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Molecular structure and pathophysiological roles of the Mitochondrial Calcium Uniporter☆

Cristina Mammucari [a,*], Anna Raffaello [a,*], Denis Vecellio Reane [a], Rosario Rizzuto [a,b,**]

[a] Department of Biomedical Sciences, University of Padua, 35131, Italy
[b] Neuroscience Institute, National Research Council, Padua 35131, Italy

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ABSTRACT

Mitochondrial Ca^{2+} uptake regulates a wide array of cell functions, from stimulation of aerobic metabolism and ATP production in physiological settings, to induction of cell death in pathological conditions. The molecular identity of the Mitochondrial Calcium Uniporter (MCU), the highly selective channel responsible for Ca^{2+} entry through the IMM, has been described less than five years ago. Since then, research has been conducted to clarify the modulation of its activity, which relies on the dynamic interaction with regulatory proteins, and its contribution to the pathophysiology of organs and tissues. Particular attention has been placed on characterizing the role of MCU in cardiac and skeletal muscles.

In this review we summarize the molecular structure and regulation of the MCU complex in addition to its pathophysiological role, with particular attention to striated muscle tissues. This article is part of a Special Issue entitled: Mitochondrial Channels edited by Pierre Sonveaux, Pierre Maechler and Jean-Claude Martinou.

1. Introduction

Ca^{2+} is a highly versatile intracellular signal that operates to regulate a vast repertoire of cellular processes [1]. The first evidence that mitochondria can uptake Ca^{2+} dates back to the 1960s, when pioneering studies demonstrated that energized mitochondria can rapidly and efficiently accumulate Ca^{2+} [2,3]. Remarkably, these studies preceded the chemiosmotic theory of Ca^{2+} accumulation [4].

In the following years, the basic properties of mitochondrial Ca^{2+} transport were clarified: the rapid accumulation of Ca^{2+} into the mitochondrial matrix through the ion-impermeable inner mitochondrial membrane (IMM) occurs via an electrogenic pathway, that relies on the driving force of a steep electrochemical gradient attracting the divalent ion. At the same time, two main efflux pathways (i.e. the Na^{+}/Ca^{2+} (mNCX) and H^{+}/Ca^{2+} (mHCX) exchangers) are active, ensuring that Ca^{2+} accumulation does not proceed until electrochemical equilibrium is achieved [5].

However, when the mitochondrial Ca^{2+} uptake system was functionally characterized, its affinity was found to be too low to accumulate the cation, not only in resting conditions but also during the transient cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) increases generated by cell stimulation. Thus, the plasma membrane and the endoplasmic reticulum became the major players in the Ca^{2+} signaling scene while mitochondria were neglected [1]. The scenario completely changed with the development of genetically encoded Ca^{2+} probes specifically targeted to the mitochondrial matrix, allowing direct measurement of mitochondrial Ca^{2+} uptake in living cells [6]. It was shown that a large $[Ca^{2+}]_{mt}$ peak occurs dynamically in parallel to agonist-induced $[Ca^{2+}]_{cyt}$ increases. The speed and the amplitude of Ca^{2+} accumulation into mitochondria greatly exceed the values that were previously predicted on the basis of MCU properties in isolated mitochondria. In addition, upon agonist stimulation, $[Ca^{2+}]_{mt}$ reaches much higher values than those in the cytosol [6]. The discrepancy between this prompt response and the low affinity of the Ca^{2+} transporter was reconciled by the demonstration that mitochondria form close contacts with the source of Ca^{2+} accumulation, i.e. the endoplasmic reticulum. Hence, they are exposed to microdomains of high $[Ca^{2+}]$ that substantially exceed the values in the bulk cytosol [7,8,9].

Given these groundbreaking discoveries, the study of mitochondrial Ca^{2+} signaling became the primary goal of several laboratories. Indeed, it was demonstrated that mitochondrial Ca^{2+} activates three matrix dehydrogenases of the Krebs cycle, with consequent stimulation of oxidative phosphorylation and ATP synthesis [10], and that mitochondrial Ca^{2+} overload is associated with cell death [11].

However, for many years the study of the physiological roles of mitochondrial Ca^{2+} uptake has been severely limited by the lack of the molecular identity of the Mitochondrial Calcium Uniporter (MCU),

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* Corresponding authors.
** Correspondence to: R. Rizzuto, Department of Biomedical Sciences, University of Padua, 35131, Italy.
E-mail addresses: cristina.mammucari@unipd.it (C. Mammucari), anna.raffaello@unipd.it (A. Raffaello), rosario.rizzuto@unipd.it (R. Rizzuto).

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the IMM channel responsible for mitochondrial Ca^{2+} uptake. The first step forward was the direct measurement of the MCU current (I_{MCU}) by patch clamping of the inner mitochondrial membrane [12]. Finally, in 2011 our laboratory and that of V. Mootha simultaneously identified CCDC109A as the pore-forming subunit of the Mitochondrial Calcium Uniporter [13,14].

From that moment, the Ca^{2+} signaling field witnessed an explosion of discoveries aimed to clarify both the composition and functionality of the MCU complex and the pathophysiological role of mitochondrial Ca^{2+} uptake. In particular rapid progress was attained in characterizing the role of MCU in cardiac and skeletal muscle.

In this contribution, we summarize the main findings on the molecular structure and regulation of the Mitochondrial Calcium Uniporter complex and its role in pathophysiology, with particular attention to striated muscle tissues.

2. Molecular components of the Mitochondrial Calcium Uniporter complex

The uniporter complex, the highly selective channel responsible for mitochondrial Ca^{2+} uptake, consists of both pore-forming and regulatory subunits (Fig. 1 and [15]). MCU was the first structural component of the pore to be identified in 2011 [13,14]. The MCU gene is well conserved in all eukaryotes, except for yeast, encoding a 40 kDa protein composed of two coiled-coil domains and two transmembrane domains separated by a short hydrophilic loop enriched in acidic residues (the "DIME" loop). MCU was shown to be sufficient per se to mediate Ca^{2+} transport. Indeed, reconstitution of MCU protein in a planar lipid bilayer gave rise to Ca^{2+} currents with the main electrophysiological properties previously recorded for the Mitochondrial Calcium Uniporter [12,13]. In agreement with known uniporter properties, this current was inhibited by ruthenium red and Gd^{3+} [13]. A critical amino acid residue responsible for this inhibition was identified in the loop region [14]. In addition, isolated mitochondria from different tissues of the MCU knockout mouse show no mitochondrial Ca^{2+} uptake [16], and silencing of MCU inhibits mitochondrial Ca^{2+} uptake in many cell types and physiological conditions [14,17–20].

MCU exists in a large protein complex (~480 kDa) [14], suggesting that many other proteins contribute to the channel formation. Two proteins were indeed shown to be part of the pore-forming subunit, MCUb [21] and EMRE [22].

MCUb is an MCU related gene located on *Mus musculus* chromosome 3 (chromosome 4 for the *Homo sapiens* ortholog). The gene is present in vertebrates but absent in other organisms in which MCU is present (e.g., plants, kinetoplastids, Nematoda, and Arthropoda). The encoded protein (330 amino acids long) is highly conserved among all species and shares a 50% similarity with the MCU. It has two predicted transmembrane domains similar in sequence to the MCU although critical amino-acid substitutions in the pore region prevent MCUb from forming a Ca^{2+}-permeable channel, thus acting as a dominant-negative subunit [21]. Interestingly, MCUb/MCU ratio varies greatly between tissues, suggesting that it might contribute to the spatio-temporal control of mitochondrial Ca^{2+} uptake. Strikingly, this correlates with Ca^{2+} current measurements by patch-clamp of different tissues [23]. Indeed, tissues characterized by low mitochondrial Ca^{2+} transients, such as the heart, show a higher MCUb/MCU ratio, compared to tissues such as skeletal muscle.

Another protein associated with the pore-forming subunit is the recently identified EMRE ("essential MCU regulator", previously known as C22orf32), although whether or not EMRE is required to form a functional Ca^{2+} channel is still debated. EMRE was identified by SILAC-based quantitative mass spectrometry of affinity-purified MCU complex [22]. EMRE is a 10 kDa, metazoan specific protein with a single transmembrane domain that spans the IMM and a highly conserved C-terminus rich in aspartate residues. EMRE silencing completely abrogates MCU activity in cells, even though MCU expression and oligomerization are preserved [22]. However, in a planar lipid bilayer, MCU alone is sufficient to give rise to Ca^{2+} currents, as discussed above [13]. It was proposed that EMRE is required for the interaction of MCU with MICU1 and MICU2, fundamental regulators of the channel (see below), since EMRE silencing prevents MICU1 and MICU2 to coimmunoprecipitate with MCU [22]. However, this data is in contrast with bilayer experiments that showed that MICU1 is sufficient to induce MCU channel activity [24]. It is important to note that MCU and MICU1 are conserved in plants, fungi and protozoa whereas EMRE is not present. Consistently, in yeast and in mammalian cells, *Dictyostelium discoideum* MCU homolog conducts Ca^{2+} in the absence of an EMRE homolog while, in these systems, human MCU requires the presence of EMRE to act as a functional channel [25]. Recently, new discoveries were made, uncovering a previously unrecognized role for matrix [Ca^{2+}] in the regulation of MCU through EMRE [26]. According to Vais et al. EMRE acts as Ca^{2+} sensor on both sides of the IMM, with a mechanism that requires MICU1 and MICU2. Thus,

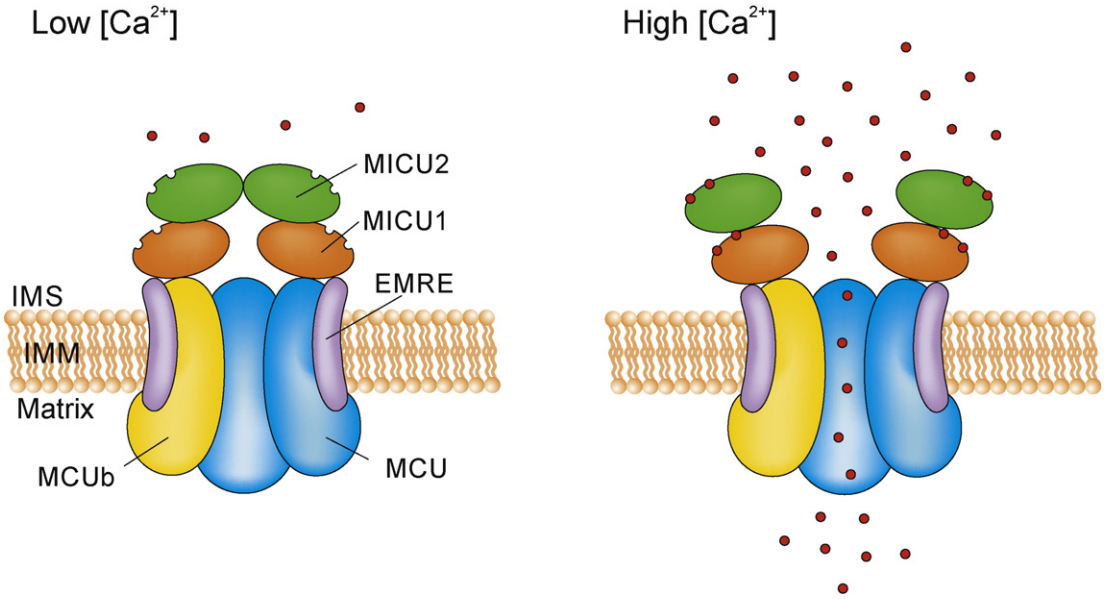


Fig. 1. MCU comprises pore-forming and regulatory proteins.

EMRE plays a dual role, according to $[Ca^{2+}]_{cyt}$, protecting the mitochondria both from Ca^{2+} depletion and from Ca^{2+} overload [26].

One of the key features of mitochondrial Ca^{2+} uptake is the sigmoidal response to extra-mitochondrial Ca^{2+} concentrations. In resting conditions, mitochondrial Ca^{2+} uptake is inhibited despite the huge driving force for matrix cation accumulation. This mechanism prevents vicious cycling of cation accumulation, conspicuous energy drain, morphological alterations and release of apoptotic cofactors [1]. On the other hand, mitochondrial Ca^{2+} rapidly rises when the $[Ca^{2+}]$ in the close proximity of mitochondria reaches a threshold value (>10 μM) [8,27,28]. Given the topology of the MCU, with only a small loop facing the intermembrane space and the N- and C-termini residing in the matrix [14,29], it seemed unlikely that MCU itself could be responsible for these fundamental properties. Thus, the existence of a highly sophisticated gatekeeping mechanisms was predicted, including both negative modulators acting at low $[Ca^{2+}]$, and activators able to induce Ca^{2+} currents during cell stimulation.

It is now widely accepted that the components of the MICU family of mitochondrial EF-hand containing proteins are responsible for this vital property of mitochondrial Ca^{2+} uptake [30]. MICU1 (formerly known as EFHA3) was the first member of this family to be described, even before the molecular identification of MCU [31]. A mitochondrial compendium of 1098 genes [32] was searched for membrane proteins evolutionarily conserved in vertebrates and in kinetoplastids but absent in yeast, and MICU1 was identified by an RNAi screen of the selected genes. MICU1 is a 54 kDa protein that possesses two EF-hand domains and a putative transmembrane domain. Whether or not MICU1 is a membrane protein is still being debated. It was originally shown to be associated with the IMM [31] but its topology is still an open issue, since a number of studies have reached different conclusions [22,33–35]. Recent proteomic experiments strongly demonstrate that the components of the MICU family reside in the intermembrane space [22,36,37]. As for the function, MICU1 was initially shown to be necessary for mitochondrial Ca^{2+} uptake [31,38]. However, the group of M. Madesh demonstrated that mitochondria lacking MICU1 are constitutively loaded with Ca^{2+} [33]. It was therefore hypothesized that MICU1 is fundamental to prevent Ca^{2+} uptake at low $[Ca^{2+}]_{cyt}$, while playing a minor role at higher $[Ca^{2+}]_{cyt}$. Complexity was added by the study performed by G. Hajnóczky's laboratory [35]. The gatekeeper role of MICU1 at low $[Ca^{2+}]_{cyt}$ was confirmed, but it was also shown that in the absence of MICU1 the cooperativity of mitochondrial Ca^{2+} uptake is lost, suggesting that MICU1 plays a dual function depending on $[Ca^{2+}]_{cyt}$. Coherently, studies in human patients, in which loss of function mutations in MICU1 cause a disease phenotype characterized by proximal myopathy, learning difficulties and a progressive extrapyramidal movement disorder, showed increased mitochondrial Ca^{2+} load. This increase was observed both at basal cytosolic Ca^{2+} concentrations and after spontaneous, low-frequency, transient increases in cytoplasmic Ca^{2+} levels [39].

However, these studies preceded the identification of MICU1 paralogs [40], MICU2 (formerly known as EFHA1) and MICU3 (formerly known as EFHA2), also characterized by the presence of two highly conserved EF-hand domains. MICU2 and MICU3 likely arise from gene duplication in vertebrates and show highly different expression profiles in mouse tissues. Indeed, MICU3 expression is restricted to the central nervous system and, at lower levels, to skeletal muscle [40], while MICU2 shares a similar tissue distribution and the intermembrane space localization with MICU1 [36]. MICU2 forms an obligate heterodimer with MICU1 through a disulfide bond that interacts with MCU in the DIME loop, i.e. the short intervening region between the two transmembrane domains (Fig. 1 and [24]). MICU2 inhibits the channel activity of purified MCU in planar lipid bilayers and reduces channel opening at resting $[Ca^{2+}]$ in intact HeLa cells, thus ensuring minimal Ca^{2+} accumulation. Importantly, MICU2 protein stability depends on the presence of MICU1 [24,40,41], suggesting that the loss of the gatekeeping property of MCU in MICU1 silenced conditions [33,35] is due to the concomitant reduction of MICU2 expression. In addition, as $[Ca^{2+}]_{cyt}$ increases upon cell stimulation, Ca^{2+}-dependent conformational changes of the MICU1-MICU2 dimer release MICU2 dependent inhibition (Fig. 1). At the same time MICU1 stimulates MCU activity, acting as the cooperative activator of the channel (Fig. 1). Accordingly, a MICU2 mutant unable to bind Ca^{2+} acts as gatekeeper also in conditions of high $[Ca^{2+}]_{cyt}$ while, in the same conditions, MICU1 mutant loses the capability to cooperatively activate the channel [24].

As for MICU3, its specific function is still unknown. Moreover, its restricted expression in the CNS and skeletal muscle [40] suggests a tissue specific mitochondrial Ca^{2+} uptake mechanism that still needs to be thoroughly investigated.

Another protein whose role is not yet definitive is the Mitochondrial Calcium Uniporter Regulator 1 (MCUR1, formerly known as coiled-coil domain-containing 90A or CCDC90A) that was discovered thanks to an RNAi screen of mitochondrial genes that regulate mitochondrial Ca^{2+} uptake [42]. MCUR1 is a 40 kDa inner membrane protein that possesses two transmembrane spanning helices. MCUR1 was shown to be required for uniporter activity [42]. Indeed, its silencing abrogates mitochondrial Ca^{2+} uptake while its overexpression enhances MCU activity. This protein was shown to interact with MCU [42], although mass spectrometry of MCU interactors failed to identify MCUR1 [22]. In yeast and human cells, suppression of MCUR1 causes a specific cytochrome c oxidase (COX) assembly defect and decreased mitochondrial membrane potential ($\Delta\psi_m$), with consequent reduction in mitochondrial Ca^{2+} uptake [43]. This observation, together with the evidence that yeast MCUR1 orthologous lacks uniporter activity, suggests an indirect function of MCUR1 in regulating mitochondrial Ca^{2+} uptake. However, measurements of MCU-mediated Ca^{2+} currents by patch clamp electrophysiology of mitoplasts showed that MCUR1 knockdown diminishes MCU Ca^{2+} currents independently of differences in $\Delta\psi_m$ [44]. Thus, further experiments are necessary to clarify the role of MCUR1.

3. The physiological role of mitochondrial Ca^{2+} uptake in non-muscle tissues

Before the molecular identification of the uniporter complex components, the established roles of mitochondrial Ca^{2+} signaling included stimulation of ATP production, buffering of cytosolic Ca^{2+} transients and regulation of cell death [1]. In parallel with the elucidation of the molecular identity and functions of the components of the MCU complex, a number of studies have been performed aimed to elucidate the biological functions of Ca^{2+} transport. In this part of the review we will summarize the principal findings on the role of mitochondrial Ca^{2+} homeostasis in non-muscle cells.

The first studies, in chronological terms, examined the role of MCU in glucose-induced ATP production in pancreatic β-cells [15,35]. In these cells, the glucose-dependent stimulation of oxidative metabolism elevates cytosolic [ATP], responsible for the closure of ATP-sensitive K^+ (KATP) channels in the plasma membrane, causing cell membrane depolarization and Ca^{2+} influx via voltage-gated Ca^{2+} channels. These events trigger the first phase of insulin secretion. In addition to this triggering pathway, glucose also activates an essential metabolic amplifying pathway that increases the action of Ca^{2+} on exocytosis (for review, see [47]).

Immediately before the discovery of the MCU, it was demonstrated that mitochondrial $[Ca^{2+}]$ increases, caused by Ca^{2+} entry through the plasma membrane, are necessary for the amplification of sustained glucose-dependent insulin secretion [48]. In this model, glucose is sufficient to induce ATP synthesis in the first phase of insulin secretion but mitochondrial Ca^{2+}-dependent ATP production is required for the more pronounced sustained phase. The discovery of MCU and MICU1 allowed a finer manipulation of mitochondrial Ca^{2+} entry in β-cells. Silencing of MCU [19,45,49] and MICU1 [46] confirmed that Ca^{2+} entry through the plasma membrane triggers mitochondrial Ca^{2+} uptake via the MCU complex that further accelerates oxidative phosphorylation and hence ATP production, contributing to sustained

insulin release. Interestingly, insulin secretion stimulated by tolbutamide, a potassium channel blocker used in the management of type II diabetes, is dependent on MCU activity [45]. Since defects in the glucose sensitivity and function of β-cells are crucial aspects of type 2 diabetes (T2D) and alterations of β-cell Ca^{2+} homeostasis are observed in T2D [50], it is plausible that pharmacological modulation of the MCU complex could represent a winning strategy to regulate insulin secretion in vivo and might provide a new approach to improve glucose tolerance.

Due to the important implications in health and disease, the role of mitochondrial Ca^{2+} signaling in controlling cell death and cancer progression has been extensively investigated. In many cell types, mitochondrial Ca^{2+} overload plays a permissive role in cell death by triggering the opening of the mPTP and the release of caspase cofactors [1]. As expected, mitochondria from total MCU knockout mice show no mPTP opening upon addition of extramitochondrial Ca^{2+}. However, MEF cells derived from these animals show no difference in the kinetics or magnitude of cell death upon different stimuli compared to MEF cells derived from wild type mice [16].

Increased levels of mitochondrial Ca^{2+} uptake, either by MCU overexpression [13] or MICU1 silencing [33], sensitize HeLa cells to apoptotic stimuli. Consistent with the data correlating MCU expression and cell death, increased levels of miR-25 that specifically reduce MCU protein levels confer resistance of cancer cells to apoptotic stimuli, and this effect is reversed by anti-miR-25 overexpression [51].

However, MCU expression is induced in estrogen receptor (ER)-negative breast cancers that are associated with poor prognosis [52]. In addition, MCU silencing does not have any effect on cell apoptosis of the highly aggressive triple negative breast carcinoma cell line MDA-MB-231 [52,53], suggesting that these cells primarily utilize MCU-independent mechanisms for survival. In this cell line, only caspase-independent cell death was potentiated by MCU silencing [52]. MCU plays a critical role in MDA-MB-231 migration with a mechanism that has been ascribed to be dependent on store-operated Ca^{2+} entry [54]. We have now shown that MCU expression correlates with breast cancer progression, and that MCU depletion inhibits in vivo tumor growth and metastasis formation, with a mechanism that implies reduced ROS formation and HIF-1α expression, while the role of store-operated Ca^{2+} entry is not totally unambiguous [55].

The contribution of mitochondrial Ca^{2+} overload to cell death has been systematically investigated in cortical neurons. MCU overexpression exacerbates NMDAR-dependent increases in mitochondrial Ca^{2+}, loss of mitochondrial membrane potential and excitotoxic cell death [20]. Furthermore, MCU expression seems to be precisely suppressed by synaptic activity that promotes neuroprotection against excitotoxic insults [56], via a mechanism involving nuclear Ca^{2+} and CaM kinase-mediated induction of the transcription factor Npas4. A confirmation of the importance of mitochondrial Ca^{2+} uptake in brain comes from the discovery of a loss-of-function mutation of MICU1 in individuals with a neuromuscular disease characterized by learning difficulties and an extrapyramidal disorder [39], although surprisingly no abnormalities have been reported in the MCU knockout mice [16]. Among the several aspects that still need to be addressed, the definition of the role of MICU3, whose expression is restricted to the nervous system, will be of great interest.

4. Role of MCU in cardiac physiopathology

Cardiac mitochondria are the major source of ATP, required for heart function, and inhibition of mitochondrial ATP synthesis leads to loss of contractile capacity and cell death [57]. While the importance of mitochondria metabolism in the heart is undisputed, the role of mitochondrial Ca^{2+} uptake in shaping cytosolic [Ca^{2+}] and its consequences on heart pathophysiology remains controversial (Fig. 2).

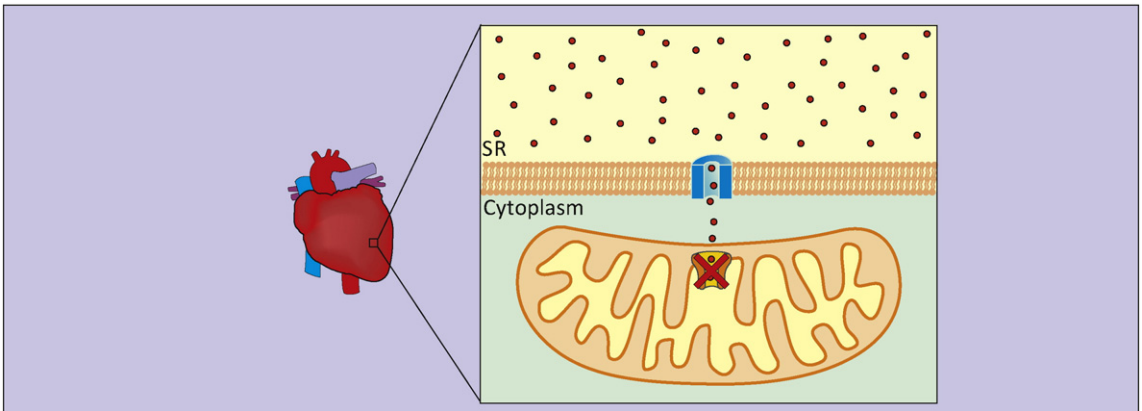
Direct patch-clamp measurements of I_{MCU} in mitoplasts leads to the unexpected finding thatI_{MCU} in mouse heart is substantially smaller than other mouse tissues. In particular heart I_{MCU} is 30 times smaller than skeletal muscle I_{MCU} [23]. Nonetheless skeletal muscle and heart mitochondrial Ca^{2+} currents share the same biophysical properties, indicating that the difference in current density is not due to differences in the proteins responsible for channel activity. These findings have multiple possible explanations: i) in the heart, mitochondria occupy up to 37% of the cell volume, thus it is plausible that mitochondrial Ca^{2+} uptake is kept low in order to not abolish the cytosolic Ca^{2+} transients necessary for heart beating, ii) frequent elevation of cytosolic Ca^{2+}, if efficiently translated into mitochondrial Ca^{2+} uptake, could lead to futile cycling of Ca^{2+} across the IMM, mitochondrial Ca^{2+} overload and damage.

The first study on neonatal cardiomyocytes after the molecular identification of MCU was aimed at understanding whether and to what extent beat-to-beat mitochondrial Ca^{2+} oscillations depend on hot spots generated on their surface, and whether mitochondrial Ca^{2+} uptake shapes cytosolic Ca^{2+} transients [58]. Indeed, whether or not mitochondrial Ca^{2+} uptake buffers systolic Ca^{2+} peaks remained an unresolved question. In addition, the dynamics of mitochondrial Ca^{2+} uptake during contraction were unclear. T. Pozzan laboratory investigated the existence and amplitude of high [Ca^{2+}] microdomains at the SR/mitochondria contacts and analyzed the role of MCU in mitochondrial Ca^{2+} uptake and on buffering of cytosolic Ca^{2+} transients. Taking advantage of a GFP-based Ca^{2+} indicator targeted at the OMM, the authors demonstrated that 20–30 μM Ca^{2+} hot spots are formed during systole. In addition, modulation of MCU levels by overexpression or silencing, reduced and increased the amplitude of beat-to-beat cytoplasmic Ca^{2+} oscillation respectively.

Analysis of the MCU knockout mouse brought surprising results that are still being debated [16,59] (Fig. 2). As expected MCU knockout cardiac mitochondria had impaired Ca^{2+} uptake and Ca^{2+}-dependent oxygen consumption. Yet, basal ATP levels were unaltered, suggesting that MCU depletion does not affect basal mitochondrial metabolism [59]. Both in 12 and in 20 month-old mice, basal heart parameters were indistinguishable from wild type animals. In addition, left ventricular cardiac output at baseline and following isoproterenol treatment were similar in MCU knockout versus wild type mice [59], even though the rise in mitochondrial [Ca^{2+}] induced by isoproterenol in wild type cardiac myocytes did not occur in MCU depleted cells [16]. Unexpectedly, while cyclosporine A (CsA), which inhibits Ca^{2+}-dependent cell death mediated by mPTP opening, protected wild type hearts from ischemia–reperfusion injury, infarct area in MCU knockout hearts was similar to control hearts, and CsA did not exert any protection. This suggests that Ca^{2+}-independent death pathways take place in the absence of MCU [16]. Also, upon transverse aortic constriction (TAC) overall survival as well as histologic parameters like hypertrophy and fibrosis were similar among the two genotypes [59].

The mild phenotype observed in MCU knockout hearts could be partially explained by the observation that resting free [Ca^{2+}] in knockout mitochondria is only partially reduced [16]. These data suggested the existence of alternative mechanisms for Ca^{2+} accumulation, although MCU knockout mitochondria and intact cells were not capable of any energy-dependent Ca^{2+} uptake [16]. Of note, MCU knockout mice are viable only on a mixed genetic background, and not even at Mendelian ratio, while deletion of MCU on the inbred C57BL/6 background is embrionically lethal [60]. Thus, in the mixed background, compensatory mechanisms that justify animal viability could in principle take place.

Further studies were conducted in a transgenic mouse model in which MCU activity was inhibited by the overexpression of a dominant-negative form of MCU under the control of αMHC promoter (DN-MCU) [61,62] (Fig. 2). DN-MCU mitochondria were unable of taking up Ca^{2+}, but no differences were observed in terms of cardiac and mitochondria morphology and baseline heart function. Despite the lack of differences in basal heart rate and weight, O_2 consumption rates (OCR) were increased in DN-MCU isolated perfused hearts, but not in isolated mitochondria or permeabilized fibers. Additionally,



	MCU$^{-/-}$ mouse	DN-MCU^{TG} mouse	Conditional cardiac specific MCU$^{-/-}$ mouse
Basal heart parameters	Normal	Normal	Normal
Fight or flight response	No effect	Impaired response	Impaired response
Ischaemia-reperfusion injury	No effect	No effect	Protection
TAC-induced damage	No effect	N.d.	No effect

Fig. 2. Cardiac phenotypes of MCU mouse models.

in agreement with the buffering properties of mitochondria relative to cytosolic Ca^{2+}, in DN-MCU hearts increases in cytoplasmic [Ca^{2+}] were observed, that were partially reversed by ATP dialysis. These data indicate that inhibition of MCU activity results in: i) reduced heart performance and ii) enhanced energetic demand related to extramitochondrial Ca^{2+} signaling. Nonetheless, in agreement with findings on MCU knockout mouse, inhibition of MCU activity by DN-MCU was inefficient in protecting hearts from cell-death induced by ischemia–reperfusion injury [62].

Further insights demonstrated that the main defect of DN-MCU hearts is the impairment of the "fight or flight" response, i.e. the physiological heart rate acceleration consequent to catecholamine stimulation [61]. Specifically, prolonged P-waves, PQ and PR intervals were measured within DN-MCU mice being compared to wild type animals, while QRS and QT interval durations were similar. These data indicate a selective reduction in atrial and atrioventricular conduction velocity, while conduction velocities in the distal conduction system or in the ventricular myocardium were unaffected. MCU was shown to be essential for increased mitochondrial Ca^{2+} uptake in sinoatrial nodal (SAN) pacemaker cells, with consequent stimulation of oxidative phosphorylation. Increased ATP synthesis thus sustains SERCA activity and prompts SR refilling during heart rate acceleration [61].

Both MCU knockout and DN-MCU mouse models are characterized by constitutive modulation of MCU activity which begins during development. Adaptations to embryonic long-term loss of mitochondrial Ca^{2+} uptake cannot be excluded. Thus, a mouse model in which MCU is specifically deleted in adult cardiomyocytes was developed (Fig. 2 and [63,64]). Acute cardiac-specific deletion of MCU did not only trigger growth defects, as expected, but also did not cause lactic acidosis and phosphorylation of PDH. Analyses of heart structure and function show both similarities and differences with previous models. Ca^{2+}-induced mitochondrial Ca^{2+} uptake, but not basal mitochondrial Ca^{2+}, was drastically reduced in MCU-depleted cardiac mitochondria. Still, heart morphology and ultrastructure, as well as basal cardiac functions were unaltered. When subjected to chronic damage by transverse aortic constriction (TAC), no differences were observed in heart weight, cardiomyocyte area and fractional shortening. However, in contrast to what was previously reported for constitutive MCU inhibition mouse models [16,62], deletion of MCU in adult cardiomyocytes leads to protection from cell death induced by acute damage such as ischemia–reperfusion injury. As already observed in MCU knockout mice, acute MCU deletion caused desensitization of Ca^{2+}-induced mPTP opening. OCR and ATP synthesis were similar between wild type and acutely deleted MCU heart mitochondria in basal conditions, in line with basal mitochondrial Ca^{2+} measurements. However, MCU was required for short-term OCR increase by Ca^{2+} or β-adrenergic receptor agonist stimulation. Of note, OCR in MCU knockout adult cardiomyocytes eventually reached the values of wild type cells, suggesting that MCU is critical for acute mitochondrial Ca^{2+} uptake in conditions of increased heart work and that alternative mechanisms are responsible for slow Ca^{2+} accumulation. Similarly, performance during a treadmill protocol of strenuous work was decreased in adult cardiomyocyte-specific MCU knockout mice. However, if mice were allowed to warm-up for enough time, no differences were observed among wild type and knockout animals.

Mitochondrial Ca^{2+} uptake is regulated by various mechanisms. As for the heart, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has been proposed to induce mPTP opening-dependent myocardial death by increasing I_{MCU} [18]. Transgenic mice with myocardial expression of CaMKIIN, a CaMKII inhibitor, targeted to mitochondria, were protected against ischemia–reperfusion injury and showed reduced mitochondrial Ca^{2+} uptake and I_{MCU}. CaMKII catalytic activity was essential for increased I_{MCU} and two CaMKII target sites, required for CaMKII-induced I_{MCU}, were identified in the MCU sequence. However, I_{MCU} recordings and evidence for the CaMKII-dependent MCU regulation, reported in the study by Joiner et al. [18], have been questioned. Fieni et al. [65] highlighted that the I_{MCU} recorded in the Joiner at al. study was two orders of magnitude greater than the one previously measured, and characterized by high fluctuations incompatible with the low single-channel conductance of MCU. Further studies are required to clarify this issue.

Other post-transcriptional regulations of MCU activity have been described in cardiac cells [66]. In particular, α_1-adrenoceptor (α_1-AR) activation by the α_1-AR agonist phenylephrine triggered translocation

of activated proline-rich tyrosine kinase 2 (Pyk2) from the cytosol to the mitochondrial matrix. Once in the matrix, Pyk2 phosphorylates MCU and accelerates its tetrameric oligomerization, eventually triggering mitochondrial Ca^{2+} uptake increase. In cardiac cells, while MCU overexpression caused increased mitochondrial superoxide upon phenylephrine treatment, the opposite effect was observed by Pyk2 inhibition. In addition, the α_1-AR-Pyk2 axis was shown to initiate cell death in adult cardiomyocytes.

Great interest is now placed on the role of miRNA in cardiac pathophysiology. As noted above, MCU has been identified as a target of miR-25 and MCU protein levels inversely correlate with miR-25 expression [51,67]. Expression of miR-25 in cardiomyocytes is increased upon oxidative stress, and overexpression of miR-25 decreases mitochondrial Ca^{2+} uptake and reduces apoptotic cell death eventually protecting cardiomyocytes from oxidative damage, suggesting that the protective role of miR-25 in cardiac pathology is mediated by MCU-dependent mitochondrial Ca^{2+} signaling [67].

5. Mitochondrial Ca^{2+} signaling in skeletal muscle homeostasis

In contrast to heart tissue, only 5% of skeletal muscle cell volume is occupied by mitochondria. Possibly because they are not exposed to frequent Ca^{2+} elevations, skeletal muscle mitochondria display much higher I_{MCU} compared to the heart [23]. As mentioned above, MCU knockout mice display a mild phenotype, although skeletal muscle is the most affected tissue [16]. Stimulation of O_2 consumption by Ca^{2+} is suppressed in MCU knockout muscle mitochondria, although matrix Ca^{2+} levels are reduced only by about 75%. PDH phosphorylation is increased in MCU knockout muscles, in agreement with the Ca^{2+}-dependent regulation of PDP1 phosphatase, consequently PDH activity is decreased. In line with the role of mitochondrial Ca^{2+} uptake to stimulate ATP production required for muscle function, MCU knockout mice show reduced maximal power output (Fig. 3).

To overcome the possible compensatory effects of the MCU knockout mouse during embryonic development, the role of MCU in skeletal muscle homeostasis has been further investigated by overexpressing or silencing MCU specifically in skeletal muscle a few days after birth and in adulthood (Fig. 3). For this purpose, infection of skeletal muscle with Adeno-associated viral vectors (AAVs) was used [68]. MCU overexpression triggered skeletal muscle hypertrophy, while MCU silencing causes muscle atrophy, both during post-natal development and in adulthood. Most importantly, MCU overexpression protected muscles from the loss of muscle mass during denervation, indicating a potential therapeutic role of MCU modulation in muscle atrophy. A purely metabolic effect was excluded as a possible mechanism since PDH activity was unaffected by MCU overexpression, although as reported for the MCU knockout, PDH activity was reduced in shMCU muscles. In addition, the trophic effect was very similar between glycolytic and mitochondria-rich oxidative muscles, in which mitochondrial metabolism plays different roles. Nonetheless, mitochondria volume was modulated by MCU, suggesting a role for mitochondrial Ca^{2+} in the regulation of organelle biogenesis and morphology. As for the mechanism, established hypertrophy pathways, like IGF1-Akt/PKB and PGC-1α4, were responsible for the regulation of muscle size by MCU (Fig. 3). In addition, RNA microarray analyses demonstrated that MCU modulation controls global gene expression, thus identifying a Ca^{2+}-dependent mitochondria-to-nucleus route that links mitochondrial function to the control of muscle mass [68,69] (Fig. 3). The idea that in skeletal muscle mitochondrial Ca^{2+} uptake is fundamental for tissue homeostasis was strongly supported by the discovery of human patients harboring a loss-of-function MICU1 mutation and with a disease phenotype characterized by proximal myopathy, learning difficulties and progressive extrapyramidal movement disorder [39]. Strikingly, the increase in mitochondrial Ca^{2+} uptake was able to buffer cytoplasmic Ca^{2+} transients despite normal mitochondrial membrane potential. In addition, mitochondrial network was severely fragmented. Altogether these findings demonstrate that mitochondrial Ca^{2+} signaling in skeletal muscle is fundamental in human pathophysiology.

6. Conclusions

The study of the dynamic regulation of mitochondrial Ca^{2+} uptake and its contribution to pathophysiology greatly advanced after the discovery of the molecular identity of MICU1 [70], followed soon after by MCU [13,14], and of the other interactors in the following years. We predict that many open questions, some of which are summarized hereafter, will be answered in the near future.

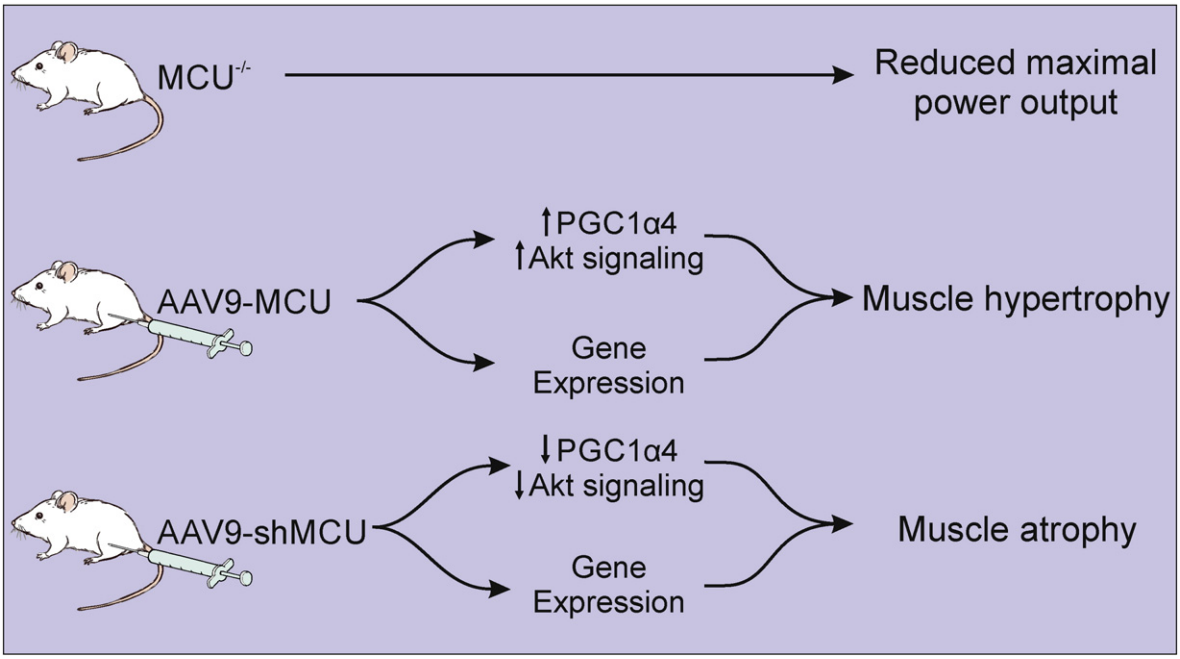


Fig. 3. In vivo skeletal muscle phenotypes.

MCU activity is regulated by conformational changes in the MCU complex and at least partially controlled by Ca^{2+} itself. MICU1 and MICU2 are the two major players in this modulation, and different studies are aimed to understand their role. However, some issues concerning the relative contribution of one to the other's function, as well as their role in the modulation of mitochondrial Ca^{2+} uptake in basal versus stimulated $[Ca^{2+}]_{cyt}$, are still under debate. In addition, the role MCUR1 in modulating channel activity is still uncertain, and the role of MICU3 in the regulation of mitochondrial Ca^{2+} uptake in the nervous system is also still largely unknown.

The expression levels of the different MCU complex components have been analyzed in various tissues and cell lines. In some cases, the relative expression of MCU and of its interactors is in line with the predicted mitochondrial Ca^{2+} uptake behavior (e.g. skeletal muscle expresses high levels of MCU, compared to other tissues). However, especially in cell lines, this paradigm is not always confirmed, indicating that other regulatory systems contribute to MCU activity. In addition, the study of gene regulation of MCU complex components is still in its early stages.

Some work has been done on the post-transcriptional regulation of MCU complex, which certainly plays an important role. However, in some cases contradictory results have been reported, indicating that further studies are required.

The surprisingly mild phenotype of the MCU knockout mouse has been discussed in many published reviews and comments. Since its description, tissue-specific and inducible knockout animals have been developed, in order to i) discern the contribution of MCU in single organs and ii) overcome possible compensatory mechanisms that may occur during development. This approach brought clarification of the role of MCU in the heart, where MCU has been shown to be involved in the fight or flight response. The same should be done for other organs and tissues, including skeletal muscle, the nervous system and pancreatic β-cells.

In parallel to defining the contribution of MCU activity to pathologies, the discovery of drugs that modulate MCU activity will be extremely important for the future development of putative MCU-targeting therapies.

The uniporter is formed by MCU protein, by the dominant negative subunit (MCUb) and EMRE. In low $[Ca^{2+}]_{cyt}$ the regulatory subunit MICU2 keeps the channel closed (left). In conditions of $[Ca^{2+}]_{cyt}$ elevation, MICU2 is inhibited, while MICU1 is activated and acts as a cooperative activator of MCU (right).

Comparison of heart phenotypes among the different mouse models in which MCU activity is inhibited.

Deletion of the MCU gene causes reduction in maximal power output. Post-natal and skeletal muscle-specific knockdown of MCU triggers atrophy, while MCU overexpression causes hypertrophy. Established muscle trophism pathways are modulated by MCU.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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