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Abstract

Mitochondrial Ca^{2+} uptake is crucial for an array of cellular functions while an imbalance can elicit cell death. In this chapter, we briefly reviewed the various modes of mitochondrial Ca^{2+} uptake and our current understanding of mitochondrial Ca^{2+} homeostasis in regards to cell physiology and pathophysiology. Further, this chapter focuses on the molecular identities, intracellular regulators as well as the pharmacology of mitochondrial Ca^{2+} uniporter complex.

Keywords

Mitochondria, Mitochondrial Ca^{2+} uptake, Mitochondrial Ca^{2+} uniporter, Pharmacology

Abbreviations

Inner mitochondrial membrane	IMM
Intermembrane space	IMS
Outer mitochondrial membrane	OMM
Endoplasmic reticulum/Sarcoplasmic reticulum	ER/SR
Reactive Oxygen Species	ROS
Adenosine triphosphate	ATP
Cytosolic Ca^{2+} concentrations	$[Ca^{2+}]_c$
Mitochondrial Ca^{2+} concentrations	$[Ca^{2+}]_m$
Extramitochondrial free Ca^{2+} concentrations	$[Ca^{2+}]_o$
Mitochondrial Ca^{2+} uniporter	mtCU
Inositol 1,4,5-trisphosphate receptor	IP_3R
Ryanodine receptor	RyR
Mitochondrial Ca^{2+} uptake 1	MICU1
Mitochondrial Ca^{2+} uptake 2	MICU2
Mitochondrial Ca^{2+} uptake 3	MICU3

Essential MCU regulator	EMRE
Mitochondrial Ca^{2+} uniporter regulator 1	MCUR1
Mitochondrial ryanodine receptor 1	mRyR1
Rapid mode of uptake	RaM
Coenzyme Q 10	CoQ10
Transient receptor potential channel 3	TRPC3
Leucine zipper-EF-hand containing transmembrane protein 1	LETM1
Voltage dependent anion channel	VDAC
Ca^{2+}/calmodulin kinase	CaMK
Ca^{2+}/calmodulin-dependent protein kinase II	CaMKII
Neuronal PAS Domain Protein 4	Npas4
Cyclic adenosine monophosphate response element–binding protein	CREB
Electronmicroscopy	EM
Nuclear magnetic resonance	NMR
α1-adrenoceptor	α_1-AR
Proline-rich tyrosine kinase 2	Pyk2
Mitochondrial permeability transition pore	mPTP
Pyruvate dehydrogenase	PDH
α-ketoglutarate/oxoglutarate) dehydrogenase	OGDH)
Isocitrate dehydrogenase	ICDH
Ruthenium 360	Ru360
Transient Receptor Potential Vanilloid	TRPV
TWIK-related Acid-sensitive K^+ channel	TASK-3
Mitogen-activated protein kinase	MAPK

1. Introduction

Mitochondria play an important role in Ca^{2+} homeostasis, which is crucial for balancing cell survival and death.[1, 2] During the 1950s it was observed that isolated mitochondria could accumulate Ca^{2+}.[3] Subsequently, an energy-driven accumulation of Ca^{2+} by isolated mitochondria was demonstrated.[4, 5] It was initially thought that mitochondrial Ca^{2+} transport consists of an active uptake and passive release process,[6] but multiple groups (reviewed by Gunter *et al* 1994)[7] showed that Ca^{2+} uptake across the inner mitochondrial membrane (IMM) is energetically favorable, while efflux requires electrogenic ion-exchange (antiport). This raised the possibility that mitochondria may play a significant role in the regulation or buffering of cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$).[8] Though, mitochondria were one of the first organelle to be associated with intracellular Ca^{2+} handling, the relative low affinity of their Ca^{2+} transport systems, led to the conclusion that they were physiologically irrelevant. It was demonstrated that in suspensions of respiring isolated rat liver mitochondria alone, the steady state extramitochondrial free Ca^{2+} concentrations ($[Ca^{2+}]_o$) of incubating solutions was about 0.5 μM.[9] Addition of microsomes, which contain endoplasmic reticulum (ER) that has Ca^{2+} transport systems with a higher affinity for Ca^{2+} than that of mitochondria, was able to reduce $[Ca^{2+}]_o$ to 0.2 μM. Similar results were obtained in digitonin-permeablized hepatocytes and thus brought forth the idea that the “set point” of $[Ca^{2+}]_c$ is established by the ER Ca^{2+} transport mechanism and not the mitochondria (at ~0.2 μM).[9] However, interest revived in mitochondrial Ca^{2+} homeostasis in the 1990s when the development of Ca^{2+} sensors that can selectively measure the changes in the mitochondrial matrix

Ca^{2+} concentrations ($[Ca^{2+}]_m$) allowed to demonstrate propagation of physiological Ca^{2+} signals from cytosol into the mitochondrial matrix. High Ca^{2+} microdomains at the ER/sarcoplasmic reticulum (SR) and mitochondria interface addressed the discrepancy between the relatively small (approximately 1 μM or less) global $[Ca^{2+}]_c$ peak levels and the much higher in vitro activation range (Kd ≅50 μM) for the mitochondrial Ca^{2+} uniporter (mtCU) in most tissues. The ER/SR, which possesses the Ca^{2+}-release channels, inositol 1,4,5-trisphosphate receptor (IP_3R), and/or ryanodine receptor (RyR), could release Ca^{2+} at the mitochondria/ER/SR junctions with concentrations sufficient to meet the threshold of the mtCU.[10, 11] These groundbreaking studies repositioned mitochondria as key players in the dynamic regulation of cellular Ca^{2+} signaling under physiological conditions.

Ca^{2+} uptake into mitochondria was mostly considered to result from a single transport mechanism mediated by a Ca^{2+}-selective channel of the IMM, the mtCU.[12] The electrophysiological characteristic of mtCU as a highly selective Ca^{2+} activated Ca^{2+} channel (I_{MiCa}) was confirmed by measuring total or single channel ionic current from the IMM of mitoplasts.[13] The discovery of the molecular identity of the mtCU protein complexes was tightly connected to the establishment of MitoCarta, a comprehensive mitochondrial protein compendium in 2008.[14] Based on the establishment of this compendium, the Ca^{2+} sensing EF-hand regulator mitochondrial Ca^{2+} uptake 1 (MICU1) was identified first in 2010 as a regulator of the channel.[15] With one or no predicted transmembrane domain, MICU1 has never been considered to form the mtCU pore. To that end, in 2011, a ~40 kDa protein with two transmembrane domains was discovered as the molecular identity of the mtCU pore termed MCU by the groups of Mootha and

Rizzuto.[16, 17] Following the identification of the MCU, other regulatory subunits were identified in the last five years. These findings open up exciting opportunities for using genetic approaches to elucidate molecular mechanisms that regulate mitochondrial Ca^{2+} uptake in a variety of cell types/tissues. Since the mechanisms for regulating mitochondrial Ca^{2+} concentrations ($[Ca^{2+}]_m$) are critical for fundamental cellular processes, the importance of understanding Ca^{2+} uptake mechanisms in physiology.[18-20] and pathophysiology[21-24] have become increasingly relevant.

In this chapter, we review the current model of the mitochondrial Ca^{2+} influx mechanism, with special focus on the molecular identity of the mtCU complex.

Furthermore, the physiological, pathophysiological and pharmacological implications of mitochondrial Ca^{2+} uptake and future directions of study are discussed.

2. Molecular identities of mitochondrial Ca^{2+} channels/transporters

2.1. Overview

Following the discovery of the pore, MCU, further regulatory subunits were identified, suggesting that the mtCU exists as a multi-protein complex capable of multiple states of MCU activity (i.e., mtCU complex).[16] Proteins in the mtCU complex include trans-membrane subunits [MCU, MCUb and the essential MCU regulator (EMRE)], and membrane-associated regulatory subunits in the intermembrane space (IMS) (MICU1-3) (Fig.1). Mitochondrial Ca^{2+} uniporter regulator 1 (MCUR1) another two-transmembrane domain coiled coil domain containing protein of the IMM was also proposed to interact with the MCU protein and to modulate the channel function;[25] however it was not

present in the ~480 kDa uniporter holocomplex coined as the “uniplex”.[26] In addition to mtCU complex, we also briefly describe other mitochondrial Ca^{2+} channels/transporters that have been reported, which includes mitochondrial ryanodine receptor 1 (mRyR1), rapid mode of uptake (RaM), mCa1 & 2, Coenzyme Q 10 (CoQ10), the transient receptor potential channel 3 (TRPC3), and the Leucine zipper-EF-hand containing transmembrane protein 1 (LETM1).

2. 2. mtCU complex

2.2.1. MCU

The MCU gene (previously known as CCDC109A) is highly conserved across eukaryotes except yeast.[16, 17] The MCU is a 40 kD protein contains a proteolytically cleaved mitochondrial import sequence, two coiled-coil domains, two transmembrane domains, and a short motif of amino acids between the two transmembrane domains critical for Ca^{2+} transport.[16, 17] MCU has been suggested to form the pore as a homo-oligomer and a recent study using nuclear magnetic resonance (NMR) demonstrated a pentameric stoichiometry.[27] Although there was originally some debate about the MCU topology, it is clear now that both the N- and C-termini face the mitochondrial matrix with a short motif of amino acids being exposed to the IMS.[28] Overexpression of MCU increases the rate of mitochondrial Ca^{2+} influx in both intact and permeabilized cells, causing a significant decrease in $[Ca^{2+}]_c$ transients in intact cells.[16] Further, the mutation of two negatively charged residues inside the highly conserved DIME motif (QxGxLAxLTWWxYSWDIMEPVTYF), in the IMS **(**D261Q/E264Q in human MCU)

completely abolishes the MCU activity.[16, 17] On the other hand, the partial knockdown of MCU greatly inhibits the rate and amplitude of mitochondrial Ca^{2+} entry[16, 17] whereas the knockout essentially eliminates rapid uptake of Ca^{2+} pulses[26, 29] and the expression of the wild-type MCU in MCU knockdown cells fully rescues Ca^{2+} uptake profile.[17] Thus, MCU is responsible for Ca^{2+} transport into the mitochondria. As of now, the essential role of MCU for mitochondrial Ca^{2+} uptake was validated in many cell types/tissues including the liver,[17] heart,[30] cardiomyocytes,[31, 32], skeletal muscles,[29] pancreatic β cells,[18] neurons[33] and mammary gland epithelial cells.[24]

2.2.2. MCUb

MCUb, originally reported as CCDC109B, is a 33-kDa protein that shares 50% similarity to MCU with the key amino acid substitutions (R251W, E256V) in the DIME motif.[34] Co-introduction of MCU and MCUb in a lipid bilayer dramatically decreases the open probability compared to only MCU incorporation. In addition, MCUb overexpression in intact cells decreases mitochondrial Ca^{2+} uptake in response to $[Ca^{2+}]_c$ increases, suggesting that MCUb interacts with MCU and acts as an endogenous dominant-negative subunit of the mtCU pore.[34] Interestingly, the ratio of the amount of MCU and MCUb mRNA varies in different tissues.[26, 34, 35] This raises the possibility that the ratio of MCU and MCUb expression may be one of the mechanisms that differentially regulate mitochondrial Ca^{2+} uptake in different tissues.

2.2.3. MICU1-3

MICU1 (previously known as CBARA1/EFHA3) is a 54-kDa protein with two highly conserved EF-hand Ca^{2+}-binding domains.[15] The submitochondrial localization of MICU1 has been a matter of debate[15, 21, 36] but recent proteomic mapping studies[37, 38

as well as interactome analysis of the intermembrane space oxidoreductase MIA40[39] indicate that the MICU1 is a soluble (or membrane associated) protein in the IMS,[23, 40, 41] but not in the matrix. MICU1 is proposed to be pivotal in both the gatekeeping and cooperative activation of mtCU; keeping the channel closed at resting conditions, but promoting cooperative activation of the channel at high Ca^{2+} [23, 42]. Alternatively, MICU1 was also proposed to only convay either of these functions (gatekeeper,[21, 36] cooperative activator).[40]

Additionally, MICU isoforms, MICU2 (known as EFHA1) and MICU3 (known as EFHA2) are also identified.[43] Both MICU2 and MICU3 possess the conserved EF-hand domains, but share only 25% sequence identity with MICU1.[43] Relative expression levels of these MICU isoforms vary across the different tissue types. MICU1 and MICU2 are ubiquitously expressed in mammalian tissues, whereas MICU3 is expressed only in the nervous system and skeletal muscle.[43] Though the role of MICU1 and MICU2 have been extensively studied by several groups, but up to date there is no report attempted to characterize the MICU3 function. MICU2 forms a heterodimer with MICU1, thus indirectly associating with the MCU.[40, 43] Moreover, the stability of MICU2 is dependent on the level of MICU1 expression.[40, 43] Importantly, MICU2 inhibits the function of the MCU at lower $[Ca^{2+}]_c$ levels both in planar lipid bilayers and in intact cells.[40, 44] These data lead to the suggestion that MICU2, would be the gatekeeper of MCU instead of MICU1, which would form a regulatory dimer with MICU2 to modulate MCU channel activity in opposite manner. On the other hand, a recent study by the Mootha group showed that upon disabling the Ca^{2+} sensing by their EF hands, MICU1 and MICU2 both would keep the channel closed and MICU1 would do this even if MICU2 was

ablated (MICU2 KO).[45] This would suggest that MICU1 alone can act as a gatekeeper but the gatekeeping activity would be lifted by lower [Ca^{2+}] than that of MICU2.[44] At low $[Ca^{2+}]_c$, the inhibitory effect of MICU2 is in dominance to safeguard minimal Ca^{2+} accumulation in the presence of a very large electromotive force for cation accumulation. At high $[Ca^{2+}]_c$, however, Ca^{2+}-dependent MICU2 inhibition and MICU1 activation warrant the mitochondria to respond rapidly for bringing adequate amount of Ca^{2+} into matrix during $[Ca^{2+}]$c oscillations so that Ca^{2+}-sensitive steps in ATP production can be stimulated efficiently. A very recent work by the Rizzuto/Raffaello group describes a splice variant of MICU1, termed MICU1.1 containing an insertion of 4 aminoacids (EFWQ) at position 181 of MICU1, that is highly expressed in the skeletal muscle with increased Ca^{2+} binding affinity.[46] This splice variant seem to convay higher sensitivity (lower threshold) for the activation of mtCU further suggesting that MICU1 is instrumental in the gatekeeping of mtCU.

2.2.4 EMRE

EMRE (known as C22ORF32) is a 10-kDa protein that contains a single transmembrane domain and a highly conserved aspartate-rich C-terminal region.[26] While MCU and MICUs are well preserved across phylum, EMRE homologs are not present in plants, fungi or protozoa, indicating that EMRE likely arose in the metazoan lineage.[26] However, within mammals, EMRE is ubiquitously expressed across tissues.[26] Importantly, it has been shown that knockdown or knockout of EMRE completely abolishes mitochondrial Ca^{2+} uptake, indicating that this protein is essential for the functional mtCU channel. EMRE interacts with MCU at the IMM and MICU1 at the IMS, acting as a retainer of MICU1/2 in the mtCU complex.[26, 47-50] A majority of evidence

suggest that the N-terminus of EMRE faces the matrix with the C-terminus facing the IMS.[48, 49, 51]

In addition to the $[Ca^{2+}]_c$ sensing via MICU, MCU may also be regulated by Ca^{2+} and Mg^{2+} from the matrix side. Recent work from the Foskett group has presented electrophysiological (mitoplast patch clamp) evidence for a biphasic (bell-shaped) Ca^{2+} regulation of mtCU from the matrix side with a matrix $[Ca^{2+}]$ activation window of ~0,01-2 μM. The acidic tail of EMRE was shown to be critical for this $[Ca^{2+}]$ regulation from the matrix side and, contrasting other works, was suggested that EMRE would rather have an N_{out}-C_{in} topology and its acidic tail would operate as the luminal Ca^{2+} sensor. Since MICU1/2 were also required for the matrix-side $[Ca^{2+}]$ regulation and considering the overwhelming evidence for EMRE's N_{in}-C_{out} topology, one could entertain an alternative mechanism for EMRE's contribution. EMRE may relay a signal from a distinct matrix Ca^{2+} sensor to the gatekeepers MICU1/2 via the interaction of its C-terminal acidic tail with a lysine-rich basic stretch of MICU1.[48] As to the matrix Ca^{2+} sensor, very recently a comprehensive molecular structure (crystallography) study has identified a Ca^{2+}/Mg^{2+} binding acidic patch on the N-terminal matrix domain of MCU that conveys Mg^{2+} dependent inactivation of the channel.[52] Further studies will be needed to clarify EMRE's role if any in this latter regulation.

2.2.5. MCUR1

MCUR1 (known as CCDC90A) is a 40-kDa protein that consists of two transmembrane domains and one coiled-coil region. The N- and C-termini of MICUR1 is proposed to face the IMS, thus the bulk of this protein exposed to the matrix.[25] Knockdown of MCUR1 not only inhibits agonist-induced mitochondrial Ca^{2+} uptake, but also decreases

basal $[Ca^{2+}]_m$. Overexpression of MCUR1 results in an increase of mitochondrial Ca^{2+} uptake, but only when MCU exists, indicating that MCUR1 is required for Ca^{2+} uptake through the mtCU complex. MCUR1 interacts with MCU, but not with MICU1, suggesting that different compositions of the mtCU complex may exist. Shoubridge and colleagues raised a question about the direct involvement of MCUR1 in the regulation of the MCU complex.[53] They demonstrated that MCUR1 knockdown causes a drop of mitochondrial membrane potential ($\Delta\Psi_m$), proposed that the effect of MICUR1 on MCU activity may be indirect through changing the driving force of Ca^{2+} entry.[53] However, it was demonstrated that MCUR1 binds to the MCU-pore and EMRE through their coiled-coil domains which stabilizes the mtCU complex and loss of MCUR1 reduces the bioenergetics and promotes autophagy.[51] However, a recent study has shown that Drosophila cells lacking the MCUR1 homologue still exhibited typical mtCU Ca^{2+} uptake.[54]

2.3. Other channels

2.3.1 Transport across the outer mitochondria membrane

In order for Ca^{2+} to interact with the mtCU it must first travel across the outer mitochondrial membrane (OMM). Initially the OMM was considered to be freely permeable to Ca^{2+} mostly by way of the highly abundant voltage dependent anion channel (VDAC). Later, a pair of studies demonstrated that increasing the permeability of the OMM via overexpression of VDAC[55] or via treatment with truncated Bid (tcBid)[56] increased the rate of Ca^{2+} influx into the mitochondrial matrix from IP3R-linked high $[Ca^{2+}]$ microdomains. Moreover, it has been shown that physiological $[Ca^{2+}]$ changes

can enhance the cation (K^+) conductance of VDAC reconstituted in bilayer and also enhance the permeability of the OMM to H^+ and ATP in permeabilized cells.[57] Nevertheless, the cation permeability of VDAC reconstituted in a lipid bilayer has been reported higher in the closed state.[58] Thus, VDAC expression levels as well as gating state can modulate mitochondrial Ca^{2+} entry.

2.3.2. mRyR1

Localized in the IMM, mRyR1 is an alternative mechanism for mitochondrial Ca^{2+} uptake in cardiac and neuronal cells.[59-61] RyRs are the largest known ion channels of about >2MDa. Three different subtypes of RyR isoforms (RyR1, RyR2, and RyR3) have been described and cloned, with different pharmacological properties and tissue-specific expression. RyR1, the primary isoform in the skeletal muscle, is considered to be the major Ca^{2+} release channel in SR;[62] RyR2 is most abundant in cardiac muscle cells[63] (and, in a lesser amount, the brain); RyR3 is widely expressed in the ER of different vertebrate tissues[64] and may be coexpressed with RyR1 and RyR2. In cardiac muscle cells RyR2 is abundantly localized in the SR,[65] but RyR1 is also detectable both at the mRNA and protein levels.[66, 67] Using immuno-gold particle and electron microscopy, we reported that a low level of RyR1 is expressed at the IMM in cardiomyocytes, and with higher Ca^{2+} conductance and higher Km for Ca^{2+} binding as compared to mtCU, mitochondrial RyR (mRyR) channels serves as a fast and high affinity Ca^{2+} uptake pathway.[60, 61] Owing to the remarkable biochemical, pharmacological, and functional similarity of RyR in cardiac mitochondria to those of RyR1 in skeletal muscle SR, we designated it as mRyR1.[61] mRyR1 showed a bell-shaped Ca^{2+} dependence of [3H]ryanodine binding with maximal binding at approximately pCa of 4.4 and complete

block at pCa 2 suggestive of RyR1. Moreover, unlike the cardiac SR-RyR2, caffeine showed hardly any effect on ryanodine binding in mitochondria and binding was inhibited by 50% in the presence of 0.33 mmol L^{-1} Mg^{2+}.[68] In permeabilized cardiomyocytes, ruthenium red at a concentration of 1–5 μmol L^{-1} blocked mitochondrial Ca^{2+} uptake with no significant effect on SR Ca^{2+} release.[69] Single channel characterization of the mRyR1 revealed a novel 225-pS cation–selective channel in heart mitoplasts, with four distinct channel conductance (100, 225, 700 and 1000 pS in symmetrical 150 mmol L^{-1} CsCl), which was blocked by high concentrations of ruthenium red and ryanodine, known inhibitors of ryanodine receptors.[70] Ryanodine showed a concentration-dependent modulation of this channel, with low concentrations (10 μmol L^{-1}) stabilizing a subconductance state while high concentrations (≥100 μmol L^{-1}) blocked the channel activity.[70]

Although both the mRyR1 and the MCU are inhibited by low concentrations of ruthenium red (1–5 μM) and Mg^{2+}, the unique single-channel characteristics of mRyR1 clearly differentiate it from previously identified mitochondrial ion channels. Further clarifications will be needed to distinct the roles of mRyR1 and mtCU in the physiological Ca^{2+} signaling activities of the cardiac muscle mitochondria. Interestingly, a recent paper shows that stimulation of IP_3R in adult cardiac myocytes with endothelin-1 causes Ca^{2+} release from the SR, which is uniquely tunneled to mitochondria via mRyR leading to stimulation of mitochondrial ATP production.[71]

2.3.3. RaM

RaM, first studied in isolated liver mitochondria, is a kinetically distinct mode of mitochondrial Ca^{2+} uptake, capable of sequestering significant amounts of Ca^{2+}

hundreds of times faster than the mtCU. RaM is activated only transiently, facilitates mitochondria to rapidly sequester Ca^{2+} at the beginning of each cytosolic Ca^{2+} pulse and rapidly recovers between pulses, which allows mitochondria to respond to repetitive Ca^{2+} transients.[72] Similar to mtCU and mRyR1, RaM was inhibited by ruthenium red, but required over an order of magnitude more than that required for the inhibition of mtCU (0.1 mmol L^{-1}). Likewise, RaM is also activated by polyamines, such as spermine, at a concentration of 0.1 mmol L^{-1} and displayed three times more of an increase in activity than mtCU.[73] In addition, a rapid mode of Ca^{2+} uptake was also proposed in isolated heart mitochondria but with some different transport features from those of liver.[74] The reset time was longer (>60 s) and with less sensitivity towards the inhibition by ruthenium red. Moreover, ATP and GTP activated RaM in liver but not in heart where RaM is activated by ADP and inhibited by AMP. Notably, RaM has always been considered to be potentially an “operating mode” of the uniporter instead of a distinct channel/transporter entity; however, there have been no studies to reconcile RaM with I_{MiCa} or with the thus far identified molecular components of the mtCU complex.

2.3.4. mCa 1&2

mCa1 and mCa2 are both voltage gated mitochondrial Ca^{2+} selective channels similar to mtCU with a maximal conductance of 10.9 and 6.56 pS, respectively, at 105 mmol L^{-1} [Ca^{2+}], and half saturating concentration (K_m) of 15.1 and 19.6 mmol L^{-1} [Ca^{2+}], respectively. They have unique single-channel characteristics and sensitivity to Ru360. mCa1 channels display higher single-channel amplitude, smaller opening time, a lower open probability (P_O=0.053), and multiple subconductance states. While, mCa2 channels have a smaller single-channel amplitude with a lower conductance, longer

openings, a higher open probability and no subconductance states. Like MCU and RaM, both mCa1 and mCa2 were activated by spermine. However, mCa2 was only partially inhibited by μmol L^{-1} concentrations of Ru360.[75] Like RaM, mCa1/2 have not been studied further in the molecular era of mtCU to explore if it was indeed a distinct channel entity or rather the result of a particular (stoichiometric) permutation and/or post-translational modification of the mtCU complex constituents.

2.3.5. CoQ

CoQ10 is an essential component of the mitochondrial electron-transport chain (ETC) with the primary role as an electron and proton transporter. It was also reported that CoQ10 is a regulator of mitochondrial Ca^{2+} and redox homeostasis. Under physiological conditions, hydroxyl CoQs can bind and efficiently transport Ca^{2+}. Hydroxyl CoQs have a very high affinity for Ca^{2+} and therefore, can function at $[Ca^{2+}]_c$ lower than 0.5 μM and potentially even at resting $[Ca^{2+}]_c$ levels.[76] This relatively slower Ca^{2+} transfer might be a component of the thus far unidentified source of small tonic Ca^{2+} accumulation observed in MCU knockout cardiac mitochondria.[77, 78]

2.3.6. LETM1 & TRPC3

LETM1, initially identified as a K^+/H^+ exchanger, was recently reported as a Ca^{2+}/H^+ antiporter. Using a siRNA genome-wide screening in drosophila, it was reported to be localized at the IMM. It transports Ca^{2+} bidirectionally across the IMM in a pH gradient-dependent manner and is inhibited by ruthenium red.[79] However, a recent study with LETM1 protein reconstituted in liposomes demonstrated LETM1 as an electroneutral $1Ca^{2+}/2H^+$ antiporter, insensitive to ruthenium red.[80]

Lastly, TRPC3 was demonstrated as an alternative mitochondrial Ca^{2+} uptake pathway. It is permeable to Ca^{2+}, Na^{+}, and K^{+} and can contribute to mitochondrial Ca^{2+} uptake during conditions with a relatively high extramitochondrial [Ca^{2+}].[81]

3. Transcriptional/post-transcriptional and post-translational regulation of the mtCU complex

As described above (see Section 2), the mtCU is a multisubunit complex with many regulators. However, the expression patterns of each component are variable in a tissue-specific manner[43, 82] for adapting to the appropriate Ca^{2+} sensitivity by intracellular signals in each tissue. Therefore, it is of interest to elucidate how the mtCU complex is differentially regulated at the level of gene expression, which is linked to its modulation of mitochondrial Ca^{2+} uptake. Accordingly, it has been reported that transcriptional and post-transcriptional mechanisms can regulate MCU expression and activity to specific functional demands.[43, 82, 83] For example, in neurons, synaptic activity suppresses MCU transcription through a nuclear Ca^{2+} signals, Ca^{2+}/calmodulin kinase (CaMK) and the transcription factor Npas4 dependent mechanism, preventing excitotoxic death.[33] In addition, the Ca^{2+}-regulated transcription factor cyclic adenosine monophosphate response element–binding protein (CREB) directly binds to the MCU promoter and stimulates MCU expression, regulating mitochondrial metabolism.[84] MCUb expression was also reported to be increased though independent of CREB activation.[84] It has been shown that MCU is also a target of microRNA-25 (miR-25), which can efficiently decrease MCU gene expression and activity.[83] Furthermore, analyses of post-translational modifications of the MCU components are ongoing. In

2012, Joiner *et al* for the first time reported two Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) phosphorylation candidate motifs at the N-terminus of MCU. CaMKII resides endogenously in the mitochondrial matrix and is highly activated during pathophysiological conditions like ischemia reperfusion and myocardial infarction; promotes myocardial death via CaMKII-mediated increases in MCU current, by phosphorylation of MCU at serine 57 and 92. However, mitochondrial CaMKII inhibition reduced MCU current and was protective against ischemia/reperfusion injury, myocardial infarction, and neurohumoral injury.[30, 85, 86] Recently, Lee *et al* showed that MCU-S92A mutant expression failed to rescue the Ca^{2+} channel activity in a MCU knockdown cell line. In addition, they also presented the crystal structure of the N-terminal region of MCU including, (S92) a potential CaMKII phosphorylation site and concluded them to be indispensable for modulation of channel activity.[87] Additionally, our group demonstrated that α1-adrenoceptor (α1-AR) signaling activates Ca^{2+} and ROS dependent proline-rich tyrosine kinase 2 (Pyk2); translocates Pyk2 into the mitochondrial matrix. Activated Pyk2 interacts with MCU and directly phosphorylates MCU tyrosine residue(s) and enhances mitochondrial Ca^{2+} uptake by promoting MCU channel oligomerization and formation of tetrameric channels.[88] However, persistent α1-AR stimulation increases ROS production, activates the mitochondrial permeability transition pore (mPTP) opening and eventually leads to cell death via Pyk2 activation in cardiomyocytes.[32]

4. Physiological roles of mitochondrial Ca^{2+} uptake

Mitochondrial Ca^{2+} has been implicated as an important regulator of fundamental cellular processes, which range from the regulation of cellular metabolism, buffering cytosolic Ca^{2+}, modulating cellular redox environments, to other cell-type specific functions. As described above, we have witnessed a rapid advance in our understanding of the role of mitochondrial Ca^{2+} uptake mechanisms in physiology and pathophysiology since the recent molecular discovery of the mtCU pore (i.e. MCU) and its regulators. Therefore, in the next sections, we summarize the role of mitochondrial Ca^{2+} uptake mechanisms highlighting the functions of the mtCU complex during physiological (Section 4) and pathological (Section 5) conditions.

4.1. Mitochondrial Ca^{2+} and energy metabolism

Mitochondrial Ca^{2+} uptake serves as one of the major factors for regulating cellular bioenergetics.[89, 90] Denton and McCormick in 1980's demonstrated that mitochondrial Ca^{2+} plays an important role in regulating three Ca^{2+} dependent dehydrogenases: pyruvate dehydrogenase (PDH), α-ketoglutarate (also called oxogluterate) dehydrogenase (OGDH) and NAD-isocitrate dehydrogenase (ICDH)[91, 92] that are the rate-limiting enzymes in substrate supply for ATP synthesis.[93] Of the three dehydrogenases, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase are activated through the binding of Ca^{2+} [94] whereas, pyruvate dehydrogenase activation depends on Ca^{2+}-dependent phosphatase mediated dephosphorylation step.[95] Increase in mitochondrial Ca^{2+} uptake can activate oxidative metabolism via activated matrix dehydrogenases, resulting in an increased supply of reducing equivalents to drive

respiratory chain activity and ATP synthesis.[92] Mitochondrial matrix Ca^{2+} also regulate bioenergetics by S100A1 mediated direct Ca^{2+}-dependent activation of F_o-F_1ATP synthase activity.[96, 97]

Surprisingly, mouse embryonic fibroblasts or isolated mitochondria from MCU-knockout mice have apparently well-maintained basal mitochondrial metabolic function and energetics, albeit with decreased Ca^{2+} uptake and lower resting Ca^{2+} levels.[15-17, 25] Even more surprisingly, this lack of energetic phenotype extends to the beating heart *in vivo* under physiological conditions (approximately 500 beats/min), either in germline or inducible cardiac-specific MCU knockout mice.[29, 77, 78, 98] Likewise, though global MCU knockout displayed no evidence of Ca^{2+} uptake in mitochondria yet, basal ATP levels were not evidently altered, indicating that lack of MCU does not have marked impact on basal mitochondrial metabolism.[99] However, skeletal muscle showed a minor defect in muscle strength after endurance training.[29] The mild phenotype of MCU knockout mice could be due to some kinds of adaptation in these animals.[98] Similarly, in a cardiac specific MCU knockout mouse, there is no energetic phenotype *in vivo* under normal physiological conditions. However, these mice displayed a decreased β-adrenergic receptor-mediated fight or flight response for increased workload under stress and a decreased ischemia-reperfusion injury.[77, 78] Similar results have been obtained via cardiac specific overexpression of a dominant negative mutant MCU.[100] These surprising findings have set a stage for seeking other compensatory or unknown mechanisms for the MCU-independent regulation of bioenergetics in beating heart.[101]

Knockdown of MCUR1 reduces mitochondrial Ca^{2+} uptake resulting in disruption of oxidative phosphorylation which activates AMP kinase-dependent pro-survival

autophagy.[25] However, in pancreatic β-cells, knockdown of MCU and MICU1 markedly reduced the mitochondrial Ca^{2+} uptake and showed that, MCU- and MICU1- mediated Ca^{2+} uptake is critical for continual ATP synthesis, glucose metabolism and insulin secretion.[18, 19] MCU silencing down-regulates the expression of respiratory chain complexes, mitochondrial metabolic activity, and oxygen consumption.[102] In addition to MCU, absence of LETM1 decreased basal mitochondrial oxygen consumption, discernible inactivation of complex IV activity and a drop in ATP production.[103] We recently reported that RyR1-overexpressing cardiac cells had higher mitochondrial ATP under basal conditions with augmented $[Ca^{2+}]_c$-dependent ATP production,[104] supporting our previous finding of a low respiratory control index in RyR1 knockout mice and insensitivity to $[Ca^{2+}]_c$ stimulation of O_2 consumption in mice.

4.2. Cytosolic Ca^{2+} buffering

Apart from mitochondria's role as the main energy supplier, its implication in cytosolic Ca^{2+} buffering is becoming increasingly apparent. Mitochondria can directly influence the $[Ca^{2+}]_c$ by importing Ca^{2+} through the MCU and efflux through the Na^+/Ca^{2+} exchanger or H^+/Ca^{2+} exchangers.[7, 12, 105-107] Since the resting $[Ca^{2+}]_c$ values are ~100 nM and the $\Delta\Psi m$ is ~-180 mV, the prediction is that at electrochemical equilibrium, theoretical $[Ca^{2+}]_m$ values could be higher than 0.1 M.[108] However, the low affinity of the MCU to Ca^{2+} (Kd around 10–50 μM), the presence of mitochondrial efflux mechanisms and the decrease of $\Delta\Psi m$ upon the cation influx would avert the attainment of electrochemical equilibrium. Therefore, particularly under resting conditions,

mitochondria may not uptake any Ca^{2+}. Based on these considerations, the evident discrepancy between the low affinity of MCU, the low concentration of global cytosolic Ca^{2+} signals and the amplitude of $[Ca^{2+}]_m$ rises was resolved in 1990's by the concept of a microdomain of high $[Ca^{2+}]_c$ between ER/SR and mitochondria contact areas.[109, 110] According to which, mitochondria are strategically located in close proximity to ER/SR through tethering proteins,[111] and these close contact sites provide mitochondria preferential access to a much higher $[Ca^{2+}]$ than that measured in the bulk cytosol during Ca^{2+} release from ER/SR and able to activate the MCU. These local $[Ca^{2+}]$ exposures of the mitochondrial surface have been measured to be ~10 μM in average by means of "hotspot" mapping of OMM-targeted Ca^{2+} sensor proteins[112] or Ca^{2+} sensors directly targeted to the SR/ER-OMM focal contact areas utilizing a drug-inducible heterodimerization strategy.[113] In addition, there are reports that VDAC in the OMM, and IP3 receptors in the ER are enriched at the mitochondria–ER interface, facilitating a Ca^{2+} transfer from the ER to the mitochondria.[114-116] Several functional and morphological studies further suggested that mitochondria can form close contacts not only with ER/SR[110, 117, 118] but also the Golgi apparatus[119] and the plasma membrane.[120-122] However, among these interactions, the ER/SR-mitochondria connections have gained much attention, and various proteins have been proposed to link mitochondria to the ER/SR such as MIRO, MFN2, and the Mmm1/Mdm10/Mdm12/Mdm34 complex.[123, 124] Therefore, ER/SR-mitochondria communication also serve as a highly localized Ca^{2+} buffering system. This in turn can modify the activity of any nearby Ca^{2+}-dependent proteins. Such regulation has been reported for IP_3R that display isoform-specific biphasic dependence on $[Ca^{2+}]c$. Depending on the dominating IP3R isform, local Ca^{2+}

clearance by mitochondria can either suppress IP3R activation (and Ca^{2+} release from the ER) via reducing the local [Ca^{2+}] (and so IP3 sensitivity) over IP3R clusters;[125, 126] or do the opposite by decreasing [Ca^{2+}] from high inhibitory to stimulatory range.[127] By similar principles, local mitochondrial Ca^{2+} clearance has also been implicated in sustaining the activation of I_{CRAC}/Orai channels during store operated Ca^{2+} entry by relieving local feedback inhibiton of the channels by Ca^{2+}.[128-130]

4.3. Reactive oxygen species (ROS) generation

Mitochondria are a major source of ROS in the cell. It has been well recognized that $[Ca^{2+}]_m$ enhance ROS generation by stimulating the TCA cycle and oxidative phosphorylation[131, 132] and/or triggering opening of mPTP,[133, 134] which plays an important role in the regulation of cellular function. For example, a recent study identified that mtCU-mediated mitochondrial Ca^{2+} uptake triggers mitochondrial ROS production and transient opening of the mPTP, which promotes wound repair and organismal survival.[20] In addition, it has been shown that mitochondrial Ca^{2+}-mediated ROS production modulates neural differentiation through activation of the Wnt/β-catenin pathway.[135] However, excess Ca^{2+} uptake by the mtCU can be detrimental for a cell, triggering excessive ROS generation and initiating cell death pathways such as apoptosis.[21-24] Therefore, mitochondrial Ca^{2+} uptake can be either beneficial or detrimental depending on the amount of Ca^{2+} uptake and cellular conditions. We will discuss the pathological role of mitochondrial Ca^{2+} uptake in Section 5.

5. Pathological roles of mitochondrial Ca^{2+} uptake

As shown in Section 4.2, mitochondrial Ca^{2+} uptake significantly contributes to buffering cytosolic Ca^{2+} under physiological Ca^{2+} release from ER/SR. However, intensive long-lasting pathophysiological release of Ca^{2+} from ER/SR causes persistent mitochondrial Ca^{2+} accumulation, which consequently triggers excessive ROS generation followed by ATP depletion, the opening of the mPTP[136, 137] and apoptotic/necrotic cascade.[137] Accordingly, MCU-overexpressing and MICU1-knockdown human cell lines leads to increased sensitivity to apoptosis.[16, 21] Moreover, human genetic disease associated with MICU1 null mutations exhibiting central nervous system (extrapyramidal symptoms, learning difficulties) and skeletal muscle (fatigue) phenotypes have been recently identified.[138, 139] Liver-specific knockout of MICU1 has been recently shown to severly impair liver regeneration after partial hepatectomy, which phenotype could be almost completely rescued by administration of NIM811, a non-immunosuppressant mPTP inhibitor.[140] In addition, MCU overexpression in *T. brucei* are also sensitized to apoptotic stress.[22] However, MCU overexpression in a human breast adenocarcinoma cell line[24] and MCU-knockout mouse embryonic fibroblasts[29] show no difference in sensitivity to apoptosis.

As discussed above, although, mitochondrial Ca^{2+} increase has been associated with apoptosis in many pathological conditions[141] however, very little is known about the roles of mitochondrial Ca^{2+} signaling in cancer. Marchi *et al.* 2013, showed that microRNA-25 (miR-25) expression can decrease in MCU gene expression and activity.[83] Specifically, miR-25 is up-regulated in human colon and prostate cancers, which leads to decreased MCU levels followed by reduced mitochondrial Ca^{2+} uptake

and resistance to Ca^{2+}-dependent apoptotic challenges.[83] Consistent with these results, overexpression of MCU or knockdown of MICU1 in HeLa cervical cancer cells results in constitutive mitochondrial Ca^{2+} influx and increases HeLa cell sensitivity to hydrogen peroxide and ceramide toxicity.[16, 21] In other cancer paradigms like in triple-negative breast cancer MCU has been identified as a promoter of progression/invasiveness by supporting the mitochondrial Ca^{2+}-ROS-HIF-1α signaling axis.[142] Thus, the suppression of the MCU expression by miRNA provides initial clues to the relevance of this pathway in human cancers.

Recent studies show that genetic and molecular manipulation of the mtCU complex can also affect cell-type specific functions such as neurotransmission, growth and development. MCU overexpression increases NMDA receptor-dependent excitotoxicity in mouse neurons via enhanced mitochondrial calcium uptake resulting in aggravated mitochondrial depolarization and neuronal injury. However, MCU knockdown protects neurons against NMDA receptor-mediated excitotoxic cell death.[33]

6. Pharmacological modulators of the MCU

Despite the well-known role of the MCU as a key controller of Ca^{2+} homoeostasis, there is little information about its pharmacological regulation. Although, several pharmacological inhibitors have been described to modify the activity of the MCU, their lack of specificity and cellular permeability has limited their application (Table 1). One of the most widely studied and effective inhibitors is the hexavalent polysaccharide stain, ruthenium red or its derivate Ru360.[13, 143] In 2011, De Stefani *et al*. demonstrated the

MCU role as the channel-forming subunit, permeable to Ca^{2+} and inhibited by ruthenium red, in an isolated mitochondria. They reconstituted MCU in lipid bilayers and recorded ruthenium red-sensitive Ca^{2+} current with 6-7-pS single-channel activity.[16] These findings was very recently supported by another patch-clamp experiment by Chaudhuri *et al* (2013). They showed parallel changes in the mitochondrial Ca^{2+} current in a MCU knock-down and overexpression system. In addition, by exploiting the inhibitory characteristic of ruthenium red they further confirmed MCU as a pore-forming subunit of the channel complex. They demonstrated that a single point mutation (S259A) in the putative pore domain conferred resistance to ruthenium red[17, 144] without changing current magnitude indicating that that ruthenium red directly targets the channel.

However, ruthenium red binds to and inhibits a wide variety of plasma membrane and intracellular Ca^{2+} and K^+ channels like Transient Receptor Potential Vanilloid (TRPV),[145, 146] TWIK-related Acid-sensitive K^+ channel (TASK-3) [147] and RyR.[148] Ru360, a purified form of ruthenium red, is more effective than ruthenium red with an IC_{50} 5 nM vs 1 µM, respectively.[149] Ru360 also demonstrates better specificity for the MCU over other Ca^{2+} channels in cardiac muscles.[16, 17, 143] Earlier studies have reported a number of drugs exhibiting MCU inhibition such as the cardioactive drugs quinidine, alprenolol, propranolol, oxyfedrine, and tetracaine,[150] the diuretic, ethacrynic acid, amiloride analogs and derivatives,[151] and the antibiotic gentamicin.[152] Minocycline, a tetracycline-derived antibiotic that has been used clinically to treat bacterial infections, is also a potent inhibitor for MCU.[153] Mg^{2+}, an antagonist of mitochondrial Ca^{2+} uptake also inhibits the MCU at physiological concentrations.[154] Lanthanides such as La^{3+}, Gd^{3+} and Pr^{3+} are also well known competitive inhibitors and at low concentrations they may

activate the uniporter's activation site and facilitates the transport of other ions.[155] However, they inhibit a variety of other Ca^{2+} channels and pumps too. Thiourea derivate KBR7943, originally an inhibitor of the plasma membrane Na^{+}/Ca^{2+} exchanger is also reported to have an inhibitory effect on the MCU.[156] In addition, MCU activity is also inhibited by adenine nucleotides; ATP being the most potent inhibitor (EC_{50} 0.6 mM) followed by ADP > AMP. Interestingly, AMPPNP, a non-hydrolysable analog of ATP was also found to be as efficient as ATP, suggesting that inhibitory action does not require ATP hydrolysis.[157] On the other hand, uniporter activity is known to be activated by inorganic phosphate (Pi), which can accelerate the Ca^{2+} uptake rate by precipitating with Ca^{2+} in the mitochondrial matrix, and thereby lowering the $[Ca^{2+}]_m$.[158] The Ca^{2+} influx rate and affinity for Ca^{2+} is modulated by protein kinases. Specifically, the ζ isoform of protein kinase C, will activate, whereas the β/δ isoforms inactivate MCU.[159] Knock-down studies of p38 mitogen-activated protein kinase (MAPK) have resulted in an increase of mitochondrial Ca^{2+} uptake suggesting either itself or its downstream targets can inhibit MCU.[160, 161] Likewise, SB202190, an inhibitor of p38 MAPK, significantly activates mitochondrial Ca^{2+} uptake, both in intact and in permeabilized cells.[162] Other pharmacological activators include, natural plant flavonoids (e.g. genistein, quercetin, kaempferol),[163] polyamines such as spermine and spermidine[164, 165] and estrogens receptor agonists (4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT)).[166] Lastly, MCU mediated Ca^{2+} uptake also displays allosteric positive regulation by cytosolic Ca^{2+} in a calmodulin-dependent manner[167, 168] which was shown to be inhibited by calmodulin inhibitors.[169]

Table: 1 Pharmacological modulators of MCU

Compound	Effect(s)	References
Ruthenium compound: ruthenium red, Ru360	Inhibitor	13, 143
Lanthanides: La^{3+}, Gd^{3+} and Pr^{3+}	Inhibitor	170
Cardioactive drugs: quinidine, alprenolol, propranolol, oxyfedrine, and tetracaine	Inhibitor	150
Amiloride analogs and derivatives	Inhibitor	151
Mg^{2+}	Inhibitor	154
KBR7943	Inhibitor	156
Minocycline	Inhibitor	153, 171
Polyamines: spermine and spermidine	Activator	165
Estrogen receptor agonists: 4,4′,4″-(4-propyl [1H]-pyrazole-1,3,5-triyl)trisphenol (PPT)	Activator	166
The p38 MAP kinase inhibitor: SB202190	Activator	162
Flavonoids	Activator	163

7. Conclusions

Ca^{2+} uptake into the mitochondrial matrix plays a vital role in the regulation of multiple physiological and pathological processes, ranging from cytoplasmic Ca^{2+} signaling to bioenergetics and cell death. Mitochondria can uptake Ca^{2+} via multiple channels and pathways, however, the mtCU complex is the most prominent and well-characterized pathway. In this chapter, we have focused on the recent identification of the components of the mtCU complex as well as the other mitochondrial ion channels. Our understanding about the molecular complexity of mtCU gradually evolved from the concept of a single protein to macromolecular signaling complexes, which includes a Ca^{2+} pore forming component and regulatory components controlling channel activity. We discussed the means by which multiple cell types and tissues regulate and use

these channels to best-function for their physiological role in an organism, as well as how the dysfunction of this system can lead to pathophysiological conditions.

The recent characterization of the mtCU complex has opened up the possibility for precise crystal and cryo-electronmicroscopic (EM) structural information of the individual proteins as well as the complete complex. Finally, future insight into the transcriptional, post-transcriptional, and post-translational modifications of the multi-protein mtCU complex will contribute to the development of more specific pharmacological tools and potentially therapeutic drugs.

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Figure Legend:

Figure 1. The Molecular Structure of the mtCU complex. Composed of MCU and MCUb (the channel forming subunits) together with essential mtCU regulators, EMRE, MCUR1 and intermembrane space proteins, MICU1 and MICU2.

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Figure 1.