Intracellular Ca^{2+} storage in acidocalcisomes of *Trypanosoma cruzi*

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The use of digitonin to permeabilize the plasma membrane of *Trypanosoma cruzi* allowed the identification of a non-mitochondrial nigericin- or bafilomycin A_1 -sensitive Ca^{2+} -uptake mechanism. Proton uptake, as detected by ATP-dependent Acridine Orange accumulation, was also demonstrated in these permeabilized cells. Under these conditions Acridine Orange was concentrated in abundant cytoplasmic round vacuoles. This latter process was inhibited (and reversed) by bafilomycin A_1 , nigericin and NH_4Cl in different stages of *T. cruzi*. Ca^{2+} released Acridine Orange from permeabilized cells, suggesting that the dye and Ca^{2+} were being accumulated in the same acidic compartment and that Ca^{2+} was taken up in exchange for protons. Addition of bafilomycin A_1 ($5\text{ }\mu\text{M}$), nigericin ($1\text{ }\mu\text{M}$) or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; $1\text{ }\mu\text{M}$) to fura 2-loaded epimastigotes increased their intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Although this effect was more noticeable in the presence of extracellular Ca^{2+} , it was also observed in its absence. Addition of NH_4Cl ($10\text{--}40\text{ mM}$) to different stages of *T. cruzi*, in the nominal absence of extracellular

Ca^{2+} to preclude Ca^{2+} entry, increased both $[\text{Ca}^{2+}]_i$ in fura 2-loaded cells, and intracellular pH (pH_i) in 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxymethyl ester (BCECF)-loaded cells. Treatment of the cells with the Ca^{2+} ionophore ionomycin under similar conditions (nominal absence of extracellular Ca^{2+}) resulted in an increase in $[\text{Ca}^{2+}]_i$ and a significantly higher increase in $[\text{Ca}^{2+}]_i$ after addition of NH_4Cl , nigericin or bafilomycin A_1 , all agents which increase the pH of acidic compartments and make ionomycin more effective as a Ca^{2+} -releasing ionophore. Similar results were obtained when the order of additions was reversed. Taking into account the relative importance of the ionomycin-releasable and the ionomycin plus NH_4Cl -releasable Ca^{2+} pools, it is apparent that most of the Ca^{2+} stored in different stages of *T. cruzi* is present in the acidic compartment thus identified. Taken together, these results are consistent with the presence of a $\text{Ca}^{2+}/\text{H}^+$ exchange system in an acidic vacuole, which we have named the 'acidocalcisome' and which appears to be a unique organelle present in trypanosomatids.

INTRODUCTION

Trypanosoma cruzi is a protozoan parasite that infects a wide variety of vertebrates and is the agent responsible for Chagas' disease in humans. It is an obligate intracellular parasite, which implies the adaptation to life inside cells with a very different ionic composition to the external milieu. The intracellular forms of *T. cruzi* live, proliferate and differentiate in an environment where the free Ca^{2+} concentration is very low (of the order of $0.1\text{ }\mu\text{M}$) as compared with that experienced by extracellular forms (of the order of about 1.0 mM). This dramatic difference in free Ca^{2+} to which these cells are adapted suggests that Ca^{2+} homeostasis should differ between the parasites and the host cells, and that other mechanisms should be operative in these parasites to maintain their intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$).

Two intracellular Ca^{2+} -uptake mechanisms have been described in different stages of *T. cruzi* [1–7]. One has characteristics typical of mitochondria in other eukaryotic cells [8], such as inhibition by antimycin A, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and Ruthenium Red, stimulation by respiratory substrates, phosphate and acetate, a high capacity and low affinity for Ca^{2+} and the ability to buffer external Ca^{2+} concentrations ($[\text{Ca}^{2+}]_e$) in the range $0.6\text{--}0.7\text{ }\mu\text{M}$ [1–7]. The second uptake mechanism has features similar to endoplasmic reticulum in other eukaryotic cells [9], such as inhibition by

sodium orthovanadate, an inhibitor of P-type ATPases, and by high concentrations of anti-calmodulin agents, stimulation by ATP, a low capacity and high affinity for Ca^{2+} , and the ability to buffer $[\text{Ca}^{2+}]_e$ in the range $0.05\text{--}0.1\text{ }\mu\text{M}$ [1,3,5–7].

Using permeabilized cells, we have recently discovered the presence of a third intracellular Ca^{2+} -uptake mechanism in the related trypanosomatid *T. brucei* [10]. Ca^{2+} transport by this mechanism is sensitive to vanadate and to bafilomycin A_1 , a specific inhibitor of vacuolar-type H^+ -ATPases [11]. The results [10] were consistent with the presence of a $\text{Ca}^{2+}/\text{H}^+$ -ATPase system pumping Ca^{2+} into acidic vacuoles, easily revealed by fluorescence microscopy, which we named 'acidocalcisomes' [10]. The presence of an H^+ -countertransporting Ca^{2+} -ATPase involved in Ca^{2+} sequestration that resides in acidic vacuoles has only been described in *Dictyostelium discoideum* [12], although vanadate-sensitive Ca^{2+} -uptake systems associated with acidic compartments have been found in rat parotid [13] and pancreatic [14] acinar cells. In addition, by using energy-dispersive X-ray elemental mapping [15], large accumulations of Ca^{2+} have been localized in vacuoles identifiable by scanning transmission electron microscopy throughout the body of *T. cruzi* epimastigotes, with major concentrations in both the anterior and posterior regions.

Taking into account all these considerations, we investigated the possible presence of Ca^{2+} in an acidic compartment in *T.*

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; $[\text{Ca}^{2+}]_e$, extracellular Ca^{2+} concentration; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; fura 2, 1-(2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl)-2-(2'-amino-5'-methylphenoxy)ethane-*NNN'*-tetra-acetic acid; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein; pH_i , cytosolic pH.

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cruxi using intact fura 2-loaded cells or permeabilized cells. We show that the different stages of *T. cruzi* possess an acidic compartment which is rich in Ca^{2+} and that most of the releasable Ca^{2+} in these cells is located in this compartment.

MATERIALS AND METHODS

Culture methods

T. cruzi trypomastigotes and amastigotes (Y strain) were obtained from the culture medium of L_6E_9 myoblasts by a modification of the method of Schmatz and Murray [16] as we have described previously [5,6,17]. The final concentration of trypomastigotes and amastigotes was determined by using a Neubauer chamber. The contamination of trypomastigotes with amastigotes and intermediate forms, or of amastigotes with trypomastigotes or intermediate forms, was always less than 5%. *T. cruzi* epimastigotes (Y strain) were grown at 28 °C in a liquid medium consisting of brain–heart infusion (37 g/l), haemin chlorohydrate (20 mg/l dissolved in 50% triethanolamine), and 10% heat-inactivated newborn calf serum [1]. At 5 days after inoculation, cells were collected by centrifugation. All the cells were washed twice with Dulbecco's PBS (138 mM NaCl, 2.7 mM KCl, 1.2 mM KH_2PO_4 , 8.1 mM Na_2HPO_4 , pH 7.2). The protein concentration was determined by the biuret assay [18] in the presence of 0.2% deoxycholate. L_6E_9 myoblasts were cultured as described previously [19].

Chemicals

ATP, A23187, ionomycin, sodium orthovanadate, FCCP, Triton X-100, Dulbecco's modified Eagle's medium, fetal- and newborn-calf serum, succinate, arsenazo III, EGTA, thapsigargin and digitonin were purchased from Sigma Chemical Co. Bafilomycin A_1 was obtained from Dr. K. Altendorf (University of Osnabrück, Osnabrück, Germany). Acridine Orange, 1-(2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl)-2-(2'-amino-5'-methylphenoxy)ethane-*NNN'*-tetra-acetic acid acetoxymethyl ester (fura 2/AM) and 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) were from Molecular Probes. All other reagents were of analytical grade.

Determination of Ca^{2+} movements

Variations in free (extravesicular) Ca^{2+} concentrations were followed by measuring the changes in the absorbance spectrum of arsenazo III [20], with an SLM Aminco DW2000 spectrophotometer at the wavelength pair 675–685 nm. No free-radical formation from arsenazo III occurred under the conditions used [21–23].

Proton-pump activity

The acidification of vesicles in digitonin-permeabilized cells was monitored by measuring changes in the absorption spectrum of Acridine Orange [24], with the SLM DW2000 instrument at the wavelength pair 493–530 nm.

Spectrofluorimetric determinations

Fura 2 determinations were performed essentially as described by Philosoph and Zilberstein [25]. After harvest of the cells, they were washed twice at 6000 *g* for 10 min at 4 °C in buffer A, which contained 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO_4 , 5.5 mM D-glucose and 50 mM Hepes, pH 7.2 [25]. Cells were resuspended to a final density of 10^9 cells/ml in loading buffer which contained

116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO_4 , 5.5 mM glucose, 1.5% sucrose and 50 mM Hepes, pH 7.2, and 6 μM fura 2/AM. The suspensions were incubated for 30 min in a 30 °C water bath with mild agitation. Subsequently, the cells were washed twice with ice-cold buffer A to remove extracellular dye. Cells were resuspended to a final density of 10^9 cells/ml in buffer A and were kept in ice [25]. For fluorescence measurements, a 125 μl sample of the cell suspension was diluted into 2.5 ml of buffer A (5×10^7 cells/ml final density) in a cuvette placed in a thermostatically regulated (30 °C for epimastigotes, 37 °C for amastigotes and trypomastigotes) Hitachi F-2000 spectrofluorimeter. Excitation was at 340 and 380 nm and emission was at 510 nm. The fura-2 fluorescence response to $[\text{Ca}^{2+}]$, concentration was calibrated from the ratio of 340/380 nm fluorescence values after subtraction of the background fluorescence of the cells at 340 and 380 nm as described by Gryniewicz et al. [26]. For intracellular pH measurements, the cells were incubated in the presence of 6 μM BCECF-AM in the same loading buffer for 30 min at 30 °C. The cells were then washed twice in buffer A to remove extracellular dye, and suspended in the same buffer to a final density of 10^9 cells/ml. Experiments were performed in cuvettes placed in a thermostatically regulated Hitachi F-2000 spectrofluorimeter with 2.5 ml of incubation medium and samples (125 μl) of the cell suspensions as described above. The fluorescence ratio, with wavelengths for excitation set at 505/440 nm and for emission at 530 nm, were recorded and translated into pH values on the basis of the ratios obtained at various pH values [27]. Other experimental conditions and calibrations were as described previously [1–6]. $[\text{Ca}^{2+}]$ was calculated by titration with different concentrations of Ca-EGTA buffers [1–6]. Concentrations of the ionic species and complexes at equilibrium were calculated by using an iterative computer program as described previously [1–6].

RESULTS

Presence of a non-mitochondrial nigericin-sensitive Ca^{2+} compartment in *T. cruzi* epimastigotes

In order to detect endogenous Ca^{2+} in these parasites, digitonin-permeabilized epimastigotes were incubated with antimycin A and oligomycin to prevent mitochondrial Ca^{2+} uptake [1,2,4] and in the presence of sodium orthovanadate and absence of ATP, to prevent Ca^{2+} uptake by the endoplasmic reticulum [3]. Evidence has already been presented that mitochondria *in situ* do not contain a significantly high amount of endogenous Ca^{2+} in *T. cruzi* [3]. Accordingly, Figure 1 (trace *a*) shows a very slow release of endogenous Ca^{2+} during the time of observation, which could come from the endoplasmic reticulum or other organelles [3]. This was followed by a fast Ca^{2+} release upon addition of the Ca^{2+} ionophore A23187. Trace (*b*) shows that, after the cells were incubated for a period of about 5 min in the presence of the K^+/H^+ exchanger nigericin (1 μM), there was release of about 30% of the endogenous Ca^{2+} . The remaining Ca^{2+} was then completely released by the addition of A23187 on top of nigericin (trace *b*). The ability of nigericin to release some non-mitochondrial endogenous Ca^{2+} strongly suggests that it is stored in an acidic compartment.

Bafilomycin A_1 inhibits Ca^{2+} uptake in *T. cruzi* epimastigotes

Acidic compartments are characterized by the use of an ATP-driven H^+ pump to maintain their interior at a pH lower than that of the cytoplasm [28]. These H^+ pumps are usually bafilomycin A_1 -sensitive [11]. Epimastigotes were incubated as above, except that vanadate was omitted, which allowed the uptake of

Figure 1 Presence of a non-mitochondrial nigericin-sensitive Ca^{2+} compartment in permeabilized epimastigotes

The cells (0.4 mg of protein/ml) were added to the standard reaction medium (125 mM sucrose, 65 mM KCl, 10 mM Hepes, pH 7.2, 2.5 mM potassium phosphate, 1 mM MgCl_2) containing 2 $\mu\text{g}/\text{ml}$ oligomycin, 1 $\mu\text{g}/\text{ml}$ antimycin A, 500 μM sodium orthovanadate and 40 μM arsenazo III. Trace (a) is control without nigericin. Trace (b) is with the addition of nigericin (NIG, 1.0 μM) where indicated. Digitonin (D, 20 μM) and 1 μM Ca^{2+} ionophore (A23187, traces a and b) were added where indicated. Traces are representative of three independent experiments conducted on separate cell preparations.

Figure 2 Effect of bafilomycin A_1 and sodium orthovanadate on Ca^{2+} uptake by non-mitochondrial Ca^{2+} compartments of epimastigotes

The cells (0.4 mg of protein/ml) were added to the standard reaction medium, as in Figure 1, containing 2 $\mu\text{g}/\text{ml}$ oligomycin, 1 $\mu\text{g}/\text{ml}$ antimycin A, 40 μM arsenazo III and 20 μM digitonin. Trace (a) is control; trace (b) is in the presence of 4 μM bafilomycin A_1 . ATP (1.0 mM, added 3 min after digitonin), vanadate (VAN, 1 mM) or 10 μM Ca^{2+} ionophore A23187 were added where indicated. Traces are representative of six independent experiments conducted on separate cell preparations.

Ca^{2+} by intracellular compartments once ATP was added (Figure 2, trace a). Incubation with 4 μM bafilomycin A_1 (~ 10 nmol/mg of cell protein) decreased this uptake by $30 \pm 8\%$ (mean $\pm$ S.D. from 6 separate experiments; trace b). Uptake was halted by addition of 1 mM sodium orthovanadate and reversed by A23187, and was inhibited, by an extent similar to that with bafilomycin A_1 , by 5 μM nigericin (results not shown). A Ca^{2+} -ATPase has recently been characterized in *T. brucei* which is 90% inhibited by 0.1 μM thapsigargin [29]. We found no effect of this compound, at 0.6 μM , on Ca^{2+} uptake either alone or in the presence of 4 μM bafilomycin A_1 (results not shown).

ATP-dependent vacuolar acidification in permeabilized epimastigotes: effects of nigericin, NH_4Cl , bafilomycin A_1 and Ca^{2+}

In order to investigate the role of H^+ -ATPases in the maintenance of a nigericin-sensitive Ca^{2+} pool in *T. cruzi*, we measured Acridine Orange uptake by permeabilized epimastigotes. Acridine Orange is a tertiary amine that becomes concentrated in acidic compartments within cells and changes both its absorbance

Figure 3 ATP-driven Acridine Orange accumulation by digitonin-permeabilized epimastigotes

The cells (0.4 mg of protein/ml) were added to the standard reaction medium containing 2 $\mu\text{g}/\text{ml}$ oligomycin, 1 $\mu\text{g}/\text{ml}$ antimycin A, 3.0 μM Acridine Orange, 1 mM EGTA and 20 μM digitonin and preincubated for 4 min before starting absorbance measurements. ATP (1.0 mM), 1.0 μM nigericin (NIG), 1.4 mM CaCl_2 , 5 μM bafilomycin (BAF) or 20 mM NH_4Cl was added where indicated. Traces are representative of three independent experiments conducted on separate cell preparations.

and fluorescence properties as a consequence of its accumulation and subsequent dimerization [24]. Addition of ATP to a reaction medium containing antimycin A, oligomycin and permeabilized epimastigotes caused a significant time-dependent decrease in the absorbance of Acridine Orange (Figure 3, trace a). The addition of bafilomycin A_1 (BAF, trace b) caused a reversal of the absorbance decrease, which was completed by addition of NH_4Cl . Figure 3 (trace c) shows that addition of CaCl_2 partially reversed the change in absorbance and that the reversion was completed by addition of NH_4Cl . Under all experimental conditions tested, the decrease in Acridine Orange absorbance induced by ATP was reversed by nigericin or NH_4Cl . The latter compound, in the form of the weak base ammonia, can freely cross membranes but, once inside an acidic compartment, it becomes protonated and thereby trapped, and consequently raises the organellar pH [30,31]. The nigericin and NH_4Cl effects confirm that the decrease in Acridine Orange absorbance was caused by its accumulation in acidic compartment(s). This could also be directly observed by using a fluorescence microscope. Under the conditions of Figure 3 (trace a), *T. cruzi* epimastigotes accumulated Acridine Orange in multiple round vacuoles of varying size located mainly in the posterior end of the cell, although some were also seen in the anterior end, and which fluoresced bright orange. That these vacuoles were acidified by H^+ -ATPases was suggested by the disappearance of their fluorescence upon treatment of the permeabilized cells with 1.0 μM nigericin. In agreement with the results shown in Figure 3, bafilomycin A treatment (5 μM) only partially decreased the fluorescence of the vacuoles, probably indicating a slower mechanism of Acridine Orange release (results not shown). Similar results were obtained after incubation of intact epimastigotes with Acridine Orange (Figure 4). Fluorescence of the vacuoles disappeared after treatment with either 1.0 μM nigericin or 20 mM NH_4Cl , and was greatly decreased with 5 μM bafilomycin A (results not shown).

In conclusion, it appears that the bafilomycin A_1 -sensitive H^+ -ATPase is present in vacuoles which accumulate Ca^{2+} , acidi-

Figure 4 Accumulation of Acridine Orange in acidic vacuoles of epimastigotes

Cells (10^8 cells/ml) were incubated in buffer A containing $3 \mu\text{M}$ Acridine Orange. The suspensions were incubated for 15 min at 30°C in a water-bath with mild agitation. Note the highly fluorescent vacuoles that appear to be clustered at the posterior end of the cells (large arrows, yellow–orange). The small arrows indicate the kinetoplast (green). Cells were photographed with a Leitz Laborlux 12 fluorescence microscope fitted with a fluorescein filter set and a Kodak Ektachrome 400 Elite film. Magnification $\times 1000$.

fication due to the proton pump being balanced by de-acidification caused by $\text{Ca}^{2+}/\text{H}^+$ exchange.

ATP-dependent vacuolar acidification in permeabilized amastigotes and trypomastigotes

Figure 5(a) (trace *a*) shows that the addition of Acridine Orange to permeabilized trypomastigotes in the presence of ATP, under similar conditions to those used for the experiments shown in Figure 3, was followed by a time-dependent decrease in absorbance of the dye, which levelled out after 2 min. Addition of 20 mM NH_4Cl reversed the change in absorbance (trace *a*). The addition of bafilomycin A_1 (BAF, dashed trace) caused an almost complete reversal of the absorbance decrease, which was completed by addition of nigericin. Figure 5(a) (trace *b*) shows that addition of CaCl_2 partially reversed the change in absorbance and that the reversion was completed by addition of bafilomycin A_1 and nigericin. Similar results were obtained with *T. cruzi* amastigotes. When Acridine Orange was added to a reaction medium containing permeabilized amastigotes in the presence of ATP under similar conditions, a time-dependent decrease in absorbance of the dye also occurred (Figure 5b). This decrease was significantly inhibited by inclusion of 20 mM NH_4Cl or $1 \mu\text{M}$ bafilomycin A_1 . Addition of more ATP after bafilomycin A_1 did not result in any further change in Acridine Orange absorbance, and addition of NH_4Cl reversed the change. NH_4Cl and bafilomycin A_1 also caused reversion of the changes in dye absorbance if they were added after the absorbance had levelled out. Addition of CaCl_2 to these amastigote preparations also caused almost complete release of Acridine Orange (results not

Figure 5 Accumulation of Acridine Orange in acidic vacuoles in permeabilized trypomastigotes (a) and amastigotes (b)

(a) The cells (trypomastigotes; 0.3 mg of protein/ml) were added to the standard reaction medium containing $2 \mu\text{g}/\text{ml}$ oligomycin, $1 \mu\text{g}/\text{ml}$ antimycin A, $3.0 \mu\text{M}$ Acridine Orange, $14 \mu\text{M}$ EGTA and $20 \mu\text{M}$ digitonin and preincubated for 4 min before starting absorbance measurements. ATP (0.5 mM), $1.0 \mu\text{M}$ nigericin (NIG), $40 \mu\text{M}$ CaCl_2 , $5 \mu\text{M}$ bafilomycin A_1 or 20 mM NH_4Cl was added where indicated. (a) Control; dashed line shows the addition of $5 \mu\text{M}$ bafilomycin A_1 (BAF) and $1.0 \mu\text{M}$ nigericin where indicated; (b) shows the addition of $40 \mu\text{M}$ CaCl_2 , $5 \mu\text{M}$ bafilomycin A_1 and $1.0 \mu\text{M}$ nigericin where indicated. (b) The cells (amastigotes; 0.3 mg of protein/ml) were added to the standard reaction medium containing $2 \mu\text{g}/\text{ml}$ oligomycin, $1 \mu\text{g}/\text{ml}$ antimycin A, $3.0 \mu\text{M}$ Acridine Orange, 0.5 mM EGTA and $40 \mu\text{M}$ digitonin and preincubated for 4 min before starting absorbance measurements. ATP (0.5 mM), 10 mM NH_4Cl or $5 \mu\text{M}$ bafilomycin A_1 was added where indicated. Traces are representative of three independent experiments conducted on separate cell preparations.

shown). Bafilomycin A_1 solvent (dimethyl sulphoxide) had no effect at the concentration used (results not shown).

Increase in $[\text{Ca}^{2+}]_i$ by bafilomycin A_1 , nigericin and FCCP in amastigotes and trypomastigotes of *T. cruzi*

Since a significant amount of the endogenous Ca^{2+} appears to be accumulated in an acidic compartment in *T. cruzi*, we studied the effect of reagents which disrupt pH gradients on the $[\text{Ca}^{2+}]_i$ of intact cells loaded with the fluorescent Ca^{2+} indicator fura 2. Addition of bafilomycin A_1 greatly increased $[\text{Ca}^{2+}]_i$ in both amastigotes (Figure 6a) and trypomastigotes (Figure 7). This increase also occurred in the nominal absence of extracellular Ca^{2+} (in the presence of 1 mM EGTA; Figure 6a, and results not shown), indicating that the bafilomycin A_1 -induced rise in $[\text{Ca}^{2+}]_i$ is due, at least in part, to release of Ca^{2+} from intracellular stores. Similar results were obtained when nigericin or FCCP was used instead of bafilomycin A_1 , although the rise in $[\text{Ca}^{2+}]_i$ was not as great (Figures 6b, 6c and 7).

Release of Ca^{2+} by NH_4Cl and ionomycin: effects on cytosolic pH (pH_i)

Changes in pH_i have been postulated to regulate $[\text{Ca}^{2+}]_i$ in *T. brucei* bloodstream trypomastigotes [32]. We therefore investigated whether there was any correlation between changes in pH_i and changes in $[\text{Ca}^{2+}]_i$ in different stages of *T. cruzi*. The effects of reagents on pH_i were monitored with BCECF-loaded cells (Figure 8). Experiments were performed in the nominal absence of extracellular Ca^{2+} (in the presence of 1 mM EGTA) in order to prevent refilling of intracellular Ca^{2+} pools by Ca^{2+} entry. Exposure of *T. cruzi* trypomastigotes (or amastigotes; results not shown) to 20 mM NH_4Cl resulted in a rapid increase in pH_i (Figure 8a). In the continued presence of NH_4Cl , pH_i gradually

Figure 6 Effect of bafilomycin A₁, nigericin and FCCP on [Ca²⁺]_i in amastigotes

Cells were loaded with fura 2/AM as described in the Materials and methods section and suspended in buffer containing 116 mM NaCl, 0.8 mM MgSO₄, 5.5 mM glucose, 50 mM Hepes, pH 7.2, and 1 mM CaCl₂, or 1 mM EGTA where indicated. Bafilomycin A₁ (BAF; 5 μ M), nigericin (NIG; 1 μ M) or FCCP (1 μ M) was added where indicated. Traces are representative of three independent experiments conducted on separate cell preparations.

Figure 7 Effect of bafilomycin A₁, nigericin and FCCP on [Ca²⁺]_i in trypomastigotes

Cells were loaded with fura 2/AM as described in the Materials and methods section and suspended in buffer containing 116 mM NaCl, 0.8 mM MgSO₄, 5.5 mM glucose, 50 mM Hepes, pH 7.2, and 1 mM CaCl₂. Bafilomycin A₁ (BAF; 5 μ M), nigericin (NIG; 1 μ M) or FCCP (1 μ M) was added where indicated. Traces are representative of three independent experiments conducted on separate cell preparations.

returned to basal levels over a 7–8 min period. As illustrated in Figure 8(b), 20 mM NH₄Cl also induced a concomitant increase in [Ca²⁺]_i, which returned to basal levels during the same period. Addition of equimolar NaCl or choline chloride (20 mM) in the absence of an isotonic correction increased neither pH_i nor [Ca²⁺]_i in either *T. cruzi* trypomastigotes or amastigotes (results not shown), thus indicating that the effect was not due to changes in osmotic pressure. The effect of NH₄Cl on [Ca²⁺]_i and pH_i was dose-dependent (5, 10, 20 mM), and NH₄⁺ was concluded to be the active ion, since (NH₄)₂SO₄ produced identical results (not shown). These results indicate that the NH₄Cl-induced rise in [Ca²⁺]_i is due, at least in part, to release of Ca²⁺ from intracellular stores. The correlation, if any, between pH_i and Ca²⁺ was tested

Figure 8 Effect of NH₄Cl and ionomycin on pH_i and [Ca²⁺]_i

Trypomastigotes were loaded with either fura 2/AM (b, d) or BCECF/AM (a, c) as described in the Materials and methods section and suspended in buffer containing 116 mM NaCl, 0.8 mM MgSO₄, 5.5 mM glucose, 50 mM Hepes, at pH 7.2, and 1 mM EGTA. NH₄Cl (20 mM) or ionomycin (ION; 1 μ M) was added where indicated. Traces are representative of three independent experiments conducted on separate cell preparations.

further with the addition of weak organic acids, which readily enter the cytoplasm, but are excluded from acidic compartments. Addition of 20 mM sodium propionate to either trypomastigotes or amastigotes resulted in a 0.4 unit decrease in pH_i, but no changes in [Ca²⁺]_i were detected (results not shown). In contrast, addition of ionomycin resulted in a 0.2 unit decrease in pH_i that was accompanied by an increase in [Ca²⁺]_i, as illustrated in Figures 8(c) and 8(d). Thus a decrease in pH_i was not necessarily associated with an increase in [Ca²⁺]_i, as has been proposed to occur in *T. brucei* bloodstream forms [32].

NH₄Cl and ionomycin act synergistically to release Ca²⁺ from acidic compartments

The cellular origin of the mobilized Ca²⁺ was investigated by repeating the above experiments with sequential addition of

Figure 9 Synergistic effect of NH_4Cl and ionomycin on Ca^{2+} release from intact amastigotes

Amastigotes were loaded with fura 2/AM and suspended in the same buffer described in Figure 8. NH_4Cl (20 mM) or ionomycin (ION; $1 \mu\text{M}$) was added where indicated. Traces are representative of three independent experiments conducted on separate cell preparations.

Figure 11 Comparative size of the Ca^{2+} -containing acidic compartment in epimastigotes (a) and trypomastigotes (b)

Cells were loaded with fura 2/AM and suspended in the same buffer described in Figure 8. NH_4Cl (20 mM) or ionomycin (ION; $1 \mu\text{M}$) was added where indicated. Traces are representative of three independent experiments conducted on separate cell preparations.

Figure 10 Synergistic effect of bafilomycin A_1 or nigericin and ionomycin on Ca^{2+} release from intact amastigotes

Amastigotes were loaded with fura 2/AM and suspended in the same buffer described in Figure 8. Bafilomycin (BAF; $5 \mu\text{M}$), nigericin (NIG; $1 \mu\text{M}$) or ionomycin (ION; $1 \mu\text{M}$) was added where indicated. Traces are representative of three independent experiments conducted on separate cell preparations.

NH_4Cl and ionomycin. Adding ionomycin ($0.26 \mu\text{M}$) to *T. cruzi* amastigotes (Figure 9a) previously exposed to NH_4Cl (20 mM) caused a second, higher, rise in $[\text{Ca}^{2+}]_i$ without any concomitant

change, or with only a small decrease, in the pH_i (results not shown). If the order of additions was reversed (Figure 9b), ionomycin caused a transient increase in $[\text{Ca}^{2+}]_i$, accompanied by a small decrease in pH_i , as indicated above (Figures 8c and 8d). Addition of NH_4Cl , which elevated pH_i (results not shown; see Figure 8b), caused a secondary increase in $[\text{Ca}^{2+}]_i$ which was significantly higher than in the absence of ionomycin (Figures 8b and 9a). The results suggest that the Ca^{2+} mobilized by ionomycin in the absence of NH_4Cl comes from different internal pool(s) from that released by NH_4Cl with or without ionomycin. This is because ionomycin binds essentially no Ca^{2+} below pH 7.0, and it cannot carry Ca^{2+} out of acidic compartments because of competition from protons at the inside face of the membrane [33]. In the absence of NH_4Cl , it releases a relatively small amount of Ca^{2+} only from neutral or alkaline compartments, but releases much more Ca^{2+} after NH_4Cl has elevated the pH of acidic compartments. These results are compatible with the pH of the most important Ca^{2+} -containing intracellular compartment of different stages of *T. cruzi* (Figure 9, and see below) being acidic.

Effects of nigericin and bafilomycin A_1 on $[\text{Ca}^{2+}]_i$

To demonstrate further the presence of an intracellular Ca^{2+} pool present in an acidic compartment of *T. cruzi*, we added ionomycin to fura 2-loaded amastigotes incubated in the presence of bafilomycin A_1 (Figure 10a) or nigericin (Figure 10c) in a Ca^{2+} -free medium (with addition of 1 mM EGTA). In all cases there was a significant increase in $[\text{Ca}^{2+}]_i$, and similar results were observed when the order of additions was reversed (Figures 10b and 10d). Similar results (not shown) were observed with fura 2-loaded epimastigotes and trypomastigotes. Taken together, these results indicate the presence of an acidic compartment, sensitive to H^+/K^+ exchangers (nigericin), and inhibitors of the vacuolar H^+ -ATPase (bafilomycin A_1), that contains a significant amount of Ca^{2+} .

Comparative size of the Ca^{2+} -containing acidic compartment (acidocalcisome) in different stages of *T. cruzi*

To compare the amounts of Ca^{2+} stored in the acidic compartment of different stages of *T. cruzi*, we also added ionomycin and NH_4Cl to fura 2-loaded epimastigotes (Figure 11a) and trypomastigotes (Figure 11b). The results indicate that most of the releasable Ca^{2+} present in different stages of *T. cruzi* (amastigotes, Figures 9 and 10; trypomastigotes and epimastigotes, Figure 11) is located in an acidic compartment. It is noteworthy that epimastigotes (Figure 11a) have a higher basal $[\text{Ca}^{2+}]_i$ (about 50 nM) than trypomastigotes (Figure 11b) or amastigotes (Figure 9) (about 20 nM), but these values increased in the same proportion (about 9-fold) after addition of ionomycin and NH_4Cl to the different stages.

DISCUSSION

The presence of an acidic Ca^{2+} -storage vacuole in *T. cruzi* (acidocalcisome) is suggested by various lines of evidence. First, using permeabilized cells, we have demonstrated the release of Ca^{2+} by the K^+/H^+ ionophore nigericin (Figure 1) and the inhibition of its uptake by nigericin or by the vacuolar H^+ -ATPase inhibitor bafilomycin A_1 (Figure 2). Also using permeabilized cells, we have shown that Ca^{2+} inhibits acidification of intracellular vesicles (as measured by Acridine Orange accumulation; Figures 3 and 5) and that Acridine Orange is taken up by round vacuoles present in the anterior and posterior ends of epimastigotes (results not shown). The distribution of these vacuoles in epimastigotes (Figure 4) is consistent with the distribution of vacuoles containing large accumulations of Ca^{2+} as detected by dispersive X-ray elemental mapping [15]. In intact cells assayed with fura 2, nigericin and bafilomycin A_1 promoted Ca^{2+} release from intracellular compartments, as did the proton ionophore FCCP (Figures 6 and 7). NH_4Cl , a weak base which elevates organellar pH, also released Ca^{2+} (Figure 8), but was much more effective when combined with ionomycin (Figures 9 and 11), a Ca^{2+} ionophore. Ionomycin alone, however, could not mobilize much Ca^{2+} , as it does not bind Ca^{2+} at acidic pH [34], but required the presence of NH_4Cl (Figures 9 and 11) or other agents (bafilomycin A_1 or nigericin; Figure 10) to elevate the pH of the acidic compartment before it could transport the Ca^{2+} out of such organelles. Most of the stored free Ca^{2+} in this organism is stored in an acidic compartment (Figures 9–11).

There are several reports of acidic compartments in trypanosomatids [34–39]. In the bloodstream forms of *T. brucei*, specific receptors mediate the endocytosis of host low-density lipoproteins (LDL) that are subsequently degraded in acidic vacuoles by thiol-proteases [34,35]. LDL receptors are apparently also present in procyclic trypomastigotes [35]. The weak base chloroquine accumulates in acidic vacuoles of these parasites, decreases the growth rate of procyclic trypomastigotes *in vitro*, and slows down the increase in parasitaemia, prolonging the survival time of infected mice [35]. An intracellular acidic compartment has also been implicated in the lysis of *T. brucei* by normal human serum [36,37]. Acidic vacuoles have been postulated to rupture upon accumulation of a minor subclass of human serum high-density lipoproteins (HDL), called trypanosome lytic factor [37]. A role for Ca^{2+} release from these vacuoles in the toxicity of HDL has not been considered. These findings indicate the potential chemotherapeutic importance of these organelles. A pre-lysosomal vacuole has also been identified in *T. cruzi* and was designated as 'reservosome' [38,39]. This is also acidic and rich in cysteine protease, and does not contain acid phosphatase or other lysosomal membrane proteins [39].

Acidic vacuoles containing Ca^{2+} have been described only in *D. discoideum* [12], and *T. brucei* [10], although vanadate-sensitive Ca^{2+} -uptake systems associated with acidic compartments have been found in rat parotid [13] and pancreatic [14] acinar cells. In *D. discoideum* some acidic vacuoles contain a high-affinity vanadate-sensitive Ca^{2+} uptake activity that is stimulated by the pH gradient formed by the H^+ -ATPase [12]. It has been suggested [40] that these Ca^{2+} -sequestering acidic vacuoles could be fragmented contractile vacuole membranes. In this regard, the presence of a contractile vacuole apparatus in trypanosomatids [41], including *T. cruzi* [42], has been described, and a role of these vacuoles in osmoregulation cannot be ruled out.

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