Review

Trypanosomes and the solution to a 50-year mitochondrial calcium mystery

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The ability of mitochondria to take up Ca^{2+} was discovered 50 years ago. This calcium uptake, through a mitochondrial calcium uniporter (MCU), is important not only for the regulation of cellular ATP concentration but also for more complex pathways such as shaping Ca^{2+} signals and the activation of programmed cell death. The molecular nature of the uniporter remained unknown for decades. By a comparative study of mitochondrial protein profiles of organisms lacking or possessing MCU, such as yeast in the former case and vertebrates and trypanosomes in the latter, two groups recently found the protein that possesses all the characteristics of the MCU. These results add another success story to the already substantial contributions of trypanosomes to mammalian biochemistry.

Mitochondrial discovery

Mitochondria (Glossary) have a central role in intracellular Ca^{2+} homeostasis, and it is well-established that intramitochondrial Ca^{2+} concentration can reach micromolar values of tens to hundreds upon a few micromolar rise in cytosolic Ca^{2+} [1,2]. This is because mitochondria are exposed to microdomains of high Ca^{2+} concentration in proximity to sites of Ca^{2+} release at the endoplasmic reticulum (ER), or to Ca^{2+} channels at the plasma membrane [1–6]. This Ca^{2+} uptake is important for shaping the amplitude and spatiotemporal patterns of cytosolic Ca^{2+} increases [7–9] and for regulating the activity of three mitochondrial dehydrogenases. Intramitochondrial Ca^{2+} stimulates a pyruvate dehydrogenase phosphatase that activates the pyruvate dehydrogenase or allosterically activates 2-oxoglutarate- and isocitrate-dehydrogenases, resulting in increased ATP production [10–15]. Activation by Ca^{2+} of metabolite carriers on the external face of the mitochondrial inner membrane also facilitates this stimulation of energy production [16,17]. Excessive Ca^{2+} uptake, however, favors the formation of the 'permeability transition pore', leading to the release of pro-apoptotic factors in the cytosol and cell death (reviewed in [18]).

Under physiological conditions, mitochondrial Ca^{2+} uptake occurs by a uniport mechanism driven electrophoretically by the negative-inside membrane potential without direct coupling to ATP hydrolysis or transport of other ions [19]. The activity of this mitochondrial calcium uniporter (MCU) was found 50 years ago [20,21], and the biophysical properties of this Ca^{2+}-selective channel were extensively characterized [19,22]. However, the molecular nature of the channel was only recently identified as a result of progress in genome sequencing and the knowledge of the distribution of the uniporter in different eukaryotes [23,24]. Trypanosomes had a fundamental role in this discovery.

Discovery of the mitochondrial calcium uniporter (MCU) of trypanosomes

For many years after discovery of the MCU in mammalian mitochondria [20,21] it was thought that less-complex lifeforms such as plants, insects and other invertebrates [25] or unicellular organisms, such as yeast [26], lacked a specific uptake pathway. This situation was rectified in 1989 [27,28] when it was reported that epimastigotes of *Trypanosoma cruzi*, the etiologic agent of Chagas disease, possess a MCU with characteristics similar to those described in mammalian mitochondria: electrogenic transport, sensitivity to

Glossary

Acidocalcisomes: acidic calcium stores rich in polyphosphate present in different organisms from bacteria to humans.
Aequorin: fluorescent protein from the jellyfish *Aquora victoria* used to detect calcium *in vivo.*
Antimycin A: potent inhibitor of the respiratory chain at the level of cytochrome b–c_1.
Aspartate-glutamate carrier: transporter that exchanges aspartate for glutamate located at the mitochondrial outer membrane.
ATP-Mg-Pi carrier: transporter that exchanges ATP-Mg for Pi located at the mitochondrial outer membrane.
Bcl-2 (B cell lymphoma 2) family: a family of apoptosis regulator proteins.
Caspases: proteases involved in cell death.
Excavata: a supergroup of unicellular eukaryotes that include many human parasites.
Isocitrate dehydrogenase: enzyme that catalyzes the conversion of isocitrate to succinate in the mitochondrial matrix.
Mitochondria: membrane-enclosed organelles found in most eukaryotic cells. Only one mitochondrion per cell is present in trypanosomes. As in other eukaryotes, its compartments include the outer membrane, the intermembrane space, the inner membrane, and the matrix.
Oligomycin: inhibitor of the mitochondrial ATP synthase.
Petite: yeasts and trypanosomes that have lost most or all of their mitochondrial DNA.
Pyruvate dehydrogenase: enzyme that catalyzes the conversion of pyruvate into acetyl-CoA.
Ruthenium red: potent inhibitor of the mitochondrial calcium uniporter.
Ruthenium 360: potent inhibitor of the mitochondrial calcium uniporter related to ruthenium red.
Thapsigargin: potent inhibitor of sarcoplasmic–endoplasmic reticulum (SERCA) calcium ATPase.

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ruthenium red, and low affinity for the cation. As occurs with mammalian mitochondria, addition of Ca^{2+} to digitonin-permeabilized *T. cruzi* epimastigotes in the presence of mitochondrial substrates, such as succinate, and absence of ATP, stimulates respiration (Figure 1a), and this is accompanied by ruthenium red-sensitive Ca^{2+} uptake (Figure 1b) [28]. Successive Ca^{2+} addition reveals the high capacity of these mitochondria to accumulate Ca^{2+} (Figure 1b) [28]. Ca^{2+} uptake also results in a small decrease in membrane potential in agreement with its electrophoretic transfer into the mitochondria (Figure 1c) [29].

This MCU was later described in other trypanosomatids such as *Leishmania braziliensis* [30], *Leishmania mexicana, Leishmania agamae, Crithidia fasciculata* [31], *Leishmania donovani* [32], in the infective stages of *T. cruzi* [33,34], and finally in *Trypanosoma brucei* [35–37]. The finding of a MCU uniporter in the bloodstream (BS) stage of *T. brucei* [38] was surprising because these stages lack a respiratory chain. However, Lehninger *et al.* had described in 1963 [39] that Ca^{2+} uptake into rat liver mitochondria under favorable conditions could be energized by ATP in the absence of respiration, in which case it was inhibited by oligomycin, and not by inhibitors of the respiratory chain. This is also what happens in BS trypanosomes: the mitochondrial membrane potential is dependent on hydrolysis of ATP by the ATP synthase which acts as an ATPase [38,40–42], allowing Ca^{2+} still to be electrophoretically transported by the MCU [38]. Figure 1d shows that the membrane potential of BS trypanosomes is collapsed by oligomycin. Ca^{2+} uptake by BS trypanosomes has three characteristics: (i) it takes place until the ambient free Ca^{2+} concentration is lowered to 0.6–0.7 μM, (ii) it is inhibited by oligomycin, and (iii) it is associated with the depolarization of the inner membrane energized by ATP. These results indicate that Ca^{2+} uptake is mediated by the ATPase-dependent energization of the inner mitochondrial membrane [38].

Discovery of the MCU Protein

The evolutionary conservation of a MCU in vertebrates and kinetoplastids, and its absence in yeast, was utilized to identify proteins required for Ca^{2+} uptake [43]. From an inventory of 1098 mouse mitochondrial proteins from 14 tissues, 1013 of which mapped to human genes (MitoCarta [44]), 18 fit the following criteria: (i) localization in the

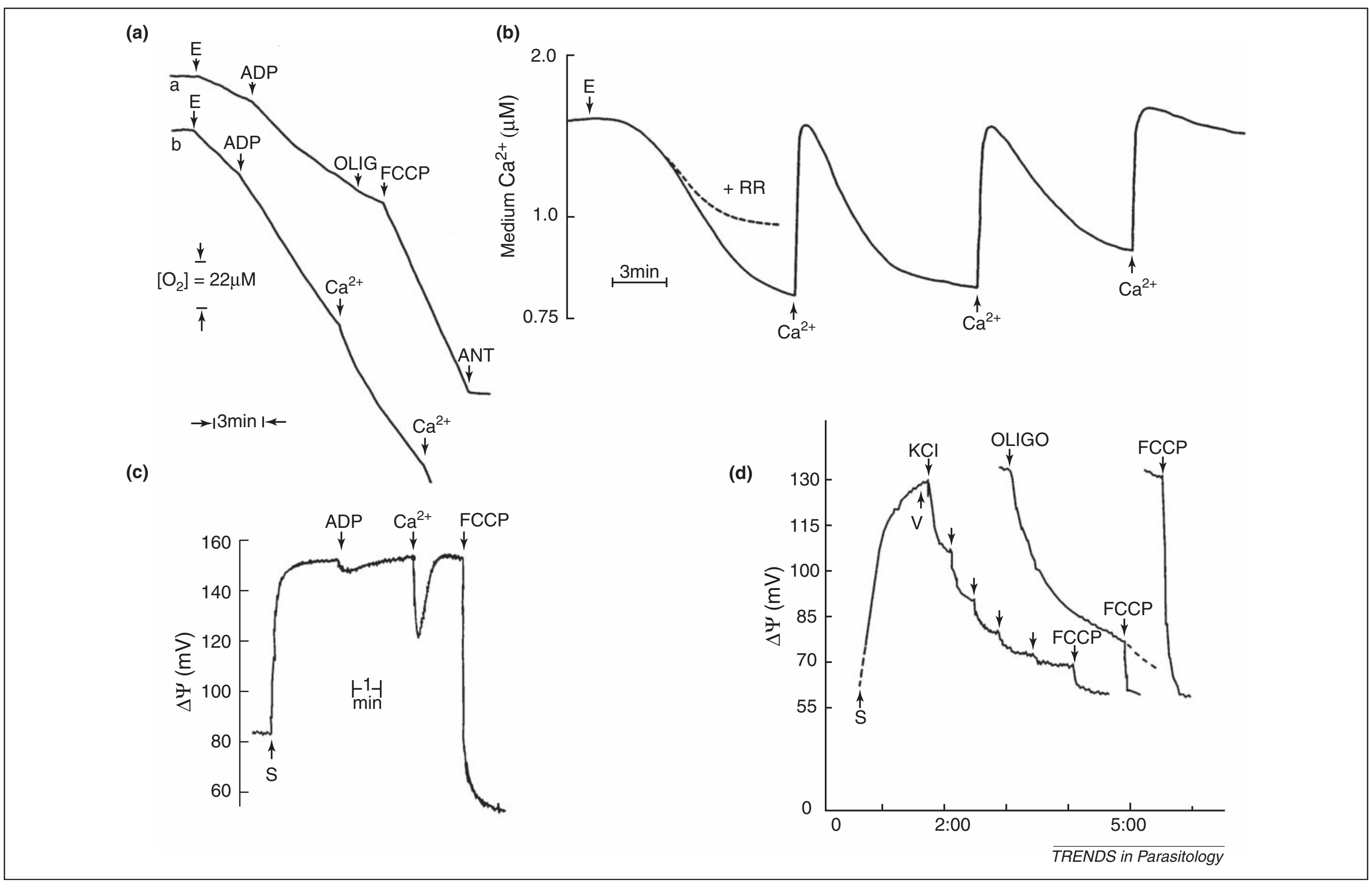

Figure 1. Evidence for a mitochondrial calcium uniporter (MCU) in *Trypanosoma cruzi*. **(a)** Trace a: this shows that oxygen uptake by digitonin-permeabilized epimastigotes (E) in the presence of succinate increases after the addition of ADP, indicating oxidative phosphorylation. The rate of nonphosphorylating respiration was obtained by the addition of oligomycin (OLIG) and the maximal rate of respiration was induced by addition of the uncoupler carbonyl cyanide p-trifluoromethoxylhydrazone (FCCP). Antimycin A (ANT) completely abolished respiration. Trace b: shows that addition of $CaCl_2$ (Ca^{2+}) to these preparations stimulates respiration, indicating its electrophoretic transport into the mitochondria. **(b)** Successive Ca^{2+} addition to these mitochondria results in Ca^{2+} uptake until their capacity to take up Ca^{2+} is exhausted. This uptake is inhibited by ruthenium red (RR). **(c)** The mitochondrial membrane potential in digitonin-permeabilized epimastigotes in the presence of succinate can be measured with safranine (S). After safranine addition there is an increase in absorbance that indicates stacking of the dye to the energized mitochondrial membrane. A membrane potential value of 140–150 mV was calculated using the Nernst equation. Addition of $CaCl_2$ to these preparations results in a decrease in membrane potential, compatible with the electrophoretic influx of Ca^{2+} into the mitochondria. **(d)** Determination of the mitochondrial membrane potential of BS trypanosomes *in situ*. The increase in absorbance after S addition is reversed by the subsequent addition of oligomycin (OLIGO) or FCCP. Titration of $\Delta\Psi$ was performed by the addition of known concentrations of KCl (arrows) in the presence of valinomycin (V). A membrane potential value of 130 mV was calculated. Reproduced with permission from [28] **(a,b)**, [29] **(c)** and [38] **(d)**.

inner mitochondrial membrane, (ii) expression in the majority of mammalian tissues, and (iii) having homologs in vertebrates and kinetoplastids but not in the yeast *Saccharomyces cerevisiae* [43]. An RNAi screen of the top 13 candidates allowed identification of the mitochondrial calcium uptake 1 (MICU1) protein, an MCU regulator. Use of a similar exclusion method and examining proteins with at least two transmembrane domains that are not expressed in yeast but conserved in kinetoplastids, one protein (NP_001028431 in *Mus musculus*) was identified and named MCU [23]. Figure 2 shows that MCU has two highly conserved transmembrane domains that are present in several eukaryotes including trypanosomatids. Real-time PCR demonstrated a universal tissue expression of the MCU protein and coexpression with MICU1 in mice [23]. Working with HeLa cells, silencing MCU by RNAi revealed a role of this protein in mitochondrial Ca^{2+} uptake independent of changes in the mitochondrial membrane potential. Overexpression of the gene increased the speed of Ca^{2+} uptake and mitochondrial Ca^{2+} concentration, and sensitized the cells to cell death following H_2O_2 or ceramide treatment due to Ca^{2+} overload. The recombinant protein was purified and showed channel activity in lipid bilayers, and mutagenesis of charged amino acids (glutamines) in the presumed pore-forming region of MCU abolished its channel activity. In parallel, another study performed complementary computational analyses to predict proteins functionally related to MICU1 and essential for mitochondrial Ca^{2+} uptake – and spotlighted the same protein CCDC109A (NM_138357.1 in *Homo sapiens*) which was also named MCU [24]. RNAi experiments were also performed in HeLa and HEK-293 cells, as well as in mouse liver, to investigate the role of MCU in mitochondrial Ca^{2+} uptake. In contrast to the results of De Stefani *et al.* [23], overexpression of MCU by Baughman *et al.* [24] failed to stimulate Ca^{2+} uptake; their topology experiments suggested that the N- and C-termini of MCU face the matrix rather than the intermembrane space, and a large complex was needed to induce Ca^{2+} transport rather than MCU alone. These discrepancies will need to be worked out in the future.

Roles of mitochondrial Ca^{2+} in trypanosomes

The roles of mitochondrial Ca^{2+} in trypanosomes are apparently more limited than in mammalian cells. None of the dehydrogenases stimulated by Ca^{2+} in vertebrates [45] have been studied in detail in trypanosomatids. There is no evidence that the pyruvate dehydrogenase E1 subunit, whose gene was identified in *T. cruzi* [46], is activated by dephosphorylation, as is the mammalian orthologous enzyme, although it seems to possess phosphorylation sites with similarity to those of the mammalian enzyme [46]. The mitochondrial isocitrate dehydrogenase present in trypanosomatids is NADP-dependent [47], in contrast to the Ca^{2+}-regulated mammalian NAD-dependent isocitrate dehydrogenase. The FAD-glycerol phosphate dehydrogenase, which is activated by Ca^{2+} in vertebrates and invertebrates but apparently not in yeast and plants [45] is, as in these latter organisms, devoid of the Ca^{2+}-binding EF-hands domains and is presumably insensitive to Ca^{2+}. In addition, BS *T. brucei* probably do not express these dehydrogenases, although they possess a MCU [38]. Although there are sequences with homology to the aspartate-glutamate carrier (AGC) and ATP-Mg-Pi carriers (SCaMCs), which in mammalian cells are known to be regulated by Ca^{2+} [17], the orthologs in trypanosomes lack EF-hand domains that are present even in the *S. cerevisiae* homolog [48], and are therefore presumably also Ca^{2+} insensitive.

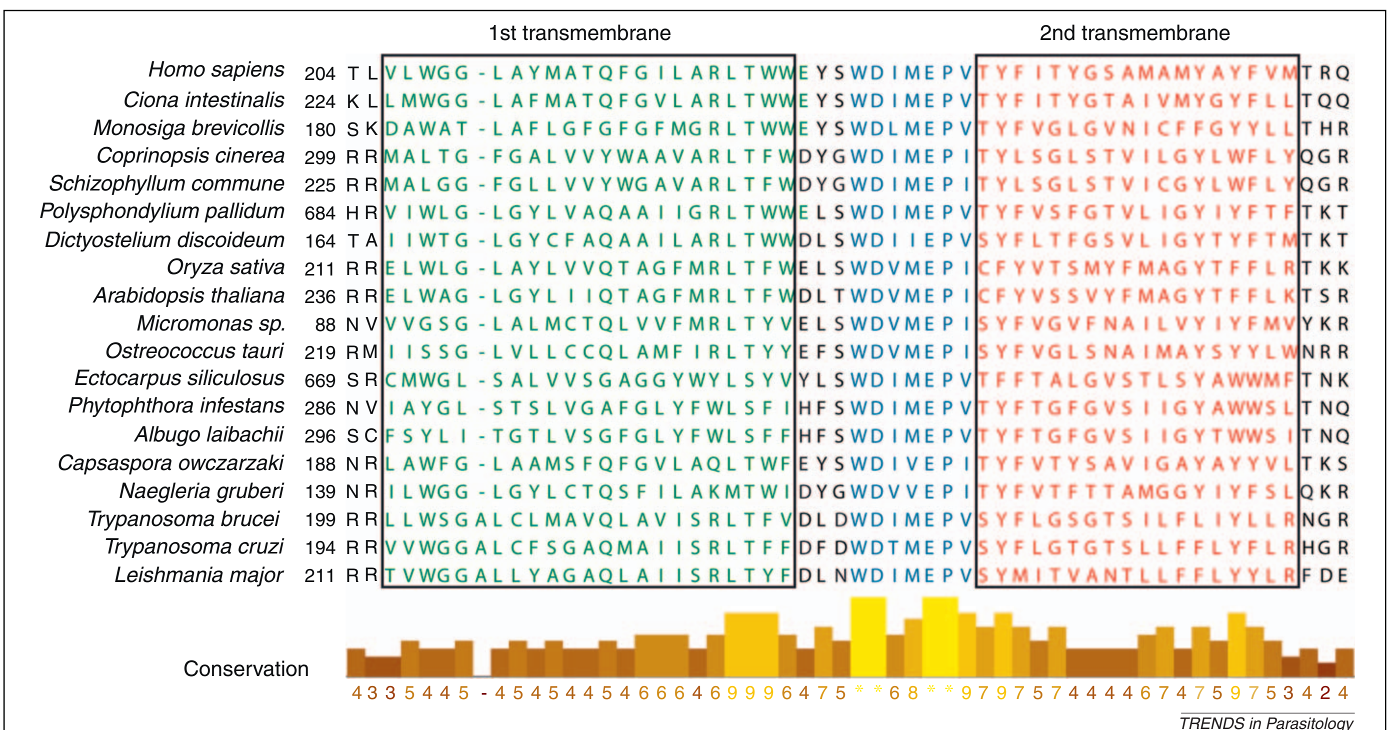

Figure 2. The mitochondrial calcium uniporter includes two highly conserved transmembrane domains. The alignment is of the putative transmembrane domain and pore region of MCU proteins from 19 eukaryotes including several trypanosomatids. The graph indicates the sequence conservation.

Experiments using aequorin targeted to the mitochondria of *T. brucei* revealed that intramitochondrial Ca^{2+} concentrations in *T. brucei* can reach values much higher than cytosolic Ca^{2+} rises when Ca^{2+} influx through the plasma membrane or Ca^{2+} release from acidic calcium stores (acidocalcisomes) are stimulated [37], just as in mammalian cells [1,2]. In fact, membrane potential-dependent Ca^{2+} uptake into the mitochondrion of *T. brucei* can be induced, as occurs in the human organelle, at both nano- and micromolar concentrations [49]. These results suggest a very close proximity of these organelles and the presence of microdomains of high Ca^{2+} concentration in the vicinity of the plasma membrane or acidocalcisomes [37]. Because the sarcoplasmic–endoplasmic reticulum Ca^{2+}-ATPase (SERCA) of *T. brucei* has low sensitivity to thapsigargin, a microdomain of high Ca^{2+} concentration between the ER and the mitochondria could not be established in these studies [37]. However, these results suggest that one of the main functions of the MCU in trypanosomes would be, as in mammalian mitochondria [7–9], to shape the amplitude and spatiotemporal patterns of cytosolic Ca^{2+} increases. In mammalian cells, the clustering of the outer mitochondrial membrane voltage-dependent anion channels (VDACs) at the ER/mitochondrial contact sites and in close contact with the inositol 1,4,5-trisphosphate receptor (IP_3R) appears to be limiting for the Ca^{2+} uptake capacity of the organelle when Ca^{2+} is released from the ER [50]. Trypanosomes possess a single VDAC ortholog, porin, which is required for mitochondrial metabolite transport and is essential under growth conditions that depend on oxidative phosphorylation [51,52], but the localization of their IP_3R-like proteins is unknown [53].

Mitochondrial Ca^{2+} is a recognized contributor to programmed cell death (PCD), or apoptosis, in trypanosomatids. Morphological features that can be attributed to PCD, such as shrinkage, membrane blebbing, mitochondrial alterations and chromatin condensation were described in *T. cruzi* as early as 1977 [54]. Trypanosomatids, however, lack some of the key regulatory or effector molecules involved in apoptosis in mammalian cells, such as the tumor necrosis factor (TNF)-related family of receptors, Bcl-2 family members, and caspases [55,56]. Mitochondrial Ca^{2+} overload with changes in mitochondrial membrane potential, reactive oxygen species (ROS) generation and release of cytochrome *c* have been observed upon different triggers of cell death in trypanosomatids [57]. In *T. brucei*, the production of ROS impairs mitochondrial Ca^{2+} transport, leading to its accumulation in the nucleus, causing cell death [58]. In *Leishmania*, a mitochondrial endonuclease G is released and translocated to the nucleus [59] leading to stimulation of a caspase-independent, apoptosis-like cell death (reviewed in [57]). *T. cruzi* appears to be highly resistant to mitochondrial permeability transition [27], and apoptosis-like death upon mitochondrial Ca^{2+} overload is dependent on superoxide anion generation [60].

In summary, mitochondrial Ca^{2+} uptake in trypanosomatids appears to have a role in shaping the amplitude of cytosolic Ca^{2+} increases after influx through the plasma membrane or release from acidocalcisomes, and in apoptosis-like death, but apparently not in the regulation of ATP production.

How mitochondrial Ca^{2+} is released in trypanosomes

The mitochondrial Ca^{2+} efflux pathway in mammalian cells appears to promote the exchange of matrix Ca^{2+} by external Na^+ (in excitable cells) or H^+ (in non-excitable cells) [61]. A gene encoding the Na^+/Ca^{2+} exchanger NCLX was recently identified [62] and the encoded protein was shown to possess all of the characteristics of the Na^+/Ca^{2+} exchange activity described years ago [61]. The exchanger is located in the inner mitochondrial membrane and is inhibited by CGP-37157, which was originally discovered as an inhibitor of this activity in 1988 [63]; its overexpression enhances Na^+/Ca^{2+} exchange activity, and its silencing reduces it. However, there are no orthologs of this gene in trypanosomatids. Evidence for a Ca^{2+} efflux pathway in *T. cruzi* has been presented [27] and, in agreement with those results, trypanosomatids possess an ortholog to the Letm1 protein, which has recently been described as encoding a mitochondrial Ca^{2+}/H^+ exchanger [64]. Surprisingly, the mammalian exchanger is blocked by ruthenium 360, and partially inhibited by CGP-37157. This finding is puzzling because the insensitivity of mitochondrial Ca^{2+} exchangers to ruthenium red had been established before [61]; further work is necessary to confirm, or exclude, the direct role of Letm1 in mitochondrial Ca^{2+} handling [50].

Uniqueness of the trypanosome mitochondrion

Trypanosomes harbor peculiar mitochondria. As members of Excavata, recently viewed as the most basal eukaryotic supergroup [65], they retain some putatively very primitive features, in particular the unusual biogenesis of cytochrome *c* [66] and highly simplified protein-import machinery [67]. This machinery probably evolved immediately subsequent to endosymbiosis, qualifying kinetoplastids as strong candidates for one of the earliest extant eukaryotic lineages [68].

The existence of a single mitochondrion per cell in either active or repressed form (see below), along with the availability of high quality mitoproteome of procyclic form (PF) *T. brucei* [69], combined with our rather advanced knowledge of the kinetoplastid organelle, qualify it as a very suitable model mitochondrion, already successfully explored in several ways.

The trypanosome mitochondrion as a model organelle

We have so far presented an elegant use of trypanosomes in elucidating the molecular basis of mitochondrial Ca^{2+} influx. Similarly, dissection of the replication and maintenance of the mitochondrial DNA in kinetoplastids (kDNA) network, the first extranuclear DNA ever observed, was very instrumental for studies of less abundant organellar DNAs in other eukaryotes, and provided one of the key insights into the topology of circular DNA molecules ([70,71]) for recent reviews). Another landmark, achieved by studying this organelle in *T. brucei*, *Leishmania tarentolae* and *Crithidia fasciculata*, was the discovery of RNA editing ([72,73] for recent reviews). More recently, it was the conspicuous absence of several genes in the genomes of trypanosomatids and a few other eukaryotes that was instrumental for the identification, through phylogenetic profiling, of novel subunits of human NADH dehydrogenase (respiratory complex I) [44].

T. brucei is particularly suitable for studies of processes that control the activity of its single mitochondrion. Although the organelle in the PC stage is metabolically and physiologically similar to the conventional eukaryotic mitochondrion, it transforms into a highly suppressed form in the BS stage [74]. Proteins involved in kDNA replication, mitochondrial RNA editing and processing, and tRNA import and translation, are present and essential throughout the life cycle [75–79]; however, the morphology and metabolism of the organelle undergo extensive remodeling [74]. The ability to obtain fully functional PF mitochondria, as well as the downregulated vesicles from the BS stage, makes them very attractive for studies of differential expression and/or import of mitochondrial proteins.

As mentioned above, another major difference between the PC and BS mitochondria is that F_oF_1-ATP synthase produces ATP in the former, but consumes it in the latter organelle, being essential in both [41]. The dramatic switch between the antagonistic activities of F_oF_1-ATP synthase during the trypanosome life cycle strikingly resembles the frequently lethal switch of orthologous synthase in the mitochondria of human heart during myocardial ischemia. This is not the only peculiar and unexpected similarity between the human and *T. brucei* mitochondria. Despite its uniquely simple protein-import machinery [67,68], the *T. brucei* organelle readily accepts complex human mitochondrial import signals, making functional analyses of human proteins fairly straightforward in this background [79,80]. Moreover, it is worth noting that mitoribosomes in humans and trypanosomes are the most protein-rich and rRNA-poor ribosomes known [69,81], thus it is possible that they are subject to similar, but presently unknown, selective pressures.

Another interesting phenomenon observed in the African trypanosomes is that some lineages are prone, in nature or in the laboratory, to lose parts of their kDNA, with some mitochondria being totally devoid of kDNA [82,83]. Their host strains, *T. brucei evansi*, are in fact 'petite' mutants [83], which spread out of Africa due to their acquired independence from the tsetse fly as a vector [84]. These trypanosomes are particularly suitable for analyses of the interactions between the mitochondrion and cell nucleus because organellar transcription and translation are absent without the requisite mitochondrial-encoded genes. It is somewhat counterintuitive that proteins responsible for kDNA replication and RNA metabolism continue to be imported [83,85], and the same was recently shown for import of nuclear-encoded tRNAs into the mitochondrion [76,77]. It will be exciting to examine further the extent of this apparent lack of communication between the autonomous mitochondrion and the nucleus.

Concluding remarks

The inner mitochondrial membrane of trypanosomatids possesses a uniport carrier for calcium (MCU). This carrier allows the electrogenic entry of the cation driven by the electrochemical gradient generated by respiration in most trypanosomes, or by ATP hydrolysis in *T. brucei* BS forms (Figure 3). Calcium efflux, however, takes place via a different pathway which appears to catalyze the electroneutral exchange of internal calcium by external protons, probably undertaken by an ortholog of Letm1. Biochemical

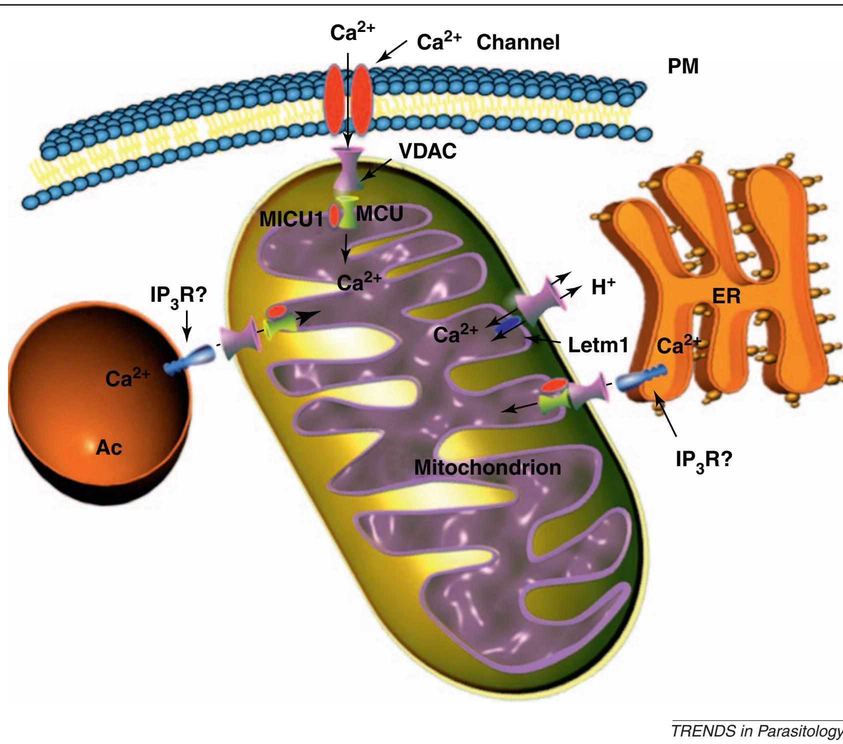

Figure 3. Mitochondrial Ca^{2+} transport in trypanosomes. The scheme depicts the molecules mediating Ca^{2+} influx and efflux (MICU1, MCU, Letm1) across the mitochondrial membrane at areas of the plasma membrane-, acidocalcisome (Ac)- or ER-mitochondrial association in trypanosomes. Abbreviations: ER, endoplasmic reticulum; IP_3R, inositol 1,4,5-trisphosphate receptor (location unknown), Ca^{2+} channel (unidentified); MCU, mitochondrial calcium uniporter; MICU1, mitochondrial calcium uptake 1; PM, plasma membrane; VDAC, voltage-dependent anion-selective channel.

evidence for Ca^{2+} uptake and for Ca^{2+}-release channels is available for several trypanosomatids. The discovery of a functional MCU in trypanosomes, as well as knowledge of its wide distribution in other eukaryotes and absence in yeast, not only led to finding the molecular nature of this channel in mammalian mitochondria, but also demonstrates the valuable contribution of an organelle of a unicellular parasite in dissecting the functions of mitochondrial proteins in general.

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