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Mitochondrial Calcium Transport in Trypanosomes

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Abstract

The biochemical peculiarities of trypanosomes were fundamental for the recent molecular identification of the long-sought channel involved in mitochondrial Ca^{2+} uptake, the mitochondrial Ca^{2+} uniporter or MCU. This discovery led to the finding of numerous regulators of the channel, which form a high molecular weight complex with MCU. Some of these regulators have been bioinformatically identified in trypanosomes, which are the first eukaryotic organisms described for which MCU is essential. In trypanosomes MCU is important for buffering cytosolic Ca^{2+} changes and for activation of the bioenergetics of the cells. Future work on this pathway in trypanosomes promises further insight into the biology of these fascinating eukaryotes, as well as the potential for novel target discovery.

Keywords

Acidocalcisome; Calcium; Inositol 1, 4, 5-trisphosphate receptor; Mitochondrial calcium uniporter

1. Introduction

Trypanosomatids belong to one of the oldest branches of eukaryotic cells that possess mitochondria [1]. These organelles have very unusual properties in these cells. A special structure, known as the kinetoplast, was the first extranuclear DNA ever described [2] and consists of thousands of concatenated DNA minicircles and a few DNA maxicircles encoding a few gene products and having a very complex mechanism of replication (reviewed in [3]). Most mitochondrial mRNAs are subjected to editing by a process first discovered in these cells [4] (reviewed in [5]). The mitochondrial genome of trypanosomes does not contain tRNAs and the whole set of these tRNAs needs to be imported by a mechanism that share components with the protein import machinery [6, 7]. Some respiratory complexes are incomplete or absent in some trypanosomatids, such as in

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Phytomonas spp. [8], and in the bloodstream stages of the *Trypanosoma brucei* group [9]. These species are also characterized by the presence of an alternative oxidase [10, 11] similar to those present in plants and fungi, by the presence of an ATP synthase functioning in reverse, as an ATPase, to maintain the mitochondrial membrane potential [12, 13, 8, 14, 15], and by a partially functional, in *Phytomonas* spp. [8, 16], or absent, in the case of bloodstream forms of *T. brucei* [16], tricarboxylic acid cycle.

Despite these peculiarities, trypanosomatids are one of the eukaryotic groups that have conserved a mitochondrial Ca^{2+} transport mechanism (mitochondrial calcium uniporter or MCU) with similarities to those of animal cells, as first demonstrated in *Trypanosoma cruzi* in 1989 [17, 18]. Interestingly, this finding together with the reported absence of the uniporter described in *Saccharomyces cerevisiae* [19], and the availability of sequenced genomes of many species, led to the discovery of the molecular identity of the MCU [20, 21] and one modulator of the uniporter, the mitochondrial calcium uptake 1 or *MICU1* [22]. The early history of mitochondrial Ca^{2+} transport [23], and of the studies that led to the discovery of the uniporter [24–26] have been reviewed elsewhere.

2. The components of the mitochondrial Ca^{2+} uniporter

The ability of mitochondria to take up Ca^{2+} was discovered more than 50 years ago when it was found that rat kidney mitochondria were able to take up large amounts of Ca^{2+} [27, 28] and that this process was energized by coupled respiration [28]. The properties of this process were soon identified: Ca^{2+} uptake is inhibited by respiratory chain blockers and oxidative phosphorylation uncouplers [28] and does not require ATP hydrolysis, except when the respiratory chain is blocked, and in this case it is inhibited by oligomycin [29]; other divalent cations, such as Mn^{2+} [30, 31] and Sr^{2+} [32], can be taken up by this mechanism, while Mg^{2+} is a competitive inhibitor [33]; Ca^{2+} uptake is saturable and accompanied by H^+ extrusion [34] and could be accompanied by phosphate that can precipitate in the matrix [35]; the uniporter is inhibited by the dye ruthenium red [36] and its derivative, RU360 [37], and is a gated, Ca^{2+} -selective, ion channel [38].

Since the discovery of the molecular nature of the uniporter [20, 21] there has been a flurry of activity to identify all the components of the mitochondrial calcium uniporter complex (MCUC or uniplex).

HeLa cells MCU is a 40 kDa protein that loses its cleavable target sequence during mitochondrial import resulting in a 35 kDa mature form [20]. The protein has two transmembrane domains and topology studies have convincingly demonstrated that both its *N*- and *C*-terminal domains span into the mitochondrial matrix [39] while these two domains are connected in the intermembrane space by a short loop containing the DIME motif, which is highly conserved [20, 21] (Fig. 1). It has been suggested that the protein forms oligomers, probably tetramers, as part of a larger complex of about ~480 kDa [20], with eight helices lining the putative pore region where the DIME motif is, and charged residues in proximity of the pore favoring Ca^{2+} flux [40]. The reconstitution of MCU into planar lipid bilayers [21] and patch-clamp studies of mitoplasts (mitochondria devoid of outer membranes) [41] have demonstrated that this protein is the pore-forming subunit of the uniporter complex.

Homologs of MCU are found together with homologs to MICU1 in nearly all metazoa, including plants, as well as some fungi (i.e., *Cryptococcus neoformans*, *Neurospora crassa*) that do not have a MICU1 homolog, and other protists (i.e., trypanosomatids, ciliates, *Naegleria gruberi*, *Dictyostelium discoideum*, *Chlamydomonas reinhardtii*) and in some bacteria of the Bacteroides/Chlorobi group, but are absent in Apicomplexan (i.e., *Plasmodium spp.*, *Toxoplasma gondii*, *Cryptosporidium spp.*, *Eimeria spp.*) and in organisms lacking classical mitochondria (i.e., *Giardia intestinalis*, *Trichomonas vaginalis*, *Entamoeba histolytica*) [42]. Downregulation of *MCU* expression leads to autophagy [43] while overexpression leads to mitochondrial Ca^{2+} overload [21], which in turn leads to mitochondrial membrane permeabilization, and apoptosis [44]. Interestingly, expression of *D. discoideum MCU* alone in *S. cerevisiae* is sufficient to reconstitute MCU activity, while expression of the human *MCU* requires the co-expression of another component (essential MCU regulator or EMRE, see below) [45]. Surprisingly, *MCU* knockout mice are viable, although smaller in size, and with marked reduced ability to perform strenuous work, potentially linked to alterations in the phosphorylation of pyruvate dehydrogenase (PDH) [46].

MCU has a paralog, named MCUB, which in HEK-293T cells is a 35-kDa protein whose primary sequence is 50% similar to that of MCU and, as MCU, possesses two transmembrane domains (Fig. 1). MCUB has key mutations in the predicted pore-forming region and does not transport Ca^{2+} when inserted in planar lipid bilayers [40]. *MCUB* has lower expression level and a different expression profile from *MCU*, being more abundantly expressed in heart and lung and appears to be a subunit of the complex with inhibitory properties. MCUB is inserted into the MCU oligomer and exerts a dominant-negative effect [40] (Fig. 2A). Direct patch-clamp recordings from the inner mitochondrial membrane of different tissues of mice have indicated that the activity of the MCU varies greatly between them [47] and the variable presence of MCUB could be contributing to the different activity of the uniporter in these tissues [47].

MICU1 was discovered before MCU and was proposed as a modulator of the uniporter [22]. The HeLa cells MICU1 is a 54-kDa protein with two EF-hand Ca^{2+} -binding domains (Fig. 3). Two paralogs were found, MICU2 and MICU3, of which MICU3 is present mainly in neural tissues and skeletal muscle [48]. All of them have N-terminal mitochondrial targeting signals [48]. HeLa cells MICU2 is a 45-kDa protein with a ~27% sequence identity to MICU1 and two EF-hands domains (Fig. 3). MCU, MICU1 and MICU2 reside within a complex and cross-stabilize each other's protein expression in a cell-type dependent manner: loss of MICU1 leads to loss of MICU2 protein (but not mRNA) [49, 48], and in some cells (i.e., mouse liver), to decreased MCU expression [48]. Most authors agree that MICU1 and MICU2 localize to the mitochondrial intermembrane space [50, 49, 22], although in the case of MICU1 this has been disputed [51]. Their presence in the intermembrane space would allow these EF-hand containing proteins to sense cytosolic Ca^{2+} changes and control Ca^{2+} affinity of the complex [52] (Fig. 2A). MICU1 and MICU2 form a 95-kDa dimer bridged by a disulphide bond between MICU1-Cys465 and MICU2-Cys410 and interact with MCU through the short DIME loop of MCU located in the intermembrane space [49]. According to recent reports [53, 49] MICU1 and MICU2 would play non-redundant roles in the

regulation of MCU and would act as gatekeepers, inhibiting its activity at low cytosolic Ca^{2+} concentrations preventing mitochondrial overload, and stimulating its activity at high cytosolic Ca^{2+} concentrations. MICU1 [50, 43], MICU2 [49] or both MICU1 and MICU2 operating together [53] have been proposed as the gatekeepers. It is possible that the use of different cell types, and experimental protocols are responsible for these discrepancies. The crystal structure of Ca^{2+} -free and Ca^{2+} -bound MICU1 has recently been reported and suggest that Ca^{2+} -free MICU1 exists as a hexamer, and rearrange in multiple oligomers upon Ca^{2+} binding [54]. Interestingly, mutations of *MICU1* were found in human patients with a disease phenotype characterized by proximal myopathy, learning difficulties and a progressive extrapyramidal movement disorder, associated to a primary defect in mitochondrial Ca^{2+} signaling [55].

Three other regulators of the MCU recently described are MCUR1 (mitochondrial Ca^{2+} uniporter regulator 1) [56], EMRE [57], and the solute carrier 25 member 23 (SLC25A23) [58] (Fig. 2A). HeLa cells MCUR1 is a 40-kDa protein with two transmembrane domains and one coiled-coil region that is ubiquitously expressed in mammalian tissues [56]. The *N*- and *C*-terminus face the intermembrane space while ~250 amino acids are in the matrix. The protein interacts with MCU but not with MICU1. Downregulation of MCUR1 decreases mitochondrial Ca^{2+} uptake and appears to regulate the expression of MCU, while overexpression results in enhanced mitochondrial Ca^{2+} uptake [56]. Human EMRE is a 10-kDa protein with a single transmembrane domain located in the inner mitochondrial membrane. EMRE is ubiquitously expressed in mammalian tissues and required for the interaction of MCU with MICU1/MICU2, acting as a bridge. Its downregulation reduces mitochondrial Ca^{2+} uptake. As occurs with the MICU1 and MICU2, MCU cross-stabilizes EMRE and in MCU-depleted cells, EMRE abundance is greatly decreased [57]. SLC25A23 is a solute carrier belonging to the ATP-Mg/ P_i group, which transports adenine nucleotides in response to Ca^{2+} [59]. This is a multi-transmembrane, mitochondrial resident protein with two EF-hand motifs and two paralogs (SLC25A24 and SLC25A25). It was found that HeLa cells SLC25A23 interacts with MCU and MICU1, and that knockdown of its expression (but not of its paralogs) decreases mitochondrial Ca^{2+} uptake, and protects cells from oxidative stress [58]. These results [58] and a previous quantitative mass spectrometry study of affinity-purified MCUC that recovered MCU, MCUB, MICU1/MICU2, EMRE, and the phosphate carrier SLC25A3 [57] suggest the existence of associations of MCU and carriers in supercomplexes or transportomes [58]. Fig. 2A shows a schematic representation of the MCUC organization in mammalian cells.

3. Other potential mitochondrial Ca^{2+} uptake mechanisms

Before the discovery of MCU several proteins were proposed to have a role in mitochondrial Ca^{2+} uptake, such as the ryanodine receptor 1 (RyR1) [60, 61], the uncoupling proteins 2 and 3 (UCP2/3) [62], and the leucine zipper EF hand-containing transmembrane protein 1 (Letm1) [63]. The lack of correlation between RyR1 expression and MCU activity suggested that this mechanism may be specific only for muscle cells [64], while the role of UCP2/3 was questioned based on studies carried out with UCP2/3 knockouts [65]. On the other hand, Letm1 was previously reported to be a mitochondrial K^+/H^+ exchanger [66] and its role as a $\text{Ca}^{2+}/\text{H}^+$ exchanger [63, 67] has also been questioned [64, 68]. A more recent

contributor to mitochondrial Ca^{2+} uptake was identified as the canonical transient receptor potential 3 channel (TRPC3) [69]. The report that mice knockout for *MCU* are viable and that the mitochondrial calcium levels are reduced but not absent in the mitochondria derived from *MCU*^{-/-} mice suggest that there is some slow mechanism for mitochondrial Ca^{2+} uptake that is not dependent of MCU [46]. It is also possible that the mitochondria of some tissues may have more than one mechanism and more work is needed to clarify these discrepancies.

4. Mitochondrial Ca^{2+} release

Early biochemical experiments discovered that Na^+ released Ca^{2+} from isolated heart mitochondria [70]. This process was later characterized as a carrier mediated antiporter that exchanged Na^+ for Ca^{2+} [23] and was specifically inhibited by the benzothiazepine CGP-37157 [71]. The molecular identity of this exchanger was defined only a few years ago with the identification of *NCLX* (or *SLC25A6*) [72]. *NCLX* is particularly active in the mitochondria of excitable tissues, while other tissues appear to have a $\text{Ca}^{2+}/\text{H}^+$ exchanger. *NCLX* operates non-electroneutrally with a 1 Ca^{2+} per 3 Na^+ stoichiometry, while there is no consensus about the stoichiometry of the $\text{Ca}^{2+}/\text{H}^+$ exchanger (1 Ca^{2+} per 2 or 3 H^+) [73]. The molecular identity of the mitochondrial $\text{Ca}^{2+}/\text{H}^+$ exchanger is unknown.

5. Role of mitochondrial Ca^{2+} uptake

In many cells there are quasi-synaptic contacts between mitochondria and the endoplasmic reticulum (ER), the sarcoplasmic reticulum (SR), or the plasma membrane [74, 75] and Ca^{2+} release from the ER/SR or Ca^{2+} entry through plasma membrane channels create Ca^{2+} microdomains that can be rapidly removed by mitochondria, thereby buffering cytosolic Ca^{2+} increases and regulating the activity of the Ca^{2+} channels. Mitochondria can also act as a Ca^{2+} sink in polarized cells preventing diffusion of a Ca^{2+} wave to a different portion of the cell [74].

In addition to this cytosolic Ca^{2+} buffering role, mitochondrial Ca^{2+} uptake is important for regulation of cellular bioenergetics [76]. Intramitochondrial Ca^{2+} stimulates a PDH phosphatase that activates the PDH by dephosphorylation of its $\text{E1}\alpha$ subunit, or directly activates 2-oxoglutarate- and isocitrate-dehydrogenases, resulting in increased NADH production and ATP generation through oxidative phosphorylation. Ca^{2+} has been suggested to also directly stimulate the F_0F_1 -ATPase [77] and from the cytosol can stimulate mitochondrial solute carriers like the aspartate/glutamate and the ATP-Mg/Pi exchangers, which possesses EF-hand domains facing the cytosol [78, 79].

Mitochondrial Ca^{2+} has also important roles in regulation of life-death decisions. Mitochondrial Ca^{2+} overload has a permissive role in apoptosis and has also been associated to necrotic cell death, while decreased mitochondrial Ca^{2+} transport has been associated to autophagy, as a survival mechanism (reviewed in [74]).

6. Mitochondrial Ca^{2+} transport in trypanosomatids

The lack of a MCU in yeast [19] led some authors [80, 81] to believe that MCU was present only in vertebrates, but absent in plants, insects and other invertebrates, or in unicellular organisms. This situation was rectified in 1989 when we reported that epimastigotes of *Trypanosoma cruzi* have a MCU with similar properties to those described in mammalian mitochondria: electrogenic transport, sensitivity to ruthenium red, and low affinity and high capacity to take up Ca^{2+} [17, 18]. These studies were later confirmed in other life cycle stages of *T. cruzi* [82, 83] as well as in other trypanosomatids, including *Leishmania braziliensis* [84], *L. mexicana*, *L. agamiae* and *Crithidia fasciculata* [85], *L. donovani* [86], *T. brucei* [87, 15, 88], and *Herpetomonas* sp. [89]. The presence of a MCU in the bloodstream stages of *T. brucei* [15], which are devoid of a functional Krebs cycle, respiratory chain and oxidative phosphorylation, was unexpected, as it was clear at the time [81] that Ca^{2+} transport in vertebrate mitochondria was important for stimulation of three matrix dehydrogenases and, as a consequence, of oxidative phosphorylation. How Ca^{2+} could be transported in the absence of a respiratory chain-dependent mitochondrial membrane potential (Ψ_{μ}) was solved because Nolan and Voorheis have just discovered that the Ψ_{μ} of these bloodstream forms is generated by the ATP synthase acting in reverse, as an ATPase (Nolan and Voorheis, 1992), in agreement with previous reports [29] indicating that Ψ_{μ} can be maintained by ATP when the respiratory chain is blocked. This reverse activity of the ATPase was confirmed more recently using genetic tools [12, 14]. Fig. 4 shows that while mitochondrial Ca^{2+} transport by permeabilized procyclic forms requires respiratory substrates (succinate), mitochondrial Ca^{2+} transport by permeabilized bloodstream forms requires ATP. In both cases this activity is inhibited by ruthenium red.

Our studies also indicated the presence of separate pathways for Ca^{2+} influx and efflux as judged by the response of these mitochondria to the additions of Ca^{2+} and EGTA [18]. It was later found in other trypanosomatids [89] that the Ca^{2+} efflux mechanism is Na^{+} -independent and possibly due to a $\text{Ca}^{2+}/\text{H}^{+}$ exchanger. This is in agreement with the absence of orthologs for $\text{Na}^{+}/\text{Ca}^{2+}$ exchangers in early eukaryotes [90].

The discovery of the molecular nature of the mammalian MCU allowed the identification of the trypanosome orthologs. One gene encoding the putative trypanosome MCU was found in each trypanosomatid investigated (Fig. 1). As the mammalian counterpart, the proteins possess two predicted transmembrane domains and a mitochondrial targeting signal. For example, the open reading frame of the *T. brucei* MCU predicts a mature protein of 30 kDa, preceded by a 48 amino-terminal mitochondrial targeting signal. The putative Ca^{2+} -specific selectivity filter (DIME motif) is conserved in the loop between the two transmembrane domains at the C-terminal region [91].

Trypanosomatids also possess orthologs to MICU1-MICU2 (Fig. 3) and MCUB (Fig. 1) but there are no identifiable orthologs to MICUR1 or EMRE, which appear to be more recently acquired components of the MCUC [57] (Fig. 2B). Interestingly there are no orthologs to MICU2 in *Leishmania* spp. In addition, although there are sequences with homology to the aspartate-glutamate and ATP-Mg/Pi carriers that are regulated by Ca^{2+} in mammalian cells [79], none of the carriers in trypanosomes contain EF-hand domains [92] and are therefore

presumably Ca^{2+} insensitive. Other potential mitochondrial Ca^{2+} uptake mechanisms are probably absent in trypanosomes, as there is no evidence of the presence of orthologs to uncoupling proteins [92], ryanodine receptors, or TRPC-type channels [93], while the ortholog to Letm1 appears to function as a K^+/H^+ exchanger [94].

7. Role of mitochondrial Ca^{2+} uptake in trypanosomes

Experiments in *T. brucei*, in which the expression of *MCU* was down-regulated by RNAi or by conditional knockout, demonstrated that MCU is the sole responsible transporter for mitochondrial Ca^{2+} uptake in permeabilized cells and that this activity is essential for *in vitro* and *in vivo* growth of the parasites [91]. This work was the first to report the essentiality of MCU for the survival of a eukaryotic organism.

Downregulation of *TbMCU* results in decreased mitochondrial Ca^{2+} uptake without altering their membrane potential, as it was reported in mammalian cells [20, 21]. Importantly, down-regulation of *TbMCU* in procyclic stages increases the AMP/ATP ratio in the cells and autophagy, as determined by the increase in the number of autophagosomes and quantitative measurements of the autophagy marker Atg8-II [91], which is the ortholog of LC3-II in mammalian cells [95]. This is also in agreement with results reported in vertebrate cells in which blocking the mitochondrial Ca^{2+} uptake by inhibitors like RU360 [96] or knockdown of *MCU* [56] were shown to increase autophagy, as a survival mechanism. Interestingly, these effects are more pronounced when procyclic trypomastigotes are grown in a medium rich in proline and poor in glucose. Under these conditions, which are prevalent in the *tse tse* fly, procyclic forms increase the rate of proline consumption and are more sensitive to oligomycin supporting the view that oxidative phosphorylation becomes essential [97]. Proline is metabolized to glutamate through two mitochondrial reactions. The first is catalyzed by proline dehydrogenase or proline oxidase that oxidizes L-proline to 1 -pyrroline-5-carboxylate, which is in tautomeric equilibrium with glutamic- γ -semialdehyde, and can be hydrolyzed by 1 -pyrroline-5-carboxylate dehydrogenase to produce L-glutamate [98]. Glutamate is then deaminated by transaminases or dehydrogenases to be converted into α -ketoglutarate, which through some of the Krebs cycle reactions is converted into succinate. As the proline dehydrogenase of trypanosomatids possesses a unique EF-hand domain not present in mammalian proline dehydrogenases, it was proposed that mitochondrial Ca^{2+} could be important for the activation of this pathway [91]. In contrast, under glucose-rich conditions usually present in regular culture medium mitochondrial Ca^{2+} could be stimulating the PDH phosphatase thereby activating pyruvate oxidation. In this regard, the PDH E1 α subunit of trypanosomes seems to possess phosphorylation sites with similarity to those of the mammalian enzyme [99], suggesting that, as the mammalian enzyme, trypanosome PDH could be activated by Ca^{2+} -stimulated dephosphorylation. The mitochondrial PDH is important for the generation of acetyl-coenzyme A (acetyl-CoA) that is required for the formation of acetate, which translocates to the cytosol and is used for fatty-acid synthesis [100]. Fig. 5 shows the possible reactions occurring in procyclic trypomastigotes.

Overexpression of *TbMCU* in procyclic stages leads to increased mitochondrial Ca^{2+} uptake without changes in Ψ_m and to mitochondrial Ca^{2+} overload. These changes lead, similarly

to what occurs in vertebrate cells [21, 44] to increased sensitivity to pro-apoptotic agents such as C2-ceramide and H₂O₂, and to ROS generation, which results in cells death [91]. It is interesting to note that mitochondrial Ca²⁺ overload in trypanosomes has been linked to necrosis or programmed cell death mediated by ROS [101–103].

Down-regulation of *TbMCU* by conditional knockout in *T. brucei* bloodstream forms results in complete loss of mitochondrial Ca²⁺ uptake energized by ATP, while their membrane potential remains unaffected [91]. Interestingly, these bloodstream forms conserve PDH activity, albeit at lower levels than procyclic forms and evidence supporting a role for Ca²⁺ in stimulating this activity is suggested by the partial rescue of the lethal effect of *TbMCU* down-regulation by addition of threonine to the culture medium [91]. Bloodstream forms possess a threonine dehydrogenase [104] able to bypass the need of a Ca²⁺-stimulated step in the mutant parasites for generation of acetyl-CoA. These results are in agreement with the report that knockdown of PDH does not result in growth defect in *T. brucei* bloodstream forms unless the medium is depleted of threonine or threonine dehydrogenase is ablated simultaneously [105]. In bloodstream forms the acetyl-CoA generated from pyruvate or threonine could be used for intramitochondrial fatty acid synthesis (FAS II). This pathway is known to result in the generation of lipoic acid and myristic acid, and is essential in these trypanosomes [106]. Alternatively, acetyl-CoA could be used to generate acetate [107] that could be transferred to the cytosol where is converted back to acetyl-CoA by acetyl-CoA synthetase [100] and can be used for fatty acid synthesis, which appears to be essential for the bloodstream forms when they are in their mammalian hosts [108]. Fig. 6 shows a scheme of the reactions that could occur in bloodstream trypomastigotes.

The trypanosome mitochondria have also a role in buffering cytosolic Ca²⁺ increases. Ca²⁺ release from acidic stores (acidocalcisomes) or Ca²⁺ entry through the plasma membrane results in rapid mitochondrial Ca²⁺ uptake that reaches intramitochondrial levels that are much higher than cytosolic Ca²⁺ rises [109]. The recent discovery of an inositol 1,4,5-trisphosphate (IP₃) receptor in acidocalcisomes [110] and of a close proximity of acidocalcisomes and the mitochondria of trypanosomatids [111] are compatible with a role of IP₃-stimulated Ca²⁺ release from acidocalcisomes and mitochondrial Ca²⁺ uptake as a requirement for efficient mitochondrial metabolism and normal cell bioenergetics [91], as it has been described for the endoplasmic reticulum and mitochondria of vertebrate cells [96].

8. Concluding remarks

In conclusion, the mitochondrial Ca²⁺ uniporter complex (MCUC) presence in trypanosomes, which turned out to be so important for the discovery of the molecular identity of MCU, appears simpler than that of mammalian cells and composed by a pore subunit (MCU) a dominant negative subunit (MCUb), and a regulatory couple formed by MICU1 and MICU2. However, we cannot rule out the existence of highly divergent or entirely different regulators. In contrast to what happens in mice, MCU is essential for survival in *T. brucei*. Mitochondrial Ca²⁺ efflux is probably through a Ca²⁺/H⁺ antiporter and there is no evidence for the presence of Ca²⁺-regulated carriers. Mitochondrial Ca²⁺ is probably important for the regulation of the activity of PDH E1 α phosphatase in *T. brucei* bloodstream forms, and proline dehydrogenase in procyclic stages, and for shaping the

amplitude of cytosolic Ca^{2+} increases in the proximity of acidocalcisomes and the plasma membrane. Some of the questions that remain to be answered in trypanosomes is how the bioenergetics in general and activities such as PDH and proline dehydrogenase in particular are directly regulated by Ca^{2+} and whether, as occurs in vertebrate cells [96], constitutive generation of IP_3 with stimulation of the IP_3R and Ca^{2+} release from intracellular stores (acidocalcisomes in case of trypanosomes), followed by mitochondrial Ca^{2+} uptake regulates mitochondrial activity. Further studies in this area could provide important insights into the biology of these parasites, the evolution of these transport mechanisms, and ultimately novel targets for trypanocidal drugs.

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Highlights

Discovery of MCU in trypanosomes led to the finding of the *MCU* gene in mammals while *MCU* is essential in trypanosomes it is not essential in mice

Other components of the trypanosome MCU complex are MCUb, MICU1 and MICU2

Mitochondrial Ca^{2+} is essential to regulate trypanosome bioenergetics.

Fig. 1.

Domain organization of MCU and MCUB proteins highlighting two highly conserved transmembrane domains and one putative pore region, from *Trypanosoma brucei* (TbMCU, Tb427tmp.47.0014; TbMCUB, Tb427.10.300), *Trypanosoma cruzi* (TcMCU, TcCLB.503893.120; TcMCUB, TcCLB.504069.4), *Leishmania major* (LmMCU, LmjF.27.0780; LmMCUB, LmjF.21.1690), and *Homo sapiens* (HsMCU, NP_612366.1; HsMCUB, NP_060388.2). Two critical conserved substitutions from MCU to MCUB are boxed and indicated with arrows. MTS, mitochondrial targeting sequence; coil, coiled-coil domain; TM, transmembrane domain; DIME, functional “DIME” motif, the putative Ca²⁺ selectivity filter.

Fig. 2. Schematic representation of the mitochondrial calcium uniporter complex (MCUC) organization of mammals (A) and trypanosomes (B), was based on our interpretation of published data, and modified from Pendin et al. (52) and Sancak et al. (57). IMS: intermembrane space. Black balls represent Ca^{2+} .

Fig. 3.

Domain organization of MICU1 and MICU2 proteins highlighting two conserved EF hands, from *Trypanosoma brucei* (TbMICU1, Tb427.08.1850; TbMICU2, Tb427.07.2960), *Trypanosoma cruzi* (TcMICU1, TcCLB.511391.210; TcMICU2, TcCLB.510525.130), *Leishmania major* (LmMICU1, LmjF.07.0110), and *Homo sapiens* (HsMICU1, NP_006068.2; HsMICU2, NP_689939.1). MTS, mitochondrial targeting sequence; EF, EF-hand domain for Ca^{2+} binding

Fig. 4.

Representative tracings of Ca^{2+} uptake by digitonin-permeabilized wild-type procyclic (A) and bloodstream (B) forms of *T. brucei*. (A). The reaction buffer contained 2 mM succinate, and 1 μM Calcium Green 5-N. After several pulses of Ca^{2+} (5 μM final concentration), 5×10^7 procyclic forms were added to the reaction medium (2.45 ml) and the reaction was started adding 50 μM digitonin. Blue arrow indicates addition of digitonin to permeabilize the cells. No Ca^{2+} uptake was detected in the absence of succinate (not shown). (B) The reaction containing 2×10^8 bloodstream forms with 1 μM Calcium Green 5-N was started adding 40 μM digitonin in the presence of 1 mM ATP and 500 μM sodium orthovanadate. Ruthenium red (RR, 40 μM , red tracing), FCCP (5 μM) or oligomycin (Oligo, 2.5 $\mu\text{g/ml}$) were added where indicated. No Ca^{2+} uptake was detected in the absence of ATP (not

shown). A decrease in fluorescence indicates decreasing medium Ca^{2+} or increasing mitochondrial Ca^{2+} . Other conditions as in reference 91.

Fig. 5.

Scheme of the energy metabolism in procyclic trypomastigotes. Arrows indicate steps of glucose, proline, and threonine metabolism and glycerolipid biosynthesis; dashed arrows indicate steps for which no evidence of flux is available. Abbreviations: A, ATPase; AcCoA, acetyl-CoA; Cit, citrate; CoASH, coenzyme A; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; G3P, glycerol 3-phosphate; KG, 2-ketoglutarate; Mal, malate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Resp. Ch., respiratory chain; TCA, tricarboxylic acid cycle. Enzymes are: 1, pyruvate dehydrogenase; 2, acetyl-CoA thioesterase; 3, acetate:succinate CoA-transferase; 4, succinyl-CoA synthetase; 5, F₀F₁-ATP synthase; 6, acetyl-CoA synthetase; 7, threonine dehydrogenase; 8, proline dehydrogenase. Activities potentially stimulated by Ca²⁺ are indicated. Reprinted with permission from ref. 91.

Fig. 6. Scheme of the energy metabolism in bloodstream trypomastigotes. Abbreviations and symbols as in Fig. 5, except for: A, ATPase; FAS II, type II fatty acid biosynthesis pathway; GPDH, glycerol 3-phosphate dehydrogenase; UQ, ubiquinone; TAO, trypanosome alternative oxidase. Pyruvate is mostly excreted out of the cells. Activity potentially stimulated by Ca^{2+} is indicated. Reprinted with permission from ref. 91.