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Calcium at the center of cell signaling: interplay between endoplasmic reticulum, mitochondria and lysosomes

Anna Raffaello^{1,*}, Cristina Mammucari^{1,*}, Gaia Gherardi¹, and Rosario Rizzuto^{1,2,*}

¹ Department of Biomedical Sciences, University of Padua, 35131, Italy

² Neuroscience Institute, National Research Council, Padua, 35131, Italy

Abstract

In recent years, rapid discoveries have been made relating to Ca^{2+} handling at specific organelles, which have important implications for whole cell Ca^{2+} homeostasis. In particular, the structures of the endoplasmic reticulum (ER) Ca^{2+} channels revealed by electron cryomicroscopy (cryo-EM), the continuous update on the structure, regulation and role of the mitochondrial calcium uniporter (MCU) complex, and the analysis of lysosomal Ca^{2+} signaling are milestones on the route towards a deeper comprehension of the complexity of global Ca^{2+} signaling. In this Review, we summarize recent discoveries on the regulation of inter-organellar Ca^{2+} homeostasis and its role in pathophysiology.

INTRODUCTION

The second messenger Ca^{2+} regulates numerous cellular processes, including, but not limited to, muscle contraction, exocytosis and gene transcription. The endoplasmic reticulum (ER) (sarcoplasmic reticulum, SR, in striated muscles) is the largest Ca^{2+} store in the cell. While the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) at rest is about 100 nM, ER $[\text{Ca}^{2+}]$ can reach 1mM, depending on the cell type. Upon physiological stimuli, Ca^{2+} is released from the ER/SR, which in turn stimulates Ca^{2+} influx from the plasma membrane. This rapid and sustained $[\text{Ca}^{2+}]_{\text{cyt}}$ increase regulates Ca^{2+} -dependent effector proteins (e.g. calpains, kinases, phosphatases, ion channels) and Ca^{2+} -dependent functions. Eventually, Ca^{2+} is pumped back to the ER and $[\text{Ca}^{2+}]_{\text{cyt}}$ returns to resting values. Specific proteins and channels contribute to the fine-tuned regulation of the whole cycle. In addition, close contacts between the ER and the plasma membrane, and the other intracellular organelles, participate to the control of Ca^{2+} homeostasis. In particular, mitochondria were the first organelles shown to be capable of taking up Ca^{2+} , even before the chemiosmotic theory for mitochondrial Ca^{2+} accumulation (for a review see [1]). Many roles have been ascribed to mitochondrial Ca^{2+} uptake [1]. Indeed, three dehydrogenases of the Krebs cycle have been shown to be modulated by Ca^{2+} [2], thus controlling overall cellular metabolism.

* correspondence: anna.raffaello@unipd.it, cristina.mammucari@unipd.it, rosario.rizzuto@unipd.it.

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Furthermore, mitochondrial Ca^{2+} accumulation shapes cytosolic Ca^{2+} dynamics and mitochondrial Ca^{2+} overload has been associated with the apoptotic process [1].

More recently, lysosomes have been suggested to participate to the regulation of Ca^{2+} homeostasis acting as Ca^{2+} stores, but their contribution to cellular Ca^{2+} signaling is still debated.

In recent years important discoveries have been made relative to different aspects of Ca^{2+} homeostasis and to the interplay between Ca^{2+} stores and cytosolic Ca^{2+} pool. Here we summarize the main aspects that have emerged, focusing on the mechanisms and regulation of Ca^{2+} signaling in ER, mitochondria and lysosomes.

ER Ca^{2+} release

As mentioned above, ER/SR represents the main Ca^{2+} store. Rapid release of Ca^{2+} from this compartment ensures sustained $[\text{Ca}^{2+}]_{\text{cyt}}$ rises required for specific cell functions. Recent work contributed with mechanistic details on inositol 1,4,5-trisphosphate (InsP_3)-mediated Ca^{2+} dynamics and on the molecular structure of InsP_3 receptor 1 ($\text{InsP}_3\text{R1}$) and of ryanodine receptor 1 (RyR1).

Activation of phospholipase C triggers the release of InsP_3 from phosphatidylinositol 4,5-bisphosphate ($\text{PtdIns}(4,5)\text{P}_2$). Upon interaction of InsP_3 with its receptors (InsP_3Rs), located at the ER membrane, Ca^{2+} is released to the cytosol (Fig. 1). Ca^{2+} itself regulates InsP_3R open probability (P_o), activating InsP_3R at increasing $[\text{Ca}^{2+}]$ up to a specific threshold of $[\text{Ca}^{2+}]$, while playing an inhibitory role at higher $[\text{Ca}^{2+}]$. In addition, oscillatory cytosolic Ca^{2+} rises are characteristic responses to agonist-induced InsP_3 release. The frequency of such oscillations depends on stimulus strength and determines the fine control of downstream metabolic responses. The mechanism underlying InsP_3 -dependent $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations has been clarified only recently. Indeed, work in the Thomas lab has demonstrated that InsP_3 and $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations are mutually determined, and that InsP_3 fluctuations are essential for the generation of $[\text{Ca}^{2+}]_{\text{cyt}}$ spikes and propagation of Ca^{2+} waves, in agreement with the so-called cross-coupling hypotheses [3].

The InsP_3R family comprises three isoforms, $\text{InsP}_3\text{R1}$, $\text{InsP}_3\text{R2}$ and $\text{InsP}_3\text{R3}$, which are expressed at different levels in different tissues. Of note, $\text{InsP}_3\text{R1}$ is mostly abundant in neuronal cells of the central nervous system (CNS) and mutations in this gene are associated with severe CNS disorders, including ataxia. $\text{InsP}_3\text{R2}$ is expressed in different tissues and it represents the most abundant isoform present in the cardiac muscle. InsP_3Rs form heterotetramers, whose activity is not simply the sum of the single isoforms constituting the channel, but rather have unique properties and responsiveness to ATP, Ca^{2+} and InsP_3 [4,5].

In striated muscles, Ca^{2+} is released from the SR by the opening of RyRs . As for InsP_3Rs , the RyR family includes three isoforms; that is, RyR1 -3. All isoforms are abundant in CNS. In addition, RyR1 and RyR2 are highly expressed in striated muscles. In detail, RyR1 is particularly enriched in skeletal muscle, while RyR2 in cardiac muscle. RyR3 is expressed in most tissues.

In skeletal muscle, RyR1 opens upon coupling with the voltage-gated Ca^{2+} channel (Cav1.1), also known as the Ca^{2+} -dependent L-type calcium channel dihydropyridine receptor (DHPR), thus ensuring excitation-contraction (EC) coupling. In the myocardium, L-type Ca^{2+} channels ensures entrance of Ca^{2+} which triggers the opening of RyR2 by a Ca^{2+} -induced Ca^{2+} release mechanism.

Mutations in RyR1 are associated with malignant hyperthermia susceptibility, central core disease, and minicore myopathy with external ophthalmoplegia [6]. In this respect, the compound AICAR has been proposed for prophylactic treatment of heat-induced sudden death based on his ability to interact with RyR1 and reduce Ca^{2+} leakage independently of activation of its target AMP-activated protein kinase (AMPK) [7]. Mutations in RyR3 gene have been associated to central core disease as well. RyR2 mutations are linked with stress-induced polymorphic ventricular tachycardia and arrhythmogenic right ventricular dysplasia type 2.

Recently, the electron cryomicroscopy (cryo-EM) structures of InsP_3R 1 and of RyR1 have been described. InsP_3Rs and RyRs are both characterized by an analogous tetrameric structure and share similar activation mechanisms. Indeed, besides the specific role of InsP_3 on InsP_3Rs activation, both InsP_3Rs and RyRs are activated by low $[\text{Ca}^{2+}]$ and inhibited by high $[\text{Ca}^{2+}]$. The structure of InsP_3R 1, solved with a resolution of 4.7 Å [8], revealed that, while the ion-conduction pore is similar to homologous tetrameric ion channels (e.g., RyRs), the gating of the channel is ensured by unique C-terminal domains facing the cytosol, which interact with the InsP_3 -binding domain of neighbouring subunits causing an allosteric rearrangement. These results are in agreement with previous studies based on microsomal membrane preparations expressing recombinant InsP_3R isoforms [9]. In parallel to InsP_3R , the structure of RyR1 was also solved by cryo-EM, revealing a 6-transmembrane ion channel characterized by an EF-hand domain for Ca^{2+} -mediated allosteric gating and a huge cytoplasmic domains on top of each transmembrane domain [10–12]. Comparison between InsP_3R and RyR structures revealed important similarities between the two channels. Of note, although the cytoplasmic domain of RyR is much larger than the one of InsP_3R , the location of N-terminal regions, required for the formation of functional tetramers, relative to the transmembrane channel regions of the receptors, is very similar. A novel ER Ca^{2+} channel has been identified to be encoded by the TMCO1 gene (Transmembrane and coiled-coil domains 1) [13]. At physiological $[\text{Ca}^{2+}]$ TMCO1 is present as inactive monomers in the ER membrane. Above a certain $[\text{Ca}^{2+}]$ threshold, TMCO1 monomers form active tetrameric channels, which extrude Ca^{2+} from the ER matrix, thus preventing ER Ca^{2+} overload. Mutations in the TMCO1 gene are associated to human cerebrotendinous xanthomatosis (CTD) dysplasia spectrum, which is mainly characterized by craniofacial dysmorphism, skeletal anomalies, mental retardation and ataxia [13].

Store-operated Ca^{2+} entry (SOCE)

Once Ca^{2+} has been released from the ER, the enhanced $[\text{Ca}^{2+}]_{\text{cyt}}$ is sustained by Ca^{2+} influx from the extracellular milieu through store-operated Ca^{2+} entry (SOCE). SOCE facilitates Ca^{2+} refilling into the ER, which is a necessary event to stop Ca^{2+} entry and to restore resting $[\text{Ca}^{2+}]$. The mechanism of store-operated Ca^{2+} entry (SOCE) is ensured by

the coordinated activity of two families of proteins, STIM and ORAI. STIM1 and STIM2 are single trans-membrane ER proteins that have their C-terminal domains facing the cytosol [14]. The ORAI family comprises cell membrane proteins that form the conductive pore of the Ca^{2+} release-activated Ca^{2+} (CRAC) channels and is composed of ORAI1-3, each of which contains 4 transmembrane domains. For ORAI1-3, both N- and C-terminal domains face the cytosol [14]. Upon ER Ca^{2+} depletion, STIM moves to ER-plasma membrane junctions where it interacts with ORAI, thus inducing Ca^{2+} influx to the ER (Fig. 2). Both loss- and gain-of-function mutations in STIM and ORAI (resulting in decreased and increased Ca^{2+} influx, respectively) have been detected in human patients [15]. Loss-of-function mutations result in severe combined immunodeficiency (SCID)-like syndrome, autoimmune diseases, myopathy and ectodermal dysplasia, which results in defects in sweat glands function and teeth development [15]. Gain-of-function mutations cause different syndromes, all associated with myopathy and platelet defects [15].

ER Ca^{2+} uptake

ER Ca^{2+} uptake exerts a buffering effect on $[\text{Ca}^{2+}]_{\text{cyt}}$, ensuring the maintenance of resting $[\text{Ca}^{2+}]_{\text{cyt}}$ in the nanomolar range by the activity of the sarco(endo)plasmic reticulum calcium ATPase (SERCA) (Fig.1). SERCA activity is increased upon ER/SR Ca^{2+} store release, allowing rapid re-uptake of cytosolic Ca^{2+} . In addition, it has been proposed that coupling between SOCE, SERCA and InsP3 mediates Ca^{2+} signaling between spatially distant effectors [16]. According to this model, Ca^{2+} entering through SOCE is taken up in the ER by SERCA, to be released again by InsP3R to activate distal Ca^{2+} -activated Cl channels (CaCCs), which contribute to the regulation of cell membrane potential.

SERCA family comprises three isoforms, SERCA1-3, encoded by ATP2A1-3 genes respectively. SERCA1 is exclusively expressed in skeletal muscles, SERCA2 is expressed in skeletal and cardiac muscles, in brain and in other tissues, and SERCA3 is ubiquitously expressed. Moreover, SERCA activity is modulated by transmembrane proteins; specifically, phospholamban in the heart and sarcolipin in skeletal muscle [17]. Mutations in SERCA1 are linked to Brody disease, characterized by defects in relaxation upon exercise, stiffness and cramps.

Defects in the expression levels and activity of SERCA2a, one of the two SERCA2 isoforms, specifically expressed in slow-twitch myofibers and in cardiac muscle, are apparent in heart failure and phospholamban mutations have been identified in patients with cardiac hypertrophy and decreased ejection fraction [18].

Recently, the role of a peptide named DWORF in enhancing SERCA activity in striated muscles has been reported [19]. Intriguingly, DWORF is encoded by a genomic region that is annotated as a long noncoding RNA. DWORF displaces inhibitory proteins from SERCA, thus increasing muscle performance. So far, DWORF is the only known direct activator of SERCA, and potential therapies for heart disease could possibly arise from positive modulation of DWORF activity.

ER-mitochondria contact sites

That mitochondria accumulate high $[Ca^{2+}]$ upon physiological stimulation has long remained counterintuitive given that, in these conditions, $[Ca^{2+}]_{cyt}$ rises only from 0.1 μM to about 2-3 μM . This $[Ca^{2+}]_{cyt}$ is too low to allow Ca^{2+} uptake by the low affinity MCU. However, this model was revised when the existence of high $[Ca^{2+}]$ microdomains ($[Ca^{2+}] > 10 \mu M$) at the site of ER-mitochondria contacts was demonstrated [1]. Both interorganelle distance and contact site size have been proven to be critical parameters for Ca^{2+} transfer. Purification of a subcellular fraction corresponding to the ER/mitochondria contacts (referred to as mitochondria-associated membranes, MAM) led to the identification of proteins enriched in these membrane domains [20].

Among them, Mitofusin 2 (Mfn2) is located at both ER and mitochondria membranes where it forms homo- and heterotypic interactions, the latter with Mitofusin 1 (Fig.1). Mitofusin 2 has been reported to strengthen ER-mitochondrial contacts and to facilitate mitochondrial Ca^{2+} uptake [21,22]. However, this model has been challenged by a quantitative electron microscopy analysis which demonstrated that, in Mfn2-knockout (KO) cells, ER-mitochondrial contacts are increased, rather than decreased [23]. Along these lines, analysis of light microscopy images that takes into account the changes in organelle morphology upon Mfn2 deletion further demonstrated the increase in percentage of the mitochondrial perimeter colocalizing with the ER [24]. The reduction in mitochondrial Ca^{2+} uptake previously observed in Mfn2 KO cells [21] was suggested to be due to reduced MCU expression levels in these cells [24]. In addition, acute silencing of Mfn2, which did not affect MCU protein levels, triggered an increase in mitochondrial Ca^{2+} uptake [24]. Thus, the authors concluded that Mfn2 acts as a negative regulator of ER-mitochondrial tethers which, by reducing the number of contacts, avoids toxic Ca^{2+} accumulation [24]. Overall, the role of Mfn2 at the ER/mitochondria contacts is still highly debated.

For the sake of brevity, we will not discuss here the many chaperones and other proteins involved in mitochondrial dynamics that participate to the regulation of ER/mitochondria Ca^{2+} transfer, these have been reviewed elsewhere recently [20].

MITOCHONDRIA

The molecular characterization of the uniporter complex began with the discovery of one of its regulatory subunits, mitochondrial Ca^{2+} uptake 1 (MICU1) [25], soon followed by the identification of CCDC109A, now known as MCU, as the pore-forming subunit of the uniporter complex [26,27] (Fig. 3). In the following five years, we have witnessed an explosion of discoveries on the composition of the MCU complex and, more importantly, on the physiopathological roles of mitochondrial Ca^{2+} entry. Great advancements in the understanding of the regulation of the channel comes from the description of the structural properties of its components [28–30], although the complete architecture of the MCU complex is far from being solved.

Molecular components of the Mitochondrial Calcium Uniporter complex

Recent studies have shown that the Ca^{2+} permeant pore is composed by three proteins: MCU, MCUB and EMRE [31] (Fig.3).

MCUB (previously known as CCDC109B) is an MCU isoform, conserved in most of the vertebrates and in many plants but absent in other organisms where MCU is present [32]. MCU and MCUB share 50% sequence similarity and each possesses two transmembrane domains separated by a short loop that slightly differs from MCU. More importantly, this isoform has a crucial amino acid substitution in the loop region (E256V) that has an impact on the channelling properties. Indeed, in living cells, overexpression of MCUB reduces the amplitude of $[\text{Ca}^{2+}]_{\text{mit}}$ transients evoked by agonist stimulation, whereas MCUB silencing elicits the opposite effect, indicating that MCUB acts as a dominant negative subunit that incorporates into the uniporter channel and reduces its activity [32]. Still unclear is the function of this protein in physiopathology, although the ratio of MCU to MCUB expression, which varies greatly between tissues, might contribute to the differences in the amplitude of mitochondrial Ca^{2+} uptake in different tissues, as recently demonstrated by patch-clamp [33].

More debated is the role of EMRE in the regulation of MCU channel activity [34]. EMRE is a 10 kD protein that is widely expressed in most mammalian tissues and is composed of a single predicted transmembrane domain with a highly acidic carboxyl-terminus [35]. EMRE was shown to play a dual function in the regulation of MCU activity. First, it seems necessary for MCU channel activity since its silencing abrogates Ca^{2+} entry into mitochondria [35], although purified MCU is sufficient to give rise to Ca^{2+} currents in a planar lipid bilayer [27]. Second, EMRE seems to also mediate the interaction between MCU and the regulatory subunits MICU1 and MICU2 [35]. Of note, EMRE is not present in plants, fungi and protozoa, where MCU and MICU1 are expressed, suggesting that it represents a metazoan specialization. Coherently, in yeast only the heterologous expression of human MCU--but not *Dictyostelium discoideum* MCU--requires EMRE for proper MCU functioning [36].

One of the peculiar properties of mitochondrial Ca^{2+} uptake is the sigmoidal response to $[\text{Ca}^{2+}]_{\text{cyt}}$. Indeed, despite the steep mitochondrial membrane potential, in resting conditions Ca^{2+} uptake is inhibited to prevent matrix Ca^{2+} overload and the dissipation of membrane potential that would prevent ATP synthesis. In turn, when cells are stimulated, mitochondria have to respond promptly by exponentially increasing the Ca^{2+} carrying capacity [37–39]. This property does not reside with MCU itself but rather with its regulators, the Ca^{2+} -binding EF hand-containing proteins MICU1 and MICU2 [34] (Fig.3). MICU1 silencing was initially proposed to inhibit mitochondrial Ca^{2+} uptake [25]. However, the Mallilankaraman and Csordás laboratories [40,41] showed that both transient and constitutive loss of MICU1 leads to basal mitochondrial Ca^{2+} accumulation. This observation was confirmed by the identification of a loss of function mutation in the MICU1 gene in patients affected by brain and muscle disorders [42] that causes a constitutive elevation of resting $[\text{Ca}^{2+}]_{\text{mit}}$. Recently, homozygous deletion of exon 1 of MICU1 in children affected by fatigue and lethargy has been reported to induce a strong reduction of mitochondrial Ca^{2+} uptake. However, a concomitant decrease in Pyruvate Dehydrogenase

(PDH) phosphorylation, which is regulated by the Ca^{2+} -dependent PDH phosphatase, was detected [43], suggesting that the reduction in Ca^{2+} uptake of $\text{MICU1}^{-/-}$ cells represents a consequence of increase in basal matrix Ca^{2+} , which would reduce the driving force for Ca^{2+} accumulation [43].

Csordás and coauthors also proposed that, at high $[\text{Ca}^{2+}]_{\text{cyt}}$, MICU1 is necessary to ensure maximum Ca^{2+} uptake [41], thus acting as both gatekeeper and cooperative activator of the channel. The discovery of the MICU1 paralog, MICU2 [44], revolutionized the concept of the mechanism responsible for the sigmoidal response of MCU to extramitochondrial $[\text{Ca}^{2+}]$ [31,34,45]. Importantly, silencing of MICU1 causes the concomitant destabilization also of MICU2 protein expression [44–46], thus opening the possibility that the effect of MICU1 silencing were in part due to the effect of MICU2 disappearance. Indeed, while it was confirmed that MICU1 cooperatively activates the channel at high $[\text{Ca}^{2+}]$, MICU2 was discovered to act as the genuine gatekeeper of the MCU at low $[\text{Ca}^{2+}]$ [45]. In this model, MICU1 and MICU2 form an obligate heterodimer through the formation of a disulfide bond [45], which is regulated by the mitochondrial oxidoreductase Mia40 [47]. At resting $[\text{Ca}^{2+}]_{\text{cyt}}$, the inhibitory role of MICU2 keeps the channel closed, while an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ causes conformational changes of the MICU1-MICU2 dimer releasing MICU2-dependent inhibition of the channel thus ensuring the cooperative activation of MCU by MICU1 [45]. Thus, the loss of MCU gatekeeping in cells lacking MICU1 [40,41] was likely due to the concomitant loss of MICU2.

Recently, Foskett and coauthors proposed a model in which the conserved acidic residues in the matrix-localized carboxyl tail of EMRE sense matrix $[\text{Ca}^{2+}]$ and, together with MICU1 and MICU2, ensure the inhibition of uniporter activity under normal conditions [48]. However, when $[\text{Ca}^{2+}]_{\text{cyt}}$ increases due to cell stimulation, Ca^{2+} can enter mitochondria because, under basal conditions, MCU has a small but detectable probability of being open, thus relieving EMRE-mediated matrix $[\text{Ca}^{2+}]$ inhibition of uniporter activity [48]. These data were questioned by two other studies demonstrating an opposite topology of EMRE, with its N- and acidic C-termini projected into the matrix and the inter membrane space, respectively [49,50].

Other proteins have been reported to be part of the MCU complex [34]. One of the most debated is MCUR1, an IMM integral protein that was initially shown to bind to MCU and to be required for mitochondrial Ca^{2+} uptake, although it was not identified by mass spectrometry of affinity purified MCU complex [35] and yeast MCUR1 ortholog lacks uniporter activity. An indirect mechanism was thus proposed [51] in which loss of MCUR1 induces a decrease in mitochondrial membrane potential through a specific defect in cytochrome c oxidase (COX) assembly, thereby indirectly leading to decreased mitochondrial Ca^{2+} uptake [51]. However, the Foskett group demonstrated that loss of MCUR1 significantly lowered MCU Ca^{2+} currents [52]. Furthermore, recently the Madesh group confirmed that a lack of MCUR1 in cardiomyocytes and endothelial cells lowers MCU current (I_{MCU}) and showed that MCUR1 interacts with MCU and EMRE, but not with MICU1 or MICU2, functioning as a scaffolding factor [53]. Finally, a screening of *Drosophila* cells identified MCUR1 as one of the proteins required for the regulation of the Ca^{2+} threshold for the permeability transition [54], although the mechanism is not clear yet.

Of course, future experiments will be necessary to clarify whether or not MCUR1 is an integral part of the MCU complex and its role in mitochondrial physiology.

Physiological roles of the Mitochondrial Calcium Uniporter complex

Total MCU^{-/-} mice had an unexpected phenotype [55]. These animals are viable and fertile, if maintained in a outbred CD1 strain, although isolated mitochondria from these animals lack Ca²⁺ uptake [55]. More consistent with expectations, MCU deletion results in embryonic lethality in the C57BL/6 strain [56] and [114]. This suggests the existence of a compensatory mechanism that allows mitochondrial Ca²⁺ uptake in CD1 mice. Surprisingly, MCU^{-/-} CD1 mice display no changes in basal metabolism but increased levels of PDH phosphorylation were observed in their skeletal muscle and, coherently with a reduced ATP production in these animals, their skeletal muscles show reduced maximal power output [55]. These data have been confirmed by the acute overexpression and silencing of MCU in skeletal muscles of mice [57]. This study demonstrated that the overexpression of MCU causes hypertrophy, while its silencing causes muscle atrophy and, importantly, the overexpression of MCU blocks denervation-induced atrophy [57]. As for the mechanism, MCU overexpression causes the activation of known hypertrophy pathways, such as IGF1-Akt/PKB and PGC-1 α 4. Furthermore, global nuclear skeletal muscle gene expression is controlled by mitochondrial Ca²⁺, suggesting a precise Ca²⁺-mediated mitochondria-to-nucleus route to control muscle trophism [57,58].

The analysis of MCU^{-/-} hearts revealed puzzling results [55,59]. As expected, mitochondria in MCU^{-/-} hearts do not exhibit any Ca²⁺-induced opening of the mitochondrial permeability transition pore (PTP), the key nodal point in mediating cell death [60]. The PTP, a large-conductance channel located at the IMM, opens in the presence of mitochondrial Ca²⁺ overload and whose opening results in inner membrane potential collapse, halt of mitochondrial ATP synthesis, mitochondrial swelling, rupture, release of cytochrome c and cell death [61]. Surprisingly, in a model of ischemia/reperfusion, where [Ca²⁺]_{cyt}, and hence [Ca²⁺]_{mit}, are elevated, thus leading to PTP opening [62], the levels of cell death were indistinguishable between WT and MCU^{-/-} hearts [55,56], suggesting that alternative death pathways take place in absence of mitochondrial Ca²⁺ overload. Furthermore, several other cardiac features were indistinguishable between WT and MCU^{-/-} animals, including basal ATP levels, left ventricular cardiac output at baseline and following isoproterenol treatment, and hypertrophy and fibrosis upon transverse aortic constriction (TAC) [55,59], processes to which Ca²⁺ was thought to be tightly linked [62].

Many other models have been created to clarify the role of mitochondrial Ca²⁺ uptake in the heart [63]. The analysis of a transgenic mouse overexpressing a dominant-negative form of MCU in the heart (DN-MCU) confirmed the analysis of the MCU^{-/-} mouse on basal properties [64]. In addition, DN-MCU hearts present impaired energetic responsiveness to acute sympathetic stress, namely fight-or-flight response, by lowering ATP below a critical threshold [65].

To circumvent the possible compensatory mechanisms of constitutive loss of MCU during development, inducible cardiac-specific MCU knockout mice were generated [66,67]. Similarly to what observed for MCU^{-/-} and DN-MCU models, acute deletion of MCU in

adult cardiomyocytes did not alter heart morphology, ultrastructure, basal cardiac functions or TAC outcomes [66,67], while defects in the fight-or-flight response were observed [66,67]. However, contrary to constitutive knockout models, acute loss of mitochondrial Ca^{2+} uptake protects from cell death induced by ischemia–reperfusion injury [66,67], although disparities in methodology might account for the different results observed [55,66,67].

The role of MCU in cell death and cancer progression is also debated [63]. Consistent with the well-known role of mitochondrial Ca^{2+} overload in triggering PTP opening [60], knockout MCU mice show no PTP opening upon addition of extramitochondrial Ca^{2+} [55]. Surprisingly, mouse embryonic fibroblast cells derived from these mice do not differ in the magnitude of cell death upon treatment with apoptotic stimuli [55]. In contrast with these data, cells either overexpressing MCU or silenced for MICU1 show increased susceptibility to cell death [27,40]. In addition, a reduction in MCU protein levels through increased expression of miR-25, which specifically targets MCU, confers resistance of colon cancer and cancer derived cells to apoptotic stimuli [68]. However, in breast cancer patients, MCU overexpression correlates to poor prognosis [115]. Furthermore, in the highly aggressive triple negative breast carcinoma cell line MDA-MB-231, caspase-independent cell death was potentiated by MCU silencing [69]. It was recently shown that MCU expression correlates with patients' breast tumor size and lymph node infiltration. In addition, deletion of MCU greatly reduces in vivo tumor growth and metastasis formation via suppression of ROS production and inhibition of HIF-1 α signaling [70]. A study of the mechanism underlying the negative effect of MCU down-regulation on breast cancer cell migration, suggested that MCU controls SOCE-dependent cell migration [71]. However, detailed analysis in three different breast cancer cell lines demonstrated that MCU silencing causes similar reduction in cell motility independently of the cell line tested, while it exerts different effects on cytosolic $[\text{Ca}^{2+}]$ and SOCE depending on the cell line [70]. These data indicate that the reduction in cell migration triggered by MCU silencing is due to specific inhibition of mit Ca^{2+} uptake.

Differently from what observed from the MCU knockout animals, constitutive ablation of MICU1 in mice leads to lethality within hours of birth [72], probably due to decreased numbers of neurons in the nucleus ambiguus, thus leading to defective regulation of basic functions postnatally. Analysis of mitochondrial Ca^{2+} homeostasis in MEF cells derived from these animals revealed enhanced mitochondrial Ca^{2+} uptake at resting $[\text{Ca}^{2+}]_{\text{cyt}}$ or during prolonged $[\text{Ca}^{2+}]_{\text{cyt}}$ elevations [72], in accord with data in cells [40,41,45] and in patients harboring MICU1 loss-of-function mutations [42]. Liver specific MICU1 knockout model highlighted no gross functional, morphological and/or histological differences compared to wild type animals but MICU1^{-/-} livers failed to regenerate after partial hepatectomy due to PTP opening caused by sustained mitochondrial Ca^{2+} overload [72], since treatment of MICU1^{-/-} mice with NIM811, an inhibitor of PTP, enhanced hepatocyte proliferation.

Recently, Finkel and collaborators generated CRISPR-mediated MICU1 knockout mice that result in high, but not complete perinatal death [73]. Furthermore, analysis of the surviving animals revealed marked abnormalities comparable to what observed in human patients

harboring MICU1 loss-of-function mutations [42] with were partially ameliorated with time and the deletion of one allele of EMRE.

Overall, the analysis of different animal models in which mitochondrial Ca^{2+} homeostasis is perturbed just started to clarify the importance of mitochondrial Ca^{2+} uptake. The analysis of skeletal muscle, liver and heart suggests that mitochondrial Ca^{2+} uptake plays a prominent role in stress rather than in physiological conditions and the fact that total $\text{MCU}^{-/-}$ animals are viable and present no gross phenotype remains a mystery that needs further investigation.

LYSOSOMES

The lysosomal Ca^{2+} pathway is a highly debated field. Here we summarize the main unknowns and hypotheses.

In the 1990s nicotinic acid adenine dinucleotide phosphate (NAADP), a contaminant in commercial stock of NADP, was discovered as the most potent mobilizer of Ca^{2+} from stores physically separated from those sensitive to inositol-1,4,5-trisphosphate (InsP_3) and cyclic ADP-ribose (cADPR) [74,75]. This result was also confirmed in mammalian cells [76–78]. Later, it was shown that NAADP releases Ca^{2+} not from the ER but from reserve granules, the functional equivalent of the endolysosomal system [79,80], suggesting that these organelles may function as Ca^{2+} stores.

Several physiological stimuli have been shown to increase NAADP in mammalian cells through G protein-coupled receptors and tyrosine-kinase linked receptors [81], although the intracellular synthesis of NAADP is still highly controversial. ADP-ribosyl cyclases and CD38 have been shown to catalyze the formation of NAADP in some cell types but the localization of these enzymes inside organelles and at the plasma membrane argues against these enzymes being responsible for NAADP synthesis [81].

The endolysosome system is essential for a variety of cellular functions including membrane trafficking, protein transport, autophagy and signal transduction [82]. While the endocytic and hydrolytic functions of the endolysosomal system are well established [82], the Ca^{2+} signaling function of these organelles is still in its infancy and a number of unknowns and controversial results still exists [81]. Indeed, still debated is whether NAADP mediates Ca^{2+} release specifically from the endolysosomal system. Indeed, it has been shown can also mediate Ca^{2+} efflux from SR/ER stores by directly activating RyRs. Indeed, in heart and skeletal muscle, NAADP has been shown to activate ryanodine receptor/calcium release channels [83,84]. Furthermore, in pancreatic acinar cells and in T-lymphocytes, NAADP causes the release of Ca^{2+} from the same thapsigargin-sensitive pool that is also the target of InsP_3 and cADPR [85–88].

A unifying hypothesis is that a Ca^{2+} -mediated functional coupling exists at the lysosome–ER interface. Indeed, it has been shown, in sea urchin eggs and arterial smooth muscle, that NAADP mobilizes Ca^{2+} via a 2-pool mechanism, named the “trigger hypothesis” [81,89]. In this model, NAADP mobilizes localized Ca^{2+} signals from lysosome-related Ca^{2+} stores that initiate global Ca^{2+} waves through the recruitment of ryanodine receptors on the

sarcoplasmic reticulum via Ca^{2+} -induced Ca^{2+} release since it has been shown that lysosomes co-localize with RyRs [81,89] (Fig. 4). Interestingly, in cardiac myocytes, NAADP has been shown to participate to the β -adrenoreceptor signal transduction by inducing NAADP-dependent microdomains of high $[\text{Ca}^{2+}]$ between lysosomes and SR [90]. Recent evidence also suggests that not only do acidic organelles signal to the ER through Ca^{2+} , but that the ER itself can signal to acidic vesicles in a retrograde fashion, since ER Ca^{2+} depletion ablates NAADP response [91–93]

Beside the existence and specificity of a Ca^{2+} endolysosome release factor, the release of Ca^{2+} by the lysosomes requires high $[\text{Ca}^{2+}]$ in the organelle. The measurement of Ca^{2+} inside acidic stores has been challenging because many fluorescent probes for measuring Ca^{2+} are sensitive to pH [94]. pH-corrected measurements showed that in macrophages, lysosomes contains high $[\text{Ca}^{2+}]$, ranging from 400 to 600 μM and it is highly sensitive to pH [91,95,96], although these measurements are far from being conclusive. The energy required for Ca^{2+} entry into the lysosomes by $\text{H}^+/\text{Ca}^{2+}$ exchange is provided by the activity of the vacuolar (V)-type H^+ -ATPase [82]. As for the release pathway, the mucolipin family of Transient Receptor Potential (TRPML) and the Two-Pore (TPC) channels have been proposed to be involved [97], although the role of these channels in the regulation of lysosomal ion balance requires further investigation (Fig. 5).

TPCs channels are predicted to comprise two repeats of a 6 transmembrane pore-forming domain [98] and extensive research supports the hypothesis that they are Ca^{2+} channels targeted by NAADP [99]. Subsequent studies questioned these findings (for a review see [100]). Indeed, it was proposed that TPCs are Na^+ -selective channels activated by $\text{PI}(3,5)\text{P}_2$ or voltage [101–103]. Therefore, further investigation of TPC permeation is required.

All three members of the TPC family (TPC1-3) localize to the endo-lysosomal system. Specifically, only TPC2 colocalizes with the lysosomal marker LAMP2 while TPC1 and TPC3 are predominantly expressed in endosomal compartments [104]. Interestingly, TPC2 has been demonstrated to be responsible for recruiting Ca^{2+} -induced Ca^{2+} release channels to lysosome-ER junctions [104]. Furthermore, inhibition of TPC2 was able to correct the exaggerated NAADP-evoked Ca^{2+} signals and the defects of lysosomal morphology in fibroblasts derived from familial Parkinson disease patients [105].

Still controversial is whether the three isoforms of TRPML, TRPML1-3, that resides within the endocytic pathway, are additional NAADP receptors [106]. The best-characterized member of the TRPML family is TRPML1, also called Mucolipin-1 (MCOLN1) because mutations in this protein are associated with mucopolipidosis type IV (MLIV). MLIV is an autosomal recessive neurodegenerative disease [107], in which trafficking defects within the endolysosomal system contributes heavily to the pathogenesis of the disease [108,109]. TRPML1 is expressed in most tissues and colocalizes exclusively with late endosomal and lysosomal markers but its dependence on NAADP is still highly debated [110].

One of the most important roles of localized Ca^{2+} release from acidic stores is the fusion between endocytic vesicles to maintain organelle homeostasis [111], but the molecular mechanism linking lysosomal Ca^{2+} and autophagy has been hypothesized only recently

[112,113]. Indeed, it has been proposed that during starvation, Ca^{2+} is released from the lysosome through Mucolipin-1, thus establishing a microdomain of high $[\text{Ca}^{2+}]$ in the vicinity of the lysosome leading to calcineurin (Cn) activation and transcription factor EB (TFEB) dephosphorylation. Dephosphorylated TFEB is no longer able to bind 14-3-3 proteins and translocates to the nucleus where it activates the transcription of the lysosomal/autophagic pathway gene expression.

Nevertheless, further insights are needed to better define the lysosomal-calcium pathway. In particular, what is still unclear is the $[\text{Ca}^{2+}]$ inside lysosomes (because the commonly used probes are highly sensitive to pH), the intracellular source of NAADP, and its molecular targets.

Concluding Remarks

Cellular Ca^{2+} is a universal signal acting on several physiological processes. Recent discoveries have deepened our knowledge on the contribution of several organelles in shaping the spatiotemporal dynamics of Ca^{2+} signaling. Of particular note is the determination of the electron cryo-microscopy structures of $\text{InsP}_3\text{R1}$ and of RYR1 which revealed important architectural and functional similarities between the two families of receptors. After the molecular identification of the mitochondrial uniporter complex components, continuous efforts are being placed in the determination of their interplay in the determination of the pathophysiological role of mitochondrial Ca^{2+} uptake in different tissues and organs. Finally, the endolysosomes contribution to intracellular Ca^{2+} homeostasis that is starting to emerge. A great number of unresolved questions need to be addressed (see Outstanding Questions) concerning the tight interplay between ER, mitochondria and the endolysosome system in the regulation of global Ca^{2+} homeostasis.

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Outstanding Questions Box

- Which hints will be unraveled by the determination of the ultrastructure of still unsolved ER Ca^{2+} channels on the specificity of their function and regulation?
- How are mitochondria/ER contact sites determined? What is the precise role of Mnf2?
- What is the precise crosstalk between MCU regulators (MICUs, EMRE, MCUR1) in the control of channel activity?
- How do post-translational modifications affect MCU activity?
- How is gene expression of MCU channel components and regulators controlled? Which transcription factors are involved?
- What would be the phenotype of organ-specific MCU deleted animal models?
- What is the precise NAADP biosynthetic pathway?
- Does NAADP play a role in ER Ca^{2+} release?
- Which channels are responsible for endo-lysosomal Ca^{2+} uptake and release?
- Which other pathophysiological roles are played by endo-lysosomal Ca^{2+} , besides regulation of autophagy?
- How does the interplay between ER and endo-lysosomes contribute to global Ca^{2+} homeostasis?

Trends Box

- Ca^{2+} signalling is regulated by the intimate interconnection between intracellular organelles and plasma membrane channels.
- The ultrastructures of ER and mitochondria Ca^{2+} channels have been disclosed.
- Great advancement has been made on the understanding of the function and regulation of the Mitochondrial Calcium Uniporter.
- Studies on endo-lysosomal Ca^{2+} signalling unravel a potential contribution of this compartment to global Ca^{2+} homeostasis.

FIGURE 1. Endoplasmic Reticulum (ER) Ca²⁺ store and ER-mitochondria contact sites

Following ligand binding to a GPCR, subsequent activation of PLC triggers the production of InsP₃ and DAG from PIP₂. InsP₃ binds to InsP₃Rs at the ER membrane, thus Ca²⁺ is released from the ER into the cytosol. Conversely, Ca²⁺ is withdrawn into the ER by SERCA activity. The proximity between ER and mitochondria ensures the formation of high [Ca²⁺] microdomains, allowing mitochondria to rapidly take up Ca²⁺. VDACS are responsible for the rapid transfer of Ca²⁺ through the OMM, then accumulation of Ca²⁺ into the mitochondrial matrix occurs via MCU. A number of regulatory proteins contribute to the regulation of ER-mitochondria contact sites. Red dots indicate Ca²⁺.

GPCR: G-protein coupled receptor

PLC: phospholipase C

PIP₂: phosphatidylinositol 4,5-bisphosphate

InsP₃: inositol 1,4,5-trisphosphate

DAG: diacylglycerol

InsP₃Rs: inositol 1,4,5-trisphosphate receptors

ER: endoplasmic reticulum

SERCA: sarco(endo)plasmic reticulum calcium ATPase

VDACs: voltage-dependent anion channels

OMM: outer mitochondrial membrane

FIGURE 2. Schematic representation of store operated Ca^{2+} entry (SOCE)

Immediately following a decrease in ER $[\text{Ca}^{2+}]$ (left hand side) STIM1 form multimers. STIM1 multimers translocate to the plasma membrane-ER contact sites where they recruit ORAI1 channels, leading to Ca^{2+} entry from the extracellular space. In resting conditions (right hand side), when the ER $[\text{Ca}^{2+}]$ is high, Ca^{2+} is bound to the STIM1 EF-hand domain. In this conformation of STIM1 is not able to form multimers, and thus SOCE is inactivated. Red dots indicate Ca^{2+} .

ER: endoplasmic reticulum

SOCE: store-operated Ca^{2+} entry

FIGURE 3. Schematic representation of the MCU complex

Mitochondrial calcium uptake is controlled by a multiprotein complex composed of the pore forming subunits MCU and MCUb together with MICU1, MICU2 and EMRE. In resting conditions (left hand side), MICU1/MICU2 heterodimers act as the MCU gatekeeper, due to the inhibitory effect of MICU2. Once Ca^{2+} signaling is activated (right hand side), the increase in cytosolic $[\text{Ca}^{2+}]$ induces a conformational change in the dimer that releases MICU2-dependent inhibition. At the same time, MICU1 acts as a cooperative activator of the channel, and thus stimulates the channel activity. Red dots indicate Ca^{2+} .

FIGURE 4. Trigger hypothesis of NAADP-induced Ca^{2+} release

NAADP evokes a Ca^{2+} -induced Ca^{2+} release response from the ER. NAADP acts at TPCs on lysosome-related Ca^{2+} stores to elicit a local Ca^{2+} release (middle). The increase in $[\text{Ca}^{2+}]$ at the endolysosome-ER interface sensitizes the InsP_3R , thus evoking a global Ca^{2+} wave (right hand side). Red dots indicate Ca^{2+} .

ER: endoplasmic reticulum

NAADP: nicotinic acid dinucleotide phosphate

TPCs: Two-Pore channels

InsP_3R : inositol 1,4,5-trisphosphate receptor

FIGURE 5. Ion channels and transporters in the endolysosome

TRPML and TPC channels are involved in the release of Ca^{2+} from the endolysosome.

Conversely, the activity of the vacuolar (V)-type H^+ -ATPase provides the energy required for Ca^{2+} entry into the lysosome by H^+ /Ca²⁺ exchanger.

TRPML: Mucolipin family of Transient Receptor Potential

TPC: Two-Pore channel