The Ins and Outs of Mitochondrial Calcium

Toren Finkel, Sara Menazza, Kira M. Holmström, Randi J. Parks, Julia Liu, Junhui Sun, Jie Liu, Xin Pan, Elizabeth Murphy

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 thought 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 past 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. (Circ Res. 2015;116:1810-1819. DOI: 10.1161/CIRCRESAHA.116.305484.)

Key Words: calcium signaling ■ cell death ■ mitochondria
Mitochondrial Calcium Uptake

The first clear description of the ability of mitochondria to rapidly uptake calcium came in the early 1960s, with the work of Vasington, Murphy, De Luca, and Engstrom (reviewed in the study by Carafoli). During 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 and its more specific form Ru360. Mitochondrial calcium uptake was reported to have a low affinity (high Km) of 5 to 10 μmol/L. High extramitochondrial calcium and calmodulin antagonists were also shown to inhibit calcium uptake into the mitochondria. 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. To explain these and other results, several different molecules have been implicated as potential mediators of mitochondrial calcium uptake. For instance, the skeletal ryanodine receptor 1, the primary calcium release channel in skeletal sarcoplasmic reticulum, was reported to be present in mitochondria and ryanodine, an inhibitor of ryanodine receptor 1, was shown to block mitochondrial calcium uptake. 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 mitochondrion 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 Efflux

In steady state, mitochondrial efflux must equal mitochondrial uptake. Mitochondrial efflux is primarily attributed to the function of the Na+/Ca2+–Li+ exchanger (NCLX), which can be inhibited pharmacologically by CGP37157. 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. Matrix Na+ is reported to be lower than cytosolic Na+ because of 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. Although the pH gradient in isolated mitochondria is typically measured at pH 7 units (often in nonphysiological buffers), it seems 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 Ca2+ ion. 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 and the Na+ gradient is favorable (see Mitochondrial Calcium Efflux section of this article).

In addition to the confusion as to what protein might mediate mitochondrial calcium uptake, there was for a long period of time, considerable confusion about how this process could occur. Measurements of basal cytosolic calcium were thought to be in the range of 100 nmol/L and peak calcium transients were <1 μmol/L, given the measured low affinity (~5–10 μmol/L) for calcium uptake, it initially seemed 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 endoplasmic/sarcoplasmic reticulum) and that the concentration of calcium in these microdomains is in fact high enough to allow calcium uptake via the uniporter.

Mitochondrial Calcium Efflux

In steady state, mitochondrial efflux must equal mitochondrial uptake. Mitochondrial efflux is primarily attributed to the function of the Na+/Ca2+–Li+ exchanger (NCLX), which can be inhibited pharmacologically by CGP37157. 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. Matrix Na+ is reported to be lower than cytosolic Na+ because of 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. Although the pH gradient in isolated mitochondria is typically measured at pH 7 units (often in nonphysiological buffers), it seems 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 Ca2+ ion. 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 extramitochondrial Na+ (or Li+) 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. The NCLX was recently identified 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.

In isolated mitochondria, matrix calcium can vary widely depending on the extramitochondrial calcium level.

<table>
<thead>
<tr>
<th>Nonstandard Abbreviations and Acronyms</th>
</tr>
</thead>
<tbody>
<tr>
<td>DN</td>
</tr>
<tr>
<td>EMRE</td>
</tr>
<tr>
<td>MCU</td>
</tr>
<tr>
<td>NCLX</td>
</tr>
<tr>
<td>WT</td>
</tr>
</tbody>
</table>
Measurement of in situ matrix calcium in cardiomyocytes is reported in the range of 100 to 200 nmol/L at low pacing rates and increases to 500 to 800 nmol/L with β-adrenergic stimulation. It is controversial as to whether mitochondrial matrix calcium transients actually track the changes in cytosolic calcium 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 mechanism.29,30 Although recent data question this hypothesis,31 it is not clear whether in situ mitochondrial calcium operates in the range in which matrix calcium is buffered by phosphate (above or near maximal stimulation under basal conditions).

Buffering of Matrix Calcium
Mitochondrial calcium buffering can also affect the level of free matrix calcium. As shown by several groups,32–34 at levels below ≈10 nmol Ca2+/mg of mitochondrial protein, uptake of mitochondrial calcium leads to an increase in free matrix calcium. Thus below 10 nmol Ca2+/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 >10 nmol/mg matrix-free calcium remains stable in the range of 1 to 5 μmol/L because of buffering by calcium phosphate. In fact, Nicholls35 showed that with total mitochondrial calcium levels above or =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 or =10 nmol Ca2+/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.36 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 1980s, Denton and McCormick36 showed that calcium could regulate the activity of 3 mitochondrial dehydrogenases, such as pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase. 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 nicotinamide adenine dinucleotide, fueling electron transport and increasing the output of ATP.37 More recently, Territo et al38 demonstrated that calcium also leads to activation of complex V, the F1F0 ATPase, an enzyme that converts the mitochondrial protomotive force into ATP. Their observations were that in larger species with slower heart rates (eg, 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 Pn/m mole 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 of bioenergetics 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 after ischemia and reperfusion.\textsuperscript{50-44} 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.\textsuperscript{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 <1.5 kDa; this channel is referred to as the mitochondrial permeability transition pore (mPTP).\textsuperscript{46} Calcium uptake via the MCU is thought to be the primary mechanism to activate the mPTP, and inhibitors of MCU have been reported to protect cells from chemical hypoxia\textsuperscript{47} and reduce cerebral and cardiac ischemia-reperfusion injury in vivo.\textsuperscript{48} Chalmers and Nicholls\textsuperscript{13} also reported that total mitochondrial calcium rather than free matrix calcium was the trigger for opening the mPTP. Later studies showed that cyclosporin A could inhibit the mPTP, which reinforced the concept that mPTP opening was not because of just nonspecific membrane rupture.\textsuperscript{49,50} Mitochondrial cyclophilin D was soon identified as the target of cyclosporine A.\textsuperscript{50} The identification of an inhibitor of the mPTP allowed studies to show that addition of cyclosporine A reduced ischemia-reperfusion injury.\textsuperscript{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 after ischemia and reperfusion.\textsuperscript{52} A small proof of concept clinical trial has also reported that administration of cyclosporine A to patients with myocardial infarction is beneficial.\textsuperscript{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.\textsuperscript{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.\textsuperscript{55} 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 2 groups.\textsuperscript{3,4} Both identified an approximate 40 kDa inner mitochondrial membrane protein with 2 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.\textsuperscript{3} Moreover, mutations in the acidic linker domain, between the 2 transmembrane domains, resulted in markedly diminished calcium uptake, with the appearance of a protein having a dominant negative phenotype.\textsuperscript{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.\textsuperscript{4} Nonetheless, this conclusion is not universally accepted.\textsuperscript{55} There is also some disagreement on the orientation and topology of MCU in vivo seems to exist as a tetramer.\textsuperscript{56} Nonetheless, it would seem that the most probable configuration has the linker region of MCU facing into the intermembrane space, whereas the N-terminal and C-terminal portions of the protein are in the matrix.\textsuperscript{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) to allow for live-cell proteomics.\textsuperscript{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. Although electrophysiological studies in MCU-knockout mitoplasts have not been performed, there seems little doubt that the 40 kDa 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.\textsuperscript{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).\textsuperscript{60} MICU1 is a 54 kDa protein containing 2 calcium-sensing EF hand domains. The identification of MICU1 was achieved through an ingenious targeted screen that used the MitoCarta database, a proteomic inventory of \( \approx 1000 \) known proteins that localize to the mitochondria.\textsuperscript{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 *Saccharomyces 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 18 altered mitochondrial calcium uptake. One candidate did, a poorly characterized gene that was subsequently renamed MICU1. The fact that silencing MICU1 seems 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 1 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 intermembrane 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 2 opposing functions based on mitochondrial intermembrane calcium levels. With low calcium levels MICU1 inhibits MCU, whereas 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 ≈15 to 20 μmol/L. Given that resting cytosolic calcium is significantly <1 μmol/L, under basal conditions, if MICU1 senses the intermembrane 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 (∼40% sequence similarity) containing 2 EF hands and thought to be located facing the matrix side of the inner mitochondrial membrane. 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, 1 recent article has proposed that MICU2 not MICU1 is actually the inhibitor of MCU current at low calcium concentrations, whereas 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 because of 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 2 paralogs act cooperatively.65 This cooperative model is based on several 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 >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), whereas expression of EF hand mutants of either MICU1 or MICU2 dramatically inhibited calcium uptake at high calcium levels. These data, therefore, support a model in which a rise in intermembrane 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 seem 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 about the exact topology and stoichiometry of the uniporter complex. On blue native gels, MCU is detected in a protein complex of ≈480 kD.68 Quantitative mass spectrometry revealed that this complex contained MCU, MICU1, MICU2 as well as an additional 10 kDa protein named essential MCU regulator (EMRE). EMRE is a transmembrane, inner mitochondrial membrane protein found in most metazoan species which when knocked down in 293T cells, seems to abrogate mitochondrial calcium uptake. In the absence of EMRE, MCU no longer seemed associated with MICU1 or MICU2, although MCU was still present and seemed 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), whereas 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, whereas expression of either component individually is insufficient. Nonetheless, the precise role that EMRE plays is unclear and lower organisms such as Dictyostelium seem 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 \approx 50% homology with MCU and can physically interact with the pore-forming unit.56 Silencing of MCUb increases calcium uptake, whereas 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 nonfunctional because of 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 over a long period of time, whereas MICU1 and or MICU2 seem responsible for acute gatekeeping functions. Finally, several additional proteins may associate with the uniporter complex. For instance, 2 separate mitochondrial proteins, MCUR1\textsuperscript{64} and SLC25A23\textsuperscript{71}, have both been suggested to modulate calcium uptake through the uniporter. Their exact function, however, has not been completely discerned, and for the case of MCUR1, recent evidence suggests that the previously observed effects may, in fact, be because of indirect modulation of the mitochondrial membrane potential and not the uniporter itself.72

Lessons From the MCU-Knockout Mice

MCU-Knockout 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 mice were recently developed and characterized.59 Mitochondria isolated from heart or liver of MCU-knockout mice showed no mitochondrial calcium uptake after addition of extramitochondrial 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 extramitochondrial 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 sarcoplasmic reticulum calcium uptake). Together, these studies confirmed the apparent complete lack of rapid mitochondrial calcium uptake in the MCU-knockout 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 measurable calcium uptake, implying that if other mitochondrial calcium uptake pathways exist, they either have a slow rate of uptake or they are not operating under these experimental conditions. However, isolated mitochondria from the MCU-knockout hearts contained significant measurable calcium (=25% of that present in the wild-type [WT] mitochondria), possibly suggesting that alternative mechanism for mitochondrial calcium accumulation (albeit reduced) do exist in the absence of MCU expression. It is, therefore, 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.

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 1 potential model, however, additional models exist including where MICU1 and MICU2 have independent and opposite functions. See text for details.
However, no differences in basal oxygen consumption were seen between WT and MCU-knockout mouse embryonic fibroblasts or with isolated mitochondria, although this may not be surprising because 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. 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. However, only in skeletal muscle and under conditions of maximum work, was there a modest, but discernable physiological difference between WT and MCU-knockout mice. In this specific setting, compared with the WT mice, the MCU-knockout mice had reduced ability to generate maximal power.

**Role of MCU in the Cardiovascular System**

**Isoproterenol Stimulation of Heart Rate and MCU**

It was also expected that β-adrenergic stimulation of work might be compromised in the MCU-knockout hearts because they would not be able to appropriately stimulate ATP production because of 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. Echocardiography measurements showed that basal heart rates were similar between WT and DN-MCU hearts. However, addition of isoproterenol increased heart rate to ≈650 bpm in WT 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 with other tissues. 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 mmol/mg, whereas ATP in atria from DN-MCU was only 75 mmol/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-knockout 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-knockout mice. 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. However, others have reported a much lower current for MCU and did not find data supporting a role for CaMKII regulation of MCU. 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-knockout mitochondria were found to be resistant to calcium activation of mPTP, it was further hypothesized that MCU-knockout hearts would have reduced cell death after ischemia and reperfusion. Surprisingly, after ischemia and reperfusion, MCU-knockout hearts had infarct size and recovery of contractile function that was indistinguishable from WT hearts. Although there was no mPTP opening in MCU-knockout 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. If mPTP activation actually does occur in MCU-knockout heart, then one would expect that cyclosporine A, an inhibitor of the mPTP, should still provide protection in the MCU-knockout hearts. This was indeed tested in the MCU-knockout mice. After ischemia and reperfusion, WT hearts were protected from necrosis by the administration of cyclosporine A, but MCU-knockout hearts were not. These data suggest that in contrast to the WT hearts, inhibition of the mPTP is not protective in the MCU-knockout 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 upregulation 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-knockout hearts, but it is not inhibited by cyclosporine A, perhaps because the stimulus for mPTP opening is somehow greater in MCU-knockout hearts than in the WT hearts. It is well established that mPTP opening, whereas 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.52,59 Finally, the lack of protection against ischemia and reperfusion in the MCU-knockout hearts might suggest that cytosolic calcium, rather than mitochondrial calcium is the key determinant of cell death.

Summary

For >5 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, whereas in larger amounts, mitochondrial calcium was critical for initiating cell death. The past 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 unipporter complex regulated so that it only allows calcium entry under stimulated conditions? What is the topol-ogy/structure of the unipporter complex? What components are essential to its function and how is the observed tissue variation in unipporter 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 proteins 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 is unlikely we will have to wait another 5 decades for these answers.

Acknowledgments

We are grateful to Ilsa I. Rovira for help with the article.

Sources of Funding

This work was supported by NHLBI Intramural funds and a grant from the Leducq Foundation.

Disclosures

None.

References


Finkel et al  Mitochondrial Calcium 1819


