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Calcium signaling in trypanosomatid parasites

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Abstract

Calcium ion (Ca^{2+}) is an important second messenger in trypanosomatids and essential for their survival although prolonged high intracellular Ca^{2+} levels lead to cell death. As other eukaryotic cells, trypanosomes use two sources of Ca^{2+} for generating signals: Ca^{2+} release from intracellular stores and Ca^{2+} entry across the plasma membrane. Ca^{2+} release from intracellular stores is controlled by the inositol 1,4,5-trisphosphate receptor (IP_3R) that is located in acidocalcisomes, acidic organelles that are the primary Ca^{2+} reservoir in these cells. A plasma membrane Ca^{2+} -ATPase controls the cytosolic Ca^{2+} levels and a number of pumps and exchangers are responsible for Ca^{2+} uptake and release from intracellular compartments. The trypanosomatid genomes contain a wide variety of signaling and regulatory proteins that bind Ca^{2+} as well as many Ca^{2+} -binding proteins that await further characterization. The mitochondrial Ca^{2+} transporters of trypanosomatids have an important role in the regulation of cell bioenergetics and flagellar Ca^{2+} appears to have roles in sensing the environment. In trypanosomatids in which an intracellular life cycle is present, Ca^{2+} signaling is important for host cell invasion.

Keywords

Calcium; Acidocalcisome; Acidic store; Trypanosoma; Leishmania; Inositol 1,4,5-trisphosphate receptor

1. Introduction

Trypanosomatids include a large variety of protist parasites that infect plants and animals, including humans. African (*Trypanosoma brucei* group) and American (*T. cruzi*) trypanosomes are responsible for sleeping sickness and Chagas disease, respectively, different *Leishmania* spp. cause visceral, mucocutaneous and cutaneous leishmaniases, and *Phytomonas* spp. infect more than 100 plant species, mainly distributed, as occurs with *Leishmania* spp., in tropical and subtropical regions.

Trypanosomatids have a number of biochemical peculiarities that distinguish them from vertebrate cells and some of these are relevant for Ca^{2+} signaling. They possess a limited number of plasma membrane Ca^{2+} channels [1] and one putative voltage-gated Ca^{2+} channel localizes to the flagellum [2], which is also where many Ca^{2+} -binding proteins and enzymes involved in cell signaling also localize [3]. One particular intracellular Ca^{2+} channel, the inositol 1,4,5-triphosphate receptor (IP_3R) is not localized to the endoplasmic reticulum, as in most vertebrate cells, but to acidocalcisomes [4,5]. Acidocalcisomes are lysosome-related organelles, first described in trypanosomatids [6,7] and later found in a variety of cells, from bacteria to human cells [8], that are acidic and rich in phosphorus compounds (phosphate, pyrophosphate and polyphosphate) and cations. Acidocalcisomes are the main Ca^{2+} stores in these cells [9,10]. The mitochondria of trypanosomatids possess a uniporter (mitochondrial calcium uniporter or MCU) [11–13], which is essential for their survival [14], in contrast to what happens in mice, where deletion of the gene is not essential [15]. Ca^{2+} signaling is important for host cell invasion of intracellular trypanosomatids [16–18]. Fig. 1 shows a scheme of Ca^{2+} homeostasis and signaling in trypanosomatid parasites.

Here, we describe our current understanding of Ca^{2+} homeostasis and Ca^{2+} -mediated processes that occur in trypanosomatids.

2. The plasma membrane and the regulation of cytosolic Ca^{2+} concentration

The cytosolic free calcium (Ca^{2+}) concentration is in the range of 20–100 nM in different trypanosomatids [19,20] and it is therefore within the range reported for many vertebrate cells [21]. However, there is scant information on the mechanism of Ca^{2+} entry. There are no genes encoding homologues to various types of plasma membrane Ca^{2+} channels such as store-operated channel (Orai) and the endoplasmic reticulum Ca^{2+} sensor protein (STIM), ligand-operated channels, and second messenger-operated channels [1]. However, there are some genes encoding homologues of voltage-gated channels (similar to dihydropyridine-sensitive L-type Ca^{2+} channels) and transient receptor potential (TRP) channels (Table 1) [1]. In *T. brucei* bloodstream forms the putative voltage-gated channel (Tb427.10.2880, Table 1) is located in the flagellar attachment zone, the region where the flagellum attach to the cell body [2]. Although it has not been functionally studied, downregulation of this gene expression by RNAi results in flagellar detachment and deficient growth [2]. Ca^{2+} entry in *L. mexicana* is blocked by verapamil, nifedipine, and diltiazem, while BayK 8644 and sphingosine stimulate it, and it has been proposed that an L-type Ca^{2+} channel mediates this activity [22]. A transient receptor potential (TRP) channel of the mucolipin-type (Tb427.07.950, Table 1) localizes to lysosomes and has been suggested to act in iron import into the cytosol [23]. Other TRP channels (Table 1) have not yet been investigated.

As occurs in vertebrate cells a plasma membrane Ca^{2+} -ATPase (PMCA) is responsible for the regulation of the steady-state cytosolic Ca^{2+} concentration [24], while there is no evidence of the presence of $\text{Na}^+/\text{Ca}^{2+}$ exchangers involved in Ca^{2+} efflux. Three copies of PMCA-type ATPases are present in *T. cruzi*, one of them with two copies (Tca1, TcCBL.508543.90, and TcCBL.506401.170, Table 1) localizes to the plasma membrane and also to acidocalcisomes [25]. The other two distinct PMCA-type ATPases (TcCLB.510769.120 and TcCLB.509647.150, Table 1) share 28% and 32% identity to Tca1, respectively, and have

not been studied in detail. In *T. brucei*, four genes encoding PMCA-type ATPases are present. TbPMC1 (Tb427.08.1180, Table 1) preferentially localizes to acidocalcisomes while TbPMC2 (Tb427.08.1200, Table 1) localizes to the plasma membrane [26] and they share 97% identity. RNAi mutants for TbPMC1 and TbPMC2 are growth-deficient and more sensitive to high extracellular Ca^{2+} levels [26]. These ATPases, Tca1, TbPMC1, and TbPMC2 are all able to complement yeast deficient in the vacuolar Ca^{2+} -ATPase PMC1p, demonstrating that they are functional Ca^{2+} -ATPases [25,26]. The other two *T. brucei* PMCA-type ATPases with 32% identity between them (Tb427.08.1160 and Tb427.10.11620 (TbPMC3), Table 1) have 81% and 33% identities to both TbPMC1 and TbPMC2, respectively, and remain to be studied. Calmodulin (CaM) stimulates the plasma membrane Ca^{2+} -ATPase of either *T. cruzi* [27], *T. brucei* [28], or *L. mexicana* [29], but a typical CaM-binding domain could not be identified at the C-terminal of either Tca1 or TbPMC2.

3. Ca^{2+} -binding proteins

In the cytosol, Ca^{2+} interacts with soluble Ca^{2+} -binding proteins or is sequestered within different organelles in complex with storage proteins or inorganic anions. Many Ca^{2+} -binding proteins have been identified in the trypanosomatid genomes, but most of them remain uncharacterized. CaM has been purified from *T. cruzi* [27,30], and shown to contain 4 EF-hand domains that bind Ca^{2+} , and to stimulate several enzymes like the plasma membrane Ca^{2+} -ATPase [27] and a cyclic AMP phosphodiesterase [30]. TcCaM (TcCLB.507483.39, Table 2) has 89% identity with mammalian CaM, is present in several copies in the genome [31], and localizes to the spongione of the contractile vacuole complex [32,33]. In *T. brucei*, CaM has also been characterized [34,35], is found in the paraflagellar rod, an extra-axonemal structure of the flagellum, and is required for its assembly and for flagellar-cell body attachment [36]. CaM has also been studied in *Leishmania* spp. [37] where it stimulates a plasma membrane-located Ca^{2+} -ATPase [38,39] and a protein phosphatase [40] and is involved in binding to a myosin-XXI, regulating its dimerization, motility and lipid binding [41,42], and to the mitochondrial targeting sequence of a mitochondrial tryparedoxin peroxidase, regulating its transfer to mitochondria [43].

In addition, there are also a number of CaM-like proteins containing from 1 to 5 EF-hand domains (Table 2). A *T. cruzi* calreticulin (TcCLB.509011.40, Table 2) is located in the ER [44] and involved in quality control of glycoprotein synthesis [45], but the ability of trypanosomatid calreticulin to bind Ca^{2+} has not been studied, except for *L. donovani*, where it was found to also bind to RNA [46]. However, it has been shown that ER Ca^{2+} regulates the retrotranslocation of calreticulin to the cytosol of *T. cruzi* [47]. There are also several hypothetical proteins containing calcium-binding domains (Table 2).

Several genes present in multiple copies in the genome and encoding flagellar Ca^{2+} -binding proteins have been described in trypanosomatids and named flagellar calcium binding protein (FCaBP) in *T. cruzi* [48] and calflagins in *T. brucei* [49] (Table 2). Not all trypanosomatids possess homologues to FCaBPs [50]. TcFCaBPs are *N*-myristoylated and palmitoylated and associate to the flagellar membrane in a Ca^{2+} -dependent manner [51], although their function is unknown. Similar lipid modifications are found in calflagins [52].

4. Transport mechanisms of intracellular organelles

4.1. Endoplasmic reticulum (ER)

Sarcoplasmic-endoplasmic reticulum (SERCA)-type Ca^{2+} -ATPases are involved in Ca^{2+} uptake by the ER and have been studied in several trypanosomatids. The gene encoding the *T. cruzi* SERCA (TcSCA, TcCLB.509777.70, Table 1) is able to complement yeast deficient in the vacuolar Ca^{2+} pump PMC1p [44]. It also restores growth of *PMC1* mutant yeast on medium containing Mn^{2+} , suggesting a role in Mn^{2+} uptake [44]. Specific antibodies against the protein localize to the ER and the protein forms a phosphorylated intermediate in the presence of [γ - ^{32}P]ATP and Ca^{2+} . This phosphorylation of TcSCA is sensitive to cyclopiazonic acid and hydroxylamine but unaffected by thapsigargin, supporting observations that activity of the pump is thapsigargin-insensitive [44]. A second gene coding for another putative SERCA (TcCLB.506241.70, Table 1) is also in the *T. cruzi* genome, and the predicted amino acid sequence has 30% identity to TcSCA1 but has not been studied. A SERCA-type Ca^{2+} -ATPase (Tba1, Tb427.05.3400, Table 1) was also studied in *T. brucei* [53]. In *L. mexicana amazonensis* overexpression of the SERCA-type Ca^{2+} -ATPase (Lmaa1, U70620) increases the virulence of the parasites [17].

It is not clear how Ca^{2+} is released from the ER of trypanosomes. Although ER localization of an inositol 1,4,5-trisphosphate (InsP₃R) channel was proposed in *T. cruzi* [54], the immunofluorescence evidence reported is not convincing, as there is no clear reticular pattern or co-localization with a *T. brucei* ER marker, TbBiP, in the figures published [54]. In addition, proteomic analysis of the acidocalcisomes and contractile vacuole complex of *T. cruzi* provides evidence of the localization of this protein in these organelles [33], which is also in agreement with the vacuolar and punctate localization of the channel shown in that report [54]. Furthermore, this receptor has clear acidocalcisome localization in *T. brucei* [5] (see below). Further work will be needed to solve this controversy.

Presenilins have been postulated to have a function as Ca^{2+} leak channels in the ER or as modulators of SERCA pumps [55]. These intramembrane aspartyl proteases are present in the genomes of trypanosomatids (Table 1) and they could potentially have such a role. The presenilins were identified as multi-membrane-spanning proteins localized predominantly in the ER and postulated to be involved in the pathogenesis of Alzheimer's disease [56]. They form the catalytic core of the γ -secretase complex, which releases amyloid $\beta\text{A}\beta$ from the amyloid precursor protein (APP) [56].

4.2. Nucleus

The nuclear membrane of trypanosomatids is continuous with the endoplasmic reticulum and antibodies against SERCA-type Ca^{2+} -ATPases label the nuclear membrane [17,44]. In *T. brucei* changes in cytosolic Ca^{2+} levels are reflected in the nucleus [57], indicating that there is no active Ca^{2+} accumulation in this organelle.

4.3. Mitochondria

The role of trypanosomatid mitochondria in Ca^{2+} homeostasis and cell signaling has recently been reviewed [58] and we will provide here only with a brief summary and update.

Trypanosomatids possess a mitochondrial Ca^{2+} uniporter complex (MCUC) for Ca^{2+} uptake, which appears to be simpler than the one in mammalian cells [59]. The genome of trypanosomatids possess orthologs to MCU [11,14] (Table 1), which function as the pore of the complex [60], and to accessory/regulatory proteins MCUB (Table 1), MICU1 and MICU2 (Table 3), but not MCUR1 or EMRE [59]. In addition, there are no orthologs to MICU2 in *Leishmania* spp. Trypanosomatid MCUs have similar properties to those described in mammalian mitochondria: electrogenic Ca^{2+} transport, sensitivity to ruthenium red, and low affinity and high capacity for Ca^{2+} uptake [12,13].

Trypanosomatid mitochondria have separate pathways for Ca^{2+} influx and efflux as judged by the response of *T. cruzi* mitochondria to the additions of Ca^{2+} and EGTA [13]. In other trypanosomatids the mitochondrial Ca^{2+} efflux mechanism is Na^{+} -independent [61] and possibly due to a $\text{Ca}^{2+}/\text{H}^{+}$ exchanger.

Other mitochondrial Ca^{2+} uptake mechanisms are apparently absent in trypanosomes. There is no evidence of the presence of orthologs to uncoupling proteins [62], ryanodine receptors, or TRPC-type channels [1], and the ortholog to Letm1 appears to function as a $\text{K}^{+}/\text{H}^{+}$ exchanger [63].

Down-regulation of MCU expression by RNAi or by conditional knockout in *T. brucei* leads to deficient mitochondrial Ca^{2+} uptake in permeabilized cells, increase of the AMP/ATP ratio in procyclic forms, growth defects, and autophagy [14]. These effects are more pronounced when procyclic forms are grown in a medium rich in proline and poor in glucose, conditions that are prevalent in the *tse tse* fly, in which oxidative phosphorylation becomes essential [14].

On the other hand, overexpression of *TbMCU* in procyclic stages leads to increased mitochondrial Ca^{2+} uptake and mitochondrial Ca^{2+} overload. These cells increase their sensitivity to pro-apoptotic agents such as C2-ceramide and H_2O_2 , and to ROS generation, resulting in cells death [14].

The trypanosome mitochondria have also a role in buffering cytosolic Ca^{2+} increases. Ca^{2+} release from acidocalcisomes or Ca^{2+} entry through the plasma membrane results in rapid mitochondrial Ca^{2+} uptake that reaches intramitochondrial levels that are much higher than cytosolic Ca^{2+} rises [64].

4.4. Acidocalcisomes

Acidocalcisomes are ubiquitous acidic calcium stores rich in phosphorus compounds and cations like calcium, magnesium, sodium, potassium, zinc, and iron, characterized by their electron-density at the electron microscope level, and initially described in trypanosomatids but later found in a number of species from bacteria to humans [8] (Fig. 2).

Two proton pumps, a vacuolar-type ATPase (V-ATPase) and a vacuolar pyrophosphatase (VP1) maintain their acidity [8]. A $\text{Ca}^{2+}/\text{H}^{+}$ counter transporting ATPase, of the PMCA-type, is responsible for Ca^{2+} uptake [8]. Acidocalcisomes are especially equipped for the accumulation of polyphosphate, which is synthesized and translocated into the organelle by

a polyphosphate kinase named the vacuolar transporter chaperone complex (VTC complex) [65,66]. Other components of the organelle are cation exchangers ($\text{Ca}^{2+}/\text{H}^+$, Na^+/H^+), zinc, iron, and phosphate transporters, and in some cases, such as in *T. cruzi*, a water channel or aquaporin [8]. A recent proteomic analysis of the *T. brucei* acidocalcisomes [67] provided evidence for the presence of a number of these transporters. Fig. 3 shows a scheme of a typical acidocalcisome.

In *T. brucei* the IP_3R localizes to acidocalcisomes rather than to the ER [4,5]. The acidocalcisome localization was first reported using the epitope-tagged protein [4,5] and recently confirmed using specific antibodies against Tb IP_3R [67]. As indicated above (Section 4.1) evidence for ER localization in *T. cruzi* [54] is weak and contradicts proteomic analyses of acidocalcisomes and contractile vacuole complex of these parasites that provided evidence for the presence of the Tc IP_3R ortholog in these organelles [33]. The localization of this channel in *Leishmania* spp. has not been studied.

The structure of the trypanosome IP_3Rs , as suggested by bioinformatic analyses, has been described [1]. They possess several conserved domains such as putative suppressor domain-like (SD), ryanodine receptor IP_3R homology (RIH), and RIH-associated (RIAD) domains. They also have a motif for a Ca^{2+} -specific selectivity filter (GVGD) in the putative intraluminal loop between transmembrane domains at the C-terminal region [1]. Only 4 of the ten residues that form a basic pocket that binds IP_3 are conserved in trypanosomatid IP_3Rs [4,5].

The functional study of Tb IP_3R [4,5] and Tc IP_3R [54] was done by stable transfection of the respective genes in a chicken B lymphocyte cell line devoid of the genes for all three vertebrate IP_3Rs (DT40-3KO). Tb IP_3R and Tc IP_3R 1 localize to the ER of DT40-KO cells, and IP_3 is able to release Ca^{2+} from these permeabilized cells, from their microsomal vesicles, or from intact cells stimulated by anti-B cell receptor monoclonal antibodies [4,5,54]. IP_3 also binds to microsomal vesicles from DT40-KO cells expressing Tc IP_3R [54]. Tc IP_3R expressed in HeLa cells also localizes to the ER, and IP_3 is able to release Ca^{2+} from these permeabilized cells [54]. Tb IP_3R is less sensitive to IP_3 than rat IP_3R 1 (Rn IP_3R 1) transfected in DT40-3KO cells [4,5]. Uncaged IP_3 is also able to release Ca^{2+} from live *T. brucei* procyclic trypomastigotes loaded with Fluo 4-AM but not from parasites in which Tb IP_3R expression is downregulated by RNAi [4,5].

Knockdown of *TbIP3R* expression by induction of RNAi results in growth defects in both bloodstream and procyclic trypomastigotes [4,5]. It also reduces the ability of IP_3 to release Ca^{2+} from permeabilized cells and the virulence of bloodstream forms *in vivo* [4,5]. *Null* mutants in Tc IP_3R were not obtained, suggesting the essentiality of this gene, while *TcIP3R* knockdown results in deficient growth of epimastigotes, deficient metacyclogenesis (transformation of epimastigotes into metacyclic trypomastigotes), deficient host cell invasion by trypomastigotes associated with reduced Ca^{2+} release upon their attachment to the host cells, deficient replication of amastigotes, increased transformation of amastigotes into trypomastigotes, and defects in virulence *in vivo* [54].

Overexpression of *TcIP₃R* also results in deficient growth of epimastigotes and amastigotes, and deficient metacyclogenesis, suggesting that an appropriate level of this receptor is necessary for these processes [54]. Overexpression of *TcIP₃R* also results in increased host cell invasion by trypomastigotes associated with increased Ca^{2+} release upon their attachment to host cells, and decreased transformation of amastigotes into trypomastigotes, with no changes in virulence *in vivo* except for an early appearance of parasitemia [54]. In summary, these reports clearly establish the presence of a functional IP_3 receptor in both *T. brucei* and *T. cruzi* and the essentiality of this pathway in trypanosomes.

5. Ca^{2+} signaling in trypanosomatids

Ca^{2+} regulates a number of signaling pathways in eukaryotic cells by binding to proteins that decode the Ca^{2+} signal. Some of these proteins are present in trypanosomatids as for example several protein kinases and ion channels (Table 3). A protein kinase C (PKC)-like enzyme was found in *T. cruzi* epimastigotes [68,69] that requires phosphatidylserine and Ca^{2+} for activity and is stimulated by diacylglycerol. However, the trypanosomatid genomes did not assign any of the AGC kinases found to the PKC family [70]. Several genes encoding putative calcium-calmodulin-dependent kinases (CaMK) have been identified in the trypanosomatid genomes [70] (Table 3). A soluble CaMK was purified and characterized in *T. cruzi* [71–73]. Heme-induced proliferation of *T. cruzi* culture forms is mediated by activation of a CaMKII and prevented by inhibitors of this enzyme (KN-93, Myr-AIP) [74]. Activation of this CaMKII is mediated by the enhanced reactive oxygen species (ROS) formation by heme [75,76].

Genes encoding putative Ca^{2+} -activated K^+ channels are present in the trypanosomatid genomes but they have not been studied (Table 3). Among the known Ca^{2+} -stimulated enzymes present in trypanosomatids are: an adenylyl cyclase [77] (AAC61849.1), a cyclic AMP phosphodiesterase [30], and a phosphoinositide phospholipase C (PI-PLC), all from *T. cruzi* [78].

Other known Ca^{2+} -stimulated enzymes in mammalian cells are present in trypanosomatids by devoid of Ca^{2+} -binding domains suggesting lack of Ca^{2+} modulation. For example, calpain-like proteins lack EF-hand motifs observed in the domain IV of conventional calpains [79], and several mitochondrial carriers that are stimulated by Ca^{2+} in mammalian cells lack EF-hand domains [11] and are presumably Ca^{2+} -insensitive.

Complexes of Ca^{2+} /CaM control the activity of the protein phosphatase calcineurin (PP2B) in other eukaryotic cells. Calcineurin is an heterodimeric protein formed by a catalytic subunit (calcineurin A, CnA) and a regulatory subunit (calcineurin B, CnB). However, *T. cruzi* CnA lacks CaM and autoinhibitory domains, and CnB has only two out of the four EF-hand domains characteristic of other calcineurin B proteins [80]. Nevertheless, *T. cruzi* calcineurin activity requires Ca^{2+} and this effect likely occurs *via* CnB, which can stimulate CnA by binding Ca^{2+} [80,81]. Cn inhibitors like cyclosporin or cypermethrin or downregulation of CnB expression with phosphorothioate oligonucleotides strongly inhibited entry of host HeLa cells suggesting a role of this protein in host invasion [81]. A Ca^{2+} /CaM-dependent protein phosphatase was identified and partially purified from *Leishmania* spp. Its

activity is inhibited by calmodulin antagonists and is insensitive to okadaic acid suggesting that it is a PP2B-type protein phosphatase [40].

Centrins (also known as caltractins) are also Ca^{2+} -binding proteins involved in a number of cellular processes, such as DNA repair, mRNA export, organelle duplication and signal transduction [82]. They are conserved components of centrioles in animals, and basal bodies in unicellular flagellates [83]. Some centrins also associate with axonemal inner-arm dyneins and regulate cell motility. Several centrins have been identified in the genomes of trypanosomatids (Table 3). In *T. brucei* centrins are involved organelle segregation (TbCentrin1) [84], in coordination of nuclear and cell division (TbCentrin4) [82], and in flagellar motility (TbCentrin3). Three of the five centrins associate with the flagellar basal body [83]. TbCentrin2 and TbCentrin4 localize to the basal bodies that seed the flagellum, and to a bi-lobed structure important for organelle duplication and cell division through the conserved C-terminal domain [85]. Genetic manipulation of TbCentrin4 levels affects TbCentrin2 association with the bi-lobed structure [85]. Although TbCentrin2 expression level is relatively constant throughout the cell cycle TbCentrin4 level fluctuates, decreasing most during early S-phase when the bi-lobe undergoes duplication [85]. These results thus suggest a coordinated action between these two centrin proteins, where the cell cycle-dependent TbCentrin4 expression could regulate the abundance of TbCentrin2 on the bi-lobed structure [85]. TbCentrin3 is a flagellar protein and knockdown compromises cell motility [83]. Tandem affinity purification followed by mass spectrometry identified an inner-arm dynein, TbiAD5-1, as the TbCentrin3 partner, and knockdown of TbiAD5-1 caused similar cell motility defect [83]. There is an interdependence of TbCentrin3 and TbiAD5-1 for maintaining a stable complex in the flagellar axoneme [83].

Centrins have also been investigated in *Leishmania* spp. [86,87]. *L. donovani* centrin2 (LdCEN, LdBPK221260.1) localizes to the basal body and binds Ca^{2+} [87]. The levels of LdCentrin2 mRNA and protein are high during the exponential growth of the parasite in culture and decline to a low level in the stationary phase [87]. Centrin null mutants (LdCEN^{-/-}) show selective growth arrest as axenic amastigotes but not as promastigotes [86]. Mutant axenic amastigotes have a cell cycle arrest at the G(2)/M stage and show failure of basal body duplication and cytokinesis resulting in multinucleated “large” cells [86]. Growth of LdCEN^{-/-} amastigotes in infected macrophages *in vitro* is inhibited and also results in large multinucleated parasites [86]. Therefore, disruption of centrin gene displays stage-specific/cell type-specific failure in cell division in *L. donovani*.

6. Ca^{2+} role in host cell invasion, differentiation and bioenergetics

A role for Ca^{2+} signaling in host cell invasion is demonstrated by its increase in *T. cruzi* trypomastigotes or *L. amazonensis* amastigotes during their interaction with host cells. Preventing this Ca^{2+} increase in *T. cruzi* trypomastigotes (Y strain) [18] or *L. amazonensis* amastigotes [17] by loading them with Quin 2-AM or BAPTA-AM decreases invasion of host cells. Similar results are obtained with both tissue culture-derived and bloodstream *T. cruzi* trypomastigotes (Tulahuén strain) while treatment with the Ca^{2+} ionophore ionomycin, which elevates cytosolic Ca^{2+} in trypomastigotes, significantly enhances the infective ability of the parasites [88]. These results suggest that the transient Ca^{2+} increase that occurs upon

attachment of trypomastigotes to the host cell surface is associated to invasion. The mechanism and sources of the increased cytosolic Ca^{2+} have not been investigated in detail. However, treatment of trypomastigotes with antisense oligonucleotides specific to TcIP_3R decreases TcIP_3R protein levels and impairs trypomastigote invasion of host cells [89], suggesting a role of this signaling pathway in invasion.

Ca^{2+} signaling can also have a role during differentiation of *T. cruzi* epimastigotes into metacyclic trypomastigotes [90] as suggested by changes in cytosolic Ca^{2+} observed during this process. Ca^{2+} signaling is also involved in osmoregulation [91], and programmed cell death in *T. cruzi* [92].

Based on the use of Ca^{2+} ionophores, roles for Ca^{2+} in the release of the *T. brucei* bloodstream stage surface coat [93], and in the maintenance of the cytoskeleton [94] have been proposed. Changes in cytosolic Ca^{2+} levels were also reported during differentiation from bloodstream to procyclic stages of *T. brucei* [95]. The results described above with *TbIP₃R* and *TcIP₃R* knockdowns indicate that Ca^{2+} signaling through the trypanosome IP_3Rs has roles in growth *in vitro* and *in vivo*, as well as in cell differentiation. The acidocalcisome localization of the *TbIP₃R* supports a role for these organelles in Ca^{2+} signaling.

7. Ca^{2+} and the flagellum

Flagella and cilia are motile and sensory cell organelles that are involved in signal transduction [96]. A putative Ca^{2+} channel has been localized to the flagellar attachment zone of *T. brucei* bloodstream forms [2] while the flagella of *T. cruzi* trypomastigotes are strongly labeled by antibodies against the PMCA-ATPase [17]. Epitope-tagged PMCA-ATPase or antibodies against it also localize to the flagellum of *T. brucei* [26]. Together these results suggest that the flagellar membrane possess mechanisms for Ca^{2+} entry and Ca^{2+} efflux. The flagellum also possesses a number of Ca^{2+} -binding proteins like *T. cruzi* FCBP [48] and *T. brucei* calflagins [49] as well as CaM [36] and centrins [83]. Other proteins with EF-hand domains, in addition to CaM, are present in the paraflagellar rod (PFR) of *T. brucei* procyclic forms. Tb5.20, TbPFC1 (*T. brucei* paraflagellar component 1), TbPFC6, and TbPFC7 have been proposed to have EF-hand domains [97], although only TbPFC6 has clearly defined EF-hand domains (Table 2). The paraflagellar protein TbPFC15 also contains two IQ-calcium-independent calmodulin binding motifs suggesting a role for Ca^{2+} regulation in the PFR [97]. Ca^{2+} appears to be important for adhesion of the flagellum to the cell body as RNAi mutants for the Ca^{2+} channel [2] or for CaM [36] results in flagellar detachment. The flagellum seems to sense extracellular Ca^{2+} levels. The trypanosomatid *Crithidia oncopelti* shows a tip-to-base beating at low (<0.1 mM) extracellular Ca^{2+} levels and the beating direction switches to base-to-tip at higher extracellular Ca^{2+} [98]. The roles of intraflagellar Ca^{2+} in functions well described in other organisms such as motility [99] and intraflagellar protein transport [100], have not yet been investigated in trypanosomatids.

8. Outlook

Trypanosomatids have several biochemical differences with mammalian cells regarding Ca^{2+} homeostasis and signaling. They lack several types of plasma membrane Ca^{2+} channels but possess orthologs to voltage-gated and TRP channels. A PMCA-type ATPase is also localized to acidocalcisomes and, although it is stimulated by CaM, it does not have a typical CaM-binding domain in its C-terminal region. There is no evidence for the involvement of $\text{Na}^+/\text{Ca}^{2+}$ exchangers in Ca^{2+} efflux from the cells. The endoplasmic reticulum has a SERCA-type ATPase for Ca^{2+} uptake, which is thapsigargin-insensitive, and there is some controversy in *T. cruzi* regarding the presence of an IP_3R . In *T. brucei*, the IP_3R is located in acidocalcisomes, which are the main Ca^{2+} store in trypanosomes, and where Ca^{2+} is bound to polyphosphate. This receptor is essential in both *T. cruzi* and *T. brucei*. The mitochondrial Ca^{2+} uniporter complex appear simpler than the mammalian counterpart, although we cannot rule out the presence of novel components, and is essential for growth *in vitro* and *in vivo* and for maintaining cellular bioenergetics. Ca^{2+} signaling is important for host cell invasion of intracellular trypanosomatids, and for differentiation, osmoregulation, flagellar function, and trypanosome life and death.

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Abbreviations

FCaBP	flagellar calcium binding protein
VTC	vacuolar transporter chaperone

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Fig. 1.

Schematic representation of Ca²⁺ homeostasis and signaling in trypanosomatid parasites was based on our interpretation of published data. Distinct Ca²⁺ transporting systems operate in the plasma membrane, ER, mitochondria, and the acidic Ca²⁺ stores (acidocalcisomes). Ca²⁺ entry is probably through a Ca_v channel. Once inside the cell, Ca²⁺ can be translocated back to the extracellular environment by the action of PMCA. In addition, Ca²⁺ will interact with CaBP or become sequestered by the endoplasmic reticulum through a SERCA, by the mitochondrion through a MCU, by acidocalcisomes through a Ca²⁺-ATPase (PMCA), or by the nucleus through the nuclear pores. IP₃ is generated by hydrolysis of PIP₂, catalyzed by phosphoinositide-specific PLC (PI-PLCs), which also generate DAG, possibly activating PK. IP₃ binds to IP₃ receptor (IP₃ R) and stimulates Ca²⁺ release from acidocalcisomes through the IP₃ R. When Ca²⁺ influx through the plasma membrane or Ca²⁺ release from acidocalcisomes is stimulated, Ca²⁺ can be efficiently taken up by MCU through the microdomains of high Ca²⁺ concentration (gray balls) present in their vicinity. Further details for cytosolic Ca²⁺ buffering and Ca²⁺ regulation are discussed in the text. PLC, phospholipase C; PIP₂, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; PK, protein kinase; Ca_v, voltage-gated Ca²⁺ channel; PMCA, plasma membrane Ca²⁺-ATPase; CaBP, Ca²⁺-binding protein; IP₃, inositol 1,4,5-trisphosphate; IP₃ R, IP₃ receptor; MCU, mitochondrial calcium uniporter; VDAC, voltage-dependent anion-selective channel; Letm1, leucine zipper-EF-hand containing transmembrane protein 1; CHX, Ca²⁺/H⁺

exchanger; PSEN presenilin; SERCA, sarcoplasmic-endoplasmic reticulum Ca^{2+} -ATPase; ER, endoplasmic reticulum.

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Fig. 2. Morphology of acidocalcisomes. Using conventional electron microscopy acidocalcisomes (Acc) appear as spherical empty structures with a thin layer of dense material that sticks to the inner face of the membrane and with an average diameter of $\sim 0.2 \mu\text{m}$. They are distributed throughout the cell. F, flagellum, FP, flagellar pocket, N, nucleus, K, kinetoplast. Bar = 500 nm.

Fig. 3.

Scheme of a typical acidocalcisome in trypanosomes. Ca²⁺ uptake occurs in exchange for H⁺ by a reaction catalyzed by a vacuolar Ca²⁺-ATPase that can be inhibited by vanadate (Van). Ca²⁺ release is through an inositol 1,4,5-trisphosphate receptor (IP₃ R). A H⁺ gradient is established by a vacuolar H⁺-ATPase that can be inhibited by bafilomycin A₁ (Baf A₁) and a vacuolar H⁺-pyrophosphatase (V-H⁺-PPase) that can be inhibited by aminomethylenediphosphonate (AMDP). An aquaporin allows water transport. Other transporters (for example, Na⁺/H⁺ or Ca²⁺/H⁺ exchangers, and transporters for Zn²⁺, Fe³⁺, and inorganic phosphate (Pi)) are probably present. Synthesis and translocation of polyphosphate (poly P) occurs through the vacuolar transporter chaperone (VTC) complex that uses ATP. The acidocalcisome is rich in Pi, pyrophosphate (PPi), short- and long-chain poly P, and cations. An exopolyphosphatase (PPX), and a vacuolar soluble pyrophosphatase (VSPase) are also present.

Table 1

Calcium channels and pumps identified in trypanosomatid parasites.

Proteins	TriTrypDB Gene ID (TMDs)		
	<i>T. brucei</i>	<i>T. cruzi</i>	<i>L. major</i>
PMCA	Tb427.08.1160 (10)	TcCLB.508543.90 (8)	LmjF.07.0630 (8)
	Tb427.08.1180 (8)	TcCLB.506401.170 (8)	LmjF.07.0650 (10)
	Tb427.08.1200 (10)	TcCLB.510769.120 (8)	LmjF.17.0600 (6)
	Tb427.10.11620 (10)	TcCLB.509647.150 (9)	LmjF.33.1010 (8)
SERCA	Tb427.05.3400 (10)	TcCLB.509777.70 (10)	LmjF.04.0010 (7)
	Tb427tmp.244.2570 (8)	TcCLB.506241.70 (8)	LmjF.35.2080 (8)
IP ₃ receptor	Tb427.08.2770 (5)	TcCLB.509461.90 (5)	LmjF.16.0280 (5)
MCU	Tb427tmp.47.0014 (2)	TcCLB.503893.120 (2)	LmjF.27.0780 (2)
MCUb	Tb427.10.300 (2)	TcCLB.504069 (2)	LmjF.21.1690 (2)
TRPML channel	Tb427.07.950 (6)	TcCLB.508215.6 (6)	LmjF.26.0990 (6)
TRP channel	Tb427.08.850 (6)	TcCLB.510861.94 (6)	LmjF.07.0910. (6)
Ca _v channel	Tb427.10.2880 (20)	TcCLB.504105.130 (20)	LmjF.34.0480 (22)
			LmjF.17.1440 (20)
PSEN	Tb427tmp.160.3510 (7)	TcCLB.508277.50 (7)	LmjF.15.1530 (7)

TryTrypDB gene ID numbers are identified from the genome of the *T. brucei* Lister strain 427, the *T. cruzi* CL Brener, or the *L. major* strain Friedlin at the website (<http://tritrypdb.org/tritrypdb/>). The number of predicted transmembrane domains (TMDs) present in proteins is identified via TMHMM2.0 or references and indicated in parentheses. PMCA, plasma membrane Ca²⁺-ATPase; SERCA, sarcoplasmic-endoplasmic reticulum Ca²⁺-ATPase; IP₃, inositol 1,4,5-trisphosphate; MCU, mitochondrial calcium uniporter; TRP, transient receptor potential protein; Ca_v, voltage-gated calcium channel; PSEN, presenilin.

Table 2

Calcium-binding proteins annotated in trypanosomatid parasites.

Proteins	TriTrypDB Gene ID (EF-hand motifs)		
	<i>T. brucei</i>	<i>T. cruzi</i>	<i>L. major</i>
Calreticulin	Tb427.08.7410	TcCLB.509011.40	LmjF.31.2600
Flagellar Ca ²⁺ -binding protein	Tb427.08.5440 (3)	TcCLB.509391.10 (3)	LmjF.16.0910 (2)
	Tb427.08.5460 (4)	TcCLB.509391.20 (3)	LmjF.16.0920 (2)
	Tb427.08.5465 (3)	TcCLB.509391.30 (3)	
	Tb427.08.5470 (3)	TcCLB.506749.20 (3)	
Ca ²⁺ -binding protein	Tb427.06.2720 (1)	TcCLB.507925.60 (1)	LmjF.30.1240 (1)
	Tb427.04.1740 (3)	TcCLB.510879.190 (3)	LmjF.34.2950 (3)
Calmodulin (CaM)	Tb427tmp.01.4621 (4)	TcCLB.507483.30 (4)	LmjF.09.0910 (4)
	Tb427tmp.01.4622 (4)	TcCLB.507483.39 (4)	LmjF.09.0920 (4)
	Tb427tmp.01.4623 (4)		LmjF.09.0930 (4)
	Tb427tmp.01.4624 (4)		
CaM-like protein	Tb427tmp.01.1550 (4)	TcCLB.506963.90 (4)	LmjF.36.3675 (4)
	Tb427tmp.211.2540 (1)	TcCLB.504075.3 (1)	LmjF.35.3890 (1)
	Tb427tmp.02.1160 (2)	TcCLB.508731.30 (3)	LmjF.13.1160 (2)
	Tb427tmp.02.5800 (2)	TcCLB.506933.89 (3)	LmjF.28.0800 (3)
	Tb427tmp.160.4520 (3)	TcCLB.508951.50 (4)	LmjF.21.0220 (3)
	Tb427.06.4710 (3)	TcCLB.511729.9 (5)	LmjF.15.0930 (2)
		TcCLB.507483.50 (4)	LmjF.30.3360 (3)
PFC6	Tb427.03.3770 (2)	TcCLB.509997.40 (1)	

TryTrypDB gene ID numbers are identified from the genome of the *T. brucei* Lister strain 427, the *T. cruzi* CL Brener, or the *L. major* strain Friedlin at the website (<http://tritrypdb.org/tritrypdb/>). EF, E-helix-loop-F-helix motifs for Ca²⁺ binding; PFC, paraflagellar rod proteome component. The number of putative EF-hand motifs present in proteins is identified via InterPro at the website (<http://www.ebi.ac.uk/interpro/>) and indicated in parentheses.

Table 3

Proteins potentially modulated by Ca^{2+} identified in trypanosomatid parasites at the molecular level.

Proteins	TriTrypDB Gene ID (EF-hand motifs)		
	<i>T. brucei</i>	<i>T. cruzi</i>	<i>L. major</i>
Ca ²⁺ /CaM dependent PK	Tb427tmp.01.0670	TcCLB.508601.90	LmjF.28.2000
	Tb427.07.6220	TcCLB.506513.50	LmjF.17.0060
	Tb427tmp.160.0930	TcCLB.506465.40	LmjF.26.2110
	Tb427tmp.160.0500	TcCLB.503925.30	LmjF.26.2510
	Tb427.10.1940	TcCLB.506493.50	LmjF.21.0150
	Tb427.10.5310	TcCLB.506679.80	LmjF.36.0900
	Tb427.10.3900	TcCLB.503635.10	LmjF.35.0490
	Tb427.02.1820	TcCLB.509213.160	LmjF.33.1710
Ca ²⁺ activated K ⁺ channel	Tb427.07.2750	TcCLB.510525.10	LmjF.22.0810
	Tb427tmp.160.3380	TcCLB.511585.220	LmjF.01.0810
	Tb427.01.4450	TcCLB.506529.150	LmjF.20.0090
		TcCLB.511245.30	LmjF.14.0540
PI-PLC1	Tb427tmp.02.3780 (1)	TcCLB.504149.160 (1)	LmjF.22.1680 (1)
PI-PLC2	Tb427.06.2090	TcCLB.507019.80	LmjF.30.2950 (1)
			LmjF.30.0660
			LmjF.35.0040 (1)
MICU1	Tb427.08.1850 (2)	TcCLB.511391.210 (2)	LmjF.07.0110 (2)
MICU2	Tb427.07.2960 (2)	TcCLB.510525.130 (2)	
Calcineurin B subunit	Tb427.10.370 (3)	TcCLB.510519.60 (3)	LmjF.21.1630 (4)
Centrin (Caltractin)	Tb427.10.6980 (2)	TcCLB.510181.150 (2)	LmjF.36.2430 (1)
Centrin 1	Tb427.04.2260 (3)	TcCLB506559.360 (4)	LmjF.34.2390 (2)
Centrin 2	Tb427.08.1080 (3)	TcCLB.506401.90 (3)	LmjF.07.0710 (3)
Centrin 3	Tb427.10.8710 (2)	TcCLB.508727.18 (3)	LmjF.36.6110 (2)
Centrin 4	Tb427.07.3410 (4)	TcCLB.508323.70 (4)	LmjF.22.1410 (4)
Centrin 5	Tb427tmp.01.5470 (3)	TcCLB.509161.40 (2)	LmjF.32.0660 (2)

TryTrypDB gene ID numbers are identified from the genome of the *T. brucei* Lister strain 427, the *T. cruzi* CL Brener, or the *L. major* strain Friedlin at the website (<http://tritrypdb.org/tritrypdb/>). CaM, calmodulin; PK, protein kinase; PI-PLC, phosphatidylinositol phospholipase C; MICU, mitochondrial calcium uptake. EF, E-helix-loop-F-helix motifs for Ca^{2+} binding. The number of putative EF-hand motifs present in proteins is identified via InterPro at the website (<http://www.ebi.ac.uk/interpro/>) and indicated in parentheses.