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Discussion

Molecular control of mitochondrial calcium uptake

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ABSTRACT

The recently identified Mitochondrial Calcium Uniporter (MCU) is the protein of the inner mitochondrial membrane responsible for Ca^{2+} uptake into the matrix, which plays a role in the control of cellular signaling, aerobic metabolism and apoptosis. At least two properties of mitochondrial calcium signaling are well defined: (i) mitochondrial Ca^{2+} uptake varies greatly among different cells and tissues, and (ii) channel opening is strongly affected by extramitochondrial Ca^{2+} concentration, with low activity at resting and high capacity after cellular stimulation. It is now becoming clear that these features of the mitochondrial Ca^{2+} uptake machinery are not embedded in the MCU protein itself, but are rather due to the contribution of several MCU interactors. The list of the components of the MCU complex is indeed rapidly growing, thus revealing an unexpected complexity that highlights the pleiotropic role of mitochondrial calcium signaling.

The past three years have witnessed the molecular identification of the long sought channel in charge for ruthenium red-sensitive mitochondrial Ca^{2+} uptake, the Mitochondrial Calcium Uniporter (MCU) [1,2]. This discovery revived the whole field of cellular calcium signaling [3], leading to the discovery of a number of MCU interactors, including channel subunits [4] and regulators [5–9]. The notion that MCU is the key molecule in mitochondrial Ca^{2+} uptake is confirmed in a variety of cellular systems, including liver [2], cardiomyocytes [10,11], pancreatic β-cells [12,13], cancer cells [14,15] and neurons [16]. Moreover, mitochondria derived from heart and skeletal muscle of MCU knock-out mice have been shown to lack any ability to uptake Ca^{2+} [17]. However, whether MCU is *per se* sufficient to form a functional channel is still a matter of debate, although both evolutionary analyses of MCU homologues and experimental data support this hypothesis. Indeed, on one hand several proteins have been described to be necessary for MCU function *in situ*, since the knockdown of MICU1 [5], MCUR1 [6] or EMRE [8] have been shown to inhibit mitochondrial Ca^{2+} uptake. On the other hand, most of the components of the MCU complex are not strictly conserved through evolution: MCUR1 is present only in metazoans, while EMRE is lacking in plants and protozoa. Thus, some organisms show MCU mediated mitochondrial Ca^{2+} uptake even in the absence of obvious EMRE or MCUR1 homologues, as recently demonstrated by Docampo and colleagues [18]. As to MICU1, despite being the best conserved MCU regulator (even if some exceptions exist, e.g. *Neurospora crassa*, emerging evidence indicates that it is dispensable for MCU activity [19–22].

In addition to comparative genomics considerations, experimental work aimed to define the minimal requirements to form a functional channel necessarily relies on heterologous systems (e.g. planar lipid bilayers or liposomes). Our group showed that purified MCU is sufficient *per se* to form a calcium channel in planar lipid bilayer with most of the properties of the Mitochondrial Calcium Uniporter [1]. The observation that in this condition the elicited current is similar, but not identical to that recorded in patch clamp experiments of isolated mitoplasts [23] can be likely accounted for by the different lipid environment, the lack of post-translational 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 ruthenium red and Gd^{3+}) [1,23,24]. Moreover, the specificity of planar lipid bilayer measurements are supported by numerous evidences: (i) the recombinant MCU protein has been purified from both *Escherichia coli* and an *in vitro* translation/transduction system based on wheat germ lysate (where no membrane contaminants are present), yielding the same results; (ii) site-specific mutagenesis of only two amino acids abolishes Ca^{2+} currents *in vitro* and, accordingly, inhibits mitochondrial Ca^{2+} uptake in living cells [1]; (iii) expression of the closely-related endogenous dominant-negative MCU isoform, MCUb, does not elicit any current in Ca^{2+}-containing media and progressively inhibits MCU mediated Ca^{2+} current in a dose-dependent manner [4]; (iv) MCU open probability is modified by its known modulators MICU1 and MICU2 [20]. Although additional

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experiments in the presence of other components of the complex (e.g. EMRE and MCUR1) are needed, these data strongly support the hypothesis that MCU is not only necessary but also sufficient to form a functional Ca^{2+}-channel within the IMM.

As to the regulation, MCU is part of a higher order complex that migrates around 500 kDa in blue-native gel electrophoresis [2,7,8,20]. The list of interactors is growing rapidly and now includes MCUb [4], the MICU family (that includes MICU1, MICU2 and MICU3) [7], MCUR1 [6] (that likely has an isoform, i.e. CCDC90B), EMRE [8] and SLC25A23 [9]. Such complexity is peculiar among calcium channels and most likely reflects the pleiotropic role that calcium signals play within the mitochondrial matrix as well as in modulating cytoplasmic [Ca^{2+}] changes. Indeed, it is reasonable to think that the difference in mitochondrial Ca^{2+} uptake of different tissues [25] relies on the quantitative and qualitative differences in the composition of the MCU complex the molecular level. A first level of complexity derives from the pore forming components of the complex. Indeed, the closely related MCU gene, named MCUb, encodes for a protein significantly similar in structure but functionally acting as a dominant-negative channel subunit [4]. The presence of at least two key substitutions in the amino acid sequence of the loop region of the channel (W251 and V256) was predicted to impair Ca^{2+} permeation across the pore through computational modeling; moreover, *in vitro*, MCUb does not allow the transit of Ca^{2+} cations (despite being *per se* a functional channel, allowing sodium permeation) and progressively inhibits Ca^{2+} currents when included within the MCU oligomer; finally, MCUb decreases agonist-induced calcium transients in living cells, an effect that can be mimicked by introducing the R251W and E256V mutations in the MCU sequence. Most importantly, comparative analysis of the MCU and MCUb expression at the mRNA level suggests the putative biological meaning of this molecular heterogeneity. Indeed, MCU:MCUb expression ratio varies from 3:1 to more than 40:1, a piece of data that nicely correlates, at least in part, with the tissue properties in term of mitochondrial Ca^{2+} uptake. As an example, *in situ* direct measurements of Ca^{2+} currents showed that mouse heart mitochondria show a dramatically lower current density than skeletal muscle [25]. This observation is in agreement with the MCU:MCUb expression ratio, which is low (3:1) in heart and high (40:1) in skeletal muscle. Although this hypothesis needs further studies, we think that MCU:MCUb ratio may play a major role in determining the intrinsic tissue-specific signatures of mitochondrial Ca^{2+} uptake.

Another important intrinsic feature of the mitochondrial Ca^{2+} uptake machinery is its sigmoidal response to extramitochondrial [Ca^{2+}], with low channel activity at resting cytosolic Ca^{2+} levels (in order to prevent vicious cycling of the cation with consequent energy drain) and a very large Ca^{2+} carrying capacity at higher [Ca^{2+}] (to ensure rapid mitochondrial Ca^{2+} uptake, and hence stimulation of oxidative metabolism, in stimulated cells). MCU is a relative small protein, largely residing in the mitochondrial matrix [26], with only a short loop protruding into the intermembrane space (EYSWDIMEP): it is thus hard to believe that such a complex regulation could be entirely embedded within the channel only. Rather, it would require a specific Ca^{2+} sensor located in the IMS acting as an inhibitor or activator at low and high [Ca^{2+}] respectively. The best candidates for this role belong to the MICU protein family, that includes three isoforms named MICU1, MICU2 and MICU3, characterized by the common presence of at least two EF hand domains. While MICU1 and MICU2 have a broad tissue expression [7] that parallels that of MCU, MICU3 appears to be present mostly in the brain, suggesting a tissue-specific function of this isoform. MICU1 (and thus most likely also MICU2 and MICU3) is a soluble protein that interacts with MCU. It is generally recognized to be located in the IMS [7,20,21], although a recent report locates MICU1 inside the matrix [27]. Although further studies will be necessary to precisely address this issue, the idea that MICU1 regulates MCU opening according to the extramitochondrial [Ca^{2+}] argues for its location in the intermembrane space. As to the function, several hypotheses are present in literature. MICU1 was indeed the first protein regulating mitochondrial Ca^{2+} uptake to be identified [5]: in this work, MICU1 was shown to be necessary for mitochondrial uptake, since its knockdown almost abolished [Ca^{2+}] transients in the matrix. Later, Madesh and colleagues showed that the authentic role of MICU1 is that of keeping MCU close when the extramitochondrial [Ca^{2+}] is low [22]: accordingly, MICU1 knockdown leads to a higher resting [Ca^{2+}] in the matrix without affecting agonists-induced mitochondrial transients, clearly indicating the impairment of the MCU gatekeeping mechanism. Finally, Hajnoczky and coworkers added another level of complexity to this picture. They showed that not only MICU1 controls the opening threshold of the channel, but its downregulation also attenuates MCU channel activity when extramitochondrial [Ca^{2+}] rises, i.e. upon agonists stimulation [21]. Thus, MICU1 appeared to act as both a MCU activator and inhibitor depending on external [Ca^{2+}], a notion that has been also recently confirmed [19]. However, these studies preceded the identification of MICU1 isoforms [7], in particular MICU2, when it became evident that the silencing of MICU1 leads to the concomitant disappearance of MICU2, through a post-translational mechanism (since its mRNA levels are normal). This opened the possibility that the complex action of MICU1 could be at least in part due to "off-target" effects secondary to its knockdown. We have recently explored this possibility and demonstrated that MICU1 and MICU2 form a dimer and hence they exist as an individual entity bridged together by a disulfide bond. Most importantly, they exert an opposite effect on MCU activity, with MICU2 acting as a genuine inhibitor and MICU1 stimulating channel opening. As to the regulatory mechanisms, both the gatekeeper (MICU2) and the stimulator (MICU1) have conserved EF-hand Ca^{2+} binding sites in their sequence, that sense the [Ca^{2+}] increase of an agonist-stimulated cell [20]. In this context, the recent elucidation of the crystal structure of MICU1 [28] supports the notion that MICU1 undergoes large conformational changes upon Ca^{2+}-binding. Although only the resolution of the crystal structure of the whole MICU1–MICU2 complex will elucidate its structure–function relationship, the formation of Ca^{2+}-dependent higher order MICU complexes perfectly agrees with the dynamic essence of this process. Thus, according to our model, at low [Ca^{2+}], the prevailing inhibitory effect of MICU2 ensures minimal Ca^{2+} accumulation in the presence of a very large driving force for cation accumulation, thus preventing the deleterious effects of Ca^{2+} cycling and matrix overload. As soon as extramitochondrial [Ca^{2+}] increases, Ca^{2+}-dependent MICU2 inhibition and MICU1 activation guarantees the prompt initiation of rapid mitochondrial Ca^{2+} accumulation, thus stimulating aerobic metabolism and increasing ATP production (see Fig. 1). We believe that this hypothesis recapitulates most of the apparently conflicting data regarding the function of MICU proteins and their interaction with MCU: even the most recently identified component of the MCU complex, named EMRE [8], can easily fit in this picture.

Much work is still necessary to finely dissect the intrinsic complexity of mitochondrial Ca^{2+} uptake machinery. Interestingly, despite the fact we are still in the early years of the molecular understanding of mitochondrial calcium signaling, the first example of a human pathology caused by a genetic defect in this pathway has already been reported [29], and demonstrates that this complexity is necessary to maintain the proper physiological functions. The investigation of MCU and its regulators thus promises to reveal a fascinating picture of major physiological and pathological relevance.

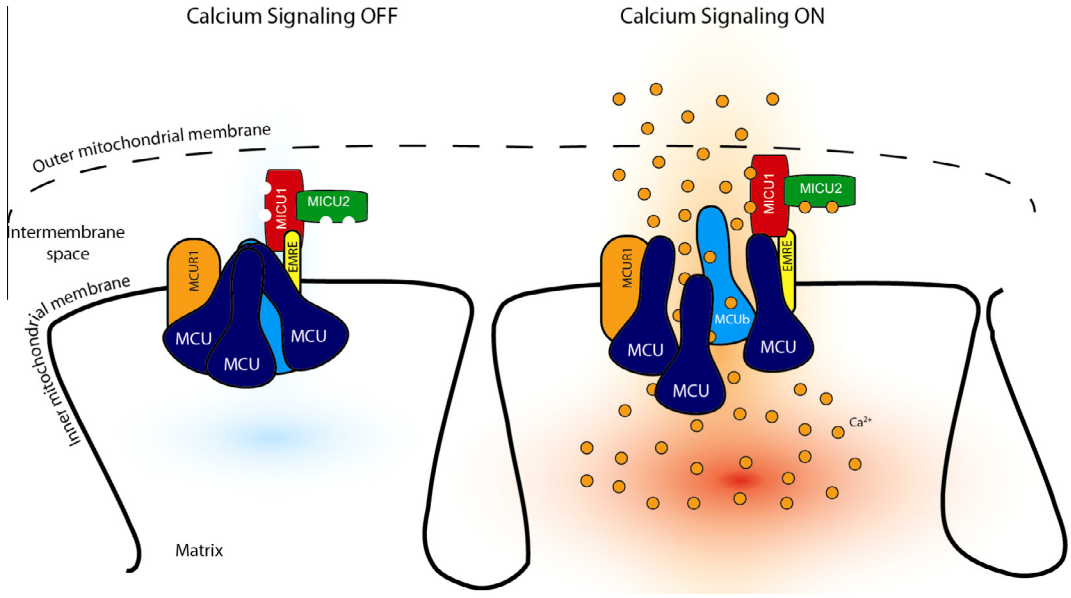

Fig. 1. Schematic representation of the MCU complex: in resting condition (on the left) mitochondrial calcium uptake is controlled by a multiprotein complex that can be composed by MCU and MCUb (the channel forming subunits) together with EMRE, MICU1, MICU2, MCUR1 and SLC25A23 (omitted for simplicity). In particular, MICU1/MICU2 heterodimers act as MCU gatekeeper, thanks to the prevailing inhibitory effect of MICU2, thus preventing vicious calcium cycles and energy sink; activation of cellular calcium signaling results in an increase of cations concentration (on the right) that induces a conformational change in the whole dimer that releases MICU2-dependent inhibition and triggers MICU1-mediated enhancement of MCU channeling activity.

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