signals on the ER surface (68, 83), we can envisage that further players will appear with properties regulating not only the Ca\(^{2+}\) release receptor channel but intrinsically also the Ca\(^{2+}\) accumulation into mitochondria (see, e.g., Refs. 47, 72). A very recent finding by A. Spät’s group, showing that communication between the ER and mitochondria is regulated by the stress-related p38 MAPK pathway opened even further prospects in placing this interaction in a physiological and pathological context (101).

Most importantly, significant further work also revealed mechanisms and consequences of how mitochondrial Ca\(^{2+}\) uptake regulates ER Ca\(^{2+}\) handling. First, by removing Ca\(^{2+}\) from the microdomain close to the IP\(_3\), Ca\(^{2+}\) release channel, mitochondria prevent the Ca\(^{2+}\)-dependent inactivation of the channel and facilitate ER Ca\(^{2+}\) release (41). This mechanism, together with the machinery by which Ca\(^{2+}\) itself triggers the relocation of mitochondria onto the ER surface (115), allows mitochondria to play a significant role in shaping the spatiotemporal patterning of \([\text{Ca}^{2+}]_{\text{c}}\) signals. In Xenopus oocytes, energization of mitochondria enhances the propagation and coordination of \([\text{Ca}^{2+}]_{\text{c}}\) waves (55), whereas in astrocytes, which express primarily IP\(_3\) type 3 receptors, energized mitochondria serve as a spatial buffer that limits the rate and extent of propagation of \([\text{Ca}^{2+}]_{\text{c}}\) waves (13). Intriguingly, studies concerning the dynamic positioning and morphological plasticity of mitochondrial structure provided important information on the role of mitochondria in shaping cellular Ca\(^{2+}\) signals. In pancreatic acinar cells, the mitochondria are concentrated into a band that isolates the secretory pole of these polarized cells, and they seem to act as a “firewall” that limits the spread of \([\text{Ca}^{2+}]_{\text{c}}\) signals from their initiation at the apical pole to the basal pole (105). Furthermore, mitochondria localized close to the basal pole are more sensitive to local Ca\(^{2+}\) influx by capacitative entry, and so it seems that the positions of mitochondria within the cell may have a profound influence on their interaction with cellular \([\text{Ca}^{2+}]_{\text{c}}\) signals (82). This latter finding highlights the fact that microdomains of \([\text{Ca}^{2+}]_{\text{c}}\) regulated by mitochondria also play a significant role in the regulation of capacitative Ca\(^{2+}\) influx (Refs. 35, 50; for review, see Ref. 81), suggesting that the mitochondria must be positioned close to the plasma membrane. The principle is very much as outlined above for the IP\(_3\)R receptor, as the Ca\(^{2+}\) influx channel is desensitised by Ca\(^{2+}\). By keeping \([\text{Ca}^{2+}]_{\text{c}}\) low in microdomains close to the channels, mitochondria keep the channels open and facilitate Ca\(^{2+}\) influx through the channels. Finally, further work, using models with redistribution of the mitochondrial network (fragmentation and perinuclear clustering) suggested an elegant scheme (33, 66), in which
bidirectional Ca\(^{2+}\) channeling between the ER and mitochondria collaborate in the regulation of capacitative Ca\(^{2+}\) influx and Ca\(^{2+}\) extrusion through the plasmamembrane Ca\(^{2+}\) ATPase (PMCA). Since the complete picture is detailed in Refs. 32, 33, 65, 110 and reviewed by our laboratory elsewhere (98), we would like now only to underline the importance of one of their findings, i.e., the lack of the subplasmamembrane mitochondrial population in hFis1 overexpressing HeLa cells led to increased Ca\(^{2+}\) cycling through the PMCA and capacitative influx channels, rendering the ER Ca\(^{2+}\) store very prone to Ca\(^{2+}\) depletion under low extracellular [Ca\(^{2+}\)] conditions. Since similar redistribution of the mitochondrial network (fragmentation and perinuclear clustering) is a major feature of several cell death processes, whereas the ER Ca\(^{2+}\) content is a key regulator of ER stress and apoptosis, undoubtedly the mechanisms discovered in these studies will find an important place in the regulation of cell fate. Similarly, ER-mitochondrial Ca\(^{2+}\) transfer appears to play an important role in induction of ER-stress-related cell death (Refs. 22, 26; Chami M, Oules B, Szabadkai G, et al., unpublished observations).

**Mitochondrial-nuclear communication through Ca\(^{2+}\) signal**

The failure of mitochondrial function is promptly signaled to the nucleus to trigger adaptive responses reorganizing cellular metabolism. The pathways involved in this signaling are best characterized in yeast, where they have been shown to be interlinked with mitochondrial DNA maintenance, nutrient sensing, and ageing-related pathways (for review, see Ref. 63). Much less is known about the underlying mechanisms in higher, multicellular eukaryotes, but, strikingly, recent evidence points to the crucial role of Ca\(^{2+}\) signaling in the process. Studies by Avadhani and coworkers on C2C12 myoblast and A549 lung cancer cell lines repeatedly showed an increase of [Ca\(^{2+}\)]\(_i\) following induction of mitochondrial failure by uncoupling, inhibitors of respiration or mtDNA depletion (12, 38). Although the pathways downstream to this long-lasting Ca\(^{2+}\) signal are at least partially characterized (e.g., activation of Ca\(^{2+}\)-/calmodulin-dependent kinases and the phosphatase calcineurin), the exact mechanism that generates the signal remains to be determined. Deregulation of cellular Ca\(^{2+}\) homeostasis due to ATP depletion and release of Ca\(^{2+}\) from the mitochondria have been proposed as principal reasons, but no detailed analysis has been performed yet on the known mitochondrial and cellular Ca\(^{2+}\) transport processes to clarify this issue.

Interestingly, mitochondrial biogenesis and consequent mitochondrial volume expansion, which can also be induced by chronic uncoupling (93) was also shown to interact with Ca\(^{2+}\) uptake and intramitochondrial Ca\(^{2+}\) distribution (11). The recent disclosure of the pathway leading to the concerted induction of nuclear-encoded mitochondrial proteins by the peroxisome proliferator-activated receptor-gamma (PPAR\(\gamma\)) co-activator-1\(\alpha\) (PGC-1\(\alpha\)) served as a useful tool to assess this effect (87). Importantly, the pathway induced by PGC-1\(\alpha\) also increased mitochondrial volume, multiplying its capacity to accumulate and buffer Ca\(^{2+}\), as evidenced by the increased distance of Ca\(^{2+}\) diffusion from the "hot spots." Overall, through this, and probably other mechanisms, PGC-1\(\alpha\)-induced mitochondrial biogenesis leads to a reduced [Ca\(^{2+}\)]\(_m\) signal. Since the expression of PGC-1\(\alpha\) is regulated partly by cellular Ca\(^{2+}\) signals (43), the questions arise as to whether feedback regulation operates between mitochondria and nucleus through the cellular and mitochondrial Ca\(^{2+}\) signaling network and, if this is the case, what are the components involved?

**Summary and Prospects**

In this review, we have attempted to cover in detail the mechanisms involved in mitochondrial Ca\(^{2+}\) homeostasis, underlining the importance of the organelle’s interactions with Ca\(^{2+}\) transport machineries in neighboring membranes (particularly the ER and the PM). These interactions are crucial in regulating the Ca\(^{2+}\) homeostasis and signaling of the whole cell and thus the numerous cellular functions regulated by the cation. Furthermore, we believe that the bidirectional signaling mediated by Ca\(^{2+}\) provides a framework by which mitochondrial biogenesis, shape, and flexible metabolic performance (“mitochondrial dynamics”) will dictate adaptive mechanisms during cell proliferation and cellular stress. This idea suggests several directions for current and future research, comprising 1) further molecular characterization of the Ca\(^{2+}\) transport routes in the mitochondria, 2) identification of molecules and their function in permitochondrial microdomains, and 3) description of the pathways involved in Ca\(^{2+}\)-dependent regulation of mitochondrial biogenesis and structure. Although isolated mitochondria will continue to be a valuable tool for the first aim, the application of molecular biological approaches and development of new tools for the investigation of mitochondrial function in a cellular context will be necessary for the further goals. Finally, systematic comparison of mitochondrial function in different cell types will help to dissect specific pathways determining how life and death of the cell and organisms rely on mitochondria.

In so short an essay, it is not possible to describe all the fascinating activity in this field and so we also apologize to those whose work is not cited here. We thank all our colleagues for the work that has gone into developing this field.

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