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Calcium transport across the inner mitochondrial membrane: molecular mechanisms and pharmacology

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Abstract

Growing evidence supports that mitochondrial calcium uptake is important for cell metabolism, signaling and survival. However, both the molecular nature of the mitochondrial Ca^{2+} transport sites and the calcium signals they respond to remained elusive. Recent RNA interference studies have identified new candidate proteins for Ca^{2+} uptake across the inner mitochondrial membrane, including LETM1, MCU, MICU1 and NCLX. The sensitivity of these factors to several drugs has been tested and in parallel, some new inhibitors of mitochondrial Ca^{2+} uptake have been described. This paper provides an update on the pharmacological aspects of the molecular mechanisms of the inner mitochondrial membrane Ca^{2+} transport.

Keywords

mitochondria; uniporter; ruthenium red; Ru360; CGP-37157; minocycline

1. Molecular mechanisms of mitochondrial calcium uptake and release

Calcium transport between the cytoplasm and the mitochondrial matrix involves the passage of Ca^{2+} across both the outer and inner mitochondrial membranes (OMM and IMM). The overall permeability of the OMM for Ca^{2+} is relatively high, presumably due to the abundant presence of the voltage dependent anion-selective channels (VDACs) that also offer high conductance for Ca^{2+} in their “closed” state. However, the IMM presents a tight barrier for Ca^{2+} . Early studies with isolated mitochondria revealed a mechanism of Ca^{2+} uptake presented by passive transport of Ca^{2+} down its electrochemical gradient without coupling Ca^{2+} transport to the transport of another ion. Therefore, this mechanism was attributed to a Ca^{2+} uniporter (reviewed in (Gunter et al., 1994; Gunter and Pfeiffer, 1990)). It was also found that upon the reverse of the driving force the uniporter can also mediate

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Ca²⁺ efflux even though in energized mitochondria, Na⁺/Ca²⁺ or H⁺/Ca²⁺ exchange mediate the exit of Ca²⁺ (Gunter and Pfeiffer, 1990). Furthermore, under various mitochondrial stress conditions such as Ca²⁺ overload, formation of a large pore, the permeability transition pore (PTP) was observed that can also support Ca²⁺ release (Bernardi et al., 1998; Szalai et al., 1999). Moreover, the PTP has been shown to “flicker” or to open and close transiently under normal conditions associated with brief depolarization transients (Duchen et al., 1998), which may also provide a rapid mechanism for mitochondrial Ca²⁺ efflux (Barsukova et al., 2011; Ichas et al., 1994). The physiological relevance of this sophisticated and high capacity Ca²⁺ transport machinery was debated because of the requirement for supraphysiological [Ca²⁺] elevations (10 – 100 μM) to stimulate the uniporter-mediated Ca²⁺ uptake of isolated mitochondria. Thus, the subsequent demonstration of a mitochondrial matrix [Ca²⁺] ([Ca²⁺]_m) rise and stimulation of the Ca²⁺ sensitive steps of oxidative metabolism in intact cells during calcium spikes and oscillations came as a major surprise (Hajnóczky et al., 1995; Rizzuto et al., 1994; Rizzuto et al., 1993). In the past 20 years, new clues were obtained as to how mitochondria sense the physiological calcium signals, including by strategic targeting of mitochondrial Ca²⁺ uptake sites close to the sites of ER Ca²⁺ release (IP₃ receptors (IP₃R) and ryanodine receptors) and plasma membrane Ca²⁺ entry sites (voltage-dependent Ca²⁺ channels and store-operated Ca²⁺ entry) (Csordas et al., 1999; Hoth et al., 1997; Korzeniowski et al., 2009; Rizzuto et al., 1998; Sanchez et al., 2001; Szalai et al., 2000). In several paradigms, mitochondrial Ca²⁺ uptake was also documented at physiological, submicromolar cytoplasmic [Ca²⁺] ([Ca²⁺]_c) elevations (reviewed in (Gunter and Sheu, 2009; Spat et al., 2008)). A fundamental electrophysiology study has clarified that the Ca²⁺ uniport across the inner mitochondrial membrane (IMM) is mediated by a channel (MiCa) (Kirichok et al., 2004). However, within the “generic” Ca²⁺ uniport category, several distinct Ca²⁺ influx mechanisms have also been isolated. These include the rapid mode uptake (RaM) (Sparagna et al., 1995) and Ca²⁺ selective conductances (mCa1&2) (Michels et al., 2009), each displaying unique characteristics in Ca²⁺ affinity, kinetics and pharmacological properties (for review see (Ryu et al.)). For instance, RaM takes up Ca²⁺ approximately 1000 times faster than that via mitochondrial uniporter. It should be noted that different cell types have very distinct intracellular Ca²⁺ profiles with Ca²⁺ transients that last from a few tens or hundreds of milliseconds (e. g. neurons and cardiomyocytes) to a few tens or hundreds of seconds (e.g. hepatocytes and glia cells), mostly due to differential expressions of various Ca²⁺ transport proteins. It is conceivable that the IMM contains multiple entities of the Ca²⁺ uniport to decode distinct intracellular Ca²⁺ signaling in various cell types.

The first proposal for a mitochondrial Ca²⁺ channel with known molecular identity in the heart was the type 1 ryanodine receptor (Beutner et al., 2005). Evidence was also presented for the involvement of uncoupling protein 2/3 in the Ca²⁺ transfer across the IMM (Trenker et al., 2007).

Subsequently, in genome-wide siRNA screen studies, LETM1 was identified as a critical component in the [Ca²⁺]_m signal (Jiang et al., 2009). However, LETM1 is an antiporter that other studies have identified as a K⁺/H⁺ exchanger (Dimmer et al., 2008; Nowikovsky et al., 2004). Currently, these candidates are still debated. While the majority of studies focused on the Ca²⁺ influx pathway, a strong candidate emerged for the Ca²⁺ efflux mediated by Na⁺/Ca²⁺ exchange. This protein, the NCLX (Palty et al., 2010) nicely reproduces the biochemical properties of a protein previously purified from mitochondria (Li et al., 1992) and uniquely displays Li⁺ promoted Ca²⁺ transport (Palty et al., 2004). Finally, during the past months some seminal studies uncovered the MCU protein that can form a Ca²⁺ channel in lipid bilayers (Baughman et al., 2011; De Stefani et al., 2011) and the MICU1, an EF hand containing, MCU binding protein that might act as an important regulator of the MCU channel (Perocchi et al., 2010). Although, additional components of the Ca²⁺ uniport might

emerge and the H^+/Ca^{2+} exchanger remains to be identified, the recently identified constituents of the IMM Ca^{2+} transport provide important means to dissect the mechanism and functional significance of the mitochondrial Ca^{2+} handling (Figure 1). The molecular composition, significance and pharmacology of the PTP have been extensively reviewed recently (Bernardi and Rasola, 2007; Halestrap, 2009), therefore the PTP will not be discussed in detail here.

2. Drugs affecting the IMM Ca^{2+} transport

The transport of Ca^{2+} across the IMM is determined by both the driving force and the capacity of transport sites. Therefore, any conditions that cause collapse of the mitochondrial membrane potential ($\Delta\Psi_m$), the dominant component of the driving force, lead to suppression of the Ca^{2+} uptake. Protonophores (also referred as uncouplers: FCCP, CCCP, and 1799), respiratory complex inhibitors (rotenone and antimycin) lead to a decrease in the $\Delta\Psi_m$ to interfere with Ca^{2+} uptake.

The Ca^{2+} uniport and the MiCa are effectively suppressed by **ruthenium red** and **Ru360**, a ruthenium derivative (Kirichok et al., 2004; Matlib et al., 1998). Although the use of ruthenium red or Ru360 is fairly straightforward in isolated mitochondria, the lack of permeation across the plasma membrane in many cell types is an important limitation of the application of these drugs in cell or tissue studies. Furthermore, ruthenium red binds to and inhibits a wide variety of Ca^{2+} and K^+ channels presenting some limitations in complex systems (reviewed in (Hajnóczky et al., 2006)). For example, ruthenium red is a potent inhibitor of the ryanodine receptors so it affects both the endo/sarcoplasmic reticulum (ER/SR) and mitochondrial components of the cellular Ca^{2+} homeostasis. Ru360 shows specificity for the Ca^{2+} uniporter over ryanodine receptors, SERCA pump, sarcolemmal Na^+/Ca^{2+} exchange, and L-type Ca^{2+} channel current in cardiac muscle (Matlib et al., 1998). Additional inhibitors of the Ca^{2+} uniport and MiCa include Mg^{2+} , that exerts inhibition in physiological concentrations (Szanda et al., 2009), lanthanides, cardioactive drugs quinidine, alprenolol, propranolol, oxyfedrine, and tetracaine, the diuretic, ethacrynic acid, amiloride analogs and derivatives, and the antibiotic gentamicin (Kirichok et al., 2004; Vinogradov and Scarpa, 1973). Pharmacological activators of the mitochondrial Ca^{2+} uniport have also been described such as polyamines (Nicchitta and Williamson, 1984; Salvi and Toninello, 2004), the p38 MAP kinase inhibitor SB202190 (Montero et al., 2002), and a range of naturally occurring flavonoids (e.g. genistein, quercetin, kaempferol) (Montero et al., 2004).

Notably, the Ca^{2+} uniport exhibits allosteric positive control by Ca^{2+} (Csordas and Hajnóczky, 2003; Kroner, 1986a, b), which is inhibited by a variety of calmodulin inhibitors (Csordas and Hajnóczky, 2003). Among the recently identified molecules of mitochondrial Ca^{2+} uptake, the MCU has been shown to be sensitive to ruthenium red, Ru360 and gadolinium (Baughman et al., 2011; De Stefani et al., 2011). An interesting clue regarding the domain that might mediate the ruthenium sensitivity is the loss of Ru360 sensitivity in the S259A MCU mutant (Baughman et al., 2011). Reconstituted LETM1-mediated Ca^{2+} flux in liposomes was also abolished by ruthenium red or Ru360 and was partially inhibited (25%) by CGP-37157 (4 μ M) (Jiang et al., 2009). Although experimental evidence is not available, based on the presence of the EF hands (though one is noncanonical), MICU1 might be a target of the calmodulin inhibitors. Thus, currently no drugs are available for effective and specific inhibition of the IMM Ca^{2+} uptake pathway in cellular systems. The genetic approach, including overexpression of dominant negative constructs (MCU-D260Q, E263Q double point mutant (De Stefani et al., 2011) and MCU E257A, D261A, E264A single point mutants (Baughman et al., 2011)) and silencing offer better options at this point.

Mitochondrial Ca^{2+} efflux was shown to be inhibited by benzodiazepine compounds (including a large group of neurotropic drugs such as clonazepam), **CGP-37157**, diltiazem, verapamil, bepridil, amiloride and amiloride analogues, which inhibit Ca^{2+} exchangers (Chiesi et al., 1987; Cox and Matlib, 1993). Diltiazem and CGP-37157 also interfere with several other Ca^{2+} transport mechanisms but 10 μM or less appears to suppress mitochondrial Ca^{2+} efflux without greatly altering other aspects of the intracellular calcium homeostasis. However, a point of discrepancy is that Neumann et al. showed less than 5 μM CGP-37157 to inhibit SERCA pumps and activate RyR (Neumann et al.), whereas Cox and Matlib reported that ≤ 10 μM CGP-37157 effectively inhibit the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchange without significantly inhibiting the plasma membrane Ca^{2+} channels, $\text{Na}^+/\text{Ca}^{2+}$ exchanger, Na^+/K^+ pump and SR Ca^{2+} pump (Cox and Matlib, 1993). Evidence has also been presented that the NCLX-mediated Ca^{2+} egress is also inhibited by CGP-37157 (5 μM : 50% inhibition, 7.5 μM : complete inhibition) (Palty et al., 2010). Although currently CGP-37157 is the sole effective and relatively specific drug to target the NCLX, it can be complemented with some potent molecular approaches (overexpression of a dominant-negative NCLX mutant, NCLX-S468T and silencing of NCLX by si/shRNA) (Palty et al., 2010).

3. Inhibition of the Ca^{2+} uniport by minocycline

Uncovering of the specific molecular components of the IMM Ca^{2+} transport will aid the design of new drugs to enhance the pharmacological approach. Recent studies of current drugs have also described some candidates for inhibition of the Ca^{2+} uptake, which may also turn out as useful tools to target the mitochondrial Ca^{2+} homeostasis. Minocycline, has received attention as a potential modulator of both the Ca^{2+} uniport and the PTP and also as a Ca^{2+} channel forming drug (Antonenko et al., 2010; Garcia-Martinez et al., 2010; Mansson et al.; Theruvath et al., 2008). To clarify the effect of minocycline on mitochondrial Ca^{2+} handling we measured cytoplasmic $[\text{Ca}^{2+}]_c$ ($[\text{Ca}^{2+}]_c$) and $[\text{Ca}^{2+}]_m$ and the $\Delta\psi_m$ in permeabilized RBL-2H3 cells.

In simultaneous measurements of $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_m$, IP_3 -evoked a rapid rise in $[\text{Ca}^{2+}]_c$ that was followed by a gradual decay (Figure 2A lower). Since IP_3 was added together with thapsigargin, a SERCA inhibitor, the decay phase was unlikely due to ER Ca^{2+} uptake. Closely following the $[\text{Ca}^{2+}]_c$ rise, the $[\text{Ca}^{2+}]_m$ also displayed an increase followed by a plateau phase (Figure 2A upper). In the presence of Ru360, the $[\text{Ca}^{2+}]_m$ increase and the decay phase of the $[\text{Ca}^{2+}]_c$ rise were inhibited (Figure 2A), indicating that these changes reflected the uniporter-mediated Ca^{2+} uptake (additional evidence was presented in (Csordas et al., 1999)). When increasing concentrations of minocycline (10, 40, 80 and 120 μM) were included in the cytoplasmic buffer, both the IP_3 -induced $[\text{Ca}^{2+}]_m$ increase and the decay of the $[\text{Ca}^{2+}]_c$ rise were dose-dependently inhibited (Figure 2A,B). Half-maximal inhibition was attained when 20–40 μM minocycline was added (Figure 2B). These effects were not due to attenuation of the ER Ca^{2+} release since the IP_3 -induced rapid rise in $[\text{Ca}^{2+}]_c$ was not suppressed by minocycline (Figure 2A lower). Furthermore, during bolus addition of CaCl_2 , the $[\text{Ca}^{2+}]_m$ rise and the clearance of the $[\text{Ca}^{2+}]_c$ increase were dose-dependently inhibited by minocycline (Figure 2CD).

To test the possibility that the Ca^{2+} uniport was inhibited by minocycline because of a decrease in the driving force of the mitochondrial Ca^{2+} uptake, $\Delta\psi_m$ was monitored simultaneously with $[\text{Ca}^{2+}]_c$ (Figure 3). Minocyclin by itself had a rapid, sustained hyperpolarizing effect possibly attributable to its ionophore nature. Up to 80 μM minocycline, the resting $\Delta\psi_m$ was maintained and no considerable depolarization was observed during IP_3 addition (Figure 3). Thus, at ≤ 80 μM minocycline, inhibition of the propagation of IP_3 -induced Ca^{2+} release to the mitochondria would reflect inhibition of the

uniporter activity. This concentration range of minocycline seems to be useful for selective inhibition of the uniporter. However, depolarization develops slowly at higher doses of minocycline upon IP₃ stimulation or rapidly at either minocycline doses upon bolus Ca²⁺ addition, indicating that under those conditions the inhibition of Ca²⁺ uptake cannot be attributed to inhibition of the uniporter per se. Ca²⁺-dependent depolarizing effect of higher doses of minocycline was probably due to either activation of the PTP (Garcia-Martinez et al., 2010; Mansson et al.) or Ca²⁺-dependent channel formation by the drug itself (Antonenko et al., 2010). Since minocycline is membrane permeable, we also tested if the IP₃-linked [Ca²⁺]_m signal was sensitive to minocycline in intact cells. Unfortunately, no significant inhibition was observed when 80 μM minocycline was applied (n=3, not shown). In summary, minocycline appears to be a useful drug for inhibition of the uniporter in subcellular systems. However, it is less potent inhibitor than ruthenium red or Ru360 and elicits multiple changes in mitochondrial bioenergetics at ≥80 μM concentration. Therefore, it remains a significant challenge for the field of mitochondrial Ca²⁺ that a selective inhibitor is still missing.

Conclusions

Few drugs are available for effective and specific inhibition of the different IMM Ca²⁺ transport mechanisms. For inhibition of the uniporter, minocycline provides an alternative to ruthenium red and Ru360 in subcellular systems but the application of all these drugs is limited in intact cells. For selective inhibition of the Na⁺/Ca²⁺ exchanger, low concentrations of CGP-37157 can be used in both subcellular models and in intact cells. The recent advance in cloning of MCU and MCU1 provide new opportunity to identify crystal structure of these proteins so that structural-basis of drug design and discovery can be pursued. Moreover, with future elucidation in the regulation and posttranslational modification of these Ca²⁺ transport proteins, it will become feasible to design small molecules for inhibiting the signaling mechanisms in such regulations. Thus, identification of the specific molecules mediating the Ca²⁺ transport will provide a new opportunity to develop more specific and flexible pharmacological tools.

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Highlights

- mitochondrial calcium uptake is important for cell metabolism, signaling and survival,
- RNA interference studies have just identified candidate proteins for Ca^{2+} uptake,
- the pharmacological aspects of the Ca^{2+} uptake mechanisms are reviewed.

Figure 1. Schematic presentation of the mitochondrial Ca^{2+} transport mechanisms and the candidate proteins

The black arrows represent the directions of Ca^{2+} transport, whereas the red arrows depict the feed-back and feed-forward effects of Ca^{2+} on the transport mechanisms. The blue arrows link the IMM transport mechanisms to the molecular candidates. The currently favoured candidates are printed in bold. Abbreviations: UCP2/3, uncoupling protein 2/3; Cyp D, cyclophilin D. For the sake of simplicity both IP3R and RyRs are depicted at the ER/SR-mitochondrial interface. Based on ultrastructural studies the RyRs are localized outside the interface area in both cardiac and skeletal muscle (Franzini-Armstrong, 2007) but sufficiently close to the mitochondria to expose them to high $[\text{Ca}^{2+}]_c$ (Garcia-Perez et al., 2008; Sharma et al., 2000).

Figure 2. Dose-dependent inhibition of the Ca^{2+} uniporter by minocycline (MC) in permeabilized RBL-2H3 cells

$[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_m$ rises associated with IP₃-induced Ca^{2+} release (A,B) or Ca^{2+} addition (CaCl_2 10 μM , C,D) were recorded in suspensions of permeabilized cells using fura2FF/AM loaded to the mitochondria and rhod2 dissolved in the cytosolic buffer (for detailed description of the measurement see (Csordas and Hajnoczky, 2001, 2003; Csordas et al., 1999)). IP₃ was applied in a saturating dose (8 μM). Analogue traces of IP₃-induced Ca^{2+} responses with or without (ctrl, black trace) MC pretreatment (120s, in the indicated doses in μM s) are shown in A. For reference, the MCU was also inhibited by the specific blocker Ru360 (10 μM , blue traces). Only increases of $[\text{Ca}^{2+}]_m$ are shown with the pre-stimulatory values shifted to zero because of a quenching artifact by MC (shown and explained in Figure 3). MC dose-inhibition curves for the $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_m$ responses associated with IP₃ stimulation are shown in B. The magnitude of the $[\text{Ca}^{2+}]_m$ rise (upper) and the extent of $[\text{Ca}^{2+}]_c$ clearance as the 55s post-peak decrease (lower) are shown. Analog traces and cumulated dose-inhibition correlation for 10 μM added Ca^{2+} are shown in C, D, respectively. Cumulated data are shown only for the initial rapid phase of the response, before the occurrence of significant depolarization (see Figure 3). For all, cumulative data are means $\pm$ S.E. from 3 experiments.

Figure 3. Effects of minocycline on the $\Delta\psi_m$ in permeabilized RBL-2H3 cells

Simultaneous recordings of $\Delta\psi_m$ (TMRE) and $[Ca^{2+}]_c$ (fura2) in suspensions of permeabilized RBL-2H3 cells are shown. TMRE was used in “dequenching mode” (depolarization increases the fluorescence). The cells were sequentially exposed to MC 0 (black) 80 (pink), 120 (purple) μM , IP_3 (8 μM), $CaCl_2$ (10 μM) and finally FCCP (Unc 10 μM) that completely dissipated $\Delta\psi_m$. MC exerted quenching of fura2 inequally at the two excitation wavelengths (more at 380 nm), resulting in a Ca^{2+} -independent rise in the ratio that also prevented proper translation of the fura2 signal to molar concentration of Ca^{2+} . The rapid increase in fura2 ratio was followed by a gradual decrease. This decrease was absent in the presence of Tg, indicating that MC might exert some stimulatory effect on SERCAs. The

rapid drop in TMRE fluorescence upon MC addition is likely to be a true hyperpolarization and not quench, since the fluorescence levels are equalized with the control after uncoupler addition.