Mitochondria as sensors and regulators of calcium signalling

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Abstract | During the past two decades calcium (Ca^{2+}) accumulation in energized mitochondria has emerged as a biological process of utmost physiological relevance. Mitochondrial Ca^{2+} uptake was shown to control intracellular Ca^{2+} signalling, cell metabolism, cell survival and other cell-type specific functions by buffering cytosolic Ca^{2+} levels and regulating mitochondrial effectors. Recently, the identity of mitochondrial Ca^{2+} transporters has been revealed, opening new perspectives for investigation and molecular intervention.

Immunological synapse
The site of functional apposition between an antigen-presenting cell (APC) and a T cell. During antigen presentation, the T cell undergoes a gross morphological rearrangement, with T cell receptors, adhesion molecules, cytoskeletal elements and organelles (including mitochondria) spatially relocating at clusters at the contact site with the APC.

Energized mitochondria
When oxidizable substrates are provided to mitochondria, electrons are fed into the respiratory chain, which couples electron flow to proton pumps across the inner mitochondrial membrane. An electrochemical proton gradient is established that drives ATP synthesis and provides the thermodynamic force for Ca^{2+} accumulation in these energized mitochondria.

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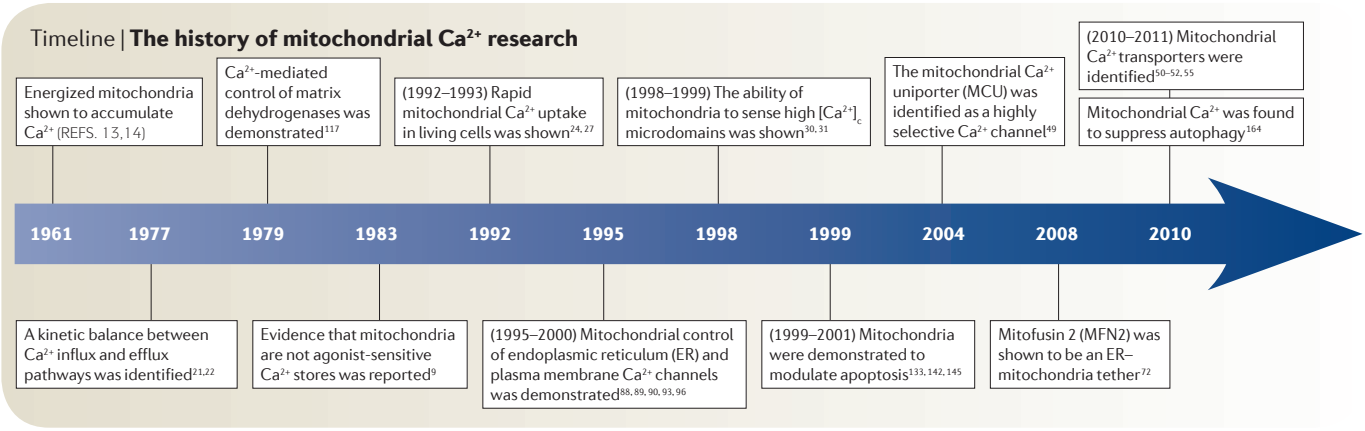
During the past century, calcium (Ca^{2+}) signalling has been characterized as a process that coordinates the inputs of various extracellular stimuli and triggers, or fine-tunes, a vast repertoire of cellular functions[1,2] (TIMELINE). The initial concept that Ca^{2+} ions control physiological events goes back to the seminal (and serendipitous) observation by Ringer *et al.* in 1883 that addition of Ca^{2+} to the perfusion buffer of isolated hearts triggered their contraction[3]. Increases in Ca^{2+} levels can be highly localized (for example, at the synaptic region, the secretory pole of an exocrine cell or the site of cell–cell interaction of a lymphocyte with an antigen-presenting cell (which is called the immunological synapse). Alternatively, local changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_c$) can diffuse across the cell as a wave and elicit an effect at a distant site. Moreover, in most cell types the increases in $[Ca^{2+}]_c$ are oscillatory[4], and the frequency of each $[Ca^{2+}]_c$ oscillation (defined as 'temporal' Ca^{2+} signature) is differentially decoded by the cell[5,6].

This spatiotemporal complexity in the regulation of $[Ca^{2+}]_c$ relies on two key requirements. The first is the cooperation of two different sources of Ca^{2+} in the generation of the $[Ca^{2+}]_c$ rise: the extracellular medium, a virtually unlimited reservoir with a $[Ca^{2+}]$ of ~1 mM, and intracellular pools (known as Ca^{2+} stores, which are endowed with a $[Ca^{2+}]$ >100 μM) that allow rapid release through store-resident channels[7–9]. Although recent work has highlighted a role also for other membrane-bound compartments (such as the Golgi apparatus[10], endosomes and lysosomes[11]), the most important intracellular stores are the endoplasmic reticulum (ER) and its specialized counterpart in muscle cells, the sarcoplasmic reticulum. The second requirement for carefully orchestrating $[Ca^{2+}]_c$ is the existence of a broad range of molecules that generate and decode $[Ca^{2+}]_c$ variations and their defined positioning within the cell[12]. Thus, pumps, channels and buffering proteins finely tune the spatiotemporal pattern of $[Ca^{2+}]_c$ rises, and targets that are located both in the cytoplasm and in different intracellular organelles sense $[Ca^{2+}]_c$ oscillations either directly via a Ca^{2+}-binding site or indirectly through the activity of Ca^{2+}-dependent enzymes (such as kinases and phosphatases) and scaffolding proteins.

In this Review article, we do not cover this broad scientific field, but rather refer to exhaustive reviews[1,2,7]. Here, we focus on the role of mitochondria and specifically discuss the basic thermodynamic principles that drive Ca^{2+} cycling across the membranes of energized mitochondria, the evidence of mitochondrial Ca^{2+} uptake in living cells and its dependence on microdomains of high $[Ca^{2+}]$. In addition, we provide molecular insight into the function of mitochondrial Ca^{2+} transporters and the regulation of Ca^{2+} uptake by mitochondria. Finally, we examine the role of mitochondrial Ca^{2+} signalling in controlling cell metabolism and cell death by necrosis, apoptosis and autophagy.

Role of mitochondria in Ca^{2+} homeostasis

Mitochondrial Ca^{2+} uptake: a historical perspective. Mitochondria were the first intracellular organelle to be associated with Ca^{2+} handling. After several studies suggested Ca^{2+} sequestration by mitochondria, Ca^{2+} uptake by isolated energized mitochondria was directly measured half a century ago[13–15] (TIMELINE). It is remarkable that this experimental observation preceded the chemiosmotic theory, which then provided the thermodynamic basis for rapid accumulation of a positively charged ion such as Ca^{2+} into the mitochondrial matrix[16].



Necrosis
A common form of cell death that frequently results from toxic injury, hypoxia or stress. Necrosis involves cell swelling, dysregulation of cell-membrane ion and water fluxes, mitochondrial swelling and the eventual release of cell contents into the interstitium. This form of cell death usually causes tissue inflammation.

Autophagy
A cellular process (also known as macroautophagy) that mediates the bulk degradation of cytosolic components. Molecules and organelles are surrounded by a double-membrane vesicle (the autophagosome). After fusion with a lysosome, an autolysosome (also known as autophagolysosome) is formed, and the content is degraded by lysosomal hydrolases. Basal autophagy has a function in quality control, whereas higher levels of autophagy are induced during starvation and other stress conditions.

Chemiosmotic theory
The energy that is required for the endergonic synthesis of ATP by the F_1F_0 ATPase is provided by the electrochemical gradient that is generated by the respiratory chain across the inner mitochondrial membrane. In respiring mitochondria, reducing equivalents are transported by carrier molecules (such as NADH and $FADH_2$) to the electron transport chain, whereas matrix protons are transported outwards. Re-equilibration of H^+ into the matrix down its electrochemical gradient is coupled to ATP production.

The mitochondrial electron transport chain translocates H^+ ions across the inner mitochondrial membrane (IMM), thereby generating an electrochemical proton gradient ($\Delta\mu H^+$) whose major component is the membrane potential difference ($\Delta\Psi$). The $\Delta\mu H^+$ drives the flow of H^+ through ATP synthase in a reaction coupled to the generation of ATP from ADP and inorganic phosphate[17]. On the basis of the Nernst equation, the resulting $\Delta\Psi$ also represents a very large driving force for Ca^{2+} accumulation.

The basic properties of mitochondrial Ca^{2+} transport were assessed in the following years. Accumulation of Ca^{2+} into the mitochondrial matrix through the ion-impermeable IMM occurs via an electrogenic pathway that involves the mitochondrial Ca^{2+} uniporter (MCU), which rapidly accumulates Ca^{2+} across the steep electrochemical gradient. The MCU is inhibited by ruthenium red[18] and lanthanides[19] and has a V_{max} of >1400 nmol Ca^{2+} per mg of protein per min (REF. 20). Ca^{2+} accumulation by the MCU is counteracted by mitochondrial Na^+/Ca^{2+} exchangers (mNCX) and mitochondrial H^+/Ca^{2+} exchangers (mHCX)[21,22], which are insensitive to ruthenium red and inhibited by benzothiazepine analogues, such as diltiazem, clonazepam and CGP-37157 (REF. 23).

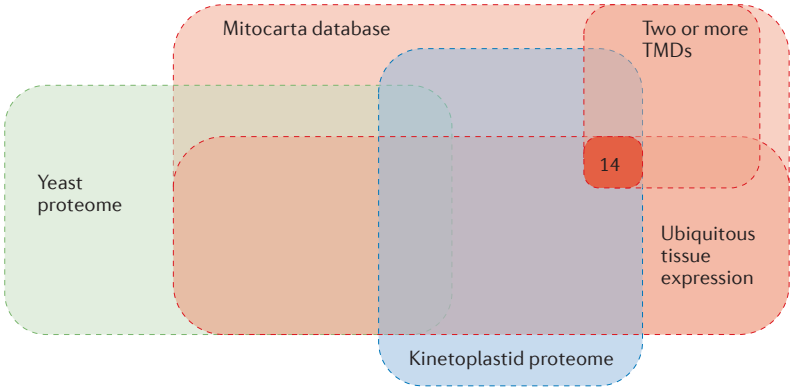
Mitochondrial Ca^{2+} uptake was directly monitored in living cells by using mitochondrial Ca^{2+} probes, including fusion proteins that carried the Ca^{2+}-sensitive photoprotein aequorin[24] and GFP-based protein constructs engineered to change fluorescence properties following Ca^{2+} binding[25,26]. Only then was it appreciated that, following stimulation with a $[Ca^{2+}]_c$-raising agonist, the speed and the amplitude of Ca^{2+} accumulation in mitochondria greatly exceed the values that were previously predicted on the basis of MCU properties in isolated mitochondria[27]. Indeed, the rise in mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mt}$) closely follows the increase of the $[Ca^{2+}]_c$[28], and the peak $[Ca^{2+}]_{mt}$ can exceed, in some cell types, 100 μM[29].

The microdomain concept. The apparent discrepancy between the amplitude of $[Ca^{2+}]_{mt}$ rises and the low affinity of the MCU to Ca^{2+} was solved by the demonstration that mitochondria are located in close proximity to the Ca^{2+} channels that elicit the rise in $[Ca^{2+}]_c$, including inositol-1,4,5-trisphosphate-sensitive channels (also known as $Ins(1,4,5)P_3$ receptors ($Ins(1,4,5)P_3Rs$)) and ryanodine-sensitive channels (also known as ryanodine receptors (RYRs)) on the ER and sarcoplasmic reticulum[30–33] as well as different classes of channels on the plasma membrane (for example, voltage-operated channels and store-operated channels)[34–37]. Therefore, mitochondria sense a microdomain of high $[Ca^{2+}]_c$ that meets the low affinity of the MCU and that dissipates rapidly, thus preventing mitochondrial Ca^{2+} overload and/or Ca^{2+} cycling across the mitochondrial membrane. This notion was supported by the close proximity of mitochondria and ER or sarcoplasmic reticulum[30,33]. Moreover, rapid mitochondrial Ca^{2+} uptake was found to be induced by perfusion of permeabilized cells with a solution containing either $Ins(1,4,5)P_3$ (which induces Ca^{2+} release from the ER) or a $[Ca^{2+}]$ 10-fold higher than that measured in the bulk cytosol[27]. More recently, the $[Ca^{2+}]_c$ reached on the outer surface of mitochondria was directly measured, thus providing reliable estimates of the microdomain $[Ca^{2+}]_c$ that is sensed by mitochondria[38,39]. The concept that mitochondria respond to a pulsatile event, which is the generation of a rapidly dissipating $[Ca^{2+}]_c$ microdomain, has major functional implications. It allows mitochondria to rapidly sense cellular Ca^{2+} signals and to act as highly localized buffers in proximity of Ca^{2+} channels, as discussed below. Thereby, mitochondrial Ca^{2+} uptake can profoundly influence cell survival and various functions, such as metabolism, secretion and signalling.

Components of mitochondrial Ca^{2+} transport

Ca^{2+} channels of the outer mitochondrial membrane. Ca^{2+} accumulation into the mitochondrial matrix requires the crossing of two membranes, the OMM and the IMM. Attention has mostly been placed on the transport systems of the ion-impermeable IMM, as the OMM is permeable to solutes that are smaller than 5 kDa and, thus, also to Ca^{2+}. OMM permeability is attributed to the abundant expression of voltage-dependent anion channels (VDACs). However, the membrane levels of OMM channels seem to be a kinetic bottleneck in the process of rapid Ca^{2+} uptake into the mitochondrial matrix following the formation of high $[Ca^{2+}]_c$ microdomains at the mouth of open ER and sarcoplasmic reticulum

Box 1 | **The long road to the mitochondrial Ca^{2+} uniporter**



Following a few unsuccessful attempts to identify the mitochondrial Ca^{2+} uniporter MCU among ruthenium red-binding glycoproteins in the 1970s[167], mitochondrially sorted ryanodine receptor 1 (RYR1) was proposed to drive mitochondrial Ca^{2+} uptake in the heart[168,169]. Although tissue distribution and electrophysiological properties exclude that RYR1 is the MCU, RYR1 could be an additional pathway cooperating with the MCU in the heart. More recently, uncoupling protein 2 (UCP2) and UCP3 were proposed to be essential components of the MCU[170]. However, mitochondria from *Ucp2*$^{-/-}$ and *Ucp3*$^{-/-}$ mice accumulate Ca^{2+} (REF. 171), and UCPs were proposed to rather act on ATP production, and thereby regulate ER Ca^{2+} loading[172]. In 2009, a small interfering RNA (siRNA) genomic screening in *Drosophila melanogaster* identified LETM1 (Leu zipper EF-hand-containing transmembrane protein 1) as a putative H^+/Ca^{2+} antiporter that mediates mitochondrial Ca^{2+} accumulation[173]. Given that LETM1 was also proposed to be a mitochondrial K^+/H^+ exchanger[174], and that the stoichiometry and ruthenium red sensitivity differ from those of Na^+/Ca^{2+} exchangers (mNCX) and mitochondrial H^+/Ca^{2+} exchangers (mHCX)[175,176], the role of LETM1 awaits further confirmation.

The MCU could not be identified by the approaches commonly used for ion channels: channel blockers (for example, ruthenium red) lacked the specificity that was necessary for direct protein identification, the use of yeast genetics was not possible owing to the absence of ruthenium red-sensitive mitochondrial Ca^{2+} uptake in *Saccharomyces cerevisiae*, and *in silico* homology searches with known Ca^{2+} channels yielded no successful hit. An alternative strategy was made possible by seminal proteomic work allowing to compile an inventory of gene products with mitochondrial localization, as proven by mass spectrometry and imaging studies (Mitocarta)[177]. As shown in the figure, the Mitocarta database was searched with unbiased search constraints, on the basis of the phylogenetic appearance of ruthenium red-sensitive Ca^{2+} uptake (which excluded the yeast proteome but included the kinetoplastid proteome), tissue distribution (which should be ubiquitous in the case of the MCU) and by minimal sequence requirements of channels (which required the existence of two or more transmembrane domains (TMDs)). At first, a 54 kDa protein, termed MICU1 (mitochondrial Ca^{2+} uptake 1), was identified in 2010, but it was shown that although this protein is necessary for mitochondrial Ca^{2+} uptake, MICU1 contains a single TMD (thus unlikely to be a channel). Then in 2011, the bona fide MCU was found to be encoded by the *CCDC109A* gene, which has two evolutionarily conserved TMDs (Supplementary information S1 (figure)).

Electrochemical proton gradient
Gradient generated by the activity of the electron transport chain that translocates H^+ across the inner mitochondrial membrane. It is composed of a membrane potential difference ($\Delta\Psi$) and a cation concentration difference (ΔpH), but the $\Delta\Psi$ component is predominant. The electrochemical proton gradient represents the driving force for H^+ entry and thus ATP synthesis.

(or plasma membrane) channels. The notion that the levels of VDAC channels at the OMM might be limiting for complete Ca^{2+} equilibration was supported by the demonstration that VDAC overexpression augments agonist-dependent rises in $[Ca^{2+}]_{mt}$, whereas VDAC downregulation has the opposite effect[40,41]. Moreover, planar lipid bilayers charged with purified VDAC proteins showed weak anion selectivity and permeability to non-electrolytes. Although VDAC becomes impermeable to negatively charged metabolites, such as ATP, and exhibits weak cation selectivity at a $\Delta\Psi$ higher than 25 mV[42], it seems unlikely that such a high membrane potential (which favours VDAC-mediated Ca^{2+} uptake) can be established across the OMM. However, the voltage gating properties of VDAC are modified by several interacting proteins and metabolites, and thus the possibility of regulation of Ca^{2+} transfer across the OMM *in situ* is plausible[43].

The potential role of VDAC in mitochondrial Ca^{2+} uptake is further complicated by the presence of different VDAC isoforms. Although the three mammalian isoforms show comparable channel properties[44], they have opposite effects on the sensitivity of a cell to apoptotic challenges that involve mitochondrial Ca^{2+} loading. This apparent discrepancy was reconciled by the demonstration that at ER–mitochondria juxtapositions mitochondrial VDAC1 establishes a protein–protein interaction with ER-located Ins(1,4,5)P_3R, thereby potentiating the transfer of low-amplitude apoptotic Ca^{2+} signals to mitochondria[45]. This observation is in accordance with previous demonstrations of isoform-specific interactions of VDAC with apoptotic regulators[46–48] and corroborates the view that VDACs are signalling hubs that participate in the control of metabolic activity and cell fate by regulating Ca^{2+} fluxes and the activity of downstream effectors.

The inner mitochondrial membrane Ca^{2+} channel. The MCU was characterized in terms of kinetic and thermodynamic properties and inhibitor specificity (with sensitivity to ruthenium red being the blueprint of MCU activity) and demonstrated to be a highly selective, low conductance Ca^{2+} channel[49]. As to its molecular identity, several candidates had been proposed until its recent discovery through an *in silico* search in the Mitocarta database (BOX 1). The electrophysiological properties of the newly identified protein, as evaluated in planar lipid bilayers, matched those previously described in isolated mitochondria, and the MCU-mediated Ca^{2+} current could be inhibited by ruthenium red and gadolinium. In addition, some insight into channel function was obtained as negatively charged residues of the amino acid sequence separating the two transmembrane domains (TMDs) of the MCU were shown to be required for channel activity[50] and, furthermore, ruthenium red-binding sites were mapped in the same region[51]. Interestingly, the inactive channel acts as a dominant-negative mutant, strongly suggesting that the MCU forms an active channel as an oligomer[50]. The channel also directly interacts with regulatory elements, such as MICU1 (mitochondrial Ca^{2+} uptake 1 protein), which is required for agonist-mediated, rapid Ca^{2+} uptake into mitochondria[52]. Ongoing work will allow the identification of all the components of this mitochondrial uniporter, but it is already clear that it is a multisubunit channel with associated regulators, which concurs with the need for tight control of the pleiotropic actions of Ca^{2+} in mitochondria.

Mechanisms of Ca^{2+} release from the mitochondria. Early biochemical experiments carried out in isolated organelles clarified that Ca^{2+} efflux from mitochondria is mediated by the ion exchangers mNCX and mHCX. Although these exchangers have been studied extensively in isolated mitochondria[53,54], their molecular

identity was not defined until a few years ago (a fate similar to that of the MCU). The molecular enigma was solved with the identification of the *NCLX* (also known as *SLC24A6*) gene product as being an isoform of a plasma membrane NCX, which localizes to mitochondria and mediates a low affinity Ca^{2+} exchange with Na^+ (REF. 55). NCLX is inhibited by the selective inhibitor of mitochondrial Na^+/Ca^{2+} exchange, CGP-37157 (REF. 23), and its downregulation inhibits mitochondrial Ca^{2+} efflux, whereas its overexpression enhances Ca^{2+} clearance from the mitochondria. Although previous reports localized NCLX to the ER or plasma membrane[56], the mitochondrial localization of NCLX and its role in Ca^{2+} efflux have been confirmed by other laboratories[57] (and G. A. Rutter, personal communication), so at least one of the molecular components of mitochondrial Ca^{2+} efflux has been elucidated. Much less is known regarding the Na^+-independent Ca^{2+} efflux, which so far has only been described in isolated mitochondria. Na^+-independent Ca^{2+} efflux seems to involve an nH^+/Ca^{2+} antiport with a predicted stoichiometry of 1 Ca^{2+} per 2 or 3 H^+ (REF. 58).

Finally, Ca^{2+} may, at least in principle, escape the mitochondrial matrix also through the opening of the mitochondrial permeability transition pore (PTP). However, the relevance of this potential efflux pathway in energized mitochondria has been questioned owing to the finding that inhibition of the PTP component cyclophilin D (CYPD) by cyclosporin A has little or no effect on mitochondrial Ca^{2+} uptake in living cells. Nevertheless, in agonist-stimulated cells, waves of membrane depolarization (which are signals for PTP opening) were reported to cause the release of Ca^{2+} from mitochondria and the amplification of agonist-dependent $[Ca^{2+}]_c$ oscillations[59]. Two recent papers have provided further evidence for a role of PTP in Ca^{2+} efflux. CYPD-deficient mice (*Ppif*$^{-/-}$ mice) display high $[Ca^{2+}]_{mt}$ in the heart, which leads to activation of matrix dehydrogenases and increased glycolysis[60]. When *Ppif*$^{-/-}$ neurons were compared with wild-type neurons, no difference was detected in the $[Ca^{2+}]_{mt}$ rise evoked by physiological stimulations, but an increased loading of mitochondria with Ca^{2+} was reported upon repeated challenges in mutant neurons[61]. In addition, in *Ppif*$^{-/-}$ neurons oxidative stress causes a smaller Ca^{2+} release from mitochondria than in wild-type cells, thus reducing cytosolic Ca^{2+} overload[62]. Therefore, it seems reasonable to conclude that the PTP can mediate Ca^{2+} efflux, a notion that could be of particular relevance in several pathophysiological conditions.

Components of ER–mitochondria junctions

As mentioned above, both GFP labelling of organelles in living cells and electron micrographs revealed the existence of close contacts (<200 nm) between mitochondria and the ER[30]. These domains, which can be biochemically purified as 'mitochondria-associated membranes' (MAMs)[63], have been implicated in housekeeping functions, including cholesterol and ceramide biosynthesis[64,65], non-vesicular transport of phospholipids (for example, by long-chain fatty-acid CoA synthases[66]) and transport of proteins, ions and metabolites[67,68]. The regions of apposition are tethered by trypsin-sensitive protein filaments, and coupling between the ER and mitochondria can be altered by experimentally manipulating the intra-organellar protein linkages[33,69]. In this multifunctional platform, Ca^{2+} transfer represents a fundamental process, given its role in energy production, cell fate and growth[70,71]. Moreover, the elucidation of the organization of these ER–mitochondrial junctions will help our understanding of the organization and function of other such 'quasi-synaptic' membrane structures[31] (FIG. 1).

Generation of mitochondria–ER contacts. Mitofusin 2 (MFN2), a component of the mitochondrial fusion and fission machinery, has been recently associated with the formation of mitochondria–ER contacts. MFN2 was shown to be enriched in MAMs, where it tethers ER and mitochondria[72]. Moreover, in cardiac muscle, the sarcoplasmic reticulum–mitochondria associations are enriched in a low-molecular weight MFN protein variant and were proposed to correspond to contact sites between the IMM and the OMM[73].

Interestingly, in fibroblasts lacking MFN2, the distance between the ER and mitochondria is increased, both organelles show altered shape, and the efficiency of mitochondrial Ca^{2+} uptake is drastically reduced[72]. This seminal observation further highlights the previously established relevance of the equilibrium between fusion and fission and Ca^{2+} homeostasis within the organellar network[74–76]. Indeed, homotypic interactions between MFN2 proteins located at the ER and the mitochondrial membrane result in a structure that mediates organellar interaction. The discovery of this new role of MFN2 was followed by the identification of MFN2-interacting proteins such as trichoplein keratin filament-binding protein (TCHP; also known as mitostatin)[77]. Thus, the control of ER–mitochondria juxtaposition requires the activity of a complex, and most likely highly regulated, tethering machinery that still needs to be completely understood.

Ca^{2+} microdomain signalling: the role of chaperones. Recent work has corroborated the view that ER–mitochondria contacts are not only morphological features, but rather specialized domains, with protein compositions that are specific for several cellular functions[78,79]. Accordingly, the repertoire of signalling molecules at these microdomains seems to be controlled by resident chaperones. The ER-resident sigma 1 receptor is highly enriched at MAMs and forms a complex with another ER chaperone, termed immunoglobulin heavy chain-binding protein (BIP; also known as GRP78 and HSPA5). After the initiation of Ca^{2+} release from the ER, sigma 1 receptor dissociates from BIP and binds to $Ins(1,4,5)P_3Rs$ at MAMs, thereby preventing the degradation of $Ins(1,4,5)P_3Rs$ and sustaining prolonged Ca^{2+} uptake by mitochondria[80]. Similarly, other ER chaperones, such as calnexin and calreticulin, are compartmentalized in MAMs[80,81] and modulate local Ca^{2+} levels both by acting as Ca^{2+} buffers through their Ca^{2+}-binding capacity and by regulating the activity of sarcoplasmic reticulum Ca^{2+}-ATPases (SERCAs)[82–84]. Calnexin activity is regulated by the cytosolic sorting protein PACS2

Membrane potential
The charge difference (measured in mV) between the two surfaces of a biological membrane that arises from the different concentrations of ions such as H^+, Na^+ or K^+ on either side. The Na^+/K^+-ATPase creates a membrane potential by using the energy stored in ATP to maintain a low concentration of Na^+ and a high concentration of K^+ inside the cell, and a high concentration of Na^+ and a low concentration of K^+ on the outside.

Nernst equation
Calculates the equilibrium potential for an ion on the basis of the charge of the ion and the concentration gradient.
$$V_{eq} = \frac{RT}{zF} \ln\left(\frac{[CAT^{z+}]_{out}}{[CAT^{z+}]_{in}}\right)$$
V_{eq} is the equilibrium potential, R the universal gas constant, T the temperature, z the valence of the ion, F the Faraday's constant and $[CAT^{z+}]$ is the concentration of any cation.

Mitochondrial Ca^{2+} uniporter
(MCU). The inner mitochondrial membrane channel responsible for mitochondrial Ca^{2+} uptake. Early biochemical work in the 1960s showed that energized mitochondria rapidly accumulate Ca^{2+} via an electrogenic mechanism with a net charge transfer of 2. Because of the lack of evidence for other ions being co-transported or exchanged during this process, this channel has been defined as a 'uniporter'.

Na^+/Ca^{2+} exchangers
(NCX). Antiporters that exchange Na^+ with Ca^{2+} at the membrane. Although fully reversible, the function of NCX at the plasma membrane is to extrude Ca^{2+} from the cytosol, while Na^+ enters down its concentration gradient. At the inner mitochondrial membrane, mitochondrial Ca^{2+} is extruded while Na^+ enters into the matrix.

H^+/Ca^{2+} exchangers
(HCX). Na^+-independent antiporter membrane protein that permits the extrusion of Ca^{2+} from the matrix and the entrance of H^+.

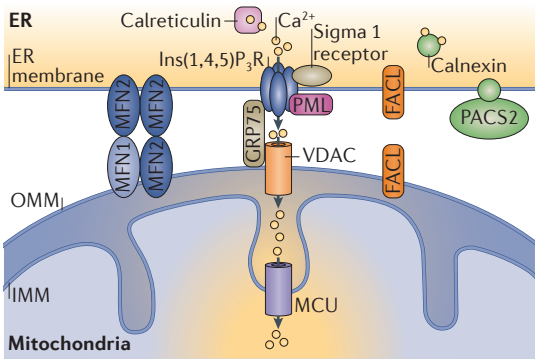


Figure 1 | **The build-up of the ER–mitochondria junctions.** Close interactions between the endoplasmic reticulum (ER) and mitochondria are essential for rapid and sustained Ca^{2+} uptake by mitochondria. Voltage-dependent anion channels (VDACs), located at the outer mitochondrial membrane (OMM), are responsible for the rapid transfer of Ca^{2+} from the ER–mitochondria apposition, and their function results in high Ca^{2+} microdomains in the mitochondria intermembrane space. Accumulation of Ca^{2+} into the mitochondrial matrix occurs via the mitochondrial Ca^{2+} uniporter (MCU), which rapidly accumulates Ca^{2+} across the steep electrochemical gradient. A number of chaperones and regulatory proteins control the formation of the ER–mitochondria junction, the clustering of signalling proteins and their modulation. Mitofusin 2 (MFN2) is involved in both mitochondrial fusion and in ER–mitochondria tethering, by both homotypic interactions and heterotypic interactions with MFN1. Chaperones modulate ER Ca^{2+} buffering (for example, calreticulin and calnexin) and control the stability or the sorting of signalling proteins. For example, sigma 1 receptor stabilizes inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) receptors ($Ins(1,4,5)P_3Rs$) when ER Ca^{2+} stores are depleted, thereby ensuring proper Ca^{2+} fluxes from the ER to the mitochondria. Phosphofurin acidic cluster sorting protein 2 (PACS2) controls the translocation of calnexin from the ER to the plasma membrane and thereby modulates ER Ca^{2+} buffering and controls ER–mitochondria appositions during apoptosis. Moreover, chaperones affect the activity of ion channels. For example GRP75 (75 kDa glucose-regulated protein), which mediates the interaction of VDAC1 with $Ins(1,4,5)P_3R$, facilitates mitochondrial Ca^{2+} uptake, and PML (promyelocytic leukaemia) protein, which regulates $Ins(1,4,5)P_3R$-mediated Ca^{2+} release from the ER, supports mitochondrial Ca^{2+} uptake and thus has a crucial role during apoptosis. The family of long-chain fatty-acid CoA ligases (FACL) is involved in lipid metabolism and is enriched in mitochondria-associated membranes (MAMs). IMM, inner mitochondrial membrane.

Aequorin
Ca^{2+}-sensitive photoprotein isolated from luminescent jellyfish that is used to detect the Ca^{2+} content in different subcellular compartments.

Inositol-1,4,5-trisphosphate-sensitive channels
($Ins(1,4,5)P_3$-sensitive channels). Ca^{2+}-selective channels that are activated by the second messenger $Ins(1,4,5)P_3$ and are mainly located in the endoplasmic reticulum (ER) membrane. Given the difference in the Ca^{2+} concentration between the ER lumen and the cytosol, opening of this channel results in a transient increase in intracellular Ca^{2+} concentration.

Ryanodine-sensitive channels
Ca^{2+}-selective channels that are mainly located in the membrane of the sarcoplasmic reticulum of skeletal muscle and heart, but they are also expressed in the endoplasmic reticulum of other tissues (in particular in the brain). They are activated by Ca^{2+} and blocked by the plant alkaloid ryanodine.

Voltage-operated channels
Plasma membrane located, highly selective Ca^{2+} channels that are activated by membrane depolarization. They are divided in several different subfamilies on the basis of their subunit composition, which also reflects their different tissue distribution, Ca^{2+}-current type and pharmacological profile. Present nomenclature uses a numerical system (that is, $Ca_v1.x$, $Ca_v2.x$ and $Ca_v3.x$), but older nomenclature uses an alphabetical system (that is, L-, P/Q-, R-, N- and T-type) according to their Ca^{2+}-current features.

(phosphofurin acidic cluster sorting protein 2) through a direct interaction between the two proteins[81]. Indeed, in physiological conditions >80% of calnexin localizes to the ER, mostly in MAMs, whereas PACS2 knockdown causes a redistribution of calnexin away from the ER to the plasma membrane, thus affecting mitochondrial and ER Ca^{2+} homeostasis.

Chaperones also seem to be involved in the direct functional interaction between ER and mitochondrial Ca^{2+} channels. Specifically, VDAC1 present on OMM was shown to interact with $Ins(1,4,5)P_3R$ on the ER through the chaperone 75 kDa glucose-regulated protein (GRP75; also known as HSPA9)[85] at the MAM. Small interfering RNA (siRNA) silencing of *GRP75* abolishes the functional coupling between $Ins(1,4,5)P_3R$ and VDAC1, thereby reducing mitochondrial Ca^{2+} uptake in response to agonist stimulation. In addition, the promyelocytic leukaemia (PML) tumour suppressor is enriched at the MAM. On the ER side, PML forms a complex with $Ins(1,4,5)P_3R$, AKT (also known as PKB) and protein phosphatase 2A (PP2A) and participates in the AKT- and PP2A-dependent modulation of $Ins(1,4,5)P_3R$ activity and hence in the regulation of ER–mitochondria Ca^{2+} transfer[86].

Overall, MAMs seem to be signalling platforms where ER and mitochondrial Ca^{2+} channels interact with several modulators. We expect that future work will expand the list of regulatory proteins that are involved in this interface, and that new findings will clarify the mechanisms that control their sorting to MAMs and the functional interactions occurring in this microdomain.

Mitochondria as cytosolic Ca^{2+} buffers

The demonstration that mitochondria rapidly accumulate Ca^{2+} upon stimulation with agonists evoking a $[Ca^{2+}]_c$ rise implied that they must be taken into account when estimating the total buffering capacity of the cytoplasm and of the specific intracellular domains where the mitochondria are clustered. In contrast to Ca^{2+}-binding proteins, mitochondria may act as fixed buffers of high capacity and sequester large amounts of Ca^{2+} from a subcellular domain. This concept was explored in several cell models, and mitochondrial Ca^{2+} buffering was shown to influence cellular Ca^{2+} signals, and hence cell function, in two conceptually different manners.

Mitochondrial Ca^{2+} buffering regulates the activity of Ca^{2+} channels. At Ca^{2+}-signalling microdomains, which are formed between mitochondria and the ER or the sarcoplasmic reticulum, or between mitochondria and the plasma membrane, energized mitochondria rapidly remove Ca^{2+} from the mouth of the open Ca^{2+} channels on the ER, the sarcoplasmic reticulum or the plasma membrane and thus modify the local $[Ca^{2+}]_c$. Following ER Ca^{2+} release, the available cytosolic Ca^{2+} provides a regulatory feedback to Ca^{2+} channels that can be activatory, inhibitory or biphasic, depending on the $[Ca^{2+}]_c$[87]. Therefore, mitochondrial Ca^{2+} uptake can modulate the activity of Ca^{2+} channels by influencing the spatiotemporal properties of the $[Ca^{2+}]_c$ (FIG. 2). For example, in *Xenopus laevis* oocytes, mitochondrial energization and the resulting increase in Ca^{2+} uptake by mitochondria coordinate the subsequent $Ins(1,4,5)P_3$-induced rises in $[Ca^{2+}]_c$ into single propagating Ca^{2+} waves of lower frequency and higher amplitude[88]. This mitochondrial buffering effect depends on the bell-shaped effect of $[Ca^{2+}]_c$ on $Ins(1,4,5)P_3R$ activity, as low and high $[Ca^{2+}]_c$ inhibit the opening of $Ins(1,4,5)P_3Rs$, whereas intermediate $[Ca^{2+}]_c$ promotes $Ins(1,4,5)P_3R$ activity. Accordingly, removal of Ca^{2+} from the vicinity of $Ins(1,4,5)P_3Rs$ inhibits channel opening (thus preventing asynchronous channel opening) but also relieves Ca^{2+}-mediated inhibition of channels that are already open, thereby further potentiating

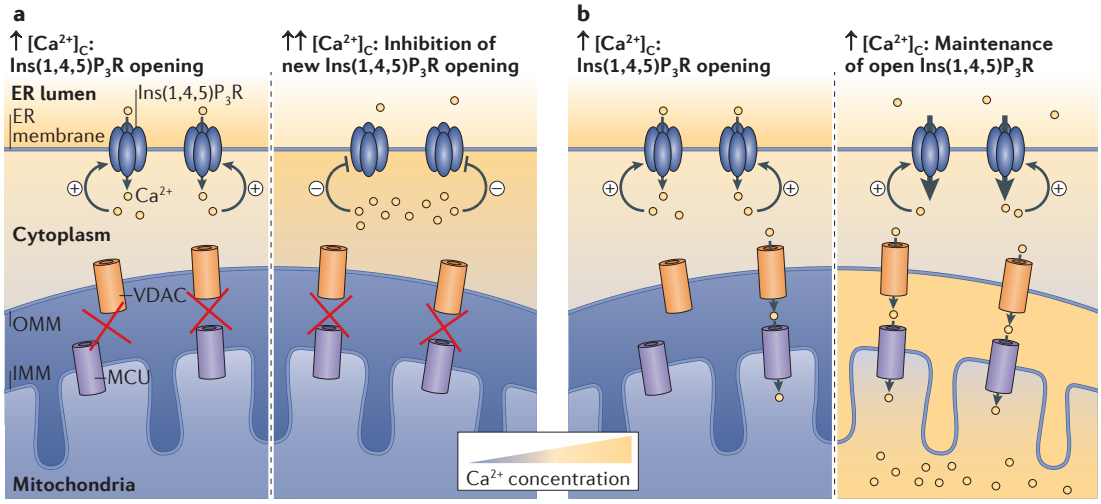


Figure 2 | **Ca^{2+} buffering by mitochondria regulates Ca^{2+} release from the ER.** This simplified model shows how the activity of inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) receptors ($Ins(1,4,5)P_3Rs$), and thus Ca^{2+} release from the ER, can be regulated by the presence of mitochondria. **a** | In the absence of mitochondrial Ca^{2+} uptake, the initial increase of cytosolic Ca^{2+} levels favours the opening of $Ins(1,4,5)P_3R$ and Ca^{2+} release from the ER (left panel). Sustained $Ins(1,4,5)P_3R$ activity leads to the build-up of a local high cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) that then inhibits $Ins(1,4,5)P_3R$ activity and blocks further Ca^{2+} release (right panel). **b** | In the presence of mitochondrial Ca^{2+} buffering activity, the initial increase of cytosolic Ca^{2+} levels again induce Ca^{2+} release from the ER (left panel). However, as mitochondrial Ca^{2+} uptake reduces the $[Ca^{2+}]_c$ at the mouth of the channel, no negative feedback by cytosolic Ca^{2+} is exerted and, thus, $Ins(1,4,5)P_3R$ activity is sustained and Ca^{2+} release from the ER is prolonged (right panel). IMM, inner mitochondrial membrane; OMM outer mitochondrial membrane; VDAC, voltage-dependent anion channel.

Store-operated channels
Highly selective Ca^{2+} channels located at the plasma membrane that open in response to depletion of internal Ca^{2+} stores. Upon endoplasmic reticulum (ER) Ca^{2+} depletion, the Ca^{2+} sensors of the STIM family, which are located at the ER surface, cause the opening of plasma membrane store-operated channels, termed ORAI. The ORAI family includes three members (ORAI1, ORAI2 and ORAI3), and the STIM family is composed of STIM1 and STIM2.

Voltage-dependent anion channels
(VDACs). Abundant and highly conserved proteins of the outer mitochondrial membrane. VDAC exists in three isoforms in mammals (termed VDAC1, VDAC2 and VDAC3) and is permeable to many solutes below 5 kDa.

Mitocarta
Inventory of 1098 mouse genes encoding proteins that are likely to be localized at mitochondria. The data were generated by mass spectrometry of mitochondria isolated from 14 tissues and protein localization was confirmed by large-scale tagging of candidate proteins with GFP and microscopy. The data were integrated with six other genome-scale data sets of mitochondrial localization.

Permeability transition pore
(PTP). High conductance inner mitochondrial membrane (IMM) channel that requires a permissive load of matrix Ca^{2+} for opening and is specifically inhibited by cyclosporin A. Persistent PTP opening irreversibly commits cells to death by causing IMM depolarization (which blocks of oxidative phosphorylation and reactive oxygen species production), matrix swelling and cristae unfolding and results in the release of stored Ca^{2+} and of apoptogenic proteins.

Ca^{2+} release[88]. In hepatocytes challenged with suboptimal $Ins(1,4,5)P_3$ concentrations, mitochondria suppress the positive feedback by Ca^{2+} on $Ins(1,4,5)P_3Rs$, thus setting the threshold for activation and shaping the cytosolic Ca^{2+} wave[89]. In rat astrocytes, the prevalent $Ins(1,4,5)P_3R$ isoform (which is $Ins(1,4,5)P_3R2$) is only positively, but not negatively, regulated by increased $[Ca^{2+}]_c$[90]. As a result, mitochondria exert a negative control on Ca^{2+} wave propagation. In cardiac cells, in which Ca^{2+} release from stores is operated by RYRs, mitochondrial Ca^{2+} uptake reduces the frequency and duration of $[Ca^{2+}]_c$ sparks, which suggests that mitochondrial Ca^{2+} uptake also modulates RYRs and controls the excitation–contraction coupling system[91].

In addition to ER-resident Ca^{2+} channels, plasma membrane-resident Ca^{2+} channels are subject to regulation by local $[Ca^{2+}]_c$ and, thus, by mitochondrial Ca^{2+} uptake. In activated T cells, mitochondria are located in proximity to ORAI channels, which mediate Ca^{2+} release-activated Ca^{2+} (CRAC) currents at the immunological synapse[92] and sustain store-dependent Ca^{2+} influx[93], thereby promoting nuclear translocation of nuclear factor of activated T cells (NFAT) and Ca^{2+}-associated gene transcription[94]. Microdomains with high $[Ca^{2+}]_c$ at the mitochondrial surface were directly detected in GH3 pituitary cells upon opening of voltage-gated Ca^{2+} channels, but not in HeLa cells upon store-operated Ca^{2+} entry (SOCE)[38]. In agreement with this observation, mitochondria were shown to be distant from the plasma membrane in rat basophil leukaemia (RBL) cells and mast cells both in resting conditions and upon stimulation[95]. Furthermore, mitochondrial Ca^{2+} uptake was shown not to affect the fast Ca^{2+}-dependent inactivation (CDI) of ORAI channels[96]. However, mitochondrial Ca^{2+} uptake participates in buffering bulk cytosolic $[Ca^{2+}]_c$ rises (as discussed below) and hence prevents slow inactivation of ORAI channels[96,97]. By this mechanism, and by controlling ER Ca^{2+} loading, respiring mitochondria regulate CRAC currents also in RBL cells and mast cells, thus revealing a complex interaction between mitochondrial Ca^{2+} uptake and SOCE.

Control of Ca^{2+} gradients through mitochondrial positioning. The mitochondrial buffering capacity may contribute to the gradual accumulation of a large amount of Ca^{2+} in a defined subcellular domain. This is, for example, observed in neurons, where the spatial distribution of mitochondria may promote large, local $[Ca^{2+}]_c$ rises (FIG. 3). In these cells, mitochondria that are located in proximity to the plasma membrane accumulate large amounts of Ca^{2+} (up to >10 mmol per kg dry weight), which reflects a high capacity storage mechanism that most likely corresponds to the formation of Ca^{2+}–phosphate precipitates[98]. Moreover, Ca^{2+} sequestration by mitochondria is crucial in defined cellular subdomains of neurons, as Ca^{2+} buffering by synapse-located mitochondria is involved in shaping $[Ca^{2+}]_c$ dynamics and strongly affects neurotransmitter release[99–103]. In this context, organelle recruitment to specific regions (for example, neuronal soma, synapses and dendritic spines) is key for regulating neuronal activity[104,105]. The emerging picture shows that both $[Ca^{2+}]_c$ and $[Ca^{2+}]_{mt}$ affect mitochondrial movements[106,107], and defects in organelle positioning have been implicated in the pathogenesis of major neurological disorders[108,109]. Also striking is the case of pancreatic acinar cells, in which

Cyclophilin D
(CYPD). Major inducer of the opening of the permeability transition pore (PTP). CYPD binds to the inner mitochondrial membrane (IMM) in a process that is regulated by Ca^{2+}, inorganic phosphate and reactive oxygen species. This interaction is prevented by cyclosporin A and by other CYPD-interacting molecules that are usually described as PTP inhibitors.

Ca^{2+} buffers
Molecules and organelles that bind or sequester Ca^{2+} and thereby act as Ca^{2+} sponges and modulate Ca^{2+} concentration in subcellular subdomains.

ORAI channels
Pore-forming subunits of store-operated channels. They are predicted to have four transmembrane domains and three family members have been identified thus far.

Store-operated Ca^{2+} entry (SOCE)
The activation of a Ca^{2+} channel in the plasma membrane in response to the depletion of Ca^{2+} levels in the endoplasmic reticulum. SOCE is also known as capacitative Ca^{2+} entry.

agonist-evoked physiological Ca^{2+} signals are normally confined to the secretory pole by the 'firewall' activity of a mitochondrial belt. Only when the buffering capacity of mitochondria is overwhelmed, for example, by supramaximal stimulation or by the action of toxic agents[110–113], can the Ca^{2+} wave diffuse to the basolateral region, thereby evoking long-term transcriptional effects in the nucleus[114].

Overall, the emerging picture defines mitochondria as efficient, high capacity Ca^{2+} buffers that shape cytosolic Ca^{2+} transients by either regulating the kinetic properties of Ca^{2+} channels or by preventing Ca^{2+} diffusion away from the area where the open channels are located. Interestingly, mitochondrial redistribution may be crucial for spatially constraining Ca^{2+} signals to defined cell domains in large cells.

Mitochondrial Ca^{2+} regulates metabolism

Ca^{2+} accumulating within mitochondria regulates intrinsic functions of the organelle, including the main mitochondrial task, which is ATP production by oxidative phosphorylation (FIG. 4). This concept dates back to the demonstration that three matrix dehydrogenases are activated by Ca^{2+}: pyruvate dehydrogenase is regulated by a Ca^{2+}-dependent phosphatase, and α-ketoglutarate- and isocitrate-dehydrogenases are regulated by direct binding of Ca^{2+} to these enzymes[115–117]. Stimulation of Ca^{2+}-sensitive dehydrogenases increases NADH availability and hence the flow of electrons down the respiratory chain, thus adjusting ATP synthesis to the increased needs of a stimulated cell[118]. In genetic disorders that affect mitochondrial function, respiratory chain defects reduce the driving force for Ca^{2+} transfer, and therefore the stimulation of ATP production[119,120].

Mitochondria seem particularly suited for decoding $[Ca^{2+}]_c$ oscillations. Single cell measurements in hepatocytes showed that $[Ca^{2+}]_{mt}$ oscillations trigger the activation of matrix dehydrogenases and an increase in NADH levels. Thus, high frequency $[Ca^{2+}]_c$ and $[Ca^{2+}]_{mt}$ oscillations represent the most effective signal for a sustained stimulation of aerobic metabolism[5]. The physiological relevance of this phenomenon was confirmed in rat cortical astrocytes, in cardiomyocytes and in pancreatic acinar cells[90,121,122].

Metabolic regulation by mitochondrial Ca^{2+} uptake is not limited to the effects on dehydrogenases. Aspartate/glutamate exchangers of the IMM (termed aralar1 and citrin (also known as aralar2)) include an EF-hand Ca^{2+}-binding domain, which allows increased metabolite transport and stimulation of ATP production in response to a rise in $[Ca^{2+}]_c$[123–125]. These Ca^{2+}-binding sites are exposed in the intermembrane space, and this implies a coordinated and complex regulation of matrix dehydrogenases and aspartate/glutamate exchangers in response to local and temporal Ca^{2+} signals.

Thus, bioenergetic studies in intact cells have demonstrated that, as predicted by the early biochemical work, mitochondrial function is under the control of the master second messenger of the cell, Ca^{2+}. The quasi-synaptic association of the mitochondria to ER and plasma membrane Ca^{2+} channels ensures prompt Ca^{2+} transfer to the organelle, which stimulates aerobic metabolism parallel to the activation of ATP-consuming processes in the cytosol. This prevents significant alterations in the energy balance of the cell.

Mitochondrial Ca^{2+} regulates cell survival

Necrosis. Mitochondrial Ca^{2+} uptake affects cell death pathways. Indeed, mitochondrial Ca^{2+} overload has long been associated with necrosis that occurs in response to ischaemia–reperfusion of the heart and with excitoxicity of neurons (for a review, see REF. 126). In ischaemia–reperfusion, cellular (and hence mitochondrial) Ca^{2+} overload, in conjunction with accumulation of reactive oxygen species (ROS), favours the sustained opening of the high-conductance PTP. This causes the rapid collapse of the membrane potential and swelling of mitochondria, with consequent loss of pyridine nucleotides and cytochrome *c*. The ensuing bioenergetic crisis and ATP depletion result in cardiomyocyte necrotic cell death[127,128].

In neuronal excitotoxicity, activation of *N*-methyl-D-aspartate receptors (NMDRs) by glutamate results in a primary increase in $[Ca^{2+}]_c$, with Ca^{2+} entering the cytoplasm directly through NMDRs[129,130]. In addition, depolarization induces the opening of voltage-gated Ca^{2+} channels, while at the same time the activity of the plasma membrane NCX is reversed. During this rise in $[Ca^{2+}]_c$, mitochondria accumulate and retain Ca^{2+} to buffer the cytosolic loading. However, this first glutamate-dependent $[Ca^{2+}]_c$ rise promotes an extensive accumulation of Ca^{2+} several hours after the toxic challenge. It was shown that necrosis is initiated by this delayed Ca^{2+} influx, which is independent of Ca^{2+} release from mitochondria but dependent on the declining activity of cytoplasmic Ca^{2+} clearing mechanisms (for example, calpain-mediated cleavage of NCX). Following necrosis initiation, mitochondria are overloaded with Ca^{2+}, the electrochemical proton gradient collapses and necrotic cell death is induced[131].

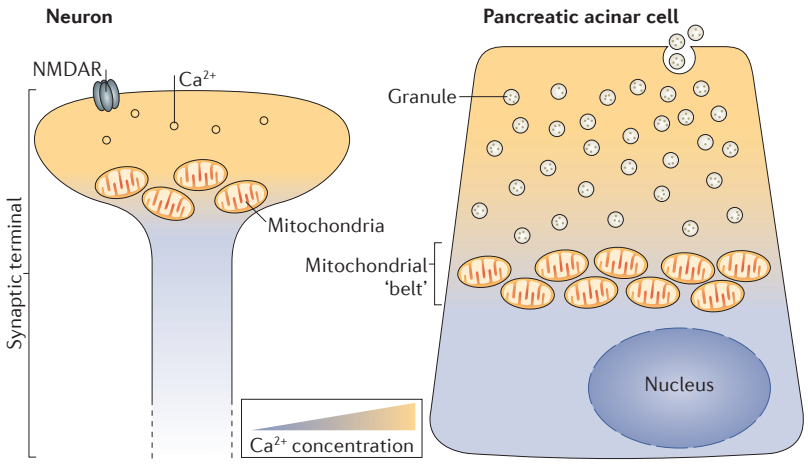


Figure 3 | **Spatial Ca^{2+} buffering by mitochondria in polarized cells.** In neurons, mitochondria localized in proximity of Ca^{2+} channels such as *N*-methyl-D-aspartate receptor (NMDAR) accumulate Ca^{2+}, and prevent the spreading of a cytosolic Ca^{2+} wave. In pancreatic acinar cells, a mitochondrial 'belt' blocks the spreading of the Ca^{2+} wave, maintaining the rise Ca^{2+} concentration confined to the apical, granule-containing region.

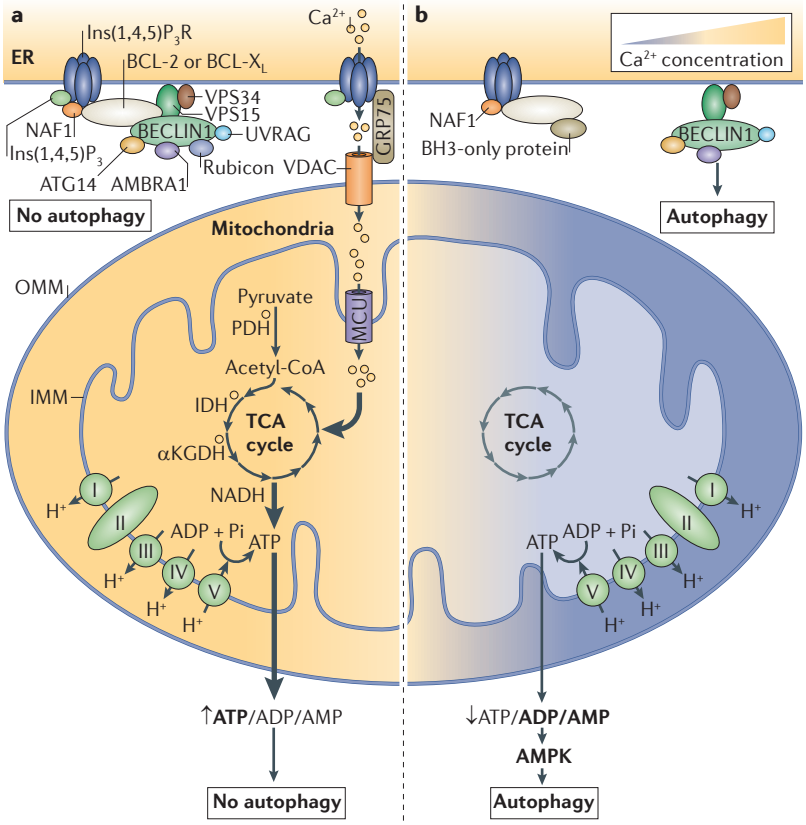


Figure 4 | **The regulation of aerobic metabolism and autophagy. a** | Conditions that inhibit autophagy. The tricarboxylic acid cycle (TCA cycle) consists of a series of reactions that produce energy through the breakdown of proteins, fatty acids and carbohydrates. In the TCA cycle, acetyl CoA is degraded, and the energy of the acetyl group is transformed into GTP and into reducing equivalents in the form of NADH. The NADH generated by the TCA cycle is then used by oxidative phosphorylation to produce ATP, and thus autophagy is inhibited. Increased mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mt}$) activates pyruvate-, isocitrate- zand α-ketoglutarate dehydrogenases (PDH, IDH and αKGDH, respectively), thus increasing the electron flux through the respiratory chain, and ATP production. Notably, inositol 1,4,5 trisphosphate ($Ins(1,4,5)P_3$) receptor ($Ins(1,4,5)P_3R$) is also independently involved in autophagy inhibition by forming protein complexes with BECLIN1 and (B cell lymphoma 2) BCL-2 or BCL-X_L. BECLIN1 is a component of the autophagic vacuolar protein sorting 34 (VPS34) complex (which also contains VPS15) and has a central role in autophagy. In its inactive state, BECLIN1 interacts with the BCL-2–$Ins(1,4,5)P_3R$ complex. **b** | Conditions that promote autophagy. Reduced Ca^{2+}-dependent stimulation of aerobic metabolism activates AMP-activated protein kinase (AMPK), and hence autophagy. Moreover, autophagy is induced following the disruption of the BCL 2–BECLIN1 interaction by BH3 only proteins, which activates BECLIN1. In addition to $Ins(1,4,5)P_3R$, several negative and positive regulators participate to the stability of BCL-2–BECLIN1 complex and to the activity of BECLIN1 (including autophagy-related protein 14 like protein (ATG14), activating molecule in BECN1 regulated autophagy protein 1 (AMBRA1), nutrient-deprivation autophagy factor 1 (NAF1), rubicon and ultraviolet irradiation resistance-associated gene (UVRAG)). GRP75, 75 kDa glucose-regulated protein; Pi, inorganic phosphate; MCU, mitochondrial Ca^{2+} uniporter; OMM, outer mitochondrial membrane.

Apoptosis. The initiation step of the intrinsic pathways of apoptosis involves the release of apoptosome components, such as cytochrome *c,* from the mitochondria. Organelle fragmentation following PTP opening[132] has a key role in this process. Despite the lack of a mechanistic understanding, numerous data reveal that the most important trigger for PTP opening is Ca^{2+}, which acts in conjunction with a variety of apoptotic signals in living cells (FIG. 5). Subthreshold apoptotic signals, such as oxidative stress or production of the short-chain ceramide analogue C2-ceramide, synergize with cytosolic Ca^{2+} waves evoked by a physiological stimulation in triggering an intracellular wave of PTP opening. This ultimately leads to apoptosis[133–135] by enabling the release of pro-apoptotic mitochondrial components. The mechanism underlying the release of pro-apoptotic proteins is still unclear, as no major swelling, and hence OMM rupture, is detected in most cell models. Rather, PTP opening was demonstrated to cause the remodelling of cristae, thus rendering cytochrome *c* stored in the cristae lumen available for release[136]. Finally, $[Ca^{2+}]_c$ rises were also shown to directly induce mitochondrial pro-apoptotic morphological modifications. Indeed, calcineurin-dependent translocation of mitochondrial fission 1 protein (FIS1) triggers mitochondrial fission[137,138] and hence cytochrome *c* release[139,140].

The role of Ca^{2+} signals in apoptosis was further reinforced by the demonstration that anti-apoptotic proteins (such as B cell lymphoma 2 (BCL-2)) lower ER Ca^{2+} levels and hence reduce cytosolic and mitochondrial Ca^{2+} responses to extracellular stimuli by increasing the ER Ca^{2+} leak[141–144]. By contrast, pro-apoptotic proteins exert the opposite effect[145]; for example, the tumour suppressor FHIT (fragile histidine triad protein) acts directly on mitochondrial Ca^{2+} channel sensitivity[146]. Interestingly, in T cells that lack the pro-apoptotic proteins BAX and BCL-2 antagonist/killer (BAK), Ca^{2+} release from the ER is reduced, and this correlates with a reduction in cell proliferation[147]. By contrast, the anti-apoptotic protein BCL-X_L directly interacts with and sensitizes $Ins(1,4,5)P_3R$, thereby partially emptying the ER (which prevents mitochondrial Ca^{2+} loading upon stimulation) and sustaining bioenergetic function via the tonic stimulation of matrix dehydrogenases[148]. In some cell types or experimental conditions, no reduction of ER Ca^{2+} levels was detected in the presence of BCL-2 (REFS. 149,150), thus raising the possibility that BCL-2-dependent control of ER Ca^{2+} leakage may depend on the $Ins(1,4,5)P_3R$ subtype or on $Ins(1,4,5)P_3R$ post-translational modifications[151,152]. Interestingly, in some of these reports a decrease in the kinetics of ER Ca^{2+} release was shown[150,153,154], which suggests reduced mitochondrial Ca^{2+} uptake. These findings highlight a dual role of mitochondrial Ca^{2+} in energy provision and induction of cell death.

Overall, a general consensus has emerged that mitochondrial Ca^{2+} loading has a permissive role, allowing several toxic challenges to cause the release of caspase cofactors from the organelle, resulting in apoptotic cell death. Furthermore, it seems that alteration of this cellular response (for a example, by tumour or viral proteins[142,145,155,156]) has a role in the pathogenesis of human disorders. At the same time, prolonged PTP opening leads to complete collapse of the membrane potential and Ca^{2+} discharge, which results in the complete loss of mitochondrial function and necrotic cell death. Accordingly, the genetic ablation of the PTP regulator CYPD was shown to impair Ca^{2+} overload and oxidative damage-induced necrotic cell death[157].

Ca^{2+}-dependent inactivation (CDI). The process whereby increases of cytosolic Ca^{2+} leads to inactivation of the Ca^{2+} release-activated Ca^{2+} (CRAC) channel.

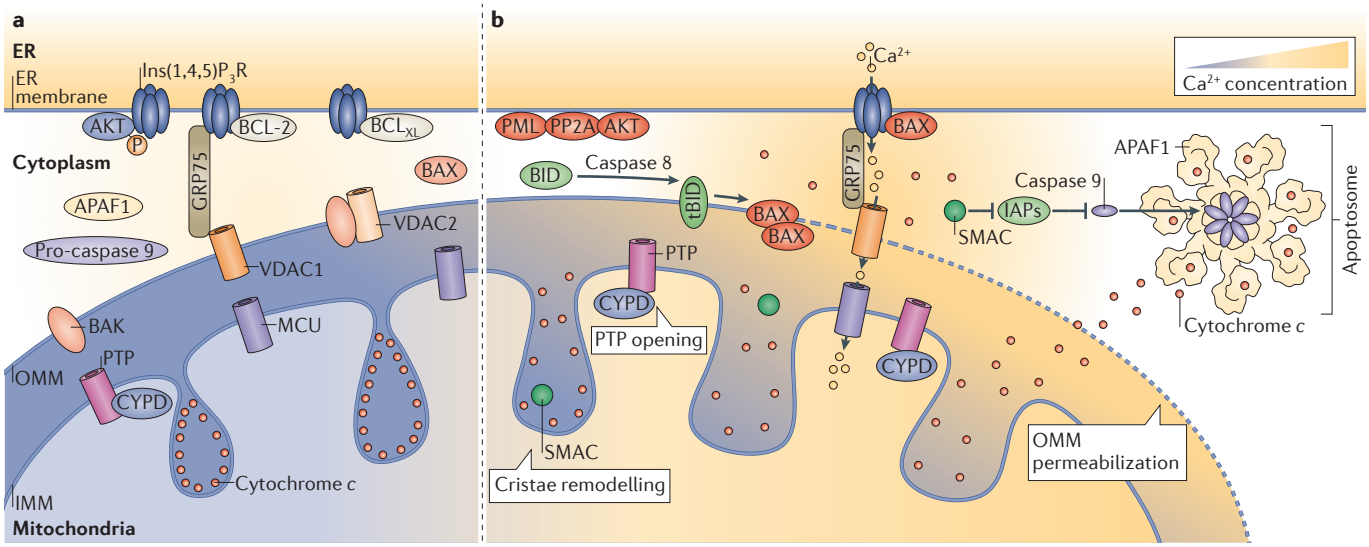


Figure 5 | **Mitochondrial Ca^{2+} signals in the regulation of cell death pathways.** **a** | Conditions that prevent massive Ca^{2+} transfer from the endoplasmic reticulum (ER) to mitochondria through the activity of the Ca^{2+} channels, which are inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) receptor ($Ins(1,4,5)P_3R$), voltage-dependent anion channel (VDAC) and the mitochondrial Ca^{2+} uniporter (MCU), generally protect cells from cell death by necrosis (not shown) and apoptosis. These conditions include overexpression of anti-apoptotic proteins such as B cell lymphoma 2 (BCL-2) and BCL-X_L, inhibition by VDAC2 of pro-apoptotic proteins such as BAK or kinases promoting cell survival proteins such as AKT. In these pro-survival conditions, the apoptotic effector caspase 9 remains in its uncleaved pro-caspase 9 form, the apototic protease-activating factor 1 (APAF1) exists in an inactive form and the caspase cofactor cytochrome *c* is stored in cristae of the inner mitochondrial membrane (IMM). Moreover, lack of the pro-apototic proteins BAX and BAK (BCL-2 antagonist/killer) result in reduced Ca^{2+} release from the ER. **b** | Following an apoptotic stimulus, BH3-interacting domain death agonist (BID) is cleaved down to truncated BID (tBID). tBID binds to the outer mitochondrial membrane (OMM), where it activates BAX and BCL-2 antagonist/killer (BAK). Activated BAX counteracts BCL-2 effects on the $Ins(1,4,5)P_3R$ and ER Ca^{2+} loading. Moreover, BAX and BAK oligomerization promotes OMM permeabilization. This leads to IMM cristae remodelling, and opening of the permeability transition pore (PTP). Furthermore, OMM permeabilization ensues and caspase cofactors such as cytochrome *c* are released into the cytosol, were they interact with the cytosolic protein APAF1 to form the apoptosome. Subsequently, pro-caspase 9 is recruited to the apoptosome, where it is activated and triggers a proteolytic cascade which, by processing specific targets, eventually results in apoptosis. SMAC (second mitochondria-derived activator of caspase; also known as DIABLO) is released from mitochondria in a caspase-dependent manner, and promotes apoptosis by binding to inhibitor of apoptosis proteins (IAPs) and thus allowing pro-caspase 9 activation. Moreover, the ER-localized pool of promyelocytic leukaemia (PML) tumour suppressor positively regulates apoptosis by regulating phosphorylation levels of the $Ins(1,4,5)P_3R$ through AKT kinase and protein phosphatase 2A (PP2A). GRP75, 75 kDa glucose-regulated protein; MCU, mitochondrial Ca^{2+} uniporter; VDAC, voltage-dependent anion channels; CYPD, cyclophilin D.

EF-hand
A highly conserved Ca^{2+}-binding domain comprising two helices (that is, E and F after the 5th and 6th helices of parvalbumin) that are linked by a short acidic Ca^{2+}-binding loop.

Excitoxicity
The pathological process in which neurons undergo cell death caused by excessive stimulation. Classically, overactivation of glutamate receptors leads to excessive Ca^{2+} entry, which causes activation of enzymes, such as calpains, that break down key cellular components leading to cell death.

Autophagy. Ca^{2+} has been proposed to have an important regulatory role in autophagy, although this role seems to be fairly complex. Ca^{2+}/calmodulin-dependent kinase kinase-β (CaMKKβ; also known as CaMKK2) activates AMP-activated protein kinase (AMPK) and hence mammalian target of rapamycin (mTOR)-dependent autophagy[158]. In addition, pharmacological compounds (such as PK11195) that increase ER Ca^{2+} loading augment vitamin D- and ATP-induced autophagy[159]. However, blockers of L-type Ca^{2+} channels also induce autophagy, which suggests an inhibitory role of cytosolic Ca^{2+} (REF. 160).

In addition, an important role in autophagy has been demonstrated for the $Ins(1,4,5)P_3R$ Ca^{2+} channels (FIG. 4). Decreasing $Ins(1,4,5)P_3$ levels by lithium treatment enhances autophagy[161], and knockdown of $Ins(1,4,5)P_3R$ stimulates autophagy via a mechanism that does not involve changes in steady-state Ca^{2+} levels in the ER or in the cytosol. As to the molecular mechanism, BCL-2 was shown to be required for $Ins(1,4,5)P_3R$-mediated inhibition of the autophagy mediator BECLIN1, and ER targeting of BCL-2 and BCL-X_L inhibits autophagy triggered by starvation or $Ins(1,4,5)P_3R$ inhibition[162,163].

Recently, the $Ins(1,4,5)P_3R$-mediated control of autophagy has been linked to increases in $[Ca^{2+}]_{mt}$ and to the regulation of cellular bioenergetics (REF. 164). $Ins(1,4,5)P_3R$-dependent mitochondrial Ca^{2+} signals were shown to suppress AMPK activation. Accordingly, in $Ins(1,4,5)P_3R1$-, $Ins(1,4,5)P_3R2$- and $Ins(1,4,5)P_3R3$ triple knockout DT40 cells autophagy was maximally activated even in the presence of nutrients. Autophagy activation did not depend on compromised BECLIN1 binding to $Ins(1,4,5)P_3Rs$, but rather on inhibition of mitochondrial Ca^{2+} loading, as MCU blockers had the same effect as $Ins(1,4,5)P_3R$ inhibition. Similar conclusions were reached by another study, in which the $[Ca^{2+}]_{mt}$-dependent pathway was proposed not to act through AMPK and thus not through a metabolic effect on ATP/ADP/AMP ratios[165]. In the attempt to reconcile the different sets of data on the role of $Ins(1,4,5)P_3R$ in

N-methyl-D-aspartate receptors
(NMDRs). Subtypes of plasma membrane ionotropic glutamate receptors that are mainly involved in memory formation and excitotoxicity. Ionotropic glutamate receptors are classified on the basis of their selective agonists (such as NMDA, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and kainate).

L-type Ca^{2+} channels
Voltage-operated channels that belong to the $Ca_v1.x$ family and that are activated by strong depolarizations. They are long-lasting and are inhibited by dihydropyridines and phenylalkylamines.

NLRP3 inflammasome
(Nucleotide-binding oligomerization domain-Leu-rich repeat- and pyrin domain-containing 3 inflammasome). A high molecular weight signalling complex that consists of a family of cytoplasmic proteins, the NLRP proteins. This complex recruits pro-caspase 1, which is then activated by autocatalytic cleavage. Active caspase 1 catalyses the cleavage of pro-interleukin1β, pro-IL-18 and pro-IL-33, resulting in the secretion of biologically active forms of these pro-inflammatory cytokines.

autophagy, a recent study reported opposite effects of $Ins(1,4,5)P_3R$ inhibition in fed and starved cells and suggested that mitochondrial Ca^{2+} signals tonically inhibit autophagy in normal feeding conditions, whereas during starvation cytosolic Ca^{2+} signals have a dominant role in autophagy induction[166].

Conclusions

The solution of the molecular enigma of mitochondrial Ca^{2+} handling came after a growing interest and repeated failures. Indeed, while mitochondrial signalling pathways, and in particular Ca^{2+} handling, were still unresolved, mitochondria were demonstrated to have key roles in cell biology and pathology. Some landmark observations include: the discovery that the intrinsic pathway of apoptosis is based on mitochondria and that the collapse of mitochondrial bioenergetics is a leading route to necrotic cell death; the finding that mitochondrial energy balance (and the subsequent regulation of ATP and NADH levels) affects cell functions and survival via specific kinases, acetylases and other protein modifying enzymes; the observation that the distribution and the three-dimensional structure of mitochondria is regulated by a complex fusion and fission machinery and that both have a key role in physiological and pathological events; and the finding that mitochondria release danger signals (including mitochondrial DNA) that activate the NLRP3 inflammasome, which links aerobic metabolism and the innate immune response. Thus, the integration of mitochondria into cellular signalling networks has become crucial for our understanding of a series of cell functions that extend well beyond bioenergetics, but insight was so far limited owing to the lack of molecular information. With the identification of the MCU, the key element of the mitochondrial Ca^{2+} signalling toolkit, a new area of research has emerged. Indeed, the availability of molecular tools (such as complementary DNAs, small interfering RNAs and dominant negative variants) and the forthcoming development of transgenic models will allow to directly address exciting topics of organ physiology and disease pathogenesis.

The large change in ATP requirements that underlies muscle activity renders Ca^{2+} transfer to mitochondria a key signalling step both in the heart and in skeletal muscle. Moreover, these tissues undergo substantial structural remodelling upon repeated exercise and/or depending on feeding conditions, and mitochondrial Ca^{2+} is involved in balancing the response to atrophic and hypertrophic challenges. Finally, apoptosis and necrosis underlie the pathogenetic mechanisms of genetic and acquired muscle and heart disorders. The lack of both molecular understanding and effective pharmacological inhibitors has so far hampered the direct investigation of the pleiotropic action of mitochondrial Ca^{2+}. In addition, mitochondrial Ca^{2+} transients are radically different in the two types of striated muscle: whereas high amplitude increases in $[Ca^{2+}]_{mt}$ underlie the function of skeletal muscle, almost undetectable $[Ca^{2+}]_{mt}$ peaks contribute to the function of the heart. What are the reasons for this different behaviour? Do expression levels of the MCU and/or MICU1 differ in these tissues? Are other regulatory mechanisms operative (for example, post-translational modifications) and what are the modifying enzymes (for example, kinases, sirtuins and others)? Finally, are other components of the machinery still missing, for example, novel regulators, possibly alternatively spliced variants or even a novel channel?

In neurons, mitochondria are now recognized as a common final pathway in neurodegeneration, and alterations in Ca^{2+} signalling have been reported in Alzheimer's disease, in Parkinson's disease and in Huntington's disease. The crosstalk between Ca^{2+} signalling and ROS production and the control of organelle structure and/or organelle turnover is still incompletely understood. It is now possible to take advantage of the available transgenic models of these diseases to test the pathogenetic mechanisms and novel pharmacological corrections.

Such studies will be supported by the characterization of the supramolecular composition of the mitochondrial uniporter and the design of specific inhibitors. As the MCU consists of only two TMDs, it is likely that it is part of a multisubunit channel. Moreover, it is possible that other regulators besides MICU1 are present in this uniporter. Finally, a challenging task is the elucidation of the crystal structure of the MCU that may provide deeper insight into this novel channel and guide the design of novel drugs. Overall, we believe that the beginning of the molecular era of mitochondrial Ca^{2+} homeostasis will provide exciting challenges for a broad set of biomedical issues, thus further integrating mitochondrial Ca^{2+} signalling in the core of cell biology.

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Competing interests statement
The authors declare no competing financial interests.

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