Enjoy the Trip: Calcium in Mitochondria Back and Forth

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Abstract

In the last 5 years, most of the molecules that control mitochondrial Ca^{2+} homeostasis have been finally identified. Mitochondrial Ca^{2+} uptake is mediated by the Mitochondrial Calcium Uniporter (MCU) complex, a macromolecular structure that guarantees Ca^{2+} accumulation inside mitochondrial matrix upon increases in cytosolic Ca^{2+}. Conversely, Ca^{2+} release is under the control of the Na^{+}/Ca^{2+} exchanger, encoded by the *NCLX* gene, and of a H^{+}/Ca^{2+} antiporter, whose identity is still debated. The low affinity of the MCU complex, coupled to the activity of the efflux systems, protects cells from continuous futile cycles of Ca^{2+} across the inner mitochondrial membrane and consequent massive energy dissipation. In this review, we discuss the basic principles that govern mitochondrial Ca^{2+} homeostasis and the methods used to investigate the dynamics of Ca^{2+} concentration within the organelles. We discuss the functional and structural role of the different molecules involved in mitochondrial Ca^{2+} handling and their pathophysiological role.

Contents

INTRODUCTION

The last 5 years have witnessed a major acceleration in the understanding of mitochondrial Ca^{2+} homeostasis. This understanding is mainly due to the molecular identification of the proteins involved in the control of Ca^{2+} flux across the inner mitochondrial membrane (i.e., exchangers, channels, and associated regulators). These proteins have been searched for by many different laboratories for more than 40 years, and in only 3 years most, if not all, of the components of this complex machinery have been molecularly identified and functionally characterized. In particular, in 2010 the group of Israel Sekler (1) identified the mitochondrial Na^+/Ca^{2+} exchanger, named Na^+/Ca^{2+} Li^+-permeable Exchanger (NCLX) (i.e., the protein that mediates organelle Ca^{2+} efflux). Shortly after, the group of Vamsi Mootha (2) identified the first protein involved in mitochondrial Ca^{2+} uptake, named MICU1 (MItochondrial Calcium Uptake 1, previously known as CBARA1 or EFHA3), and only 1 year later, two groups (3, 4) independently identified the genuine Ruthenium Red (RR)–sensitive Ca^{2+} channel of the inner mitochondrial membrane, named Mitochondrial Calcium Uniporter (MCU, previously known as CCDC109A). In the subsequent 2 years, a number of other potential regulators of mitochondrial Ca^{2+} influx were identified and partially characterized: MCUb (Mitochondrial Ca^{2+} Uniporter b) (5), the MICU family (including MICU1, MICU2, and MICU3) (6), MCUR1 (MCU Regulator 1) (7), EMRE (Essential MCU Regulator) (8), and SLC25A23 (Solute Carrier 25A23) (9). Although the role in regulating Ca^{2+} influx for some of these proteins is still debated (see the section on Molecular Components of the MCU Complex), there is a general consensus that mitochondrial Ca^{2+} uptake in live cells is mediated by a macromolecular structure, the MCU complex, the composition of which may vary depending on the cell type and different epigenetic control mechanisms.

In this review, we first discuss a few simple thermodynamic principles at the basis of the Ca^{2+} influx/efflux in mitochondria, and we describe the presently available methodologies to monitor

mitochondrial Ca^{2+} homeostasis. We then focus on the molecular machinery controlling the influx of Ca^{2+} into the mitochondrial matrix and dissect the functional role of each one of the (putative) components of the complex. We also briefly discuss the present knowledge concerning the functional and molecular characteristics of the Ca^{2+} efflux mechanism, a process that is still largely unexplored but represents the necessary counterpart of the MCU complex that ensures the physiological regulation of the mitochondrial Ca^{2+}–handling process in live cells. Finally, the present understanding of the physiological role of Ca^{2+} accumulation/release in the live cells based on the recent genetic manipulation of the molecular components of the Ca^{2+} homeostatic toolkit of mitochondria is addressed. A complete picture of MCU complex function is still missing, owing to the intrinsic novelty of many findings on the MCU complex proteins (that in some cases still require independent validation). The possibility that other modulators of the process may be discovered in the near future cannot be excluded.

THERMODYNAMICS OF MITOCHONDRIAL CA^{2+} TRANSPORT MECHANISMS

Although the identification of the molecular actors in mitochondrial Ca^{2+} uptake and release are recent discoveries, the basic functional characteristics of these processes were firmly established more than 30 years ago. Ca^{2+} influx depends on a so-called Ca^{2+} uniporter that results in the net transport of two positive charges from the medium into the mitochondrial matrix, driven by the negative potential inside the membrane; the efflux from the matrix, on the contrary, depends on two mechanisms, a ubiquitous H^+/Ca^{2+} exchange (all available evidence indicates it is an electroneutral process) and a Na^+/Ca^{2+} exchange (most likely electrogenic with 3 or 4 Na^+ ions per Ca^{2+}).

Since its discovery, Ca^{2+} uptake by isolated mitochondria has been defined as "energy driven" to indicate that Ca^{2+} is accumulated into the matrix at the expense of energy, provided by either the oxidation of a substrate or by activity of the mitochondrial H^+ ATPase (10, 11). These features clearly differentiated mitochondrial Ca^{2+} accumulation from the other Ca^{2+} transport mechanisms identified in the same years (e.g., the Ca^{2+} ATPase of the sarcoplasmic reticulum) (12). Initially, the energy dependence of mitochondrial Ca^{2+} uptake was interpreted as evidence for the existence in energized mitochondria of a high-energy phosphointermediate named X∼P. When the chemiosmotic model was eventually accepted by the majority of scientists in the field, it became clear that the driving force for Ca^{2+} accumulation is the membrane potential generated across the inner mitochondrial membrane by the extrusion of H^+ through the respiratory chain complex or by reversal of the H^+ ATPase.

The net charge transfer during Ca^{2+} uptake was briefly debated, and agreement was reached that the transport is catalyzed by a uniport mechanism with an accumulated net charge transfer of $2/Ca^{2+}$ (13). The term Mitochondrial Calcium Uniporter (MCU) was thus introduced, and this concept has not been challenged until recently. The electrochemical potential for Ca^{2+} ($\Delta\tilde{\mu}Ca$) is the sum of the chemical ($\Delta\mu Ca$) and electrical components:

$$\Delta\tilde{\mu}Ca = \Delta\mu Ca + zF\Delta\psi_m,$$

$$\Delta\tilde{\mu}Ca = RT \ln \frac{[Ca^{2+}]_m}{[Ca^{2+}]_c} + zF\Delta\psi_m.$$

If mitochondria were endowed exclusively with MCU, it is predicted that Ca^{2+} should eventually reach electrochemical equilibrium (i.e., $\Delta\tilde{\mu}Ca = 0$) with the membrane potential, and thus,

according to the Nernst equation:

$$RT \ln \frac{[Ca^{2+}]_m}{[Ca^{2+}]_c} + zF\Delta\psi_m = 0,$$

$$2.3RT \log \frac{[Ca^{2+}]_m}{[Ca^{2+}]_c} = -zF\Delta\psi_m.$$

Given a membrane potential of 180 mV, negative inside, and $z = 2$ for Ca^{2+}, the $\frac{[Ca^{2+}]_m}{[Ca^{2+}]_c}$ at equilibrium ($\Delta\tilde{\mu}Ca = 0$) should thus be:

$$\frac{[Ca^{2+}]_m}{[Ca^{2+}]_c} = 10^6.$$

That is, for a cytoplasmic Ca^{2+} of 10^{-7} M, the matrix $[Ca^{2+}]$ at equilibrium should be 10^{-1} M, a value incompatible with mitochondrial integrity and/or with any measurement of mitochondrial Ca^{2+} available at the time (14). However, the puzzle was rapidly solved with the discovery of the Ca^{2+} efflux pathways.

The key experiment that led to the identification of the efflux pathways was very simple: Isolated mitochondria were allowed to accumulate Ca^{2+} until a steady state was obtained, then the classical inhibitor of the MCU, RR, was added. Addition of RR caused Ca^{2+} efflux from mitochondria until practically all Ca^{2+} was released into the medium (15, 16). Given that RR did not modify $\Delta\psi$, this observation was compatible only with the existence of an efflux mechanism independent of membrane potential, and in liver mitochondria this efflux was identified in a $2H^+/Ca^{2+}$ antiporter (14). In mitochondria taken from excitable tissues (heart and brain in particular), it was then demonstrated that the efflux is strongly stimulated by the presence of Na^+ (or Li^+) in the medium (17). It was thus concluded that Ca^{2+} efflux is catalyzed by an Na^+/Ca^{2+} antiporter, most likely electrogenic (i.e., similar to its analog in the plasma membrane with 3 or 4 Na^+ transported in exchange to 1 Ca^{2+}). The thermodynamic predicts that, for a $2H^+/Ca^{2+}$ antiporter the equilibrium should be:

$$\log \frac{[Ca^{2+}]_m}{[Ca^{2+}]_c} = 2 \log \frac{[H^+]_c}{[H^+]_m}.$$

That is, for a ΔpH of 1 unit (the inside more alkaline), the equilibrium condition should occur when the Ca^{2+} concentration in the extramitochondrial medium is 100-fold higher than in the matrix. Under physiological conditions, therefore, this antiporter should transport Ca^{2+} from the matrix (alkaline pH and higher Ca^{2+}) into the cytoplasm. The equilibrium conditions are even more strongly shifted toward Ca^{2+} efflux for a $3(4)Na^+/Ca^{2+}$ antiporter. In this case, given that the exchanger transports one (or two) net positive charge for each Ca^{2+} moved across the membrane, the efflux of Ca^{2+} is favored by the negative membrane potential across the inner membrane.

On the basis of these simple thermodynamic considerations (and experimental data), the mitochondrial Ca^{2+} set point model was proposed: A steady state mitochondrial Ca^{2+} level is reached when the rate of Ca^{2+} accumulation via MCU is equal to the rate of Ca^{2+} efflux via the antiporters (14, 18). Any increase in the extramitochondrial Ca^{2+} level above the set point results in a net Ca^{2+} accumulation until the rate of efflux becomes identical to that of influx. This model predicts that, inevitably, in healthy, resting cells, a continuous futile cycle of Ca^{2+} across the inner mitochondrial membrane occurs, with consequent energy dissipation. However, this energy expenditure is reduced to a minimum, owing to the low Ca^{2+} affinity of the MCU under physiological conditions. Indeed, at approximately 0.1 μM extramitochondrial Ca^{2+} (i.e., that of a resting cell), the rate of Ca^{2+} influx is two orders of magnitude or more slower than the V_{max} of the MCU. This model leads to a series of other predictions, some of which have been experimentally verified: (*a*) any

increase in cytosolic Ca^{2+} (without the need of invoking the generation of Ca^{2+} hot spots; see the section on Mitochondrial Ca^{2+} Uptake/Release and Cellular Functions) causes an increase in matrix Ca^{2+}; (*b*) any change in the affinity of the MCU for Ca^{2+} (e.g., by changing the expression of MCU regulators such as MICU1 or MICU2; see the section on Molecular Components of the MCU Complex) must result in an increase (or a decrease) of Ca^{2+} in the matrix without long-term changes in the cytosolic level of the cation. These simple and well-established thermodynamic considerations are often neglected in the recent literature. For example, it has been argued that at low extramitochondrial Ca^{2+} concentrations, Ca^{2+} uptake into the organelles is catalyzed not by MCU but by $Ca^{2+}/1H^+$ or $Ca^{2+}/2H^+$ antiporters. In the first case, the equilibrium should be:

$$\Delta\tilde{\mu}\mathrm{Ca} - \Delta\tilde{\mu}\mathrm{H} = 0.$$

According to the Nernst equation, the calculation is:

$$\Delta\mu\mathrm{Ca} + 2F\Delta\psi - \Delta\mu H - \Delta\psi = 0,$$

$$RT \ln \frac{[\mathrm{Ca}^{2+}]_m}{[\mathrm{Ca}^{2+}]_c} - RT \ln \frac{[\mathrm{H}^+]_m}{[\mathrm{H}^+]_c} + F\Delta\psi = 0,$$

$$\log \frac{[\mathrm{Ca}^{2+}]_m}{[\mathrm{Ca}^{2+}]_c} = \log \frac{[\mathrm{H}^+]_m}{[\mathrm{H}^+]_c} - \frac{\Delta\psi F}{2.3RT}.$$

In mitochondria with 180 mV of membrane potential and 1 pH unit more alkaline in the matrix, the result would be:

$$\log \frac{[\mathrm{Ca}^{2+}]_m}{[\mathrm{Ca}^{2+}]_c} = -\frac{180F}{2.3RT} - 1,$$

$$\frac{[\mathrm{Ca}^{2+}]_m}{[\mathrm{Ca}^{2+}]_c} = 10^2.$$

Conversely, in the case of the $Ca^{2+}/2H^+$ antiporter, the equilibrium would be:

$$\Delta\tilde{\mu}\mathrm{Ca} - 2\Delta\tilde{\mu}\mathrm{H} = 0.$$

According to the Nernst equation, the calculation is:

$$\Delta\mu\mathrm{Ca} + 2F\Delta\psi - 2\Delta\mu\mathrm{H} - 2F\Delta\psi = 0,$$

$$RT \ln \frac{[\mathrm{Ca}^{2+}]_m}{[\mathrm{Ca}^{2+}]_c} - 2RT \ln \frac{[\mathrm{H}^+]_m}{[\mathrm{H}^+]_c} = 0,$$

$$\log \frac{[\mathrm{Ca}^{2+}]_m}{[\mathrm{Ca}^{2+}]_c} = 2 \log \frac{[\mathrm{H}^+]_m}{[\mathrm{H}^+]_c}.$$

Again, considering 1 pH unit more alkaline in the matrix, the result would be:

$$\frac{[\mathrm{Ca}^{2+}]_m}{[\mathrm{Ca}^{2+}]_c} = 10^{-2}.$$

Thus, a $Ca^{2+}/2H^+$ antiporter under physiological conditions catalyzes only Ca^{2+} efflux from the matrix, whereas a Ca^{2+}/H^+ exchanger would be compatible with Ca^{2+} accumulation in coupled mitochondria under conditions of very high Ca^{2+} in the cytoplasm and low, or no, ΔpH (for details see Reference 19).

Similarly, although the generation of Ca^{2+} hot spots on the surface of mitochondria has been proposed to explain the large and fast mitochondrial accumulation in living cells during cell stimulation (20–25), small and slow increases in the organelle matrix are predicted to occur even

in the absence of such hot spots, solely on the basis of the above-mentioned thermodynamic and kinetic considerations (24).

HOW TO MEASURE MITOCHONDRIAL Ca^{2+} UPTAKE AND RELEASE

For more than 20 years, the only method to measure the capacity of the organelles to take up or release Ca^{2+} was based on the measurement of the changes in Ca^{2+} concentration of the medium in which isolated mitochondria- or digitonin-permeabilized cells were incubated (26, 27). This methodology, technically very simple, is still commonly employed to investigate the so-called mitochondrial Ca^{2+} retention capacity (for review, see 28). The most commonly used methodologies for studying Ca^{2+} homeostasis by mitochondria are based on the use of probes [either fluorescent chemical dyes or genetically encoded indicators (GECI)] that are selectively trapped in the mitochondrial matrix (for example, see 29). A major drawback of chemical dyes is that part of the acetoxymethylester (AM) dye is also normally hydrolyzed by cytoplasmic enzymes, and the accumulation in the mitochondria is highly variable, depending on the cell type and the protocol used. Other problems, often overlooked by investigators, are the potential side effects of the matrix environment on the Ca^{2+} affinity of the trapped dye, the toxicity of the AM hydrolysis products (acetate and formaldehyde), and the increased Ca^{2+} buffering capacity in the matrix.

For information about GECI, aequorins, or green fluorescent protein (GFP)-based Ca^{2+} indicators, the reader is referred to recent reviews (30–41). The advantages of genetically encoded probes include the following: (*a*) selectivity of localization, (*b*) reduced toxicity, (*c*) availability of a large spectrum of Ca^{2+} affinities (K_d for Ca^{2+}, from nanomolar to several hundred micromolar), and (*d*) availability of probes with different chemiluminescent or fluorescent spectral properties. Drawbacks include the necessity of genetic manipulation of the cells, the effects of matrix environment on their Ca^{2+} affinity, and the sensitivity (of some probes) to pH (42–44).

All the probes described above were designed to monitor the dynamic changes of Ca^{2+} within the mitochondrial matrix. A few other probes can monitor the Ca^{2+} levels either on the intermembrane space (IMS) or on the cytosolic surface of the outer mitochondrial membrane (OMM). As to the IMS, this tool allows confirmation of the hypothesis that the Ca^{2+} level in the IMS rapidly equilibrates with that in the cytosol (20). Three types of sensors are available for measuring the changes in Ca^{2+} on the cytosolic surface of the OMM: One is based on aequorin (45) and two are GFP-based GECI (46, 47). The latter two probes have enabled direct monitoring of the generation of hot spots on the surface of mitochondria during Ca^{2+} mobilization from the endoplasmic reticulum (ER) (46, 47). These tools and protocols now permit investigation of mitochondrial Ca^{2+} handling in isolated organelles, living or permeabilized cells, tissues, and intact animal models. The choice of the best probe or protocol depends on the question addressed, the instruments available, and often, on a biased attitude of the investigator.

MOLECULAR COMPONENTS OF THE MCU COMPLEX

For the sake of clarity, in this section we describe separately the MCU complex components (and their functions) to distinguish between the core components of the membrane pore and the MCU-associated regulators (see **Figure 1**).

Core Components of the Membrane Pore

To date, three different membrane proteins have been shown to be part of the Ca^{2+} permeant pore, namely MCU, MCUb, and EMRE (for recent review, see 48–52). From the phylogenetic

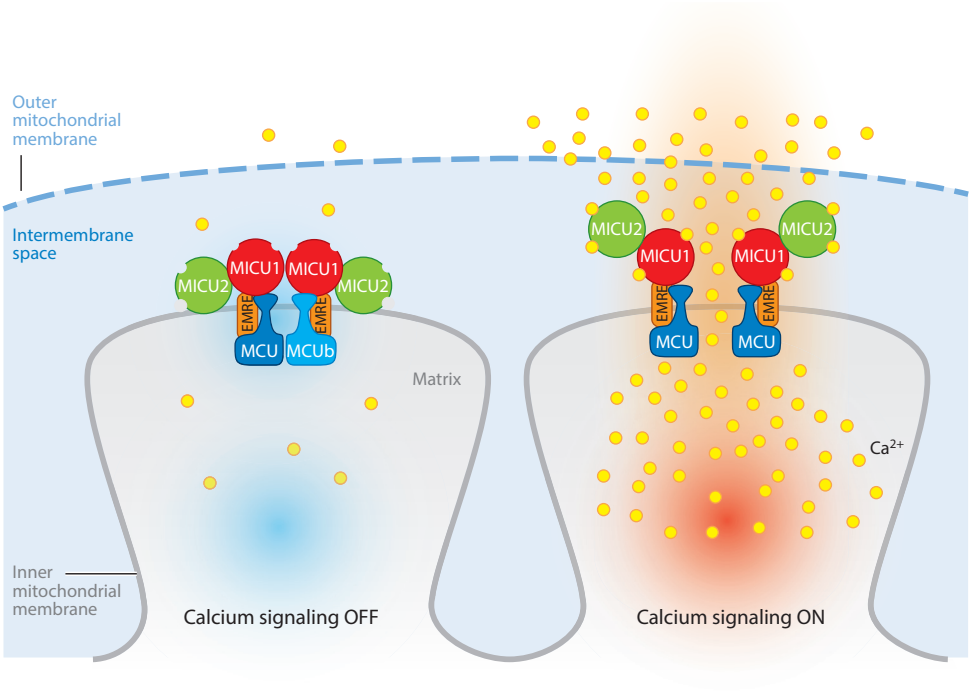

Figure 1

Schematic representation of the Mitochondrial Calcium Uniporter (MCU) complex. (*Left*) In resting condition, mitochondrial Ca^{2+} (*yellow*) uptake is controlled by a multiprotein complex that can consist of MCU and MCUb (the channel-forming subunits) together with EMRE, MICU1, and MICU2 (the other putative interactors are omitted for the sake of clarity). In particular, MICU1/MICU2 heterodimers act as MCU gatekeepers and prevent vicious calcium cycles and energy sinks. (*Right*) Activation of cellular Ca^{2+} signaling results in an increase of $[Ca^{2+}]_{cyt}$ that induces a conformational change in MICU1/MICU2 heterodimers and triggers the MCU opening. Abbreviations: EMRE, Essential MCU Regulator; MICU, MItochondrial Calcium Uptake. Reprinted with modifications from Reference 178 with permission from Elsevier.

point of view, MCU (together with MICU1) appears to be the only required component, because it is present in all organisms with an experimentally validated mitochondrial Ca^{2+} uptake. EMRE (8) is not present in fungi, protozoa, and plants, whereas MCUb (5) is restricted to most vertebrates (for reviews, see 48–52).

MCU. The MCU gene is well conserved in almost every plant and metazoa, but it is absent in yeasts and some protozoan and fungal lineages (53, 54). It encodes a 40-kDa protein (running at 35 kDa owing to the cleavage of the mitochondrial targeting signal) with two coiled-coil (CC) domains and two transmembrane domains separated by a short loop enriched in acidic residues (EYSWDIMEP). This loop is exposed to the intermembrane space, whereas the largest portion of MCU is located within the organelle matrix (55). Residues E256, D260, and E263 are critical for Ca^{2+} channeling function, because their replacement with both uncharged and positively charged amino acids results in the loss of Ca^{2+} permeation (3, 5). Moreover, substitution of serine at position 258 leads to decreased RR sensitivity (4, 56). MCU can clearly form oligomers, although the precise stoichiometry of the quaternary structure of the functional channel is still unknown.

The available evidence unambiguously supports the conclusion that MCU is the pore-forming subunit of the MCU complex. This conclusion is based on the classical approaches that have led to the identification of other channel-forming subunits: (*a*) silencing of MCU invariably leads to inhibition of mitochondrial Ca^{2+} transients in live cells (3, 4, 56), independent of the model system employed [neonatal rat cardiomyocytes (57), pancreatic β-cells (58, 59), neurons (60), breast epithelial cells (61, 62)]; (*b*) reconstitution lipid bilayers with either purified or in vitro synthetized MCU results in the development of a Ca^{2+}-specific current (3); (*c*) channels with point mutations (in the predicted channel regions), when reconstituted in lipid bilayers, present anomalous Ca^{2+} currents, and the mutant channel, when transfected, affects mitochondrial Ca^{2+} uptake in living cells (3, 5); and (*d*) analysis of isolated organelle from different tissues of MCU knockout mice revealed the lack of any mitochondrial Ca^{2+} uptake (63). As mentioned above, data in planar lipid bilayers indicate that purified MCU is sufficient per se to form a Ca^{2+} channel with most of the properties of the MCU (3). The observation that in this condition the elicited current is similar but not identical to that recorded in patch clamp experiments of isolated mitoplasts (64) can be likely accounted for by the different lipid environment, the lack of posttranslational modifications, and the absence of endogenous regulators. However, MCU reconstituted in planar lipid bilayers and MCU measured in situ share similar conductance and the same pharmacological profile (i.e., inhibition by RR and Gd^{3+}) (3, 64, 65). Also, expression of the closely related endogenous dominant-negative MCU isoform, MCUb, does not elicit any current in Ca^{2+}-containing media and progressively inhibits the MCU-mediated Ca^{2+} current in a dose-dependent manner (5), and MCU open probability is modified by its known modulators, MICU1 and MICU2 (33). Last but not least, the notion that MCU is per se sufficient to form a functional channel is supported by the evidence that overexpression of MCU alone is able to increase both organelle Ca^{2+} uptake in intact cells (2-fold) (3) as well as evidence of Ca^{2+} currents recorded through patch clamp of mitoplasts (3.4-fold) (56). However, in intact cells, assembly of a functional complex requires the presence of EMRE, a component likely involved in complex assembly (see the section on EMRE below).

MCUb. Genomic analysis has revealed a gene closely related to MCU, originally reported as CCDC109B and now known as MCUb. The encoded protein shares 50% similarity to MCU, possesses two CC domains and two transmembrane domains separated by a short loop that slightly differs from MCU (VYSWDIMEP), and has the same membrane topology. MCUb is conserved in most vertebrates but absent in many other organisms in which MCU is present. Within the MCU complex, MCU forms oligomers for self-oligomerization or hetero-oligomerization with MCUb (5, 56). More importantly, this isoform has a crucial amino acid substitution in the loop region (E256V) that is predicted to have an impact on the channel properties by removing a critical negative charge. Indeed, molecular dynamics simulations estimated that Ca^{2+} permeation through MCUb is greatly impaired. This was confirmed in both HeLa cells and planar lipid bilayers (5). On the one hand, MCUb overexpression decreased agonist-evoked mitochondrial Ca^{2+} transient in living cells (5). On the other hand, recombinant MCUb inserted in artificial membranes showed no current when Ca^{2+} was used as a permeating ion (5). In addition, concomitant expression of MCU and MCUb greatly decreased the open probability in the planar lipid bilayer, even when MCUb was present in low amounts; this finding supported the notion that the presence of MCUb within the oligomer (also at a ratio with MCU $<$ 1:1) can significantly inhibit the flux of Ca^{2+} in the channel (5). Finally, silencing of MCUb in HeLa cells caused a significant increase of mitochondrial Ca^{2+} uptake, thus underlying the inhibitory role that MCUb exerts on channel activity (5).

As to the potential physiological meaning of MCUb, we note that the ratio between MCU/MCUb varies greatly among different tissues. This variation is in line with the recent

demonstration that the overall activity of the MCU complex is highly variable among tissues (66). Most importantly, a nice correlation between the MCU/MCUb expression ratio and the recorded mitochondrial Ca^{2+}-selective currents is apparent. As an example, the MCU:MCUb expression ratio is low (3:1) in heart and high (40:1) in skeletal muscle (5). Thus, it is possible that this ratio sets the overall mitochondrial Ca^{2+} uptake capacity of different tissues.

In conclusion, MCUb appears to mediate a novel regulatory mechanism of the channel function: By inclusion of different quantities of the dominant-negative subunit in the multimeric channel, different tissues can set the maximal mitochondrial Ca^{2+} currents (5), and the MCUb isoform is inserted into the channel oligomer and alters its ion permeation properties. For example, in some potassium channels, it is known that dominant-negative and wild-type subunits can assemble, thus forming tetrameric structures and introducing large positively charged residues into the pore that alter the positioning of carbonyl oxygens and disrupt the permeation pathway. This effect underlies several disease states (e.g., Birk Barel mental retardation dysmorphism syndrome) associated with two-pore potassium channels (67). Another example is the plant silent Shaker-type subunit AtKC1, which coassembles with KAT1 and AKT2, largely inhibiting their normal activity (68). We can speculate that the modulation of the MCU-dependent Ca^{2+}influx via the controlled expression of a dominant-negative subunit represents a rapid and efficient way to modulate at will the efficacy of mitochondrial Ca^{2+} accumulation—faster and more effective than inhibition of MCU synthesis [considering also the long half-life of MCU (69)].

EMRE. As discussed above, MCU is sufficient per se to form a Ca^{2+} channel in the planar lipid bilayer, but what happens in vivo is unclear. EMRE is a broadly expressed 10-kDa protein that spans the inner mitochondrial membrane and possesses a highly conserve C terminus rich in aspartate residues; however, its membrane topology is still unclear (8). Mootha and colleagues (8) propose that this protein is required for Ca^{2+} channel activity and to keep the MICU1/MICU2 dimer attached to the MCU complex. In contrast with its essential role, EMRE homologs are not present in plants or fungi, in which MCU and MICU1 are highly conserved (8). Downregulation or knockout of EMRE totally abolishes mitochondrial Ca^{2+} uptake, even when MCU is overexpressed. It must be noted that the putative role of EMRE in mediating the binding between MCU and MICU1 is in contrast with the clear positive effect of MICU1 on MCU in the planar lipid bilayer, where no other components are present (70). However, in the absence of EMRE the MCU complex becomes smaller, as revealed by Blue Native polyacrylamide gel electrophoresis (8). This observation suggests that EMRE could be an essential protein for efficient assembly of the MCU complex. In line with this view, it was recently shown that, in a heterologous system such as yeast, EMRE is required for the formation of a functional channel with the mammalian MCU but not with MCU derived from fungi (71).

MCU-Associated Regulators

The road to discovery of MCU actually started with the identification of one of its key regulators, MICU1. Other proteins that exert a regulatory role on the channel that have been identified so far include the whole MICU family, MCUR1, and SLC25A23.

The MICU family. One key feature of the mitochondrial Ca^{2+} uptake machinery is the sigmoidal response to extra mitochondrial (i.e., cytosolic) [Ca^{2+}], with a very low rate at resting cytoplasmic [Ca^{2+}] levels (thus preventing mitochondrial Ca^{2+} overload and ion vicious cycling) and a very large Ca^{2+} carrying capacity at higher [Ca^{2+}] levels, that ensures prompt responses to cell stimulation. This property could be in principle due to the channel per se or to a set of different regulators

that keep the channel almost close at resting condition and activate it at high Ca^{2+} concentration. However, MCU exposes in the intermembrane space only a small loop, with the vast majority of the protein residing within the matrix. Hence, this sigmoidicity is unlikely to be a property of the channel per se but is likely conferred to MCU by different regulators located in the intermembrane space. In the last 4 years different groups showed that MICU's family has a role in the sigmoidal response of MCU to external Ca^{2+} levels. This hypothesis appears plausible especially because these proteins have two conserved EF hand domains that presumably modulate Ca^{2+} sensitivity.

MICU1 was the first member of the MICU family discovered in 2010 by Mootha's group (2). This protein was proposed to be necessary for mitochondrial Ca^{2+} uptake, but it is unlikely to be the channel per se as it possesses only one (heavily questioned) predicted transmembrane domain. The actual localization of this protein is still controversial, but both the recent proteomic data and the resolution of its function strongly suggest that MICU1, as well as the other members of the family, is a soluble protein of the intermembrane space (55, 72). However, another group has reported that MICU1 acts as an MCU gatekeeper by keeping MCU almost close when the extra mitochondrial Ca^{2+} concentration is low. Indeed, Madesh and coworkers (73) showed that mitochondria from MICU1-silenced cells are constitutively overloaded with Ca^{2+}, thus uncovering the gatekeeping role of MICU1. Soon after, Hajnóczky and coworkers (74) added another level of complexity to the function of MICU1. Although they confirmed the MICU1 acts as an MCU gatekeeper, they also elegantly showed that, in the absence of MICU1, mitochondrial Ca^{2+} uptake is less efficient. According to their model, MICU1 not only controls the threshold of MCU opening but also cooperates in activating the channel open state at high Ca^{2+} concentration. These data suggest that MICU1 is the key regulator of the sigmoidal response of organelle Ca^{2+} uptake to the extramitochondrial $[Ca^{2+}]$.

Indeed, similar to MCU, MICU1 has two different isoforms: MICU2 (formerly known as EFHA1) (for review, see 48 and 49) and MICU3 (formerly known as EFHA2) (6). These isoforms are likely to be located in the intermembrane space as well, and their specific role is still debated. We showed (70) that MICU2 forms an obligate heterodimer with MICU1 and that MICU2 has a genuine gatekeeping function at low Ca^{2+} levels, both in living cells and in electrophysiological recordings carried out in planar lipid bilayer. Its overexpression indeed is able to decrease the agonist-evoked stimulus in HeLa cells and to decrease the open probability of the reconstituted MCU channel in the planar lipid bilayer with no effect at high Ca^{2+} concentration (70). As already reported (6), we confirmed that the stability of MICU2 is dependent on the presence of MICU1. Indeed, MICU1-silenced cells also have a drastic reduction in MICU2 protein levels, despite no effect on MICU2 mRNA amount. Hence, we suggested that the reported loss of MCU gatekeeping in the absence of MICU1 is likely due to the concomitant loss of MICU2 (70).

MICU1 overexpression increases agonist-evoked mitochondrial Ca^{2+} transients and the MCU open probability in planar lipid bilayer (70). In summary, at low $[Ca^{2+}]$ the prevailing inhibitory effect of MICU2 ensures minimal Ca^{2+} accumulation, thus preventing the deleterious effects of massive Ca^{2+} cycling or Ca^{2+} overload. As soon as the extramitochondrial $[Ca^{2+}]$ increases, Ca^{2+}-dependent MICU2 inhibition and MICU1 activation guarantees the prompt initiation of rapid mitochondrial Ca^{2+} accumulation (**Figure 1**), thus stimulating aerobic metabolism and increasing adenosine triphosphate (ATP) production. Finally, MICU3 appears to be predominantly expressed in the central nervous system (CNS), and accordingly, it most likely exerts a tissue-specific yet unknown role.

MCUR1. To identify other components of the Ca^{2+} uptake machinery, Madesh and colleagues (7) performed a direct human RNAi screen of 45 mitochondrial membrane proteins in HEK293T

cells predicted to be part of the inner mitochondrial membrane. They identified two proteins with a modest (SLC25A23) and a strong (CCDC90A) effect on mitochondrial Ca^{2+} uptake. The latter is now known as MCUR1, a 40-kDa protein of the inner mitochondrial membrane with one predicted transmembrane domain and one CC region and with the N terminus facing the intermembrane space. Madesh and coworkers (7) demonstrated that silencing of MCUR1 largely inhibited agonist-induced mitochondrial Ca^{2+} uptake but also caused a striking decrease of basal mitochondrial matrix [Ca^{2+}]. Although both MCUR1 and MICU1 could coimmunoprecipitate with MCU, MCUR1 did not interact with MICU1, thus indicating the existence of at least two qualitatively different MCU-containing complexes (7). In addition, in proteomic studies MCU, MCUb, EMRE, MICU1, and MICU2, but not MCUR1, were resolved within the same mitochondrial complex (8). This finding implies that at least a pool of the MCU can work without a gatekeeper, thus allowing Ca^{2+} uptake into the matrix even at resting cytosolic [Ca^{2+}]. Interestingly, MCUR1 also appears to have an isoform conserved in most vertebrates, named CCDC90B, whose function is still uncharacterized. Recently, Shoubridge and colleagues (75) demonstrated that the silencing of MCUR1 causes a drop of mitochondrial $\Delta\psi$ that correlates with a decrease of complex IV assembly and activity. Considering that mitochondrial membrane potential is the main driving force guiding the entrance of all cations inside organelle matrices, this finding could explain the decrease of mitochondrial [Ca^{2+}] and most of the data reported by Mallilankaraman and colleagues (7), in which differences in $\Delta\Psi_m$ were not detected. In addition, MCUR1 is supposed to have a homolog in *Saccharomyces cerevisiae* (Fmp32), an organism lacking mitochondrial Ca^{2+} uptake. It should be stressed, however, that Fmp32 and MCUR1 share only partial similarities and their functional homology has yet to be conclusively demonstrated. However, recent electrophysiological measurements of MCU-dependent Ca^{2+} currents in mitoplasts (J.K. Foskett, personal communication) suggests that MCUR1 deletion results in inhibition of the channel current also under voltage clamp conditions, i.e., independently of any effect of the protein on the respiratory chain or $\Delta\Psi$ generated by the mitochondria.

SLC25A23. The other protein identified by Madesh and coworkers (9) is SLC25A23 (also known as SCaMC-3). This protein belongs to a family of solute carriers that transport Mg-ATP/Pi across the inner mitochondrial membrane (76). It apparently participates in mitochondrial Ca^{2+} uptake, possibly through an interaction with MCU and MICU1. The effect of this EF hand–containing mitochondrial protein is likely to depend on the local [Ca^{2+}]. Indeed, expression of a mutated form of SLC25A23 (Ca^{2+} binding sites removed) results in a dominant-negative effect, i.e., it reduced mitochondrial Ca^{2+} transients (9). It needs stressing that downregulation of SLC25A23 impairs mitochondrial respiration at least at high cellular workloads (77). Thus, by acting as an Mg-ATP/Pi carrier, SLC25A23 could impact the mitochondrial membrane potential and matrix Ca^{2+} buffering capacity and consequently affect mitochondrial Ca^{2+} uptake only indirectly.

STRUCTURE OF THE MCU COMPLEX

Although conclusive information on the 3D structure of the MCU complex is still lacking, some very recent results have shed some light on this issue. In silico analysis of the 3D model of the membrane-spanning region predicts a tetrameric structure, compatible with Ca^{2+} permeation and a pore topology (5). It is interesting to note that more than 30 years ago, purely based on kinetic and thermodynamic consideration, the Ca^{2+} carrier (as it was named at that time) was hypothesized to be a tetramer (78). This model envisions the presence of a narrow-selectivity filter constituted by the short loop connecting the two transmembrane domains (EYSWDIMEP), where the conserved acidic residues are able to locate a single Ca^{2+} above the channel pore region

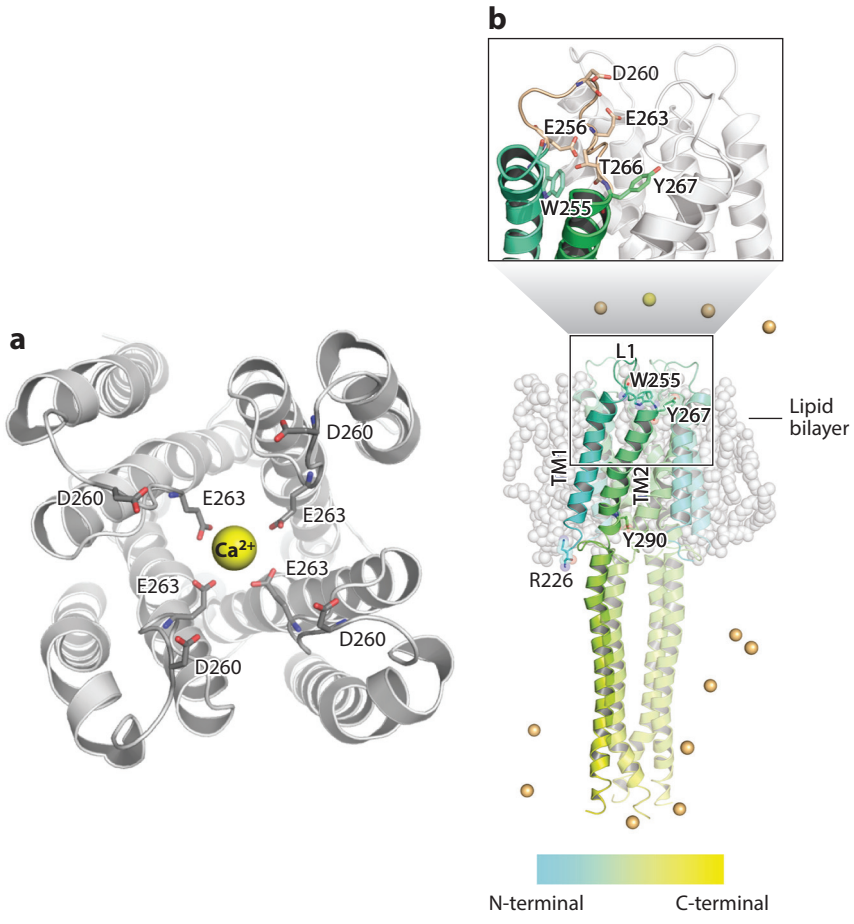

Figure 2

By combining structural bioinformatics and molecular dynamics (MD), Raffaello et al. (5) proposed a hypothetical model on the quaternary structure of Mitochondrial Calcium Uniporter (MCU) channel topology. This model of the MCU pore domain linked to its C terminus (residues 224–334) includes four identical subunits consisting of two helical membrane-spanning domains that are connected by a short loop containing a DIME motif. (*a*) Top view of the pore region of the predicted MCU tetramer. (*b*) Representation of the MCU model inserted into a lipid bilayer. An enhancement of the MCU channel region facing the intermembrane space is shown. For details see Raffaello et al. (5). Reprinted with modification from Reference 5.

(**Figure 2**). This feature is connected to a progressively widening chamber (3–5 Å) that extends underneath the channel mouth along all the MCU membrane–spanning domains (5).

The predicted structure is surprisingly similar to other Ca^{2+}-permeant channels, despite an overall very low sequence homology (79). A tetrameric structure is also supported by native gel electrophoresis of the in vitro purified MCU, which revealed an approximately 170-kDa band. However, in mammalian cells the MCU-containing complex migrates mainly with larger molecular weights, suggesting the existence of other MCU-endogenous regulators or other stoichiometries (e.g., an octamer or more).

As to X-ray data, crystal structures of MCU and MICU1 fragments are now available. Although in both cases some domains critical for protein function (the pore region and the C terminus for MCU and MICU1, respectively) are lacking from the final crystallographic structure, some preliminary conclusions can be drawn. In MCU, crystals of a fragment corresponding to the N-terminal region (aa 75–185) have been obtained. This soluble portion entirely resides in the mitochondrial matrix and contains a potential phosphorylation consensus site for CaMKII (S92), previously reported to regulate channel activity (80). It is formed by three α-helixes and six β-strands, arranged in a 3D structure that resemble either the ubiquitin-like (Ub) β-grasp fold (β-GF) or the immunoglobulin-like fold (81). From the functional point of view, MCU mutants lacking this domain can insert into the MCU complex but can only partially rescue Ca^{2+} channel activity when expressed in MCU-silenced cells.

More information is available for the crystal structure of MICU1, which has been crystallized in both Ca^{2+}-free and Ca^{2+}-bound forms (82). Unfortunately, the incorporation of the C terminus of MICU1, a region containing a cysteine residue critical for protein dimerization (70), leads to protein aggregation that prevents crystallization. However, the structure of a significant portion of the protein (aa 97–476) has been obtained with a resolution of 3.2 Å. Overall, MICU1 consists of four different domains: the N-domain, the N-lobe, the C-lobe, and the C-helix. The N-domain (residues 103–177) consists of three α-helices and three antiparallel β-strands. The N-lobe (residues 183–318) and the C-lobe (residues 319–445) contain the EF hand domains and are composed of six and seven α-helices, respectively. Other two helix-loop-helix structural units (pseudo-EF2 and pseudo-EF3) are present in these regions but do not bind Ca^{2+}. The C-helix (residues 446–476), the last domain, is a long helix that mediates interaction with MCU. According to the proposed model, when Ca^{2+} is absent MICU1 forms hexamers (organized in a trimer of MICU1 dimers) that bind and inhibit MCU. Binding of Ca^{2+} to the two EF hands triggers a large conformational change with the disassembly of the MICU1 hexamer into multiple oligomers (82). Interestingly, the affinity of MICU1 for Ca^{2+} is approximately 15–20 μM, a value compatible with the $[Ca^{2+}]$ that is reached in ER/mitochondria microdomains (46, 47).

Overall, these studies provide an initial framework for understanding the structure/function relationship of the MCU complex, hopefully enabling the rational design of compounds that selectively target mitochondrial Ca^{2+} uptake.

REGULATION OF MCU COMPLEX EXPRESSION

It is widely accepted that the versatility and universality of Ca^{2+} as intracellular messenger is guaranteed, at least in part, by the fine compartmentalization of its changes in different parts of the cytoplasm and within the organelle lumen (24). In this context, mitochondrial Ca^{2+} homeostasis plays a central role by regulating both specific organelle functions (e.g., regulation of ATP production and release of caspase cofactors) and global cellular events (e.g., shaping of cytoplasmic Ca^{2+} waves or oscillations and local Ca^{2+} microdomains) (83). In general, this versatility is also guaranteed by a cell type–specific Ca^{2+} signaling toolkit that controls specific cellular functions. Accordingly, this specificity also applies to the composition of the MCU complex, and indeed, the expression of the different components of the MCU complex can vary significantly among tissues. Unfortunately, at the moment the availability of good and reliable antibodies is limited; thus, a rigorous assessment of real protein levels is still lacking. Still, comparison of mRNA can provide interesting insight. As an example, **Figure 3** shows the expression of the bona fide MCU complex components in human tissues, as reported in a publicly available RNA sequencing database (**http://www.ebi.ac.uk/gxa/experiments/E-MTAB-513**).

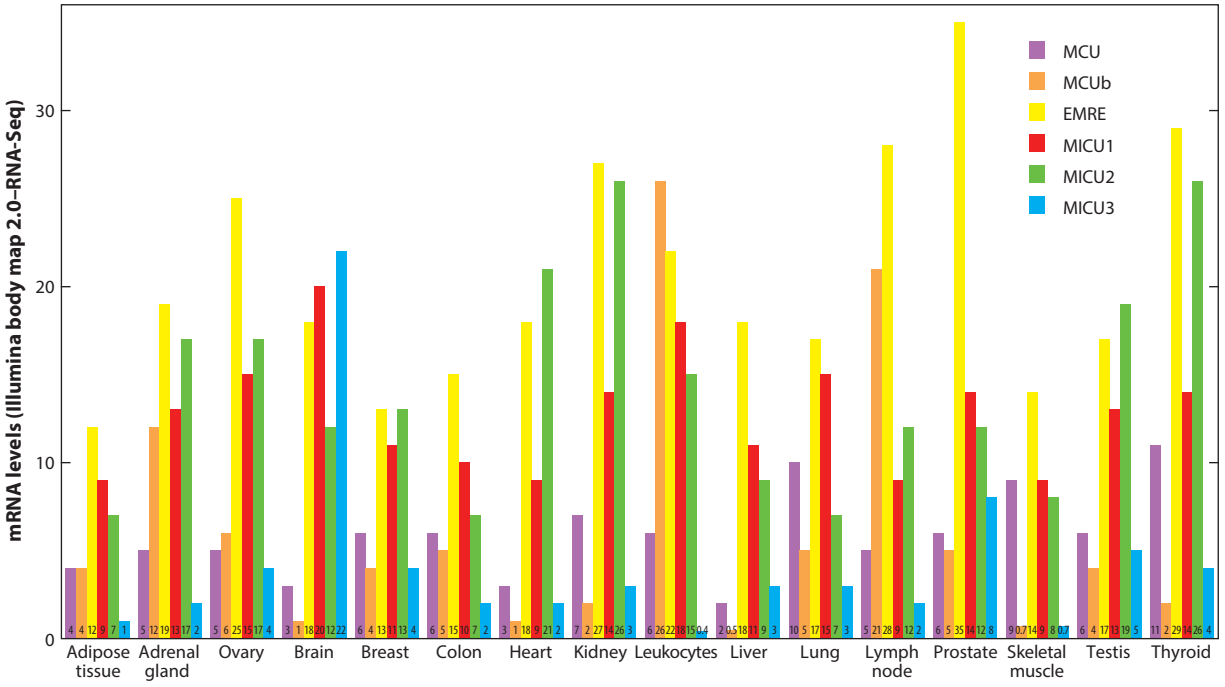

Figure 3

Tissue expression of Mitochondrial Calcium Uniporter (MCU) complex components. mRNA levels, quantified through RNA sequencing (RNA-Seq), of MCU, MCUb, EMRE, MICU1, MICU2, and MICU3 in the indicated human tissues. Data were obtained from Illumina's Human BodyMap 2.0 project and downloaded from **http://www.ebi.ac.uk/gxa/experiments/E-MTAB-513**. Abbreviations: EMRE, Essential MCU Regulator; MICU, MItochondrial Calcium Uptake.

According to these data, which recapitulate most of the findings already reported in mouse tissues (3–6, 8), MCU, EMRE, MICU1, and MICU2 are expressed in all tissues; thus, these data suggest that these components are part of the basic machinery and are required, at least in mammals, for the proper regulation of the MCU complex in vivo. On the contrary, MCUb and especially MICU3 appear to be absent in some organs, pointing out a tissue-specific function of these modulators.

MCU is constitutively expressed in all mammalian tissues at comparable levels, with highest abundance in skeletal muscle, lung, and thyroid tissues and lowest in the heart and liver. MCUb is generally expressed at very low levels in most mouse tissues, although the expression levels in humans generally appear to be significantly higher. MCUb is surprisingly highly expressed in leukocytes, but expression differences among the different subpopulations are not yet available. However, the abundant MCUb mRNA reported in lymph node tissues indicates that at least T cells express high MCUb transcripts. EMRE is present in all tissues at much higher levels compared with MCU and MCUb, and its expression poorly correlates with the other membrane components of the complex. The members of the MICU family have generally higher mRNA levels, as expected when dealing with soluble proteins with a faster turnover. Indeed, in several cell lines, the half-life is approximately 60 hours for MCU and less than 20 hours for MICU1 and MICU2 (69; D. De Stefani & R. Rizzuto, unpublished results), suggesting that the regulators, rather than the channel itself, could represent potential targets for the control of mitochondrial Ca^{2+} uptake in the fast

and medium timescales. Despite differences in the absolute levels, the expression of MICU1 and MCU nicely correlates among tissues, with the highest MICU1/MCU ratio appearing in brain and liver tissues and the lowest in skeletal muscle. Interestingly, MICU1 and MCU genes are located on the same chromosome, one next to the other, in opposite orientation, and this peculiar location is conserved in all vertebrates. This not only clearly indicates that the two genes evolved together (53) but also suggests that there could be some common transcription factors coregulating their expression. At the mRNA level, the mean expression ratio between MICU1 and MICU2 is approximately 1 (lowest in heart and kidney, higher in brain and prostate), indicating that these two genes are similarly expressed among all tissues and are both required for proper MCU gating. Conversely, the third member of the MICU family, MICU3, is generally marginally expressed in most of the tissues (at least when compared with MICU1 and MICU2), except for brain tissues. Thus, as for MCUb, MICU3 is probably a neuron-specific MCU modulator, but its genuine function is still unknown.

Despite the constitutive steady-state expression levels, much less is known about the regulation of MCU complex transcripts. Although a detailed investigation of the promoter region of MCU (and of the other proteins in the complex) is not yet available, emerging evidence indicates that MCU levels can be modulated by a number of transcription factors and miRNA. In particular, phosphorylated cAMP responsive element binding protein (pCREB) has been recently shown to bind to the MCU promoter in lymphocytes, which leads to increased channel expression (84). MCUb expression was also enhanced in the same experimental setting, but it occurred independently of CREB activation [nuclear factor of activated T cell (NFAT) has been proposed, but not demonstrated, to be involved]. Interestingly, CREB activity is sustained by the activation of cytoplasmic [Ca^{2+}] (and cAMP) (85), thus suggesting the existence of a regulatory circuit where cytoplasmic and mitochondrial Ca^{2+} homeostasis are linked together (84). However, in other cell types an opposite mechanism has been described. In neurons, MCU expression is repressed by synaptic activity via a mechanism mediated by nuclear Ca^{2+} signals, CaM kinase, and the transcription factor Npas4 (60). This apparent discrepancy could be explained by the pleiotropic role of mitochondrial Ca^{2+} uptake. Mitochondrial Ca^{2+} can increase the efficiency of ATP production; however, prolonged and sustained increases of mitochondrial [Ca^{2+}] lead to activation of apoptosis (86). From the pathophysiological point of view, increased cell death could have only a marginal impact when dealing with easily replaceable cells, e.g., lymphocytes that should also be protected from mitochondrial Ca^{2+} overload thanks to high MCUb expression levels. Conversely, the increased odds of cell death are probably highly risky in the context of a terminally differentiated tissue with almost no possibility of cell replacement.

Finally, MCU transcripts appear to be under the control of miR-25, which can efficiently reduce MCU levels and consequently mitochondrial Ca^{2+} transient (87). Downregulation of miR-25 therefore enhances mitochondrial Ca^{2+} uptake and sensitizes to cell death. Interestingly, miR-25 is overexpressed in colon cancers (88, 89), most likely accounting for the reduced sensitivity to apoptosis of these transformed cells. As to the other components of the MCU complex, their expression dynamics are still unexplored.

OTHER PUTATIVE CANDIDATES FOR CA^{2+} INFLUX REGULATION

A still unresolved question in the field is whether MCU is the unique mediator of mitochondrial Ca^{2+} uptake. Few but consistent works describe at least two matrix Ca^{2+} uptake pathways: (*a*) the first is activated by relatively high extramitochondrial [Ca^{2+}], highly sensitive to RR, and capable of sequestering huge amounts of Ca^{2+}; and (*b*) the second, termed Rapid uptake Mode (RaM), is activated by lower external [Ca^{2+}], inhibited by higher RR concentrations, and enables the rapid

and transient accumulation of short Ca^{2+} pulses (90, 91). This notion was then supported by direct electrophysiological channel recordings in isolated mitoplasts (65). More recently, Graier and coworkers (92) identified three apparently different Ca^{2+} currents, the predominant MCU-mediated (here termed i-MCC) and two others, known as xl-MCC and b-MCC, both characterized by a lower sensitivity to RR. However, it is still unclear whether these different mitochondrial Ca^{2+} uptake modes take place in vivo and in which conditions. One intriguing hypothesis has been proposed by O'Rourke and coworkers (93). By measuring free matrix [Ca^{2+}] in isolated mitochondria, they showed that the different Ca^{2+} uptake modes can activate different intramitochondrial Ca^{2+}-buffering systems, thus leading to significant differences in free matrix [Ca^{2+}] (93).

The remaining (and most important) issue concerns the molecular identity of these different Ca^{2+} uptake systems. Are these modes all MCU dependent or are they mediated by different Ca^{2+} channels (94–98)? Electrophysiological recordings of Ca^{2+} currents from mitoplasts derived from MCU-KO models will help to solve these questions. It must be stressed, however, that Ca^{2+} uptake has never been detected in mitochondria from MCU-KO mice (63). Other proteins, including leucine zipper-, EF-hand-containing transmembrane protein 1 (Letm1) (96), ryanodine receptor (RyR) (94), and uncoupling protein (UCP)2/3 (95), have been proposed to contribute to MCU-independent Ca^{2+} uptake. The authors who first described Letm1 now conclude that it catalyzes an electroneutral $Ca^{2+}/2H^{+}$ exchange (99). A mitochondria-targeted RyR has been described solely in heart mitochondria, and the possibility that it represents a contamination by sarcoplasmic reticulum (SR) has not been excluded beyond doubt. And strong evidence against the primary direct role of UCP2/3 in mitochondrial Ca^{2+} accumulation has been recently provided (97, 98). In conclusion, at present MCU appears to be the only plausible molecular candidate capable of sustaining mitochondrial Ca^{2+} uptake. Recent MCU knockout data have reopened the question of whether alternative pathways to take up Ca^{2+} into the matrix can be activated when the MCU gene is deleted.

THE CA^{2+} EFFLUX MECHANISMS

As briefly discussed in the section on Thermodynamics of Mitochondria Ca^{2+} Transport Mechanisms, the existence of Ca^{2+} efflux mechanisms that oppose the tendency of Ca^{2+} to reach electrochemical equilibrium (via the MCU) with the membrane potential is an essential characteristic of the mitochondrial Ca^{2+} homeostatic machinery. An obvious prediction, yet to be experimentally tested, is that complete knockout of all Ca^{2+} efflux mechanisms would lead to mitochondrial Ca^{2+} overload and cell death. However, this remains an open question, as such knockouts are not available. The genetic approach is prevented by the fact that the molecular actors in the Ca^{2+} efflux pathway are only incompletely known, and a pharmacological total block of the Ca^{2+} efflux pathways is not possible with the available drugs.

The molecular nature of the $2H^{+}/Ca^{2+}$ exchanger is still debated, though the recent work by Clapham and coworkers (99) suggests that Letm1 can be a plausible candidate. Mutations in Letm1 are responsible for the Wolf-Hirschhorn syndrome, characterized by craniofacial defects, growth and mental retardation, muscle hypotonia, heart defects, and seizures. The conclusion of Clapham and coworkers (99) is however challenged by other investigators (100), who maintain that Letm1 is (or is part of) the K^{+}/H^{+} antiporter. The issue is not yet solved (for a recent review, see 19).

The situation with the Na^{+}/Ca^{2+} antiporter is more clear. A first hint of the molecular nature of the exchanger was provided in 2004, when Paucek & Jaburek (101) isolated from mitochondria a 60-kDA protein capable of catalyzing in reconstituted vesicles a Na^{+}/Ca^{2+} exchange process. The identification of the molecular nature of this protein is the result of the work of Seckler's group,

published in 2010 (1). The initial intuition was that a member of the plasma membrane family of Na^+/Ca^{2+} exchangers, named NCLX, had the unique characteristics of exchanging Ca^{2+} not only with Na^+ but also with Li^+ (hence the name NCLX, for Na^+/Ca^{2+} Li^+-permeable Exchanger). NCLX is ubiquitously expressed, and different spliced variants have been found. The capacity to exchange Ca^{2+} with Li^+ was known to be a typical feature of the mitochondrial Na^+/Ca^{2+} exchanger (102) but not of the classical plasma membrane transporters NCX or NCKX (for a recent review, see 103). Seckler's group (1) then demonstrated that NCLX is expressed only in internal membranes and is highly enriched in the mitochondrial fraction—more specifically, in the inner mitochondrial membrane. Because it catalyzes the exchange of 3–4 $Na^+/1Ca^{2+}$, Ca^{2+} efflux is accompanied by the net import of one (or two) positive charge(s) into the matrix; thus, it is favored by the negative membrane potential across the inner mitochondrial membrane (see section on Thermodynamics of Mitochondrial Ca^{2+} Transport Mechanisms). Although most of the available information on the role of the mitochondrial Na^+/Ca^{2+} exchanger has been obtained by the pharmacological block of the exchanger with CGP37157, the functional role of NCLX has recently been intensively investigated in different cellular models by using genetic knockdown of the protein, and a number of unexpected functional interactions of NCLX and other components of the Ca^{2+} toolkit have been identified. For example, NCLX modulates oxidative metabolism in pancreatic β cells (104, 105), automaticity in a cardiac cell line, HL-1 (106), and Ca^{2+} signaling, proliferation, and gliotransmission in astrocytes (107). The interested reader is referred to these and other recent papers for more details.

No information is yet available on the structure of NCLX. NCLX indeed shares a relatively low degree (approximately 20%) of sequence homology with other members of the Na^+/Ca^{2+} exchangers superfamily: The typical long cytoplasmic domain found in NCX is not found in NCLX, whereas the R1 and R2 regions embedded within the transmembrane domains are retained (103). However, given the similarity of functions, the 3D structure of the mitochondrial Ca^{2+}/Na^+ antiporter is likely somehow similar to that of the plasma membrane transporters. Plasma membrane transporters are predicted to possess nine membrane-spanning helices separated in two parts by a large intracellular regulatory domain. Recently, the crystal structure of NCX from an archaeobacterium, *Methanocaldococcus jannaschii*, was determined at 1.9-Å resolution (108). A remarkable finding in structure-functional studies on NCX is that the removal of the intracellular domain does not impair the exchange properties of the protein, suggesting that the transmembrane portion of the NCX (where the sequence similarity with NCLX is more conserved) possesses the basic functional unit necessary for ion transport (108). Furthermore, Marinelli et al. (109) recently used a systematic computer simulation to show that the periplasmic surface of NCX is loaded with 3 Na^+ ions, a proton, and a water molecule; this demonstrated not only that the transport of Na^+ is the electrogenic step but also that the net charge transfer in the Na^+/Ca^{2+} exchange cycle is +1.

MITOCHONDRIAL CA^{2+} UPTAKE/RELEASE AND CELLULAR FUNCTIONS

Modulation by Pharmacological Manipulation

The complex Ca^{2+} transport toolkit of mitochondria impinges on both specific organelle functions as well as on general cellular processes. Up until the discovery of the molecular nature of the proteins involved in mitochondrial Ca^{2+} uptake and release (and thus the possibility of genetically manipulating mitochondrial Ca^{2+} handling), the only approach to addressing the importance of this process was the use of drugs that interfere, more or less specifically, with the uptake or

release processes. Compounds that collapse membrane potential (i.e., uncouplers such as FCCP), respiratory chain inhibitors (antimycin A, rotenone, cyanide), blockers of mitochondrial ATP synthesis/transport (oligomycin), blockers of MCU (La^{3+}, RR, or Ru360), and inhibitors of the Na^+/Ca^{2+} exchanger (CGP37157) have been the only available tools to affect mitochondrial Ca^{2+} transport for more than 30 years. None of these inhibitors are specific, as they either modify other organelle functions (e.g., the capacity of synthetizing ATP) or affect processes other than the targeted mitochondrial function [e.g., uncouplers can decrease the plasma membrane potential and/or acidify the cytoplasm (110), oligomycin can inhibit the plasma membrane Na^+/K^+ ATPase (111, 112), and CGP37157 can inhibit plasma membrane voltage-gated channels (113)]. It needs also stressing that inhibitors of the respiratory chain in intact cells, unless coupled with oligomycin, do not usually block mitochondrial Ca^{2+} uptake, as the latter can be sustained by reversal of the H^+ ATPase (using the ATP produced by glycolysis).

As to the so-called specific MCU inhibitors, La^{3+} is known to inhibit a variety of other Ca^{2+} channels and pumps and is poorly, if at all, permeant across the plasma membrane, whereas the effects of RR and Ru360, often used in intact cells to inhibit MCU-dependent Ca^{2+} uptake, are difficult to interpret. In particular, Hajnóczky et al. (114) clearly showed that in a variety of cell types the functional effect of these two drugs are mainly, or exclusively, dependent on their action on plasma membrane channels rather than on MCU itself. This finding is not surprising, as an exavalent cation such as ruthenium and its derivatives should be practically impermeant across the plasma membrane and, if endocytosed, should not pass the endosome membrane. We have tested both RR and Ru360 in many cell models and, by directly monitoring mitochondrial Ca^{2+} uptake in live cells, we have been unable to confirm any specific or direct effect on MCU-dependent Ca^{2+} uptake using these drugs. We cannot exclude, however, the possibility that under some conditions and after many hours of incubation RR or Ru360 may eventually reach a sufficient concentration in the cytoplasm to inhibit MCU, but in most cases the evidence supporting this conclusion has been indirect. Despite these caveats, 30 years of research using these pharmacological tools have allowed a few conclusions, many of them confirmed recently by genetic manipulation of MCU complex components.

Modulation by Ca^{2+} of Mitochondrial Functions

Ca^{2+} accumulation by mitochondria is a key component in the regulation of three essential dehydrogenases (pyruvate, α-ketoglutarate, and isocitrate dehydrogenase) that are the rate-limiting enzymes in feeding electrons at complex I of the respiratory chain (115). These three enzymes are activated by Ca^{2+} in the matrix through different mechanisms: Pyruvate dehydrogenease (PDH) activation depends on the dephosphorylation of the catalytic subunit by a Ca^{2+}-dependent phosphatase (116), whereas α-ketoglutarate and isocitrate dehydrogenases are directly activated by Ca^{2+} binding (117). An increase in the matrix Ca^{2+} concentration thus results in activation of these three enzymes and potentially of ATP synthesis by the organelles. An extensive series of experiments in isolated mitochondria, followed by a number of studies in intact cells have led to this largely accepted conclusion (118); more recently, these data have been confirmed in vivo in tissues from MCU-KO animals (see section on Genetic Manipulation). It must be stressed, however, that an increase in matrix Ca^{2+} concentration is only one of the mechanisms leading to activation of these enzymes: In particular, PDH can be activated by elevation of matrix $[Ca^{2+}]$ of a few micromolar, but it is also controlled by other allosteric modulators such as pyruvate itself, ATP, NADH, and matrix pH (118).

Other specialized functions of mitochondria are modulated by Ca^{2+} accumulation in the matrix; these functions include aldosterone production in glomerulosa cells (119), glutamate production

in pancreatic β cells (120, 121), and activation of the permeability transition pore (PTP) (122), a large pore in the inner mitochondrial membrane permeable to solutes up to 1 kDa. Although the molecular nature and the mechanism of PTP activation by Ca^{2+} is still partially unsolved (122–127), there is general consensus as to the role of cyclophillin D (a specific mitochondrial cyclophillin isoform), whose activity is modulated by matrix Ca^{2+} increases and inhibited by cyclosporine A (CsA). The interested reader is referred to the original papers or to recent reviews for a detailed discussion of these topics (122–127). Before the discovery of MCU and of the other members of the mitochondrial Ca^{2+} toolkit, to the best of our knowledge, the only genetic approach that addressed directly the role of mitochondrial Ca^{2+} accumulation in modulating organelle functions was expression of an exogenous Ca^{2+} buffer protein, S100G, within the mitochondrial matrix (128). This approach has enabled the demonstration that a reduction of the matrix $[Ca^{2+}]$ increase, without affecting other mitochondrial functions, is sufficient to reduce ATP production by the organelle upon glucose stimulation and to strongly reduce insulin secretion in pancreatic β-cells (128). Similar results have been obtained in glomerulosa cells in which angiotensin II–dependent aldosterone production is strongly reduced by increasing the matrix Ca^{2+}–buffering capacity (128). Very recently, it has been demonstrated that S100G expression in the mitochondria of glomerulosa cells results in a substantial reduction of the intraorganelle production of cAMP by the Ca^{2+}-activated, soluble adenylyl cyclase (129).

Finally, there are other mitochondrial enzymes that are activated by Ca^{2+}: (*a*) glycerol phosphate dehydrogenase, particularly active in insect flight muscle, can feed electrons in the respiratory chain at complex 2 and is also involved in the redox shuttle whereby some mammalian tissues oxidize glycolytically derived NADH (130); and (*b*) two mitochondrial metabolite transporters, aralar1 and citrin, which are isoforms of the mitochondrial aspartate/glutamate carrier (131–133). In these cases, however, the Ca^{2+} binding domains face the intermembrane space and, accordingly, do not depend on the capacity of mitochondria to accumulate Ca^{2+}, instead relying solely on the changes in cytosolic Ca^{2+} and the permeability of the outer mitochondrial membrane to the cation.

Modulation by Mitochondrial Ca^{2+} of Cellular Functions

As to the general effect on cellular functions of mitochondrial Ca^{2+} handling that have been reached by the cautious use of these pharmacological tools, the most studied phenomena are the local or general Ca^{2+} buffering of the organelles and their role in cell death. In this case, the conclusions depend, at least in part, on the cell model and the drug used. Accordingly, firm and generally accepted conclusions will only be reached when more information is obtained in genetic models with altered mitochondrial Ca^{2+} uptake/release mechanisms.

The role of mitochondrial Ca^{2+} in cell death will not be specifically addressed here, and the interested reader is referred to the vast number of papers dealing with this topic (e.g., 22, 24, 28, 61, 73, 83, 114, 122, 126, 134). As to the role of mitochondria in local or general Ca^{2+} buffering, here we limit ourselves to a few examples and refer the reader to the section on Genetic Manipulation for a detailed discussion of the data obtained through the genetic manipulations of the MCU complex.

Two simple statements appear to be largely accepted regarding the general Ca^{2+} buffering effect of mitochondria during cell activation (for review, see 24): (*a*) in all cells investigated, mitochondria do accumulate Ca^{2+} during any cytosolic Ca^{2+} increase, whether large or small; and (*b*) the amount of Ca^{2+} accumulated by the mitochondria depends on the amplitude of the Ca^{2+} rises in the cytosol (local or general), the duration of the Ca^{2+} rise, the source of the Ca^{2+} rise (plasma membrane or intracellular Ca^{2+} channels), and cell-specific elements (presumably dependent on differences in the Ca^{2+} toolkit proteins expressed). The positioning of mitochondria, in turn controlled by

the highly complex process of mitochondrial mobility and shape regulation, may cluster a large mitochondrial mass in defined domains of polarized cells, thus forming a large fixed buffer opposing the diffusion of Ca^{2+} waves.

The existence of Ca^{2+} hot spots on the surface of mitochondria at sites of close apposition to ER or plasma membrane Ca^{2+} channels explains the very rapid and large Ca^{2+} uptake by mitochondria in live cells, despite the relatively small increases in bulk cytosolic Ca^{2+} and the apparent low affinity for Ca^{2+} of MCU (20, 24, 46, 83, 135–138). However, any small increase in cytosolic Ca^{2+} is predicted (and has been experimentally demonstrated) to transiently modify the steady state between Ca^{2+} influx/efflux from the organelles independently of the hot spots (24). Clearly, the larger the local or bulk Ca^{2+} increases, the faster the rate of Ca^{2+} accumulation. As to the duration of the Ca^{2+} rises in the cytosol, very brief Ca^{2+} increases in the cytosol limit the amplitude of the Ca^{2+} rises in the matrix; paradoxically, a very prolonged and large increase may first result in massive Ca^{2+} accumulation, followed by Ca^{2+} release caused by PTP opening (134, 139). Based on these considerations, the buffering capacity of mitochondria is generally of modest entity in most cultured cell lines—in other words, complete collapse of membrane potential usually results in small increases in the peak Ca^{2+} rise caused by stimulation (e.g., see 20) and in more substantial increases in neurons subjected to a train of action potentials (140–143) or in adrenal medulla cells stimulated with depolarizing concentrations of K^+ (25, 144). In pancreatic acinar cells, blockade of mitochondrial Ca^{2+} accumulation by oligomycin and antimycin allows the Ca^{2+} rise (caused by an agonist and physiologically restricted to the apical pole of the cell) to spread to the entire cell cytoplasm (145).

Most studied, but still debated, is the nature and role of Ca^{2+} uptake by mitochondria in cardiac cells. In rat neonatal cell culture, strong evidence for a beat-to-beat cycle of Ca^{2+} accumulation/release has been found (57, 146; see 147 for review), and a significant increase in the amplitude of the systolic Ca^{2+} increase was observed upon MCU knockout by siRNA (57; see also the section on Genetic Manipulation). Conflicting results have been obtained in adult cells. For example, Pacher et al. (148) showed that mitochondria can rapidly take up Ca^{2+} even during short and localized events such as Ca^{2+} sparks. Trollinger et al. (149) observed beat-to-beat changes in mitochondrial Ca^{2+} levels, whereas under similar conditions Sedova et al. (150) found no evidence for such mitochondrial Ca^{2+} oscillations. Maack et al. (151) observed the following in adult guinea pig cardiac myocytes during repetitive voltage clamp depolarizations: (*a*) synchronous cytosolic and mitochondrial Ca^{2+} increases and (*b*) reduction of mitochondrial Ca^{2+} increases and augmentation of cytosolic Ca^{2+} peaks upon prolonged incubation with the MCU blocker Ru360. The same authors (151) also found that CGP-37157 potentiates diastolic mitochondrial Ca^{2+} accumulation. For a more general discussion of the topic, see References 147, 152. For a discussion of the role of MCU in adult cardiac cells, see also the section on in vivo Genetic Manipulation.

As to a more local Ca^{2+} buffering effect, pharmacological evidence supports a role of the local mitochondrial Ca^{2+} uptake on the efficacy of inositol 1,4,5 trisphosphate (IP_3)-induced Ca^{2+} release. In particular, mitochondrial Ca^{2+} buffering modulates the effect of the local Ca^{2+} rise on the IP_3 receptor (153, 154). Ishii et al. (155) showed that inhibition of Na^+/Ca^{2+} exchange with CGP37157 reduces the refilling of the ER with Ca^{2+} during a rapid Ca^{2+} spike, thus altering the frequency and amplitude of successive Ca^{2+} oscillations. Finally, conflicting data have been obtained as to the local buffering of Ca^{2+} by mitochondria in the vicinity of plasma membrane Ca^{2+} channels. Most studies argue in favor of a major role of mitochondria Ca^{2+} sequestration strategically located close to the mouth of voltage- and glutamate-gated channels (46, 156, 157). On the contrary, different results have been obtained concerning the role of mitochondrial Ca^{2+} uptake in the proximity of the calcium release-activated calcium modulator (Orai) type of channels (46, 158–161). Our biased opinion is that the mechanism of Orai channel gating [i.e., the apposition

of ER to the plasma membrane and physical coupling of the stromal interaction molecule (STIM), the ER Ca^{2+} sensor, with Orai on the inner surface of the plasma membrane] allows no room for a close apposition of mitochondria to the Orai channel mouth. Accordingly, the local Ca^{2+} buffering by mitochondria in this microdomain is expected to be marginal (46).

As discussed above, all these studies on the general or local mitochondrial Ca^{2+} buffering have been primarily obtained using drugs that have important side effects. These studies accordingly require more direct testing by genetic manipulation of the capacity of mitochondria to take up or release Ca^{2+}. This novel and more direct approach is described next.

Genetic Manipulation

The recent discovery of MCU and of the proteins forming the complex has opened the field to genetic loss-of-function or gain-of-function approaches to unambiguously determine the role of mitochondrial Ca^{2+} uptake in organism pathophysiology. Although the studies carried out in cultured cells concern both the overexpression and downregulation of all the proteins in the complex, in vivo models are currently available only for MCU knockout and overexpression. A recent report, however, has described a human family with a missense mutation in MICU1 (162). The phenotype of the patient is quite dramatic and is described at the end of this section. We thus limit this discussion to the few recent papers addressing the effects on the pathophysiological consequences of MCU knockout (and overexpression) in isolated living cells or in vivo.

Isolated cells. Most of the recent papers on genetic manipulation of the MCU complex have concentrated on the molecular mechanisms controlling the molecular composition and the functional role of each component in modulating Ca^{2+} influx. Only a few studies have investigated the cellular functions affected by genetic manipulation of MCU or of its associated proteins. For example, in pancreatic β-cells it was confirmed that glucose-induced insulin secretion (through the stimulation of oxidative metabolism), elevation in cytosolic ATP/ADP ratios, and the closure of ATP-sensitive K^+ channels (K_{ATP}) depend on the expression of a functional MCU. Most importantly, as pointed out above, mitochondrial Ca^{2+}-dependent boosting of ATP production appears to be a critical step in this process (58, 59, 163). Silencing of NCLX enhances the kinetics of glucose-induced changes in cytosolic ATP/ADP ratios, thus enforcing the notion that mitochondrial Ca^{2+} is a central player in the regulation of β-cell functions. Along the same lines, MCU overexpression or downregulation augment or diminish, respectively, the intramitochondrial cAMP increases caused by activation of IP_3-induced Ca^{2+} release from the ER (164). The role of MCU in apoptosis is less clear. Despite the predictions, MCU has been shown to be dispensable for apoptosis in MDA-MB-231, a cellular model of breast cancer (61, 62). Conversely, cell death experiments carried out in primary cortical neurons convincingly recapitulated the expected link between mitochondrial Ca^{2+} overload and apoptosis: MCU overexpression triggers cell death not only after excitotoxic stimulation but also in untreated cells; in parallel, MCU silencing strongly protects from apoptosis (60).

In vivo. Mitochondrial Ca^{2+} has been always considered a pleiotropic signal that potentially regulates many aspects of physiology at the whole-organism level. Genetic manipulation of MCU in lower organisms fully confirmed this hypothesis. Indeed, both silencing of and conditional knockout of MCU in *Trypanosome brucei* impaired energy production, enhanced cellular recycling through autophagy, produced marked defects of growth in vitro, and reduced infectivity in mice (165). Developmental defects were also evident in *Danio rerio* injected with anti-MCU morpholinos (166). Conversely, knockout of MCU in *Caenorhabditis elegans* led to apparently viable and fertile

worms, although production of reactive oxygen species (ROS) was impaired (167). Findings in the *Drosophila* model have not yet been published.

Mouse models. As far as mammals (mice in particular) are concerned, most of the in vivo studies of MCU silencing or overexpression have concentrated on the effects on striated muscles, particularly on cardiac muscles. The first attempt to target MCU in *Mus musculus* resulted in very surprising results for most scientists in the field. In 2013, Finkel and coworkers (63) published a paper showing a viable mouse model completely lacking MCU and characterized by a very mild phenotype. MCU-KO mice are smaller (a feature shared by many knockout models) but with no other visible features or gross histological aberrations. The only substantial defect is a decrease of skeletal muscle peak performance, independent of a remodeling in muscle fiber type. No overt differences in cardiac physiology could be appreciated, either in basal condition or after increased workload induced by isoprenaline treatment (168). Most importantly, wild-type and MCU-KO mice were totally equivalent in terms of ischemia and reperfusion (I/R) injury. Remarkably, although inhibition of mPTP opening was effective in protecting hearts from I/R injury in wild-type animals, MCU-KO animals were completely insensitive to CsA treatment. This suggests that, at least in this context, mitochondrial Ca^{2+} overload is the main pathway leading to mPTP opening, but additional CsA-independent and Ca^{2+}-independent cell death pathways overrule these routes when MCU is absent. Despite these observations, the absence of a major phenotype of the constitutive MCU-KO mouse is a persistent problem.

It is indeed puzzling that the embryonic development of all organs, requiring high energy and massive apoptosis to settle, is unaffected in this animal model, especially considering that opposite results have been obtained in other organisms. Eventually, it became clear that ablation of MCU in the standard C57BL/6 strain actually leads to embryonic lethality, a notion that has been confirmed in an independent mouse model (169). Viable mice could be obtained only in a mixed C57BL/6 and CD1 background, and even in this strain the birth ratios of the homozygous MCU null mice were half of the expected number (170), thus confirming the influence of mitochondrial Ca^{2+} uptake during embryo development. Moreover, this notion is further supported by the observation that the constitutive knockout of EMRE (resulting in the loss of mitochondrial Ca^{2+} uptake) leads to embryonic lethality, according to International Mouse Phenotyping Consortium (IMPC, see **http://www.mousephenotype.org/data/genes/MGI:1916279**).

How some of these MCU-KO animals can develop without any problem remains a mystery. The obvious explanation is that some kind of compensation takes place. However, the nature of this compensation is presently unknown.

Selective knockout of MCU in the heart. New mouse models have been generated in the last 2 years. Anderson and coworkers (171, 172) have generated a heart-specific transgenic mouse model expressing a dominant-negative MCU isoform, $MCU^{D260Q,E263Q}$ (DN-MCU). Even if this mutant is not able to completely abolish organelle Ca^{2+} uptake in cultured cells (3), mitochondria from DN-MCU–expressing hearts have shown no measurable mitochondrial Ca^{2+} uptake. Most importantly, this model has a significant phenotype. Although no evident defects were present in basal conditions, these mice are incapable of physiological fight-or-flight heart rate acceleration induced by isoprenaline stimulation. In this model, MCU is dispensable for normal heart beat; however, under physiological stress, MCU-dependent increase of ATP production is necessary to sustain the activity of sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) and to maintain the proper Ca^{2+} load of the sarcoplasmic reticulum (171) in sinus atrial node (SAN) cells. Overall, the DN-MCU–overexpressing heart exhibits impaired performance (measured as left ventricle

developed pressure) at increasing workloads. In addition, DN-MCU–expressing cardiomyocytes have significantly higher diastolic cytosolic [Ca^{2+}], caused by decreased ATP availability (172). Notably, even in this model, MCU inhibition does not protect the heart from I/R injury and these transgenic mice have been generated in the same mixed genetic background of the constitutive MCU-KO model.

Molkentin and coworkers (173) generated a conditional MCU knockout model in which two LoxP sites were introduced between exons 5 and 6 ($MCU^{fl/fl}$). These animals were then crossed with mice expressing a tamoxifen-inducible Cre recombinase [MerCreMer (MCM)] driven by the cardiomyocyte-specific α-myosin heavy chain promoter. MCU gene deletion was induced in adult mice (i.e., at 8 weeks), and the cardiac function was evaluated (173). A parallel study by Elrod and colleagues (169) on the same mouse model led to similar conclusions. In particular, ablation of MCU in adult hearts led to an 80% decrease of MCU at the protein level (173), with a drastic reduction of mitochondrial Ca^{2+} uptake (although a residual slow organelle Ca^{2+} accumulation was still observed). As to cardiac functions, the lack of MCU does not lead to major defects, even 40 weeks after gene ablation, both in normal conditions and after cardiac pressure overload induced by transverse aortic constriction (173). In this genetic model, however, MCU ablation strongly protects cardiac tissue from I/R injury (169, 173), as opposed to previously reported MCU knockout in the germ line (63, 172). In addition, these studies confirm the lack of fight-or-flight responses triggered by β-adrenergic stimulation. This effect is due to an impaired stimulation of oxidative metabolism, thus confirming the pivotal role of MCU in the coupling of ATP production to the dynamics of cellular energetic demand (169). MCM-MCU-KO–derived cardiomyocytes showed normal respiration rates in basal conditions, although a decrease in oxygen consumption rate could be detected in maximal respiration (FCCP-induced) conditions or after increased workload (isoprenaline treatment). Impairment of PDH phosphorylation (and thus activity) has been reported in all MCU-KO models, although to different extents. Most relevant, in all models there is an impaired PDH stimulation after isoprenaline treatment (63, 169, 172, 173).

Of interest, when $MCU^{fl/fl}$ animals were crossed with mice constitutively germ line–expressing Cre recombinase (B6.CMV-Cre) there was complete embryo lethality, confirming the requirement for MCU during development (169).

Knockdown of MCU in other tissues. In addition to the role in cardiac physiology, mitochondrial Ca^{2+} uptake is predicted to play a major role in all tissues, particularly in those relying on the efficiency of oxidative metabolism. We thus investigated (174) the role of organelle Ca^{2+} uptake in skeletal muscle pathophysiology by both positively and negatively modulating the MCU level. To avoid putative compensatory effects operating during embryonic development, we developed adeno-associated viral (AAV) particles for both the overexpression and the silencing of MCU. In this model, manipulation of MCU levels after birth clearly demonstrated that mitochondrial Ca^{2+} homeostasis contributes to regulate skeletal muscle trophism (174). MCU overexpression and downregulation causes muscular hypertrophy and atrophy, respectively. MCU overexpression also protects from denervation-induced muscle atrophy triggered by sciatic nerve section (174). It must be stressed that these effects appear to be independent of the control of aerobic metabolism, because (*a*) PDH activity, although defective in MCU-silenced muscles, was unaffected in MCU-overexpressing muscles, (*b*) hypertrophy was comparable in both oxidative and glycolytic muscles (in the latter the contribution of mitochondrial metabolism should play a relatively marginal role), and (*c*) analysis of aerobic metabolism revealed no major alteration. Conversely, this effect relies on two major hypertrophic pathways of skeletal muscle, PGC-1α4 and IGF1-Akt/PKB, leading to marked increase in protein synthesis. These results

indicate the existence of a Ca^{2+}-dependent mitochondria-to-nucleus signaling route that links organelle physiology to the control of muscle mass.

The pleiotropic role of mitochondrial Ca^{2+} uptake has also been confirmed in human pathophysiology (162). Few families carrying a loss-of-function mutation of MICU1 have been identified. Homozygous individuals for this mutation are characterized by early-onset proximal muscle weakness with a static course, moderately or grossly elevated serum creatine kinase levels accompanied by learning difficulties, and a progressive extrapyramidal movement disorder. Fibroblasts derived from these patients show the expected impairment of MCU gating accompanied by mitochondrial fragmentation and decreased oxidative metabolism (162). Although the pathogenesis lying behind this disease is impossible to dissect at the moment, this finding underlines the importance of mitochondrial Ca^{2+} transport in humans, as a change in one of the regulatory proteins in the complex results in a devastating genetic disease.

CONCLUSIONS

The discovery of MCU has brought the field of mitochondrial Ca^{2+} homeostasis to a fascinating turning point. Indeed, it is remarkable that in the absence of any molecular definition of the process, imaging studies and indirect pharmacological approaches have unambiguously demonstrated that mitochondrial Ca^{2+} signals play a key role in numerous physiological and pathological processes, such as metabolic regulation, neuronal activity, muscle contraction, and cell death. The lack of molecular tools was frustrating, as it hampered the possibility to verify and expand these observations. Now, molecular analyses are finally possible and providing exciting results. They have revealed an amazing complexity of the Ca^{2+} uptake machinery (e.g., multiple MCU and MICU isoforms, miRNAs, and splice variants), accounting for a molecular plasticity that well meets the tissue specificity of Ca^{2+} signals and Ca^{2+}-regulated processes. At the same time, shRNA silencing in vitro and in vivo has already allowed direct assessment of the physiological role of mitochondrial Ca^{2+} homeostasis and obtainment of novel, unexpected new information (e.g., the role in muscle trophism or cancer cell migration). Finally, some bona fide transgenic models have been developed in a variety of organisms, ranging from mice to *Drosophila melanogaster*, *C. elegans*, and *Arabidopsis thaliana*.

The field is still in its infancy and requires careful models (such as conditional and tissue-specific gene ablation) and detailed investigations. Indeed, with some surprise MCU proved to be another example of an essential protein that, if knocked out at the embryo level, results in no or a very mild phenotype due to intervening, unpredicted compensatory events (e.g., see 175–177). In our biased opinion, the lack of an overt phenotype of the constitutive MCU-KO mouse is apparent. Although embryonic lethality is evident in pure genetic backgrounds, biological robustness can account for the very mild phenotype of the remaining viable animals. Among the numerous controlled gene ablations that are currently being generated in various laboratories, including our own, substantial published information is already available for the heart, with which tissue-selective and inducible-knockout models have been generated by different groups. These models have clearly demonstrated that MCU, and hence mitochondrial Ca^{2+} accumulation, are needed for a physiological cardiac response, although they now open the search for the pathways that may, under some special conditions, partially compensate for the lack of functional mitochondrial Ca^{2+} homeostatic machinery. All other MCU-ablated lines are coming down the pipeline, and these lines can then be crossed with disease and/or knockout models. Thus, we believe that much excitement lies ahead in the important topic of cellular signaling.

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