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The Ins and Outs of Mitochondrial Calcium

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Abstract

Calcium is thought to play an important role in regulating mitochondrial function. Evidence suggests that an increase in mitochondrial calcium can augment ATP production by altering the activity of calcium-sensitive mitochondrial matrix enzymes. In contrast, the entry of large amounts of mitochondrial calcium in the setting of ischemia-reperfusion injury is believed to be a critical event in triggering cellular necrosis. For many decades, the details of how calcium entered the mitochondria remained a biological mystery. In the last few years, significant progress has been made in identifying the molecular components of the mitochondrial calcium uniporter complex. Here, we review how calcium enters and leaves the mitochondria, the growing insight into the topology, stoichiometry and function of the uniporter complex and the early lessons learned from some initial mouse models that genetically perturb mitochondrial calcium homeostasis.

Introduction

Those involved in biological or medical research often have a certain degree of envy for colleagues who labor in other scientific disciplines. Take high energy physics as an example. In 1964, a group of six physicists that included Peter Higgs postulated the existence of a certain elementary particle that helped resolve a longstanding riddle in cosmology. For the next forty years, teams of physicists sought experimental evidence of this theoretical Higgs boson. The challenge, although arduous, was clearly defined. Although laudable, this degree of focus and determination is dwarfed by what can happen in mathematics. In 1637, the French mathematician Pierre de Fermat made a relatively simple mathematical conjecture in the margin of a book. For the next 358 years, mathematicians sought the elusive proof that Fermat was indeed correct. Again, with the framework of the problem clearly outlined, Fermat's Last Theorem engaged nearly four centuries of mathematicians. In contrast, most biological and medical discoveries happen either by accident (think penicillin) or are the result of a relatively short-lived race (think the structure of DNA). That is not to say the biology does not have certain enduring challenges. One such example revolves around the relatively simple question as to how calcium enters the mitochondria. While perhaps not

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rising to the level of the search for a mysterious sub-atomic particle or deriving an elusive and fundamental mathematical proof, it has been clear since the 1960's that energized mitochondria could somehow selectively allow calcium to enter[1]. Over the next five decades, pharmacological, biophysical, biochemical and electrophysiological evidence gathered for the existence of an inner mitochondrial protein termed the mitochondrial calcium uniporter (MCU). Yet, though clearly defined, for over fifty years, the molecular identity of the uniporter remained unsolved. Like all good mysteries, along the way there were some promising leads that suggested a solution had been found[2]. Then, when some had begun to question the very existence of the uniporter, two groups using different in silico approaches both identified a previously uncharacterized mitochondrial protein (CCDC109A) as the long sought inner membrane calcium pore[3, 4]. It soon became clear that the uniporter does not act alone but rather exists in a large multimeric complex, the details which are only slowly being understood. Here, we review the evidence that calcium plays a regulatory role in mitochondria-from modulating bioenergetics, to determining the threshold for cell death. We further describe what is known about the influx and efflux of calcium into the mitochondria with particular emphasis on the inner mitochondrial uniporter complex and its increasingly complex regulation. Finally, we describe the small but growing number of *in vivo* models in which mitochondrial calcium is perturbed and the sometimes surprising phenotypes that have emerged.

Mitochondrial calcium uptake

The first clear description of the ability of mitochondria to rapidly uptake calcium came in the early 1960's, with the work of Vasington, Murphy, De Luca and Engstrom (reviewed in[5]). Over the intervening 50 years, pharmacological-based approaches played a significant role, especially after the discovery that mitochondrial calcium uptake was inhibited by the compound ruthenium red[6, 7] and its more specific form Ru360[8]. Mitochondrial calcium uptake was reported to have a low affinity (high Km) of ~5–10 μM[5]. High extramitochondrial calcium and calmodulin antagonists were also shown to inhibit calcium uptake into the mitochondria[9]. Varying forms or modes of calcium uptake were described, although it was unclear whether these were mediated by the same transporter (with different regulatory factors) or multiple different transporters. For example, when rat liver mitochondria are pulsed for a few seconds with nanomolar levels of calcium, they exhibit calcium uptake with a rapid kinetics which was termed "rapid mode" calcium uptake[10, 11]. To explain these and other results, a number of different molecules have been implicated as potential mediators of mitochondrial calcium uptake. For instance, the skeletal ryanodine receptor (RyR1), the primary calcium release channel in skeletal sarcoplasmic reticulum, was reported to be present in mitochondria and ryanodine, an inhibitor of RyR1, was shown to block mitochondrial calcium uptake[12]. LETM1 (leucine zipper-EF-hand-containing transmembrane protein 1) was also proposed to function as a mitochondrial Ca^{2+}/H^{+} antiporter, thereby mediating mitochondrial calcium influx[13–15], although others have suggested that it is a mitochondrial K^{+}/H^{+} exchanger[16, 17]. The mitochondrial Na^{+}/Ca^{2+} exchanger, which normally functions as a calcium efflux pathway (see Figure 1) has also been suggested to be able to function in reverse, leading to mitochondrial calcium entry

when the mitochondrial membrane potential is dissipated and the Na^+ gradient is favorable[18] (see section below on NCLX exchanger).

In addition to the confusion as to what protein might mediate mitochondrial calcium uptake, there was for a long period of time, considerable confusion regarding how this process could occur. Measurements of basal cytosolic calcium were thought to be in the range of 100 nM and peak calcium transients were less than 1 μM[19, 20]. Given the measured low affinity (~5–10 μM) for calcium uptake, it initially appeared that under physiological conditions, cytosolic calcium would never rise to levels high enough to activate the MCU. However, it was later demonstrated that microdomains of high calcium can exist near the junction of mitochondria and the site of calcium release (the ER/SR) and that the concentration of calcium in these microdomains is in fact high enough to allow calcium uptake via the uniporter[21].

Mitochondrial calcium efflux

In steady state, mitochondrial efflux must equal mitochondrial uptake. Mitochondrial efflux is primarily attributed to the function of the Na^+-Ca^{2+}-Li^+ exchanger (NCLX[22]), which can be inhibited pharmacologically by CGP37157[23]. It was known for some time that calcium efflux from heart mitochondria was dependent on the Na^+ gradient; although it was also shown that Li^+ can stimulate Na^+ efflux[24]. Matrix Na^+ is reported to be lower than cytosolic Na^+ due to Na^+ efflux from the mitochondria involving the mitochondria Na^+-H^+ exchanger. Matrix Na^+ is primarily regulated by the mitochondrial Na^+-H^+ exchanger, which is thought to operate close to equilibrium with the pH gradient[25]. Although the pH gradient in isolated mitochondria is typically measured at ~0.7 pH units (often in non-physiological buffers), it appears to be lower for mitochondria in situ. Studies in permeabilized cardiac myocytes also suggest that mitochondrial Na^+ is lower than cytosolic Na^+. This Na^+ gradient (lower in the matrix) is used as a driving force to extrude calcium from the matrix via the NCLX. Although it is still not proven, there is some evidence suggesting that the NCLX is electrogenic, exchanging 3 Na^+ ions for 1 Ca^{2+} ion[26]. The large negative mitochondrial membrane potential (−150 to −180 mV), coupled with the inwardly directed Na^+ gradients, provide a large driving force for extruding calcium from the mitochondrial matrix. If the NCLX is electrogenic, it could only potentially function in reverse to transport calcium into the matrix under conditions where the membrane potential was dissipated. It was long known that extra-mitochondrial Na^+ (or Li^+)[24] could stimulate calcium efflux from mitochondria and that ruthenium red did not inhibit the efflux, but rather stimulated it as would be expected for an efflux pathway[24, 27]. The NCLX was recently identified[22] facilitating targeted genetic and biochemical studies of its function and characteristics that could potentially address many of the issues mentioned above. In this regard, recent studies have shown that overexpressing the NCLX protein increased mitochondrial calcium efflux and reducing expression using siRNA-mediated approaches resulted in diminished mitochondrial calcium efflux[22, 26].

In isolated mitochondria, matrix calcium can vary widely depending on the extramitochondrial calcium level. Measurement of in situ matrix calcium in cardiomyocytes is reported in the range of 100–200 nM at low pacing rates and increases to 500 to 800 nM

with β-adrenergic stimulation. It is controversial as to whether mitochondrial matrix calcium transients actually track the changes in cytosolic calcium transients on a beat-to-beat basis, or if matrix calcium somehow integrates the levels of cytosolic calcium[28]. One argument against matrix calcium transients is that the NCLX is not capable of rapidly removing calcium on this beat-to-beat time scale. It has also been proposed that a transient opening of the mitochondrial calcium permeability transition pore might, under certain conditions, serve as an additional calcium release valve[29, 30], although recent data question this hypothesis[31].

Buffering of matrix calcium:

Mitochondrial calcium buffering can also affect the level of matrix free calcium. As shown by several groups[32–34] at levels below ~10 nmol Ca^{2+}/mg of mitochondrial protein, uptake of mitochondrial calcium leads to an increase in free matrix calcium. Thus below 10 nmol Ca^{2+}/mg there is a linear relationship between total and free calcium. In this range uptake of calcium into the mitochondria will change the free calcium. However, as total calcium rises above 10 nmol/mg matrix free calcium remains stable in the range of 1–5 μM due to buffering by calcium phosphate. In fact, Nicholls showed that with total mitochondrial calcium levels above ~10 nmol/mg that uptake of calcium buffers extramitochondrial calcium at a “set point”. Thus if in situ mitochondrial calcium operates in the range in which calcium is buffered by phosphate (~above ~10 nmol Ca^{2+}/mg protein) then calcium uptake by MCU may not be a key factor for the bioenergetic signaling system. However, as changes in cytosolic calcium have been reported to lead to changes in free matrix calcium[28] it is not clear whether in situ mitochondria operate in the range where matrix calcium is buffered.

Physiological role of mitochondrial calcium

Studies in the mid-1970s suggested that mitochondrial calcium might serve to regulate or modulate cytosolic calcium or act as a source for calcium release by a physiological agonist[35]. However, the latter hypothesis became unlikely when it became apparent that the endoplasmic reticulum/sarcoplasmic reticulum is the primary source of agonist-induced calcium release. A decade later, in the 1980’s, Denton and McCormick and others showed that calcium could regulate the activity of 3 mitochondrial dehydrogenases: pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase[36]. This led to the suggestion that the role of mitochondrial calcium uptake was not to regulate cytosolic calcium, but rather to regulate mitochondrial matrix calcium and hence the activity of these calcium sensitive mitochondrial dehydrogenases. Thus, in a cardiomyocyte, under conditions of increased work, an increase in cytosolic calcium leads to an increase in matrix calcium and serves to couple the increase in work to the increase in ATP production that is needed to sustain the work. With an increase in work, matrix calcium would increase, and this in turn would activate the mitochondrial dehydrogenases to increase mitochondrial NADH, fueling electron transport and increasing the output of ATP[37]. More recently, Balaban and coworkers demonstrated that calcium also leads to activation of complex V, the F_1F_0 ATPase[38], an enzyme that converts the mitochondrial protomotive force into ATP. Their observations were that in larger species with slower heart rates (e.g. pigs and dogs), raising matrix calcium lead to activation of complex V and that this activation was retained

by the complex even after isolation[39]. It is worth noting that the baseline turnover numbers (nmoles Pi/nmole Complex) in the mouse heart were already equivalent to the maximum calcium-stimulated levels observed in the larger dog and pig species. Thus, in the mouse, there might be less calcium regulation as Complex V may be wired to be at or near maximal stimulation under basal conditions.

Pathophysiological role of mitochondrial calcium

There is a wealth of data suggesting that an increase in cytosolic calcium and the resultant mitochondrial calcium overload are primary factors leading to cell death following ischemia and reperfusion[40–44]. The exact target of calcium overload that triggers cell death has been debated, but recent data has converged to suggest that mitochondrial calcium overload is a primary trigger of necrotic cell death[45]. In the late 1970s, studies in isolated mitochondria showed that accumulation of large amounts of matrix calcium led to the opening of a large channel in the mitochondria inner membrane that subsequently results in the release of proteins and solutes less than 1.5 kD; this channel is referred to as the mitochondrial permeability transition pore (mPTP)[46]. Calcium uptake via the MCU is thought to be the primary mechanism for calcium uptake to activate the mPTP, and inhibitors of MCU have been reported to protect cells from chemical hypoxia[47] and reduce cerebral and cardiac ischemia-reperfusion injury in vivo[48]. Chalmers and Nicholls also reported that total mitochondrial calcium rather than free matrix calcium was the trigger for opening the mPTP[33]. Later studies showed that cyclosporin A could inhibit the mPTP, which reinforced the concept that mPTP opening was not due to just non-specific membrane rupture[49, 50]. Mitochondrial cyclophilin D was soon identified as the target of cyclosporine A[50]. The identification of an inhibitor of the mPTP allowed studies to show that addition of cyclosporine A reduced cardiac ischemia-reperfusion injury[51]. Confirmation of the role of cyclophilin D in mediating calcium activation of the mPTP came from studies with genetic ablation of cyclophilin D. Indeed, isolated mitochondria from mice lacking cyclophilin D were resistant to calcium activated mPTP opening and hearts from these mice showed reduced cell death following ischemia and reperfusion[52]. A small proof of concept clinical trial has also reported that administration of cyclosporine A to patients with myocardial infarction is beneficial[53].

Alterations in mitochondrial calcium have also been suggested to play a role in altered metabolism in heart failure. Heart failure has been shown to lead to an increase in cytosolic sodium which is suggested to reduce mitochondrial matrix calcium levels by enhancing efflux of matrix calcium via the NCLX[54]. The reduction in matrix calcium is reported to decrease oxidative phosphorylation and ATP production and oxidation of NAD(P)H resulting in reduced antioxidant capacity. Indeed inhibition of the NCLX with CGP37157 has been reported to be beneficial in a guinea pig model of heart failure[54]. Although reduced matrix calcium associated with increased efflux or reduced calcium uptake can have detrimental effects, it is likely that overexpression of MCU or total loss of NCLX and the resultant unrestrained accumulation of matrix calcium would also be detrimental.

MCU and its regulators MICU1 and MICU2

The past 5 years have resulted in an explosion of studies identifying mitochondrial transporter proteins. As mentioned previously, the identification of the long elusive uniporter was reported simultaneously by two groups[3, 4]. Both identified an approximate 40 kD inner mitochondrial membrane protein with two predicted transmembrane domains. Mutation of a single amino acid (serine 259) resulted in a uniporter that loses the ability to be inactivated by the classical inhibitor ruthenium red[3]. Moreover, mutations in the acidic linker domain, between the two transmembrane domains, resulted in markedly diminished calcium uptake, with the appearance of a protein having a dominant negative phenotype[3, 4]. Reconstitution of purified MCU in lipid bilayers demonstrated channel activity arguing that MCU, by itself, may be all that is required for at least some uniporter activity[4]. Nonetheless, this conclusion is not universally accepted[55]. There is also some disagreement on the orientation and topology of MCU that *in vivo* appears to exist as a tetramer[56]. Nonetheless, it would appear that the most likely configuration has the linker region of MCU facing into the inter-membrane space, while the N-terminal and C-terminal portions of the protein are in the matrix[3, 57]. This topology was more firmly established by a novel technique that targets a genetically engineered peroxidase called APEX to a specific subcellular domain (in this case the inner-membrane space) in order to allow for live-cell proteomics[58]. This strategy, if more widely adopted, should allow for refinement of the topology of other components of the uniporter complex, as well as other large protein complexes within the mitochondria. While electrophysiological studies in MCU knockout mitoplasts have not been performed, there appears little doubt that the 40 kD protein identified as MCU is indeed the uniporter, a fact further bolstered by observations that mitochondria from MCU knockout mice lack any appreciable mechanism for the rapid uptake of calcium[59].

The first component of the uniporter complex identified was actually not the pore itself, but rather another inner mitochondrial membrane protein termed MICU1 (mitochondrial calcium uptake 1)[60]. MICU1 is a 54 kD protein containing two calcium-sensing EF hand domains. The identification of MICU1 was achieved through an ingenious targeted screen that employed the MitoCarta data base, a proteomic inventory of approximately 1000 known proteins that localize to the mitochondria[61]. To identify MICU1, the authors asked for proteins that existed within MitoCarta, further localized to the inner mitochondrial membrane (the presumptive localization of the uniporter) and were not present in *S. Cerevisiae* mitochondria (an organism known to lack the uniporter). Of the thousand plus genes whose protein products are found in MitoCarta, only 18 fulfilled these criteria. Then, using an RNAi-based approach, the authors asked whether knockdown of any of these eighteen candidates altered mitochondrial calcium uptake. One candidate did, a poorly characterized gene that was subsequently renamed MICU1. The fact that silencing MICU1 appears to inhibit mitochondrial calcium uptake must now, however, be viewed with certain degree of reservation. This is because it is now clear that the stability and expression of many of the components of the uniporter complex can be affected by knockdown of their interacting partners[62]. Hence, MICU1 knockdown can, for instance, influence MCU protein stability, thereby complicating interpretation.

In their initial description of MICU1, the authors suggested that the EF hands of this protein could potentially serve as a calcium sensing mechanism, making MICU1 an ideal candidate to act as a regulator of the uniporter[60]. Such regulation would seem essential based on electrophysiological studies of the pore that have indicated the open probability of the uniporter is near unity when the mitochondria are at their resting, hyperpolarized state[63]. Nonetheless, a uniporter that was open under basal conditions would rapidly dissipate the mitochondrial membrane potential leading to the inability to generate ATP. Several subsequent studies have suggested that knockdown of MICU1 actually increases mitochondrial calcium uptake, especially at low levels of calcium[57, 64]. These studies support the notion that MICU1 binds to and inhibits MCU opening at low cytosolic calcium levels, thereby acting as a molecular gatekeeper. These reports, however, do differ in what role they place on the EF hands of MICU1. In one study, mutation of the EF hand domain abrogated the ability of MICU1 to act in its gatekeeper role[64]. The authors suggested that the high affinity EF hands sensed matrix calcium levels and thereby inhibit MCU when the EF hands of MICU1 are complexed with calcium. In contrast, the other study proposed a model in which MICU1 senses inter-membrane levels of calcium (not matrix calcium levels) and inhibits MCU channel activity when the EF hands of MICU1 are in the apo or calcium-free state[57]. These authors, in contrast, proposed that calcium binding to the EF hands of MICU1 actually results in augmentation of MCU-dependent calcium uptake. As such, in this model, MICU1 has two opposing functions based on mitochondrial inter-membrane calcium levels. With low calcium levels MICU1 inhibits MCU, while at higher calcium levels MICU1 functions to activate the channel. This property could potentially explain the sigmoidal shape curve that is seen when calcium uptake is assessed as a function of calcium concentrations over the nanomolar to micromolar range[57]. The notion that the calcium-free form of MICU1 acts as negative regulator of MCU was also supported by recent structural studies of the calcium-bound and calcium-free MICU1 crystal structures[65]. These studies suggested that in the absence of calcium, MICU1 adopts a hexamer configuration that can interact with MCU and presumably inhibit channel activity. Binding of calcium to the EF hands of MICU1 results in a large conformational change in the protein, thereby converting the hexamer into a mixture of activating oligomers. Furthermore, this study measured the affinity of the EF hands of MICU1 for calcium at approximately 15–20 μM. Given that resting cytosolic calcium is significantly less than 1 μM, under basal conditions, if MICU1 senses the inter-membrane space (that is essentially in equilibrium with cytosolic calcium levels), one would predict it would indeed be in the apo state under non-stimulated, basal conditions.

As mentioned previously, the interpretation of MICU1 knockdown experiments needs to be interpreted with caution. This was made abundantly clear with the discovery of a paralog of MICU1, now termed MICU2[62]. MICU2 is similar to MICU1 (approximately 40% sequence similarity) containing two EF hands and thought to be located facing the matrix side of the inner mitochondrialmembrane. MICU1 and MICU2 interact in the presence of calcium or EGTA, and knockdown of MICU1 dramatically reduces MICU2 expression, at least in some cell lines. This has led to concerns that the positive or negative actions on calcium uptake ascribed to MICU1 might in fact be a function of an inadvertent reduction in MICU2. Indeed, one recent paper has proposed that MICU2 not MICU1 is actually the inhibitor of MCU current at low calcium concentrations, while MICU1 acts to stimulate MCU activity at

higher calcium concentrations[66]. Thus, these authors argue, the sigmoidal shape of calcium uptake across the inner mitochondrial membrane is due to an inhibitory effect of MICU2 at low calcium levels, and a stimulatory effect of MICU1 at high calcium concentrations. These authors also suggested that MICU1-MICU2 interaction occurs through a disulfide bond (cysteine 464 in MICU1 and cysteine 410 in MICU2). In contrast to this model that ascribes separate functions to MICU1 and MICU2, others have suggested that these two paralogs act cooperatively[55]. This cooperative model is based on a number of observations using cell-based knockout strategies. For instance, a zinc finger nuclease strategy to knockout MICU1 in 293T cells eliminated MICU1 expression and correspondingly reduced MICU2 levels by more than 80%. In contrast, knockout of MICU2 eliminated MICU2 expression without significantly altering MICU1 levels. Nonetheless, both knockout cell lines had augmented calcium uptake at low calcium levels (suggesting a loss of gatekeeper function), while expression of EF hand mutants of either MICU1 or MICU2 dramatically inhibited calcium uptake at high calcium levels. These data therefore supports a model in which a rise in inter-membrane calcium allows calcium binding to the EF hands of MICU1 and MICU2, which presumably induces a structural change in the complex that is required to ultimately open the pore at elevated calcium levels (Figure 2). It should also be considered that mutation or deletion of the MCU regulatory subunits could have indirect effects on calcium uptake for example by altering mitochondrial membrane potential or matrix pH. Interestingly, there has been a recent description of loss-of-function recessive mutations occurring at the MICU1 locus in humans[67]. These individuals develop both neurological and skeletal muscle abnormalities. Primary fibroblasts from subjects with this condition appear to show elevated basal levels of calcium, again consistent with a potential gatekeeping function of MICU1. Nonetheless, it is unclear whether this increase in mitochondrial calcium is responsible for the significant clinical findings observed.

The uniporter complex grows in complexity

As noted above, significant questions remain on the precise regulatory role of MICU1 and MICU2. Furthermore, there are unresolved issues regarding the exact topology and stoichiometry of the uniporter complex. On blue native gels, MCU is detected in a protein complex of approximately 480 kD[68]. Quantitative mass spectrometry revealed that this complex contained MCU, MICU1, MICU2 as well as an additional 10 kD protein named EMRE (essential MCU regulator). EMRE is a transmembrane, inner mitochondrial membrane protein found in most metazoan species which when knocked-down in 293T cells, appears to abrogate mitochondrial calcium uptake. In the absence of EMRE, MCU no longer appeared associated with MICU1 or MICU2, although MCU was still present and appeared to form oligomers[68]. This led the authors to conclude that EMRE is essential for uniporter activity and to propose a topology where MCU and EMRE interact in the inner mitochondrial membrane (and potentially in the matrix) while EMRE was essential for MICU1 binding in the inner membrane space (Figure 3). Reconstitution of uniporter activity has been recently described in yeast, an organism that does not have an electrogenic mechanism for mitochondrial calcium uptake[69]. Interestingly, expression of human MCU and EMRE is required to endow yeast with uniporter activity, while expression of either component individually is insufficient. Nonetheless, the precise role that EMRE plays is

unclear and lower organisms such as Dictyostelium appear to have a uniporter that does not require this component[69].

The growing complexity of the uniporter complex is further exemplified by the description of MCUb, a protein that shares approximately 50% homology with MCU and can physically interact with the pore forming unit[56]. Silencing of MCUb increases calcium uptake while overexpression results in a reduction of MCU activity. This suggests that MCUb acts in a dominant negative fashion. The expression of MCUb (which is confined to vertebrate species) varies widely from tissue to tissue and may help at least partially explain the tissue-specific variation in MCU activity that has been observed[70]. In this context, when MCUb and MCU are expressed at similar levels, the pore that forms is non-functional due to the dominant negative action of MCUb, while when MCUb is not expressed at high levels, MCU complexes largely with itself to form an active uniporter. As such, it seems possible that MCUb expression may be a mechanism to reduce MCU activity stably and over a long period of time, while MICU1 and or MICU2 appear responsible for acute gatekeeping functions. Finally, a number of additional proteins may associate with the uniporter complex. For instance, two separate mitochondrial proteins, MCUR1[64] and SLC25A23[71], have both been suggested to modulate calcium uptake through the uniporter. Their exact function however have not been completely discerned, and for the case of MCUR1, recent evidence suggests that the previously observed effects may in fact be due to indirect modulation of the mitochondrial membrane potential and not the uniporter itself[72].

Lessons from the MCU-KO mice

MCU-KO mitochondria do not take up calcium

The identification of the MCU allowed for studies to test the regulation and role of mitochondrial calcium in vivo. MCU knockout (MCU-KO) mice were recently developed and characterized[59]. Mitochondria isolated from heart or liver of MCU-KO mice showed no mitochondrial calcium uptake following addition of extra-mitochondrial calcium. These studies were performed by measuring calcium accumulation in the matrix, using mitochondria loaded with a calcium sensitive fluorescent indicator, as well as by following the loss of calcium from the extra-mitochondrial medium with a fluorescent calcium indicator (Ca Green) in the extracellular space. Similar experiments were performed in permeabilized cells to probe mitochondrial calcium uptake in situ (thapsigargin was added to inhibit SR calcium uptake). Together, these studies confirmed the apparent complete lack of rapid mitochondrial calcium uptake in the MCU-KO mice. Similar results were recently obtained in permeabilized cardiomyocytes isolated from a mouse with cardiac specific expression of a dominant negative MCU (DN-MCU), containing a mutation in the pore domain[73]. Again, there was no observed calcium uptake into mitochondria obtained from permeabilized myocytes generated from these DN-MCU mice[73]. Taken together, these data suggest that in the absence of MCU activity there is no measureable calcium uptake, implying that if other mitochondrial calcium uptake pathways exist, they either have a very slow rate of uptake or they are not operating under these experimental conditions. However, isolated mitochondria from the MCU-KO hearts contained significant measurable calcium (~25% of that present in the WT mitochondria), possibly suggesting that alternative

mechanism for mitochondrial calcium accumulation (albeit reduced) do exist in the absence of MCU expression. It is therefore quite possible that additional slow calcium uptake mechanisms exist. It is also possible that calcium can enter the mitochondria, particularly in de-energized isolated mitochondria, via the "reverse mode" of the NCLX. Future studies will be needed to address this issue.

Mitochondrial calcium and metabolism

Alterations in mitochondrial calcium uptake and mitochondrial calcium levels would be expected to alter the activity of the mitochondrial dehydrogenases and thereby alter metabolism. However, no differences in basal oxygen consumption were seen between WT and MCU-KO mouse embryonic fibroblasts (MEFs) or with isolated mitochondria[59], although this may not be surprising since an increase in mitochondria calcium is likely only needed to coordinate an increase in work with increased ATP. In addition, there were no differences in total body basal oxygen consumption between the WT and $MCU^{-/-}$ mice. These observations are consistent with initial MCU knockdown experiments that noted no changes in mitochondrial respiration when MCU expression was reduced[3]. Thus, it would seem that basal metabolism is not markedly altered in the absence of MCU expression. In contrast to basal oxygen consumption, the loss of MCU largely blocked the calcium stimulated increase in oxygen consumption in isolated skeletal muscle mitochondria[59]. However, only in skeletal muscle and under conditions of maximum work, was there a modest, but discernable physiological difference between WT and MCU-KO mice. In this specific setting, compared to the WT mice, the MCU knockout mice had reduced ability to generate maximal power[59].

Role of MCU in the cardiovascular system

Isoproterenol stimulation of heart rate and MCU

It was also expected that beta-adrenergic stimulation of work might be compromised in the MCU-KO hearts because they would not be able to appropriately stimulate ATP production due to lack of an increase in mitochondrial calcium uptake by MCU. Consistent with this expectation, a recent report noted a blunted increase in heart rate in response to isoproterenol in hearts expressing the DN-MCU[73]. Echocardiography measurements showed that basal heart rates were similar between wild-type and DN-MCU hearts. However, addition of isoproterenol increased heart rate to ~650 bpm in wild type hearts, but only to ~500 bpm in DN-MCU hearts. This blunted response to heart rate was attributed to an inability of mitochondria to increase ATP production when MCU was inactivated. This relatively modest effect of MCU on heart rate after isoproterenol stimulation could possibly be explained by the reported low abundance of MCU dependent calcium current in the heart compared to other tissues[70]. The authors also reported a significant decrease in basal ATP in freshly isolated atrial tissue from DN-MCU mice; ATP in WT atria was 250 nmol/mg whereas ATP in atria from DN-MCU was only 75 nmol/mg protein.

Mitochondrial calcium and cell death

Because high levels of mitochondrial calcium uptake via the MCU leads to opening of the mPTP, it was hypothesized that mitochondria from MCU-KO mice, which do not

accumulate matrix calcium, would not undergo calcium activated mPTP. Consistent with this hypothesis, exogenous calcium levels that activated mPTP in WT mitochondria did not activate mPTP in mitochondria isolated from MCU-KO mice[59].

CaMKII phosphorylation sites on MCU have been identified and activation of CaMKII, by addition of a constitutively active CaMKII mutant, is reported to increase MCU current in mitoplast. It was further demonstrated that cardiac specific expression of a mitochondrial and membrane targeted CaMKII inhibitory protein reduced mPTP and I/R cell death[74]. However others have reported a much lower current for MCU and did not find data supporting a role for CaMKII regulation of MCU[75]. Additional studies will be needed to resolve this issue. Inhibition of CaMKII could have other targets in addition to MCU that could contribute to cardioprotection.

As MCU-KO mitochondria were found to be resistant to calcium activation of mPTP, it was further hypothesized that MCU-KO hearts would have reduced cell death following ischemia and reperfusion. Surprisingly, following ischemia and reperfusion, MCU-KO hearts had infarct size and recovery of contractile function that was indistinguishable from WT hearts. While there was no mPTP opening in MCU KO mitochondria exposed to large amounts of calcium, this did not result in measurable cardioprotection. Presumably, the absence of MCU expression does not preclude mPTP opening in a calcium-independent manner in $MCU^{-/-}$ hearts. This would be consistent with suggestions that ROS, rather than calcium, may act as the primary activator of mPTP in vivo[76, 77]. If mPTP activation actually does occur in MCU-KO heart, then one would expect that cyclosporine A, an inhibitor of the mPTP, should still provide protection in the MCU-KO hearts. This was indeed tested in the MCU knockout mice. Following ischemia and reperfusion, WT hearts were protected from necrosis by the administration of cyclosporine A, but MCU-KO hearts were not. These data suggest that in contrast to the WT hearts, inhibition of the mPTP is not protective in the MCU-KO hearts. There are several possible explanations for this surprising finding. One explanation is that in the absence of MCU expression, there are compensatory mechanisms that lead to an up-regulation of an alternative cell death pathway, one that does not involve mPTP opening. If there are compensatory mechanisms, it would be consistent with observations that acute inhibition of MCU results in cardioprotection[78]. Another possible explanation is that mPTP opening is occurring in the MCU-KO hearts, but it is not inhibited by cyclosporine A, perhaps because the stimulus for mPTP opening is somehow greater in MCU-KO hearts than in the WT hearts. It is well established that mPTP opening, while facilitated by cyclophilin D, can still occur in its absence or in the presence of cyclosporine A, as long as the stimulus is sufficiently great[52, 79]. Finally, the lack of protection against ischemia and reperfusion in the MCU-KO hearts might suggest that cytosolic calcium, rather than mitochondrial calcium is the key determinant of cell death.

Summary

For over five decades, the question of how calcium entered the mitochondria remained a biological mystery. Even in the absence of knowing precisely how calcium passed through the inner mitochondrial membrane, numerous lines of evidence still emerged demonstrating the important regulatory role of mitochondrial matrix calcium. Among these observations

was the concept that in small amounts, mitochondrial calcium was essential to fine-tune cellular energetics, while in larger amounts, mitochondrial calcium was critical for initiating cell death. The last few years has resulted in remarkable progress in obtaining a more molecular understanding of these events. This progress has been catalyzed by the discovery of MCU and its accessory proteins. Numerous questions however remain. In particular, how is the uniporter complex regulated so that it only allows calcium entry under stimulated conditions? What is the topology/structure of the uniporter complex? What components are essential to its function and how is the observed tissue variation in uniporter activity achieved? Even more important, what is the physiological role of mitochondrial calcium in areas as diverse as bioenergetics to cell death? The characterization of additional mouse models in which MCU and its interacting partners are deleted in either a whole body or tissue-specific fashion will be critical in addressing these remaining questions. Given the overall pace of late, it's unlikely we will have to wait another five decades for these answers.

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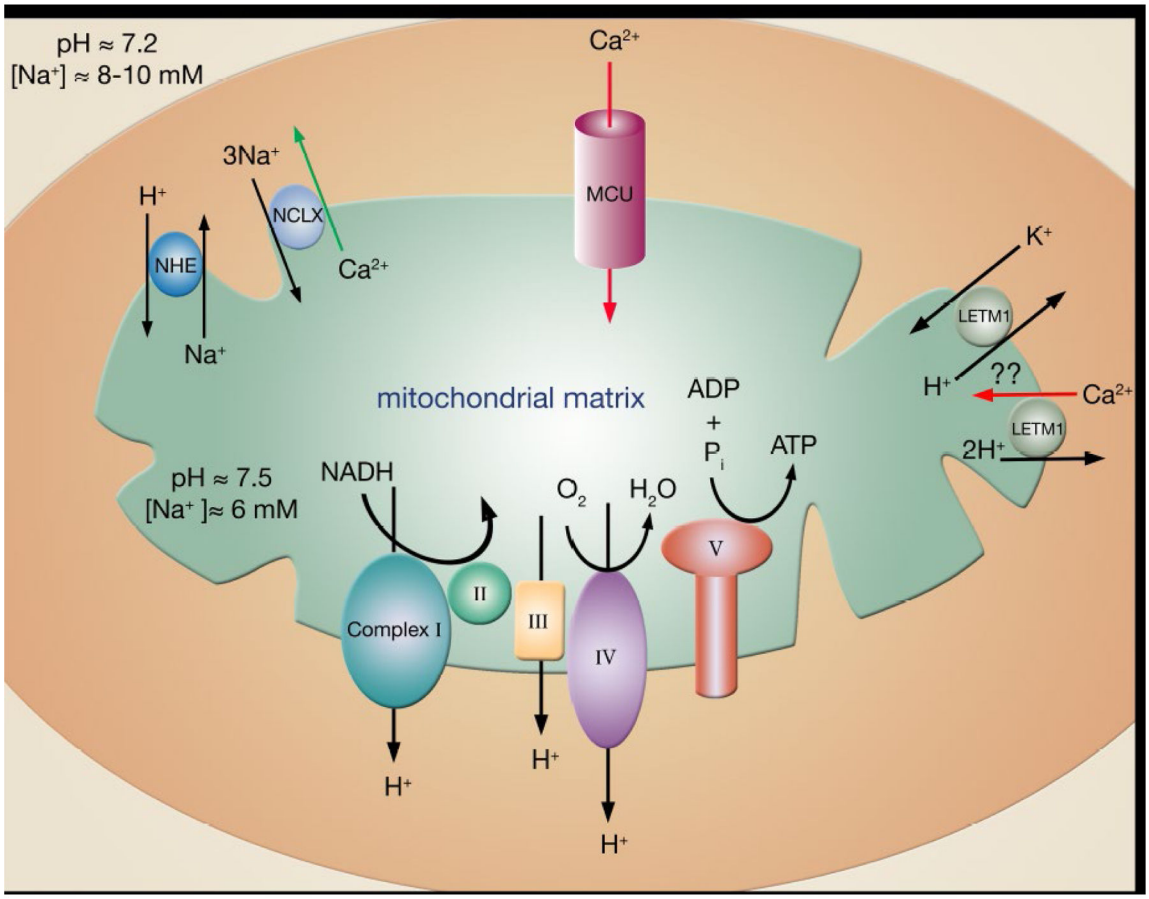

Figure 1:

Mitochondrial ion transport mechanisms involved in regulating calcium entry (red arrows) and efflux (green arrows). Calcium enters the mitochondria primarily through the mitochondria calcium uniporter (MCU). Although MCU appears to be the main calcium influx pathway, other influx mechanisms such as RyR1 (not shown) or LETM1 have also been proposed[13–15]. Questions remain however, as others have suggested that LETM1 is, in fact, a mitochondrial K^+/H^+ exchanger[16, 17]. Calcium efflux is driven by NCLX using the influx of sodium down its electrochemical gradient. The intracellular sodium is set below the cytosolic sodium by the sodium-proton exchanger (NHE), which uses the energy of the inwardly directed proton gradient to maintain mitochondrial sodium below the concentration of sodium in the cytosol.

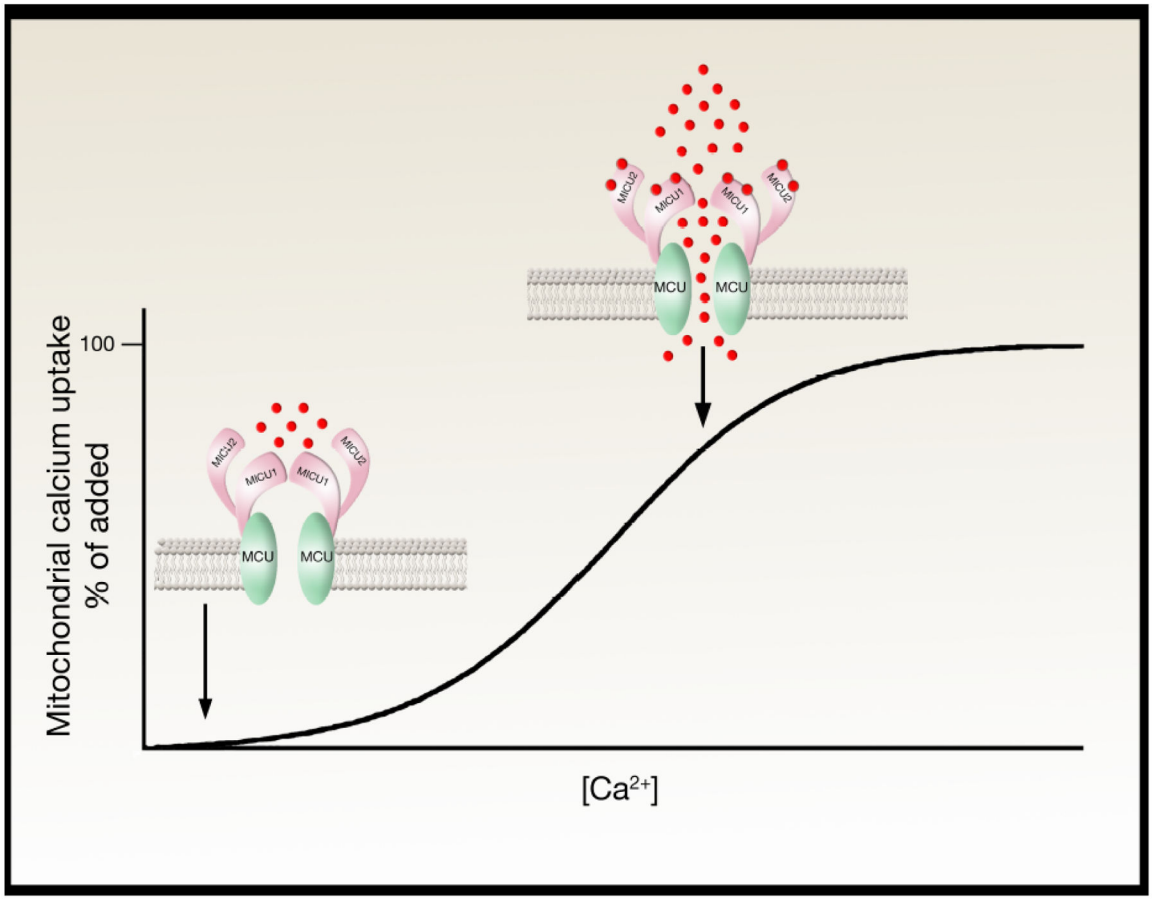

Figure 2:

Potential model to explain the observed sigmoidal calcium response curve. In this model, at low calcium concentrations (calcium ions are indicated as red circles), MICU1 and MICU2 act as a gatekeeper, preventing calcium entry. At higher calcium levels, calcium binds to the EF hands of MICU1/MICU2, resulting in a conformational change in these proteins, opening the pore and stimulating calcium entry. This is one potential model, however, additional models exist including where MICU1 and MICU2 have independent and opposite functions. See text for details.

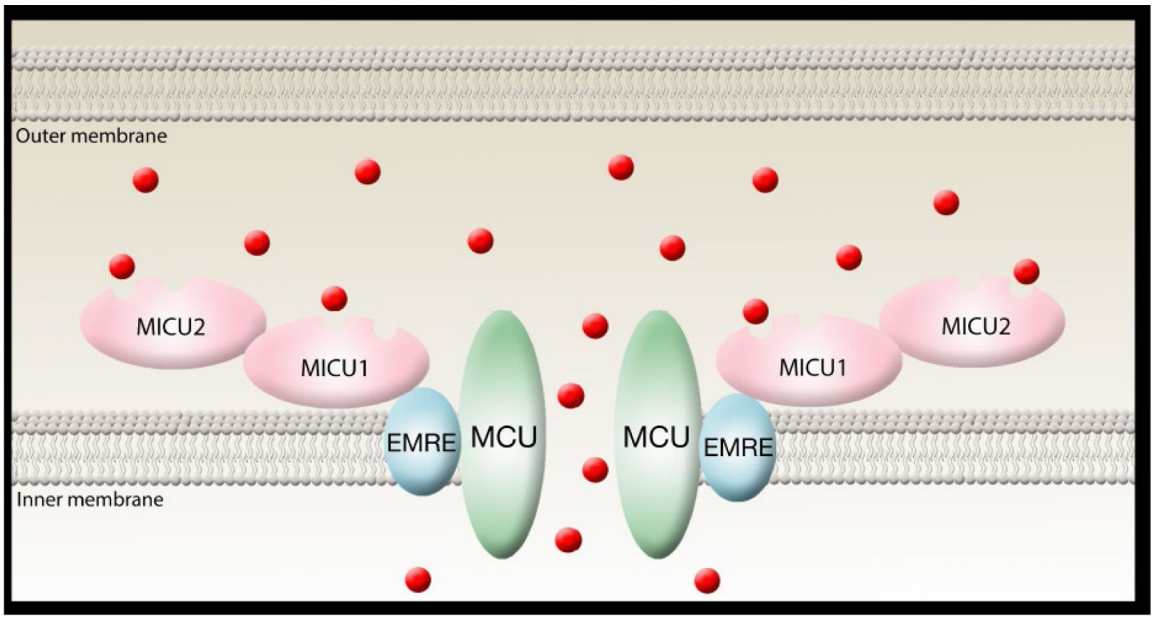

Figure 3:

Molecular components of the uniporter complex. Data strongly suggest that MCU is the pore. Most but not all experimental evidence suggests that MICU1 and MICU2 are located within the inner mitochondrial space. EMRE appears to link MCU with MICU1 and to be necessary for MCU to open and allow calcium entry (red circles) into the mitochondrial matrix. Not pictured here is MCUb, the dominant negative form of MCU that exists in various ratios with MCU. See text for further details.