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The Role of Acidocalcisomes in the Stress Response of *Trypanosoma cruzi*

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Abstract

Acidocalcisomes of *Trypanosoma cruzi* are acidic calcium-containing organelles rich in phosphorus in the form of pyrophosphate (PP_i) and polyphosphate (poly P). Acidification of the organelles is driven by vacuolar proton pumps, one of which, the vacuolar proton pyrophosphatase (V-H⁺-PPase), is absent in mammalian cells. A Ca²⁺-ATPase is involved in calcium uptake and an aquaporin is important for water transport. Enzymes involved in the synthesis and degradation of PP_i and poly P are present within the organelle. Acidocalcisomes function as storage sites for cations and phosphorus, participate in PP_i and poly P metabolism, and volume regulation, and are essential for virulence. A signaling pathway involving cyclic AMP (cAMP) generation is important for fusion of acidocalcisomes to the contractile vacuole complex (CVC), transference of aquaporin, and volume regulation. This pathway is an excellent target for chemotherapy as shown by the effects of phosphodiesterase C (PDEC) inhibitors on parasite survival.

1. INTRODUCTION

Acidocalcisomes were first identified in bacteria and named metachromatic granules (Babes, 1895) because they had the property of changing the color of basic blue dyes. They were also named volutin granules (Meyer, 1904) because of their presence in *Spirillum volutans*. Volutin granules were later found in a number of microorganisms, including trypanosomes (Swellengrebel, 1908). Polyphosphate (poly P), a linear polymer of a few to several hundred orthophosphate residues discovered by Lieberman (Lieberman, 1888), was reported in the 1940' as a component of these granules (Wiame, 1947), which then began to be known as poly P granules. More recent work in trypanosomatids and Apicomplexan parasites (Vercesi et al., 1994, Docampo et al., 1995, Moreno and Zhong, 1996) revealed that the poly P granules have proton and calcium pumps, which are responsible for their acidity and calcium content, and were given the name of acidocalcisomes. As these organelles have been found from bacteria (Seufferheld et al., 2004, Seufferheld et al., 2003) to human cells (Ruiz et al., 2004) it has been suggested that they have been conserved over evolutionary time or have appeared more than one time by convergent evolution (Docampo et al., 2010).

2. ACIDOCALCISOMES IN *T. CRUZI*

T. cruzi acidocalcisomes are electron-dense (Fig. 1A) and have a vacuolar appearance by conventional electron microscopy (Fig. 1B). At the light microscopy level they can be stained with DAPI, which labels poly P, and with dyes that accumulate in acidic

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compartments such as acridine orange (Fig. 1C) (Docampo et al., 1995), or cycloprodigiosin (Scott and Docampo, 2000). They are spherical with an average diameter of 0.2 μm , and randomly distributed in the cells (Fig. 1A).

T. cruzi acidocalcisomes are rich in orthophosphate (Pi), pyrophosphate (PPi), and poly P complexed with cations (sodium, potassium, magnesium, calcium, zinc and iron) and basic amino acids (Ruiz et al., 2001b, Rohloff et al., 2003). *T. cruzi* is especially rich in short chain poly P such as poly P₃, poly P₄, and poly P₅ (Moreno et al., 2000). On the basis of its total concentration (Ruiz et al., 2001b) and the relative volume of acidocalcisomes in the different stages of *T. cruzi* (about 1-2% of the total cell volume) (Miranda et al., 2000), it was calculated that the concentration in the organelles would be in the molar range (3-5 M) (Docampo et al., 2005). This is congruent with the detection of solid-state condensed phosphates by magic-angle spinning NMR techniques (Moreno et al., 2002), and with the very high electron density of acidocalcisomes (Scott et al., 1997, Miranda et al., 2000). Carbohydrates and lipids could be involved in maintaining these physical characteristics (Salto et al., 2008).

Some enzymatic activities have been detected in acidocalcisomes of *T. cruzi*, such as a polyphosphate kinase, and an exopolyphosphatase (Ruiz et al., 2001b, Fang et al., 2007b). Synthesis of poly P in the yeast vacuole (Hothorn et al., 2009) and in acidocalcisomes of trypanosomatids (Fang et al., 2007a) is mediated by the “vacuolar transporter chaperone” (Vtc) complex, which comprises four proteins in yeasts (Vtc 1- 4) anchored in the vacuole membrane and probably two (Vtc1 and Vtc4) anchored in the acidocalcisome membrane of trypanosomatids. Vtc4 has the catalytic activity and functions polymerizing and translocating the poly P chain through the vacuole membrane (Hothorn et al., 2009).

A scheme of all the enzymes and transporters identified in acidocalcisomes of *T. cruzi* is depicted in Fig. 2. Acidocalcisome membranes possess several pumps and at least one channel. A vanadate-sensitive Ca²⁺-ATPase was first detected in acidocalcisomes of *T. cruzi* permeabilized with digitonin (Docampo et al., 1995), and later found in the isolated organelles (Scott and Docampo, 2000). A gene encoding for the acidocalcisome Ca²⁺-ATPase was identified and used to complement yeast mutants that were deficient in the vacuolar Ca²⁺-ATPase gene *PMCI*, giving evidence of its functionality (Lu et al., 1998). This Ca²⁺-ATPase is closely related to the family of plasma membrane calcium ATPases (PMCA), although it does not have a typical calmodulin-binding domain. Two proton pumps have been detected in acidocalcisomes of *T. cruzi*. One is the multisubunit vacuolar-type H⁺-ATPase (Docampo et al., 1995) and the other is the vacuolar proton pyrophosphatases (V-H⁺-PPase) (Scott et al., 1998, Hill et al., 2000), which uses PPi instead of ATP as energy source to transport protons. The V-H⁺-ATPase was shown, by immunofluorescence and immunoelectron microscopy, to co-localize to acidocalcisomes with the vacuolar-type Ca²⁺-ATPase (Lu et al., 1998).

The K⁺-stimulated V-H⁺-PPase of *T. cruzi* was the first example of this type of enzymes found in any organism different from bacteria or plants (Scott et al., 1998). The enzyme was also found in the Golgi apparatus and plasma membrane (Martinez et al., 2002) and in the contractile vacuole complex (Rohloff et al., 2004) of *T. cruzi*, although it is predominantly localized in the acidocalcisome and can be used as a marker for this organelle purification (Scott and Docampo, 2000). The gene for the *T. cruzi* V-H⁺-PPase could be functionally expressed in yeast (Hill et al., 2000). It was found recently that the N-terminal region of the *T. cruzi* V-H⁺-PPase, when fused to other protist enzymes enhance their functional expression in yeast (Drake et al., 2010). An aquaporin has also been identified in acidocalcisomes of *T. cruzi* (Montalvetti et al., 2004). The protein acts as a water channel and is unable to transport glycerol when expressed in *Xenopus* oocytes (Montalvetti et al.,

2004). A zinc-transporter like protein was also identified by proteomic analysis and by co-localization of the tagged protein with the V-H⁺-PPase (Ferella et al., 2008).

3. VOLUME REGULATION

Any change in the intracellular and extracellular solute content generates the immediate flow of water into or out of the eukaryotic cell until equilibrium is achieved (Choe, 2009). This water flow through channels, driven by osmotic pressure gradients, causes swelling or shrinkage of the cells. Cells respond to these volume changes by activating volume-regulatory mechanisms. The processes by which swollen and shrunken cells return to a normal volume is known as *regulatory volume decrease* (RVD) and *regulatory volume increase* (RVI), respectively. Regulation of cell volume in most eukaryotic cells is by the gain or loss of osmotically active solutes that could be inorganic ions such as Na⁺, K⁺, and Cl⁻ or small organic molecules known as *organic osmolytes*, such as polyols (e.g. sorbitol, *myo*-inositol), amino acids and their derivatives (e.g. taurine, alanine, and proline), and methylamines (e.g. betaine and glycerophosphorylcholine) (Choe, 2009). Some protists, however, rely on a contractile vacuole complex (CVC) to maintain their water balance both under normal environmental conditions as well as during dramatic osmotic changes in their environment (Allen, 2009). Both mechanisms, the release of ions and osmolytes and the function of the CVC are important for volume regulation in different stages *T. cruzi* (Rohloff and Docampo, 2008). It is possible that under normal environmental conditions the CVC is responsible for most water efflux needed to maintain the steady state volume of the cells.

RVD and RVI are important homeostatic mechanisms needed by all cells under steady state conditions and in addition they are essential for the adaptation of *T. cruzi* to the diverse environmental conditions that the parasite must encounter during its life cycle. For instance, there is a considerable increase in the osmolarity in the lower digestive tract of the insect vector. Osmolarity increases slightly from the feces to the urine, from 320 to 410 mosmol.Kg⁻¹, but there is a very strong increase in the yellow rectal content, up to 1000 mosmol.Kg⁻¹ (Kollien et al., 2001). When metacyclic trypomastigotes are introduced into a new host there is again a dramatic change, back to the normal osmolarity in the tissues and blood (330 mosmol.Kg⁻¹). Entry of the trypomastigote into a parasitophorous vacuole and its release into the cytosol could potentially involve changes in osmolarity although it is difficult to have a correct estimate of the osmolarity of different regions of a cell. Therefore, the parasite is subjected to both hyperosmotic and hyposmotic stresses during its life cycle and needs mechanisms to adapt to both stress conditions.

Physiological adaptations to hyposmotic stress in different stages of *T. cruzi* have been studied more extensively. The results have indicated that a RVD mechanism is present in all stages, amastigotes, epimastigotes and trypomastigotes (Rohloff et al., 2003). The process is rapid and essentially complete in all *T. cruzi* stages by 5 min. Amino acid efflux accounts for approximately 50% of the RVD at 150 mOsm in all stages of *T. cruzi* (Rohloff et al., 2003), while the rest depends on K⁺ efflux (7%) and the function of the CVC (43%) (Rohloff and Docampo, 2008). A number of uncharged or acidic amino acids are mobilized during hyposmotic stress in all three stages and there is a marked absence of mobilization of cationic amino acids. Glu, Gly, Pro, and Ala account for nearly 90% of the total amino acids mobilized (Rohloff et al., 2003). These results suggest that amino acid efflux in *T. cruzi* occurs through anion channels/transporters as proposed in other cells (Vieira et al., 1996; Lang, 2007). Acidocalcisomes contain high concentrations of amino acids but nearly 90% of them consist of Arg and Lys, minor components of the amino acids released extracellularly during regulatory volume decrease (Rohloff et al., 2003). A rise in intracellular Ca²⁺ occurs upon hyposmotic stress which is completely dependent on extracellular calcium and,

although it plays a role in modulating the early phase of amino acid efflux, is not a key determinant of the final outcome of the regulatory volume decrease (Rohloff et al., 2003). Na^+ and phosphate are not released extracellularly. Inositol efflux to the extracellular medium is negligible (Rohloff et al., 2003).

4. THE CONTRACTILE VACUOLE COMPLEX

The contractile vacuole complex (CVC) was first described in *Paramecium* more than 200 years ago (Spallanzani, 1799), and was later found in a wide range of amoeba, photosynthetic and nonphotosynthetic flagellates, and ciliates. Clark (Clark, 1959) was the first to describe the presence of a CVC in *T. cruzi*, and reported a pulsation period (time between contractions) in epimastigotes between 1 min and 1 min and 15 sec.

The contractile vacuole of *T. cruzi* has a bipartite structure, consisting of a central vacuole or bladder and a surrounding loose network of tubules and vesicles known as the spongiome (Rohloff et al., 2004) (Fig. 1B and Fig. 3). The spongiome and central vacuole form a stable interconnected network that collapses during systole. Functional distinctions between the peripheral and central components of the contractile vacuole complex were evidenced by the localization of different proteins to each compartment. Recent proteomic analysis and fluorescence studies of GFP-tagged proteins have revealed the presence of the vacuolar H^+ -ATPase, Rab11, Rab32, AP180, VAMP1 and a putative phosphate transporter to the bladder while calmodulin, and two SNAREs are localized to the spongiome (Ulrich et al., 2010).

It has been pointed out that because no contractile vacuole from any organism characterized to date is significantly acidic (for example, one study (Stock et al., 2002) calculated the pH of the CV in *Paramecium multimicronucleatum* to be 6.4), the H^+ -ATPases most likely provides instead the primary electrochemical gradient for the movement of other ions (Allen and Naitoh, 2002). Interestingly, the CVC of *T. cruzi* also possesses a vacuolar H^+ -pyrophosphatase (Rohloff et al., 2004), which would provide a redundant mechanism for generating an electrochemical potential. The roles of the contractile vacuoles in protists, though, extend beyond regulation of cell volume to maintenance of Ca^{2+} homeostasis (Xie et al., 1996, Moniakis et al., 1999, Malchow et al., 2006) and transport of proteins to the plasma membrane (Sesaki et al., 1997), although these functions have not been investigated in *T. cruzi*. Recently, Hasne et al. (Hasne et al., 2010) demonstrated that the contractile vacuole of *T. cruzi* houses a polyamine transporter that can be transferred to the plasma membrane when the incubation media is deficient in polyamines. The CVC of *T. cruzi* also possesses an aquaporin involved in its periodic filling (Montalvetti et al., 2004, Rohloff et al., 2004).

5. ACIDOCALCISOMES AND THE CONTRACTILE VACUOLE COMPLEX

The acidocalcisome was initially postulated as an osmotically-active reservoir linked to the contractile vacuole function in both *Chlamydomonas reinhardtii* (Ruiz et al., 2001a) and *Dictyostelium discoideum* (Marchesini et al., 2002). Inorganic osmolytes, such as P_i released from hydrolyzed poly P, could be transferred from the acidocalcisome to the contractile vacuole, setting up a favorable osmotic gradient and facilitating net water flux into the CVC for subsequent water elimination (Marchesini et al., 2002). It was proposed that acidocalcisomes, which usually appear as indistinctive empty vacuoles by electron microscopy (Fig. 1B), could correspond to the “vesicles” or “vacuoles” that were identified in free-living protozoa as dynamically fusing with the spongiome portion of the contractile vacuole. More recent work established that hyposmotic stress conditions results in a significant increase in cyclic AMP (cAMP), swelling of acidocalcisomes, and displacement of green fluorescent protein (GFP)-labeled aquaporin immunofluorescent labeling from the acidocalcisomes to the CVC in a microtubule- and cAMP-dependent fashion (Rohloff et al.,

2004). Fusion of acidocalcisomes to the CVC was initially suggested by their apparent continuity in intact cells and subcellular fractions and by electron microscopy observation of similar electron-dense material in both organelles, and was detected by video microscopy (Rohloff et al., 2004). In addition hyposmotic stress induces a rapid rise in intracellular ammonia, up to 1 mM in whole cell terms, which is rapidly sequestered into acidocalcisomes (Rohloff and Docampo, 2006).

The translocation mechanism of an aquaporin from vesicles to other membranes is similar to that described in mammalian cells; a cAMP-mediated event is involved in fusion of AQP2- (Nielsen et al., 1995) and AQP8- (Garcia et al., 2001) containing vesicles to the plasma membrane. On the other hand, an acetylcholine-induced rise in Ca^{2+} induces fusion of AQP5 vesicles, probably through a protein kinase C-mediated phosphorylation event (Ishikawa et al., 1998).

6. SIGNALING PATHWAYS INVOLVED IN VOLUME REGULATION

The study of the cAMP pathway in *T. cruzi* has been limited. The predicted structure of *T. cruzi* adenylyl cyclases consists of a large presumably extracellular *N*-terminal domain, followed by a single membrane-spanning helix and an intracellular catalytic domain (Taylor et al., 1999). This is different from the typical 12-transmembrane spanning structure of G-protein coupled adenylyl cyclases. The structure suggests that these adenylyl cyclases might function as catalytic receptors, ruling out the participation of heterotrimeric G-proteins or other regulatory factors. Furthermore, heterotrimeric G-proteins have not been identified in *T. cruzi* (Parsons and Ruben, 2000). Only one adenylyl cyclase gene of the near 30 genes annotated in the genome of *T. cruzi* (TriTryp.DB) has been studied in detail. The protein product localizes to the flagellum and is activated by calcium (D'Angelo et al., 2002). The increase in cAMP levels after cells swelling might suggest the activation of either a mechanosensitive adenylyl cyclase like the one that occurs in coronary vascular smooth cells (Mills et al., 1990) or of a mechanosensitive channel (Xiao and Xu, 2010) that could lead to the influx of ions, such as Ca^{2+} , and activation of the adenylyl cyclase upon hyposmotic stress. How cAMP exerts its action is unknown. Homologs of a protein kinase A catalytic (Huang et al., 2002) and regulatory (Huang et al., 2006) subunits have been identified in *T. cruzi* but it is still uncertain whether this PKA is stimulated by cAMP. *T. cruzi* AQP1 could be phosphorylated in vitro by a bovine PKA (Bao et al., 2008) but no experiments were done with the *T. cruzi* enzyme.

Cyclic AMP phosphodiesterases (PDEs) are responsible for the termination of cyclic AMP signals by hydrolyzing cyclic AMP to 5'-AMP. There are four groups of PDEs in *T. cruzi*: **A** (two genes), **B** (two genes), **C** (one gene), and **D** (two genes). All four PDEs belong to the Class I group of PDEs, similar to the large number of Class I PDEs found in mammals (Laxman and Beavo, 2007). TcrPDEC is a novel and rather unusual PDE that, unlike all other class I PDEs, has its catalytic domain localized in the middle of the polypeptide chain (Kunz et al., 2005, Alonso et al., 2006). In contrast, PDEs have unique *N*-terminal regulatory domains and the catalytic domain is usually located near their C-terminus. TcrPDEC is the only trypanosome PDE identified to date capable of hydrolyzing cGMP, although it prefers cAMP as a substrate (cAMP K_m 30 μ M, and cGMP K_m 80 μ M) (Kunz et al., 2005). Additionally, TcrPDEC is unusual in that its *N*-terminal region contains a FYVE-type domain, a functional domain that has not been found in any PDE so far. *T. cruzi* PDEC was recently found to localize to the CVC (Schoijet, 2010). The FYVE domain was shown to be important for the activity of the enzyme and for its localization in the CVC (Schoijet, 2010). This is the PDE involved in osmoregulation since the same compounds that inhibit the activity of the recombinant enzyme (Kunz et al., 2005) inhibit CVC swelling that occurs after hyposmotic stress (Rohloff et al., 2004).

Another enzyme potentially involved in volume regulation is the class III phosphatidylinositol 3-kinase (PI3K), related to the yeast vacuolar protein sorting 34, Vps34p, and their homologs from other eukaryotes. These kinases specifically phosphorylate phosphatidylinositol to produce phosphatidylinositol 3-phosphate (PI 3-P) and are associated with a Vps15p-like protein kinase. *T. cruzi* PI3K was functionally characterized and was able to complement yeast deficient in Vsp34p (Schoijet et al., 2008). Parasites overexpressing TcPI3K showed an enlarged CVC and protection against inhibition of the RVD by the PI3K inhibitors wortmannin and LY294,000 (Schoijet et al., 2008) suggesting a role in volume regulation.

7. DRUG TARGETING OF THE VOLUME REGULATORY PATHWAY

Phosphodiesterases are validated pharmacological targets for the treatment of several human diseases, such as erectile dysfunction (sildenafil, tadalafil, vardenafil), congestive heart failure (milrinone), platelet aggregation and intermittent claudication (cilostazol), and pulmonary hypertension (sildenafil). TcrPDEC has sequence similarity to human PDE4. Since many PDE4 inhibitors are currently under development for the treatment of inflammatory diseases, such as asthma, chronic obstructive pulmonary disease, and psoriasis, as well as treating depression and serving as cognitive enhancers (Houslay et al., 2005), an extensive literature and a number of compounds with potentially little activity against human PDE4s but potentially effective against TcrPDEC are possibly available that could be tested against *T. cruzi*.

A number of compounds originally synthesized as potential PDE4 inhibitors (Zheng et al., 2008) were tested on *T. cruzi* amastigote growth, and several useful hits were obtained (King-Keller et al., 2010). An homology modeling of *T. cruzi* PDEC based on the structure of PDE4B was constructed and other potential inhibitors were obtained through virtual screening (King-Keller et al., 2010). Testing of these compounds on amastigote growth and on the recombinant TcrPDEC activity resulted in several potent inhibitors that caused no toxicity to the host cells (King-Keller et al., 2010). The TcrPDEC was chemically validated as the target for these inhibitors by following the increase in cAMP in the parasites submitted to these inhibitors and the inhibition of their response to hyposmotic stress (King-Keller et al., 2010). The lethal effect of TcrPDEC inhibitors under isosmotic conditions suggests that the cAMP signaling pathway is necessary not only to overcome dramatic changes in osmolarity that occur after hyposmotic stress but also under isosmotic steady-state conditions. Continuous cAMP oscillations, which are known to occur in other cells that have contractile vacuole mechanisms of water extrusion, such as *D. discoideum* (Monk and Othmer, 1989), could be responsible for the periodic contraction and water expulsion by the CVC that occurs under isosmotic conditions, and explain the absolute essentiality of this mechanism of water regulation in these organisms (King-Keller et al., 2010).

8. MODEL FOR VOLUME REGULATION

A model was proposed (Rohloff and Docampo, 2008) in which the stimulus of cell swelling causes a spike in intracellular cAMP through an as yet unidentified adenylyl cyclase, resulting in a microtubule-dependent fusion of acidocalcisomes with the contractile vacuole and translocation of aquaporin. A simultaneous rise in ammonia, and its sequestration in acidocalcisomes as NH_4^+ , activates an acidocalcisomal exopolyphosphatase, which cleaves poly P, releasing inorganic phosphate residues and also the various poly P-chelated osmolytes, such as basic amino acids and calcium. The resulting osmotic gradient sequesters water through the aid of the aquaporin, which is subsequently ejected into the flagellar pocket. This process is terminated by the action of a PDE (Fig. 4).

Since this model was proposed several supporting results have been reported. For instance, TcrPDEC was shown to localize to the CVC (Schoijet, 2010) and inhibitors of this enzyme affect the RVD of *T. cruzi* (King-Keller et al., 2010). This PDE has a FYVE domain, which is a phosphoinositide binding motif able to bind to PI3P, the product of a PI3K (Alonso et al., 2006, Kunz et al., 2005). Overexpression of TcPI3K was shown to affect the RVD (Schoijet et al., 2008). Several proteins important for vacuolar fusion such as SNAREs and VAMP1 were shown to localize to the CVC and a putative phosphate transporter was found in the bladder of the CVC (Ulrich, 2010). This transporter could be involved in recycling of phosphate produced by the hydrolysis of poly P during RVD (Fig. 4).

9. POLYPHOSPHATE, STRESS RESPONSE AND VIRULENCE

Rapid hydrolysis or synthesis of acidocalcisome poly P occur during hypo- or hyperosmotic stress (Ruiz et al., 2001b). Overexpression of *T. cruzi* exopolyphosphatase (TcPPX) depletes poly P and affects the RVD (Fang et al., 2007b). The use of RNAi to reduce the expression of the acidocalcisomal soluble pyrophosphatase of *T. brucei* (TbVSP1) also resulted in trypanosomes that were deficient in poly P and in their response to hyposmotic stress (Lemerrier et al., 2004). Ablation of a vacuolar transporter chaperone (VTC1) in *T. brucei* by RNAi resulted in abnormal morphology of acidocalcisomes, decrease in their poly P content, and deficient response to hyposmotic stress (Fang et al., 2007a).

In addition to its role in volume regulation acidocalcisome poly P could have a role in nutritional stress. Knockdown of a TOR-like 1 kinase in *T. brucei* using RNAi leads to growth arrest and accumulation of poly P and P_i inside acidocalcisomes, which increase in size and become heavily stained at the electron microscopy level (de Jesus et al., 2010). It has been suggested that these phenotypic changes would be similar to those occurring during nutritional stress in bacteria (de Jesus et al., 2010). This is known as the “stringent response”: amino acid starvation leads to coupled cessation of protein synthesis and stable RNA synthesis. Together with the cessation of nucleic acid synthesis and continued assimilation of P_i from the medium, this results in large accumulations of poly P (Brown and Kornberg, 2004). Knockout of the homologous gene in *Leishmania major* (LmTOR3), resulted in alteration of acidocalcisome morphology, deficient RVD, lower ability to respond to glucose starvation, and decreased virulence *in vitro* and *in vivo* (Madeira da Silva and Beverley, 2010). In contrast to the results in *T. brucei* (de Jesus et al., 2010), however, these phenotypic changes were accompanied by an apparent decrease in poly P content of acidocalcisomes although this was not detected by quantitative methods (Madeira da Silva and Beverley, 2010). No studies on this TOR-like kinase 1 (TOR3) have been done on *T. cruzi*. Interestingly, alterations in both acidocalcisomes and poly P content have been associated with reduced *in vivo* virulence in a number of parasites (Lemerrier et al., 2004, Zhang et al., 2005, Luo et al., 2005, Madeira da Silva and Beverley, 2010, Besteiro et al., 2008).

10. CONCLUSIONS

In *T. cruzi*, acidocalcisomes are rich in short chain poly P complexed with cations and basic amino acids, and possess a number of transporters (Ca²⁺-ATPase, V-H⁺-PPase, V-H⁺-ATPase, zinc transporter) and a channel (aquaporin) involved in the uptake of several ions and water, as well as enzymes involved in poly P metabolism. Acidocalcisomes and their major component, poly P, are essential for the response of *T. cruzi* and other trypanosomatids to different stress conditions. Reduced levels of poly P are associated to decreased ability to respond to osmotic or nutritional stresses and decreased virulence *in vitro* and *in vivo*. A microtubule- and cAMP-dependent signaling pathway is stimulated by hyposmotic stress and results in the transfer of the aquaporin from acidocalcisomes to the

CVC. A PI3K is also involved in the response to hyposmotic stress. Acidocalcisomes alkalize due to ammonia accumulation and also increase their volume in response to hyposmotic stress. A *T. cruzi* PDEC was localized to the CVC and demonstrated to be essential for volume regulation and survival of the parasite providing a novel target for chemotherapy.

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Abbreviations used

Ca²⁺-ATPase	calcium ATPase
cAMP	cyclic AMP
CVC	contractile vacuole complex
PDE	phosphodiesterase
PMCA	plasma membrane type Ca ²⁺ -ATPase
poly P	polyphosphate
PPi	pyrophosphate
RVD	regulatory volume decrease
RVI	regulatory volume increase
V-H⁺-ATPase	vacuolar type proton ATPase
V-H⁺-PPase	vacuolar type proton pyrophosphatase
Vtc	vacuolar transporter chaperone

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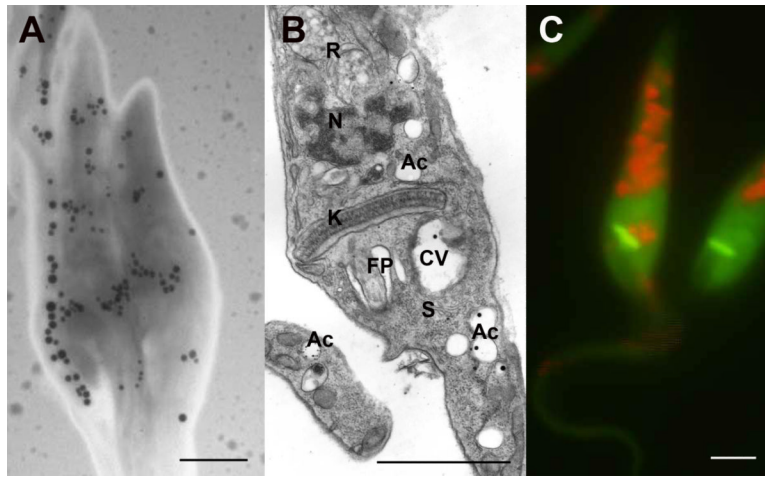


Fig. 1.

Acidocalcisomes in *T. cruzi*. (A) electron micrograph of intact epimastigotes showing the electron-dense acidocalcisomes (black granules) distributed at random; (B) electron micrograph of epimastigote sections showing the acidocalcisomes (Ac) as empty vacuoles containing some electron dense material (dots and electron dense material on the membrane of the organelle); the contractile vacuole bladder (CV) and spongione (S) are also apparent; (C) Staining of acidocalcisomes with Acridine Orange. The acidocalcisomes are shown in orange. Notations are flagellar pocket (FP), acidocalcisomes (Ac), kinetoplast (K), contractile vacuole bladder (CV), tubules forming the spongione (S), reservosomes (R), nucleus (N). Bars, A, B, C = 2.0 μm .

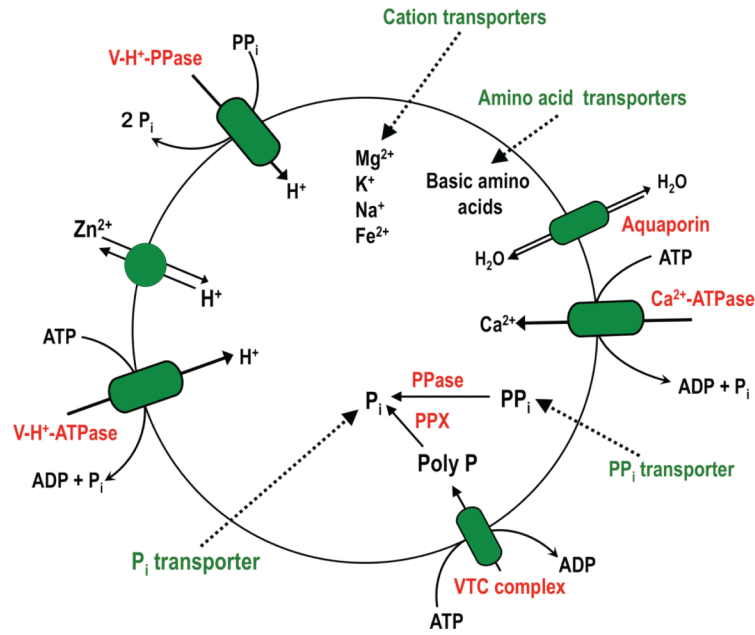


Fig. 2. Schematic representation of an acidocalcisome. A H⁺ gradient is established by a vacuolar ATPase (V-H⁺-ATPase) and a vacuolar pyrophosphatase (V-H⁺-PPase). Ca²⁺ transport is driven by a Ca²⁺-ATPase. Other transporters include a Zn²⁺/H⁺ antiporter, and a water channel or aquaporin. A vacuolar transporter chaperone (VTC) complex is involved in synthesis and translocation of poly P. Transporters for basic amino acids, P_i, PP_i, and cations are potentially present (in green). The matrix is rich in PP_i and polyphosphate (poly P) and enzymes involved in their metabolism (exopolyphosphatase (PPX), and pyrophosphatase (PPase)).

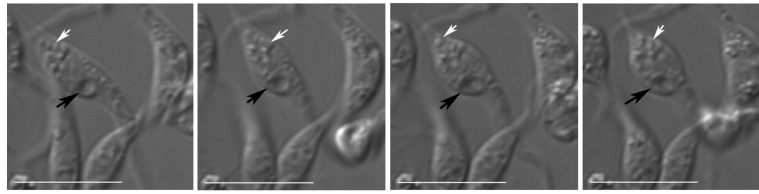


Fig. 3. Live cell imaging of the contractile vacuole. *T. cruzi* epimastigotes were submitted to hyposmotic stress (150 mOsm) to detect swelling of the contractile vacuole bladder (arrows). Note that the cells become rounded after the stress and there is also swelling of smaller vacuoles in the posterior part of the cells. Pictures were taken at 5 sec intervals after hyposmotic stress for a total time of 6 min. Initial 20 seconds of the response are shown in the figure. Bars = 10 μ m.

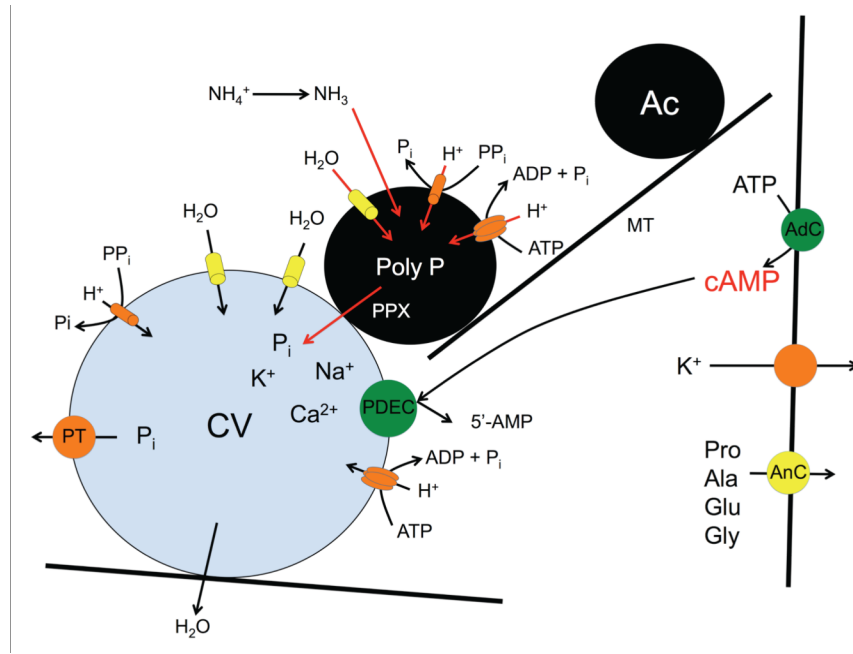


Fig. 4. Model proposed for regulatory volume decrease in *T. cruzi*. Cell swelling causes activation of an adenylyl cyclase (AdC) that results in a spike of intracellular cAMP, resulting in microtubule-dependent movement of acidocalcisomes (Ac) and fusion with the contractile vacuole (CV) with translocation of an aquaporin. A rise in ammonia and its sequestration in acidocalcisomes activates an exopolyphosphatase (PPX) that cleaves poly P, releasing inorganic phosphate residues and also various phosphate-chelated osmolytes, such as calcium and other cations. The resulting osmotic gradient sequesters water, through the aid of the aquaporin, which is subsequently ejected into the flagellar pocket. RVD is completed by the release of amino acids through an anion channel (AnC) and K⁺ through a potassium channel both localized in the plasma membrane. Termination of this cycle is by hydrolysis of cAMP through a phosphodiesterase C (PDEC) located in the contractile vacuole (CV). Phosphate is transporter back to the cytosol by a phosphate transporter (PT). Proton pumps (V-H⁺-ATPase and H⁺-PPase) maintain an electrochemical gradient in both the CV and Ac.