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Molecular identities of mitochondrial Ca^{2+} influx mechanism: Updated passwords for accessing mitochondrial Ca^{2+}-linked health and disease

Jin O-Uchi, Shi Pan, and Shey-Shing Sheu

Department of Medicine, Center for Translational Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107

Introduction

Mitochondrial Ca^{2+} homeostasis is crucial for balancing cell survival and death (Giacomello et al., 2007; Duchen et al., 2008). Mitochondrial Ca^{2+} uptake mechanisms across the inner mitochondrial membrane (IMM) are especially important for the regulation of ATP synthesis, the amplitude and spatiotemporal patterns of intracellular Ca^{2+} transients, the mitochondrial fission–fusion, dynamics, the opening of mitochondrial permeability transition pores (mPTPs), and the generation of reactive oxygen species (Gunter and Sheu, 2009; Csordás et al., 2011; Drago et al., 2011). Mitochondrial Ca^{2+} influx was dogmatically considered to result from a single transport mechanism mediated by the mitochondrial Ca^{2+} uniporter (MCU), principally a result of nearly complete inhibition by Ruthenium red and lanthanides (Gunter and Pfeiffer, 1990). However, subsequent studies have also identified additional Ca^{2+} uptake pathways, such as the rapid mode of uptake (RaM) (Sparagna et al., 1995; Buntinas et al., 2001; Bazil and Dash, 2011) and Coenzyme Q10 (Bogeski et al., 2011), which exhibit different Ca^{2+} affinity, uptake kinetics, and pharmacological characteristics from the original MCU theory.

Although the basic functional and pharmacological properties of various mitochondrial Ca^{2+} uptake mechanisms have been well studied, the molecular identities of the channels/transporters responsible for these mechanisms have not been well understood until recently. In this Perspective, we focus on the recent studies that attempted to uncover the molecular identities of mitochondrial Ca^{2+} influx mechanisms using genetic manipulations including small interfering RNA (siRNA) or knockout mice. In particular, we summarize here the recent discoveries of the molecular identity of MCU protein and also discuss the controversies of two other Ca^{2+} influx mechanisms, mitochondrial RyR type 1 (mRyR1) and leucine-zipper-EF-hand–containing transmembrane protein 1 (Letm1), together with the future directions in this research field.

Background

Mitochondria were originally found and studied simply as a cellular power-plant in the first half of the 20th century (Drago et al., 2011). Soon it was also recognized that Ca^{2+} stimulates the Krebs cycle and electron transport chain activity, which results in the stimulation of ATP synthesis (Balaban, 2009; Denton, 2009; Carafoli, 2010). Early studies in the 1960s to 1970s revealed that isolated mitochondria could take up a large quantity of Ca^{2+} (Deluca et al., 1962; Vasington and Murphy, 1962). Surprisingly, super-physiological high Ca^{2+} concentrations ($[Ca^{2+}]$) (10–100 µM) were required to activate Ca^{2+} uptake into isolated mitochondria. However, in the intact cells, less than a 10-µM $[Ca^{2+}]$ increase in the cytosol by receptor stimulation indeed propagated into mitochondria matrix (Rizzuto et al., 1992; Jou et al., 1996). This discrepancy between isolated mitochondria and intact cells was partially resolved by the finding of high cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) at microdomains between mitochondria and ER/SR, which possesses Ca^{2+}-releasing channels, inositol 1,4,5-trisphosphate (IP_3) receptor, and/or RyR because of their physical proximity (Rizzuto et al., 1998, 2009; Sharma et al., 2000; Csordás et al., 2010; Giacomello et al., 2010) (Figs. 1 and 3). The functional tight coupling between ER/SR and mitochondria is attributed to the inter-organelle tether proteins such as mitofusin 2 (de Brito

Correspondence to Shey-Shing Sheu: shey-shing.sheu@jefferson.edu

Abbreviations used in this paper: $[Ca^{2+}]_c$, cytosolic $[Ca^{2+}]$; $[Ca^{2+}]_m$, matrix $[Ca^{2+}]$; CCDC109A, coiled-coil domain–containing protein 109A; $\Delta\Psi_m$, membrane potential; HCX, H^+–Ca^{2+} exchanger; IMM, inner mitochondrial membrane; IP_3, inositol 1,4,5-trisphosphate; Letm1, leucine-zipper-EF-hand–containing transmembrane protein 1; mCa, Ca^{2+}-selective conductance; MCU, mitochondrial Ca^{2+} uniporter; mPTP, mitochondrial permeability transition pore; mRyR1, mitochondrial RyR type 1; NCX, Na^+–Ca^{2+} exchanger; RaM, rapid mode of uptake; siRNA, small interfering RNA; UCP, uncoupling protein.

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and Scorrano, 2008; García-Pérez et al., 2011) (Fig. 1). These seminal discoveries have positioned mitochondria as one of the key players in the dynamic regulation of physiological Ca^{2+} signaling.

The driving forces for mitochondrial Ca^{2+} uptake are the membrane potential ($\Delta\Psi_m$) and [Ca^{2+}] gradient across IMM. For MCU, Ca^{2+} is taken into the mitochondrial matrix down its electrochemical gradient without transport of another ion (Kirichok et al., 2004). Basically, for each Ca^{2+} transported through MCU, there is a net transfer of two positive charges into matrix resulting in a drop of $\Delta\Psi_m$, which is energetically unfavorable. However, the Ca^{2+}-stimulated respiration will not only compensate the loss of $\Delta\Psi_m$ by the efflux of H^+ through electron transport chain, but it will also produce a net gain of ATP. In addition, multiple Ca^{2+} efflux mechanisms work in concert aiming to expedite a transient and an oscillatory nature rather than a tonic and a steady-state change of matrix [Ca^{2+}] ([Ca^{2+}]$_m$).

Mitochondrial Ca^{2+} efflux mechanism is also important for cellular Ca^{2+} homeostasis, as is mitochondrial Ca^{2+} influx mechanism. The proposed mitochondrial Ca^{2+} efflux mechanisms are Na^{2+} dependent (Palty et al., 2010) and/or H^+ dependent (Jiang et al., 2009). Na^{2+}-dependent mitochondrial Ca^{2+} efflux was first documented more than 30 years ago using cardiac mitochondria (Carafoli et al., 1974), and it has been shown that Na^+–Ca^{2+} exchanger (NCX) is the primary Ca^{2+} efflux mechanism in cardiac mitochondria (Maack et al., 2006; see also Denton and McCormack, 1985; Gunter and Pfeiffer, 1990). Moreover, a strong candidate for the molecular identity of the mitochondrial NCX has been recently reported (Na^+- or Li^+-dependent Ca^{2+} transport) (Palty et al., 2010) (Figs. 1 and 3 E). On the other hand, Letm1 originally identified as a H^+–K^+ exchanger, has been recently reported to function as a critical component of a mitochondrial H^+–Ca^{2+} exchanger (HCX), but its role in Ca^{2+} extrusion is still controversial (Figs. 1 and 3 E) (see also next section). Interestingly, the protein expression of NCX is particularly robust in excitable cells including heart and brain (Palty et al., 2010), whereas the activity of the HCX is primarily found in nonexcitable cells, suggesting that there exist tissue-specific mitochondrial Ca^{2+}

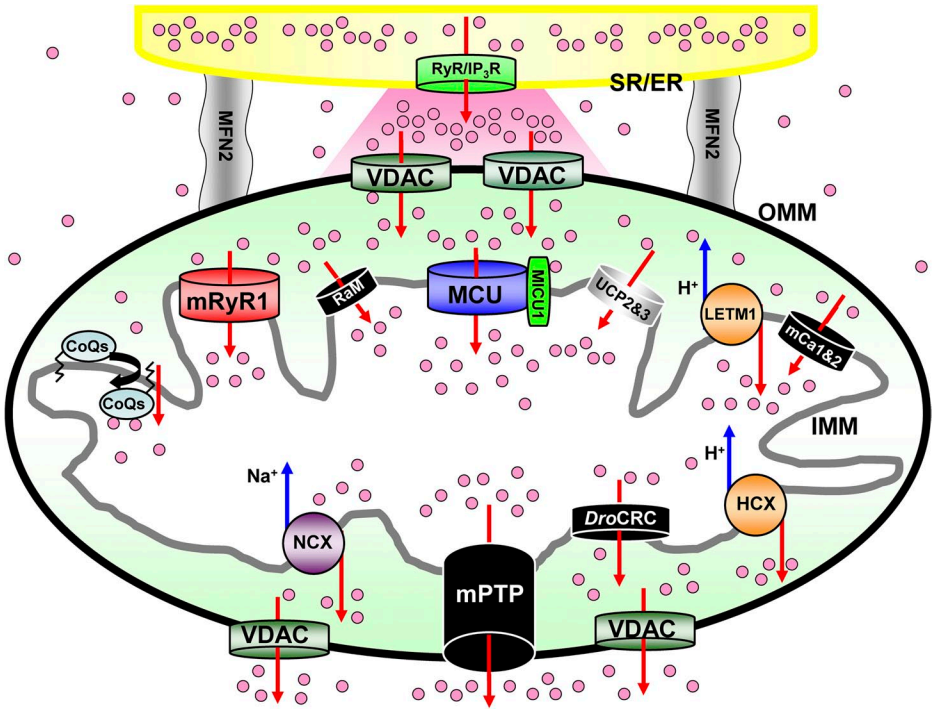

Figure 1. Mitochondrial Ca^{2+} influx and efflux mechanisms. Schematic diagram of mitochondrial Ca^{2+} channels/transporters for influx and efflux mechanisms. The functional and morphological tight coupling of ER/SR (yellow) and mitochondria is attributed to the specific structure of inter-organelle tether proteins such as mito-fusion protein 2 (gray) (de Brito and Scorrano, 2008; García-Pérez et al., 2011). Ca^{2+}-releasing sites of ER/SR, IP_3 receptors (IP_3R), or RyRs (RyR; green) are facing microdomains between mitochondria and ER/SR (shown as a pink region). Ca^{2+} release from ER/SR dramatically changes [Ca^{2+}]$_c$ at this microdomain (Csordás et al., 2010; Giacomello et al., 2010). Then, mitochondria sense the high increases of [Ca^{2+}]$_c$ at this microdomain, and [Ca^{2+}]$_c$ propagates into the mitochondria matrix through a variety of Ca^{2+} channels/transporters (Rizzuto et al., 1992). Mitochondrial Ca^{2+} influx (upper part of this figure) is determined by the MCU (blue) (Baughman et al., 2011; De Stefani et al., 2011), RaM (black) (Sparagna et al., 1995; Buntinas et al., 2001; Bazil and Dash, 2011), HCX (Letm1; orange) (Jiang et al., 2009), mRyR1 (red) (Beutner et al., 2001, 2005), hydroxyl coenzyme Q10 (CoQs) (Bogeski et al., 2011), mCa1 and mCa2 (Michels et al., 2009), and UCP 2 and UPC 3 (Trenker et al., 2007) located at the IMM. MICU1 can bind to Ca^{2+} by its EF hand, but this protein does not make the channel pore because of its single-transmembrane structure (Perocchi et al., 2010). The mPTP (black) (Giacomello et al., 2007), NCX (purple) (Palty et al., 2010), and HCX (orange) (Jiang et al., 2009) contribute to Ca^{2+} efflux (lower part of this figure) in mammalian cells. *Drosophila* mitochondria possess another selective Ca^{2+} release channel (*Dro*CRC; black) with unique featured characteristics intermediate between the permeability transition pore of yeast and mammals (von Stockum et al., 2011). Letm1 also works as a Ca^{2+} efflux pathway when [Ca^{2+}]$_c$ becomes high (Jiang et al., 2009) (see also Figs. 2 and 3 E). Voltage-dependent anion-selective channels (VDAC; dark green) provide a pathway for Ca^{2+} and metabolite transport across the outer mitochondrial membrane (OMM). The channels/transporters for which molecular identities are still unknown are shown as black. Red arrows show Ca^{2+} movements, and blue arrows show other ion movements.

efflux mechanisms. In addition, the idea that mPTP can also serve as a rapid Ca^{2+} efflux mechanism has gained appreciation as stated in several recent reviews (Gunter and Sheu, 2009; Bernardi and von Stockum, 2012). It has been shown that mPTP can open and close transiently ("flicker") at its low conductance state (Zoratti and Szabò, 1995); thus, it serves as one of the physiological Ca^{2+} efflux mechanisms (Figs. 1 and 3 E). However, under certain cellular stresses that lead to Ca^{2+} overload and/or overproduction of reaction oxygen species, mPTP can open constantly, causing the release of cytochrome c and subsequently leading to cell death (Giacomello et al., 2007). Finally, a recent report shows that *Drosophila melanogaster* mitochondria possess another selective Ca^{2+} release channel (shown as "DroCRC" in Fig. 1) with unique featured characteristics between the mPTP of yeast and mammals, such as inhibition by Pi but not by ADP and cyclosporine A (as in the mPTP of yeast mitochondria), and the existence of voltage- and redox-sensitive regulatory sites (as in the mPTP of mammalian cells) (von Stockum et al., 2011).

Through the multiple experimental approaches, several different types of mitochondrial Ca^{2+} uptake mechanisms, in addition to classical MCU, were functionally isolated, including: (a) RaM (Sparagna et al., 1995; Buntinas et al., 2001; Bazil and Dash, 2011), (b) RyR type1 (RyR1) (Beutner et al., 2001, 2005; Altschafl et al., 2007; Ryu et al., 2011), (c) Ca^{2+}-selective conductance (mCa) 1 and 2 (Michels et al., 2009), (d) I_{MiCa} (Kirichok et al., 2004), (e) Coenzyme Q10 (Bogeski et al., 2011), (f) uncoupling proteins (UCPs) 2 and 3 (Trenker et al., 2007), and (g) Letm1 (Jiang et al., 2009) (summarized in Fig. 1). Among these studies, RyR1 was found as the first mitochondrial Ca^{2+} uptake mechanism with a known molecular identity, but molecular identities of RaM (Sparagna et al., 1995; Buntinas et al., 2001; Bazil and Dash, 2011) and mCa1 and mCa2 (Michels et al., 2009) have not yet been clarified (see Fig. 1; the channels/transporters for which molecular identities are still unknown are shown in black). I_{MiCa} was recently recorded from mitoplasts (Kirichok et al., 2004), providing direct electrophysiological demonstration for the existence of a Ca^{2+}-selective ion channel, which would possibly fit to the originally predicted channel nature of MCU (Gunter and Pfeiffer, 1990). Through RNA interference studies, several groups have recently proposed novel candidate proteins that involve the mitochondrial Ca^{2+} uptake mechanism, such as Letm1 (Jiang et al., 2009) and MICU1 (Perocchi et al., 2010). Finally, two papers have come out very recently at the same time from two different groups, reporting that the coiled-coil domain–containing protein 109A (CCDC109A) is the molecular identity of MCU (Baughman et al., 2011; De Stefani et al., 2011). Because of the length limitation, only MCU, mRyR1, and Letm1 are discussed in detail in this Perspective.

MCU

UCPs 2 and 3 were proposed as the molecular identity of MCU by an siRNA study (Trenker et al., 2007). This view was soon challenged by Clapham's group, who showed that dsRNAs against *Drosophila* mitochondrial UCPs did not affect mitochondrial Ca^{2+} and H^+ concentration (Jiang et al., 2009). Interestingly, a recent report from Demaurex's group also showed that UCP3 is not an MCU, but it alters ER/SR Ca^{2+} ATPase activity by decreasing mitochondrial ATP production (De Marchi et al., 2011). Next, Mootha's group identified a protein that is an important regulator of mitochondrial Ca^{2+} uptake mechanism, using bioinformatics and siRNA screening, termed MICU1 (Perocchi et al., 2010). MICU1 has two Ca^{2+}-binding EF hands but only one putative transmembrane domain, which seems unlikely to form a Ca^{2+} channel pore and be an MCU itself (Fig. 1). After the discovery of MICU1, Mootha's group moved to whole genome phylogenetic profiling, genome-wide RNA coexpression analysis, and organelle-wide protein coexpression analysis to predict proteins being functionally related to MICU1, which is thought to be an ancillary subunit of MCU (Baughman et al., 2011). The analysis predicted that a transmembrane protein previously identified as CCDC109A is MCU. Using slightly different approaches, De Stefani et al. (2011) also identified the same protein as MCU at the same time. The characteristics of MCU found by these two groups are as follows: (a) CCDC109A (MCU) has two transmembrane domains, which seems likely to make a Ca^{2+} channel pore; (b) using RNA interference studies, knockdown of MCU dramatically reduces mitochondrial Ca^{2+} uptake in isolated mitochondria or in living cells, and this effect was rescued by overexpression of MCU; (c) MCU down-regulation itself does not affect mitochondrial O_2 consumption, ATP synthesis, $\Delta\Psi_m$, and mitochondrial morphology; and (d) site-specific mutations at the pore region in MCU show loss of function or a dominant-negative effect. Moreover, Rizzuto's group reconstituted MCU in lipid bilayers and recorded Ruthenium red–sensitive Ca^{2+} current with 6–7-pS single-channel activity (De Stefani et al., 2011). The most obvious discrepancy between these two groups' data is the topology of MCU (Drago et al., 2011). Both groups had a consensus proposal that MCU consists of two transmembrane domains and forms oligomer to be a Ca^{2+} channel. However, Rizzuto's group proposed that C and N terminals face intermembrane space (De Stefani et al., 2011), and Mootha's group proposed the opposite direction: C and N terminals face matrix (Baughman et al., 2011). The C and N terminals of the channels are generally important regions for receiving various kinds of posttranslational modifications including phosphorylation by second messengers or kinases, which would modulate the channel function (Dai et al., 2009). Therefore, the discrepancy in the topology of MCU will

need to be resolved for understanding the modulation of MCU functions by signaling molecules from cytosol or matrix.

mRyR1

One of the candidates for the mitochondrial Ca^{2+} uptake mechanism with a known molecular identity is the mitochondrial RyR in cardiac cells reported from our group (Beutner et al., 2001, 2005). Three different RyR isoforms (RyR1, RyR2, and RyR3) have been cloned, and different physiological and pharmacological properties between these isoforms have been identified (Lanner et al., 2010). In cardiac cells, intracellular Ca^{2+} release and muscle contraction were mainly controlled by isoform RyR2 located in the SR (Lanner et al., 2010). Although RyR1 is also detectable both at mRNA and at protein levels in cardiac tissue (Münch et al., 2000; Jeyakumar et al., 2002), its functional and physiological roles in the heart had not been fully understood for a long time. We first showed that a low level of functional RyR is also expressed at heart IMM (shown by high affinity binding of [^{3}H]ryanodine, immunogold staining, RT-PCR, and Western blot) and has a role of fast Ca^{2+} uptake pathway (Beutner et al., 2001, 2005) (Figs. 2 and 3 C). Furthermore, RyR in cardiac mitochondria exhibits remarkably similar biochemical, pharmacological, and functional properties to those of RyR1 in skeletal muscle SR, but not to those of RyR2 found in cardiac SR. Therefore, we termed it as mRyR1 (mitochondrial RyR1) (Fig. 1). The molecular identity of mRyR1 was carefully analyzed and confirmed by a variety of functional and biochemical experiments using not only native heart but also transgenic heart (RyR1 knockout mice) (Beutner et al., 2005).

Recently, we also performed electrophysiological experiments to directly demonstrate the existence of mRyR1, which clearly showed the predicted channel nature of skeletal RyR1 (Altschafl et al., 2007; Ryu et al., 2011). At first, we performed electrophysiological experiments using a conventional lipid bilayer system (Altschafl et al., 2007). The activity of RyR1, but not RyR2, was observed in lipid bilayers of mRyRs purified from heart IMM fraction. Neither SR nor outer mitochondrial membrane markers were detected in these mRyR1 preparations. Next, we characterized the biophysical and pharmacological properties of native single mRyR1 channels in heart mitoplast using the patch-clamp technique (Ryu et al., 2011). We observed a novel 225-pS cation–selective channel in heart mitoplasts that exhibited multiple subconductance states, which was blocked by high concentrations of ryanodine and Ruthenium red, the known inhibitors of RyRs. Ryanodine exhibited a concentration-dependent modulation of this channel, with low concentrations stabilizing a subconductance state and with high concentrations abolishing activity (Ryu et al., 2011). The channel prosperities of Ca^{2+}-dependent [^{3}H] ryanodine binding and the channel modulation by caffeine (Beutner et al., 2001, 2005) implicate that the topology of mRyR1 is the same as RyR1 at the SR (Du et al., 2002) because these agonist-binding sites are facing cytosol. Therefore, we hypothesize that C and N terminals

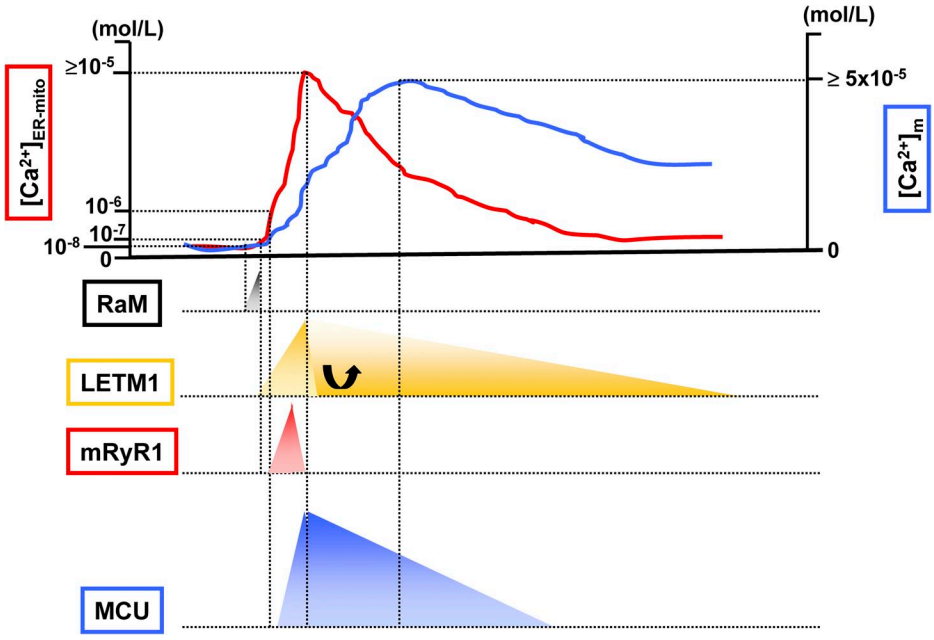

Figure 2. Mitochondrial Ca^{2+} influx mechanisms during cytosolic/mitochondrial Ca^{2+} transient. The top of the figure is an example of Ca^{2+} transient at microdomains between mitochondria and ER/SR ($[Ca^{2+}]_{ER\text{-}mito}$; red line) and Ca^{2+} transient at the mitochondrial matrix ($[Ca^{2+}]_m$; blue line). RaM (black) shows 50-fold faster Ca^{2+} transport compared with the MCU, and the activation peak is at 50 nM of extra-mitochondrial Ca^{2+}. Letm1 (orange) can be activated at ≥200 nM of extra-mitochondrial Ca^{2+}, but at high $[Ca^{2+}]_{ER\text{-}mito}$ condition, a role of Letm1 shifts to Ca^{2+} efflux rather than Ca^{2+} uptake into the mitochondrial matrix. mRyR1 (red) can start to be activated at 1 µM of extra-mitochondrial Ca^{2+}, with a fivefold faster Ca^{2+} transport compared with the MCU. 2 µM is the half-maximal concentration for Ca^{2+}-dependent activation of mRyR1, and 20 µM is the half-maximal concentration for Ca^{2+}-dependent inhibition. Thus, mRyR1 inactivates before $[Ca^{2+}]_{ER\text{-}mito}$ reaches the peak. A lower concentration of extra-mitochondrial Ca^{2+} (such as 200–300 nM) does not activate MCU, and at least >1 µM Ca^{2+} is needed for the initial activation. The estimated half-maximal concentration for the activation of MCU is ≅20 mM.

face intermembrane space (cytosolic side), and S1–S2, S3–S4, and S5–S6 linkers face the mitochondrial matrix side. However, further experiments will be needed to confirm the topology of mRyR1 by using other modulators that either act from the cytosolic side (ATP, FK-binding proteins, calmodulin, phosphorylation by protein kinase A, or Ca^{2+}/calmodulin-binding protein II) or from the matrix side (Ca^{2+}) (Lanner et al., 2010).

Collectively, our studies show the molecular and functional existence of mRyR1 in heart mitochondria and clearly distinguish it from previously identified mitochondrial ion channels. It is worthwhile to mention that unlike MCU (which is a highly Ca^{2+}-selective and low conductance ion channel as such would not strongly affect $\Delta\Psi_m$; Drago et al., 2011), RyR is a poorly Ca^{2+}-selective large cation channel (Ryu et al., 2011), and thus opening of this channel might collapse $\Delta\Psi_m$, which is

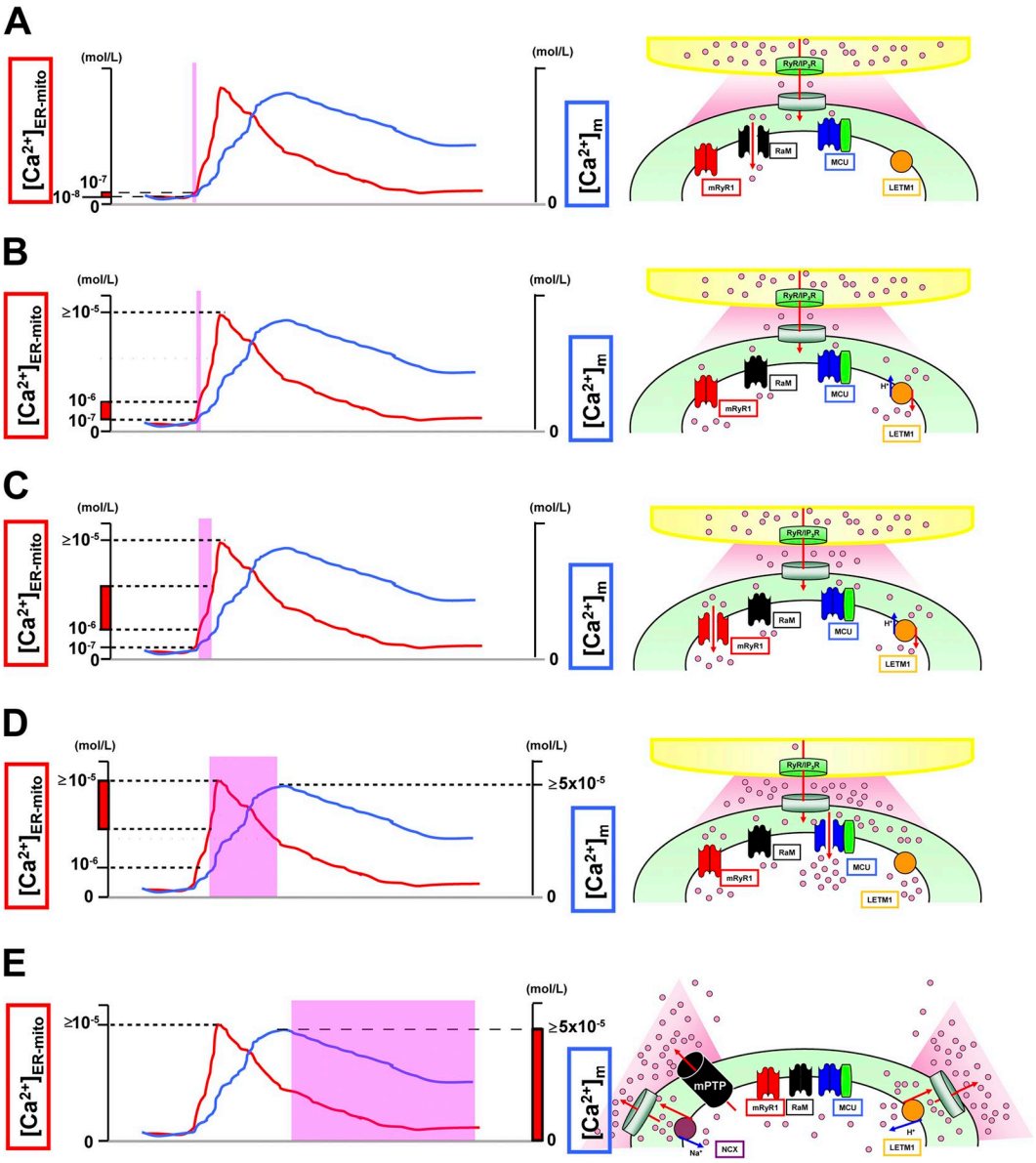

Figure 3. Activation/inactivation patterns of Ca^{2+} influx/efflux mechanisms. (A) At first, RaM (black) is activated at a very initial phase of $[Ca^{2+}]_{ER\text{-}mito}$ transient (<200 nM), with faster Ca^{2+} uptake kinetics (ms time scale). (B) Letm1 (orange) starts to uptake Ca^{2+} at ≥200 nM $[Ca^{2+}]_{ER\text{-}mito}$. (C) mRyR1 (red) starts to open at ≅1 µM $[Ca^{2+}]_{ER\text{-}mito}$, with a fivefold faster Ca^{2+} transport compared with the MCU, and inactivates before $[Ca^{2+}]_{ER\text{-}mito}$ reaches the peak. (D) Finally, MCU (blue) starts to activate at >1 µM $[Ca^{2+}]_{ER\text{-}mito}$, and the activity increases in a $[Ca^{2+}]_{ER\text{-}mito}$–dependent manner. At this point, Letm1 (orange) shifts from Ca^{2+}-uptake mode to Ca^{2+}-efflux mode. (E) mPTP (black) and NCX (purple) contribute to Ca^{2+} efflux in mammalian cells and form the decay phase of $[Ca^{2+}]_m$ transient. Letm1 also works as a Ca^{2+} efflux pathway at this phase. The channels/transporters of which the molecular identities are still unknown are shown as black. Red arrows show Ca^{2+} movements, and blue arrows show other ion movements.

energetically unfavorable. This dichotomy would be explained as follows: (a) the expression number of RyR1 in a single mitochondrion is very small (Beutner et al., 2001; Ryu et al., 2011), and thus the depolarization of $\Delta\Psi_m$ might be localized only near the site of mRyR1, and the rest of the cristae membrane preserves its voltage; (b) the bell-shape Ca^{2+} dependency with a rapid Ca^{2+} activation and inactivation profile of this channel (see also Figs. 2 and 3, C and D) would minimize the $\Delta\Psi_m$ change; and (c) any small decrease in $\Delta\Psi_m$ then can be readily compensated by the Ca^{2+}-dependent activation of dehydrogenases in tricarboxylic acid cycle and F0F1-ATP synthase. Collectively, the mRyR may be uniquely poised to sequester Ca^{2+} during a transient and rapid excitation–contraction coupling process in cardiac muscle cells.

Our first report on the identification of RyR1 in cardiac mitochondria over 10 years ago (Beutner et al., 2001) has not yet drawn high research activity on this topic, both in the cardiac and mitochondrial field, because of the following reasons: (a) the difficulty in functional separation of the very low level of (∼5%) mRyR1 from a high abundance of (∼95%) SR-located RyR2; (b) the lack of genetic approaches to dissect mRyR1 function from MCU until recently (see also above section); and (c) the sparse information about RyR1 links to human diseases, with the exception of skeletal muscle–related diseases. However, this landscape has been gradually changing by these recent exciting reports: (a) the expression of RyRs in mitochondria has been confirmed in a variety of cell types including osteoblasts (Sun et al., 2002), endothelial cells (Uehara et al., 2004), and neuronal cells (Norman et al., 2008); (b) non-skeletal muscle and non–SR-RyR phenotypes related to human pathologies are being progressively reported, such as in neurological diseases, which include HIV induction of cortical neuron injury via activation of both ER-RyR and mRyR (Norman et al., 2008; Perry et al., 2010) and positive outcome on treating patients in neurointensive care units with dantrolene (Muehlschlegel and Sims, 2009), a more selective inhibitor of RyR1 that is frequently used for treating malignant hyperthermia; (c) an intriguing clinical report, using a genome-wide association study, shows that RyR1 (not RyR2) single-nucleotide polymorphisms are associated with the risk for the development of electrocardiographic left ventricular hypertrophy (Hong et al., 2012); and (d) in a knock-in mouse model with heterozygous RyR1 (I4898T), related to a human central core disease, the ventricular chamber formation develops abnormally (Zvaritch et al., 2007).

Important future directions of cardiac mRyR research will uncover fundamental questions including: (a) What is the relative contribution of mRyR1 and other Ca^{2+} transporters in cellular Ca^{2+} homeostasis and ATP synthesis in beating heart in vivo? (b) What are the implications of mRyR in RyR1-linked diseases such as malignant hyperthermia (MacLennan, 1992), central core disease (Zhang et al., 1993), and cardiac hypertrophy (Hong et al., 2012)? (c) Does mRyR1 also exist in other excitable cells such as neuron, vascular smooth muscle cells, and skeletal muscle cells? If it does, what are the physiological and pathological implications?

Letm1 (Ca^{2+}–H^+ antiporter)

Using siRNA genome-wide screening in *Drosophila*, Jiang et al. (2009) reported that mitochondrial protein Letm1, originally known as K^+–H^+ exchanger, can play a role as Ca^{2+}–H^+ antiporter. They proposed that Letm1 is localized at the inner membrane (Fig. 1) and transports one Ca^{2+} and extracts one H^+ (Figs. 2 and 3 B). Knockdown of Letm1 abolished only the initial fast mitochondrial Ca^{2+} uptake, but it still showed sustained Ca^{2+} increase, suggesting that Letm1 works at low $[Ca^{2+}]_c$ for Ca^{2+} uptake (Figs. 2 and 3 B). Letm1 activity was inhibited by both Ruthenium red, an inhibitor of MCU, and CGP37157, an inhibitor of mitochondrial NCX. Similar data were also recently reported by Waldeck-Weiermair et al. (2011). This scenario seems like the revival story of Moyle and Mitchell (1977), which raises several points of discussion (see the Perspective by Nowikovsky et al. in this issue): (a) one Ca^{2+} for one H^+ antiporter does not favor Ca^{2+} influx physiologically, according to the electrochemical gradients of Ca^{2+} and pH; (b) Ca^{2+} influx by Letm1 might be in part mediated by the changes in $\Delta\Psi_m$ through K^+ fluxes because of Letm1 being itself a K^+–H^+ exchanger; and (c) CGP37157 had not been shown to inhibit IP_3-mediated $[Ca^{2+}]_m$ increase. It can be anticipated that new experimental results will appear in future publications to resolve this controversy.

Mitochondrial Ca^{2+} influx mechanism and human diseases

Disruption of cellular Ca^{2+} homeostasis is associated with human diseases (Berridge et al., 2003) such as cardiovascular (Bers, 2008; Lanner et al., 2010), skeletal muscle (Lyfenko et al., 2004; Lanner et al., 2010) and neurological diseases (Vicencio et al., 2010). However, the relative contributions of individual mitochondrial Ca^{2+} influx mechanisms to the disease pathogenesis are still not well understood.

As of today, around 300 mutations have been identified in RyR, and some of these mutations are directly associated with human diseases (Lanner et al., 2010). For instance, RyR1 gene mutations are involved in several debilitating and/or life-threatening muscle diseases including malignant hyperthermia (MacLennan, 1992), central core disease (Zhang et al., 1993), heat/exercise-induced exertional rhabdomyolysis (Capacchione et al., 2010), multiminicore disease (Ferreiro et al., 2002), and atypical periodic paralyses (Zhou et al., 2010). More importantly, RyR1 mutations found in human malignant hyperthermia and central core disease exhibit

abnormal Ca^{2+} regulation in cardiac mitochondria (Gross, P., N. Sokolova, S. Provazza, G. Beutner, and S.S. Sheu. 2011. 65th Annual Meeting of The Society of General Physiologists. Abstr. 30) and basal bioenergetic abnormalities in skeletal muscle mitochondria (Giulivi et al., 2011). Given the facts that mitochondria communicate closely with ER/SR, disruption of the mRyR1 Ca^{2+} influx mechanism may contribute to the initiation and development of these diseases. Similarly, Letm1 is involved in respiratory chain biogenesis and in the pathogenesis of seizures in the Wolf–Hirschhorn syndrome (McQuibban et al., 2010; Zotova et al., 2010). With the most recent discovery on the molecular identity of MCU, continued research in this field will certainly help our understating on the contribution of mitochondrial Ca^{2+} in the pathogenesis of human diseases.

Future perspective and conclusion

Historically, the mitochondrial Ca^{2+} influx mechanism has been an important topic in cell biology, despite the relatively slow progress in revealing the molecular identities of Ca^{2+}-transporting proteins. Using multiple research tools, such as gene-screening analysis, genetic manipulation, and updated biochemical, pharmacological, cell biological, and electrophysiological techniques, has led to the recent groundbreaking discoveries in MICU1, MCU, and Letm1 molecular identities. This advance in the cloning of mitochondrial Ca^{2+} channels/transporters will provide essential information for studying (a) the regulatory mechanism underlying mitochondrial Ca^{2+} uptake, such as posttranslational modifications of these channel/transporter functions; (b) the design or discovery of more specific inhibitors/activators to each channel/transporter for the potential development of therapeutic drugs; and, furthermore, (c) the molecular mechanisms underlying mitochondrial Ca^{2+}-mediated human diseases.

The long-sought mystery of the molecular identity of MCU has just been uncovered, but other studies have also identified additional Ca^{2+} uptake pathways that exhibit different function and pharmacology from MCU. The idea that more than one Ca^{2+} influx mechanism exists in mitochondria has gradually gained wider recognition because each cell type (especially excitable vs. nonexcitable cells) possesses different size/frequency of Ca^{2+} oscillations (or transients). It is reasonable to predict that different tissues coordinate mitochondria Ca^{2+} influx in different fashion by using a different combination and expression ratio of these channels/transporters. For example, mRyR1, which has a high velocity of Ca^{2+} uptake and Ca^{2+} sensitivity, is a perfect candidate to mainly regulate effective Ca^{2+}-induced ATP productions in cardiac cells in a beat to beat manner. I_{MiCa} density has begun to be recorded from different tissues using mitoplast patch clamp, and interestingly, I_{MiCa} density in the heart seems to be much smaller than in other tissues (Fieni, F., and Y. Kirichok. 2011. 65th Annual Meeting of The Society of General Physiologists. Abstr. 56A). Future studies are destined to provide new evidence regarding the diversity of Ca^{2+} influx mechanisms in different cell types/tissues, which will allow us to understand the relative contribution and/or cross talk between each mitochondrial Ca^{2+} transporter in each organ.

In conclusion, mitochondrial Ca^{2+} is crucial in governing energy production, Ca^{2+} homeostasis, and cell fate. Revealing the molecular identities of mitochondrial Ca^{2+} influx mechanisms provides us with the passwords to access a new field of study by establishing animal models to address the relationship between the mitochondrial Ca^{2+} uptake mechanism and human physiology and diseases.

This Perspectives series includes articles by Sheu et al., Zhang et al., Balaban, Santo-Domingo and Demaurex, Wei and Dirksen, Nowikovsky et al., and Galloway and Yoon.

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