Ca²⁺/H⁺ exchange in acidic vacuoles of *Trypanosoma brucei*

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The use of digitonin to permeabilize the plasma membrane of *Trypanosoma brucei* procyclic and bloodstream trypomastigotes allowed the identification of a non-mitochondrial nigericin-sensitive Ca²⁺ compartment. The proton ionophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was able to cause Ca²⁺ release from this compartment, which was also sensitive to sodium orthovanadate. Preincubation of the cells with the vacuolar H⁺-ATPase inhibitor bafilomycin A₁ greatly reduced the nigericin-sensitive Ca²⁺ compartment. Bafilomycin A₁ inhibited the initial rate of ATP-dependent non-mitochondrial Ca²⁺ uptake and stimulated the initial rate of nigericin-induced Ca²⁺ release by permeabilized procyclic trypomastigotes. ATP-dependent and bafilomycin A₁- and 7-chloro-4-nitrobenz-2-

oxa-1,3-diazole (NBD-Cl)-sensitive Acridine Orange uptake was demonstrated in permeabilized cells. Under these conditions Acridine Orange was concentrated in abundant cytoplasmic round vacuoles by a process inhibited by bafilomycin A₁, NBD-Cl, nigericin, and Ca²⁺. Vanadate or EGTA significantly increased Acridine Orange uptake, while Ca²⁺ released Acridine Orange from these preparations, thus suggesting that the dye and Ca²⁺ were being accumulated in the same acidic vacuole. Acridine Orange uptake was reversed by nigericin, bafilomycin A₁ and NH₄Cl. The results are consistent with the presence of a Ca²⁺/H⁺-ATPase system pumping Ca²⁺ into an acidic vacuole, that we tentatively named the acidocalcisome.

INTRODUCTION

There is compelling evidence that calcium ions play a crucial role in controlling many biological processes [1]. Intracellular Ca²⁺ compartmentation is governed by several transport systems operating in a highly regulated fashion [1]. Cells have evolved a variety of molecular devices, such as channels, pumps and transporters, for regulating influx and efflux of Ca²⁺ across the plasma membrane and between intracellular stores to adjust its concentration in the cytoplasm [1].

Two intracellular Ca²⁺-transport systems have been described in different trypanosomatids permeabilized with digitonin [2–10]. One transport system has characteristics typical of mitochondria in other eukaryotic cells [11] such as (i) inhibition by antimycin A, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and Ruthenium Red, (ii) stimulation by respiratory substrates, phosphate and acetate, (iii) a high capacity and low affinity for Ca²⁺, and (iv) the ability to buffer external Ca²⁺ at concentrations in the range of 0.6–0.7 μM [2–10]. The second transport system has characteristics typical of the endoplasmic reticulum of other eukaryotic cells [1] such as inhibition by sodium orthovanadate and high concentrations of anti-calmodulin agents, stimulation by ATP, a low capacity and high affinity for Ca²⁺ and the ability to buffer external Ca²⁺ at concentrations in the range of 0.05–0.1 μM [2–10]. Recently, the presence of a third Ca²⁺-transport system sensitive to changes in intracellular pH has been suggested in *Trypanosoma brucei* bloodstream trypomastigotes [12] on the basis of the changes observed in the fluorescence of Fura 2-loaded cells when high concentrations of the K⁺/H⁺ exchanger nigericin (2.75 μM) were included in the incubation medium. Since nigericin is known to uncouple oxidative phosphorylation in rat liver mitochondria when used at high concentrations (1–10 μM) [13–15], in a

previous study [16] we attributed that effect of nigericin [12] to its uncoupling action. Although we could not detect any increase in intracellular Ca²⁺ attributable to the release of Ca²⁺ from intracellular stores upon addition of moderate to high concentrations of nigericin (1–2.75 μM) to intact bloodstream trypomastigotes loaded with Fura 2, we found that nigericin was able to cause the mitochondrial membrane potential to collapse and release Ca²⁺ from the mitochondria of these cells permeabilized with digitonin [16]. However, nigericin released a larger amount of Ca²⁺ from digitonin-permeabilized *T. brucei* procyclic trypomastigotes than antimycin A [16], thus indicating that this compound could have also been releasing Ca²⁺ from a non-mitochondrial Ca²⁺ compartment of at least these developmental stages.

We report here that digitonin-permeabilized *T. brucei* procyclic and bloodstream trypomastigotes possess a non-mitochondrial, nigericin-sensitive Ca²⁺ compartment that is dependent on the pH gradient formed by a bafilomycin A₁-sensitive H⁺-ATPase. Ca²⁺ uptake by this acidic compartment, that we tentatively named an acidocalcisome, is apparently due to the presence of a Ca²⁺/H⁺-ATPase system.

MATERIALS AND METHODS

Culture methods

T. brucei procyclic forms (ILTar 1 or MITat 1.4 procyclics) were grown at 28 °C in medium SDM-79 [17] supplemented with haemin (7.5 mg/l) and 10% heat-inactivated fetal-calf serum. Two to three days after inoculation, cells were collected by centrifugation and washed twice in Dulbecco's PBS. *T. brucei* bloodstream forms (monomorphic strain 427 from clone MITat 1.4, otherwise known as variant 117) were isolated from infected mice or rats as described previously [18]. The final concentration

Abbreviations used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; LDL, low-density lipoprotein.

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of cells was determined using a Neubauer chamber. The protein concentration was determined by the biuret assay [19] in the presence of 0.2% deoxycholate.

Chemicals

ATP, calcium ionophore A23187, sodium orthovanadate, FCCP, succinate, arsenazo III, EGTA, nigericin, antimycin A, oligomycin, ammonium chloride, diltiazem, Ruthenium Red, and digitonin were purchased from Sigma. Bafilomycin A₁ was obtained from Dr. K. Altendorf (University of Osnabrück, Osnabrück, Germany). Acridine Orange was from Molecular Probes (Eugene, OR, U.S.A.). All other reagents were analytical grade.

Determination of Ca²⁺ movements

Variations in free Ca²⁺ concentrations were followed by measuring the changes in the absorbance spectrum of arsenazo III [20], using the SLM Aminco DW2000 spectrophotometer at the wavelength pair 675–685 nm. No free radical formation from arsenazo III occurred under the conditions used [21,22]. The concentrations of the ionic species and complexes at equilibrium were calculated by employing an iterative computer program as described before [6]. Each experiment was repeated at least three times with different cell preparations, and the Figures show representative experiments. The inhibitory effect of bafilomycin A₁ on the initial rate of Ca²⁺ uptake and its stimulatory effect on the initial rate of Ca²⁺ release (see Figure 3) is expressed as the relative activity with respect to the control taken as 100%, and given as mean ± S.E.M. Differences between means were considered to be significant if $P \leq 0.05$, determined by Student's *t* test.

Proton pump activity

Acidification of digitonin-permeabilized cells was followed by measuring the changes in the absorbance spectrum of Acridine Orange [23], using the SLM Aminco DW2000 spectrophotometer at the wavelength pair 493–530 nm. Each experiment was repeated at least three times with different cell preparations, and the Figures show representative experiments.

RESULTS

Presence of a non-mitochondrial nigericin-sensitive Ca²⁺ compartment in procyclic trypomastigotes

We have shown previously that nigericin releases a larger amount of Ca²⁺ from digitonin-permeabilized *T. brucei* procyclic trypomastigotes than antimycin A when these cells are allowed to accumulate the cation from a reaction medium containing respiratory substrates in the absence of ATP [16]. Under these experimental conditions antimycin A elimination of the inner mitochondrial membrane potential is followed by the release of all mitochondrial Ca²⁺ [7,16]. This suggests that the extra Ca²⁺ released by nigericin comes from a non-mitochondrial Ca²⁺ compartment sensitive to this K⁺/H⁺ ionophore. The experiment shown in Figure 1 was designed to investigate the origin of this non-mitochondrial endogenous Ca²⁺. Digitonin-permeabilized cells were incubated in the presence of antimycin A and oligomycin to prevent mitochondrial Ca²⁺ uptake [7], and in the presence of sodium orthovanadate and absence of ATP to prevent Ca²⁺ uptake by the endoplasmic reticulum [7]. Evidence has already been presented that mitochondria *in situ* do not

contain a significantly high amount of endogenous Ca²⁺ in *T. brucei* [7]. Accordingly, trace (a) shows a slow release of endogenous Ca²⁺ during the time of observation, which could come from the endoplasmic reticulum ([7], and see below). This was followed by a fast Ca²⁺ release upon the addition of the Ca²⁺ ionophore A23187. Trace (b) shows that the addition of nigericin after the cells were incubated for a period of 2 min caused a release of about 50% of the endogenous Ca²⁺. The remaining Ca²⁺ (probably coming from the endoplasmic reticulum [7]) was then completely released by the addition of A23187 on top of nigericin [trace (c)]. The broken line indicates that the proton ionophore FCCP was able to cause a slower release of Ca²⁺ from the nigericin-sensitive compartment. In contrast, addition of either Ruthenium Red (3 µM) or diltiazem (10 µM) did not release any significant amount of Ca²⁺ under these conditions (results not shown). The common ability of nigericin and FCCP to release this pool of endogenous Ca²⁺ strongly suggests that it is stored in an acidic compartment.

Inhibition of the H⁺-ATPase decreases the size and alters the initial rates of Ca²⁺ uptake and release by the nigericin-sensitive Ca²⁺ compartment

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 [24]. Bafilomycin A₁ is a recently described antibiotic which specifically inhibits these H⁺-ATPases [25]. In order to investigate the role of an H⁺-ATPase in the maintenance of a nigericin-sensitive Ca²⁺ pool in *T. brucei* procyclic trypomastigotes we measured the size of this Ca²⁺ pool under similar conditions to those used in the experiment shown in Figure 1 but after the intact cells were preincubated for 90 min in buffer with or without 14 µM bafilomycin A₁ (Figure 2). A comparison between traces (a) (bafilomycin A₁) and (b) (control) indicates that the nigericin-sensitive Ca²⁺ pool was significantly

Figure 1 Presence of a non-mitochondrial nigericin-sensitive Ca²⁺ compartment in permeabilized procyclic trypomastigotes

The cells (0.3 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 MgCl₂) containing 2 µg/ml oligomycin, 1 µg/ml antimycin A, 200 µM sodium orthovanadate, and 40 µM arsenazo III. Trace (a) is control without nigericin or FCCP. Trace (b) is with the addition of nigericin (NIG; 2.6 µM) where indicated. The broken line shows the addition of 2 µM FCCP and 2.6 µM nigericin where indicated. Digitonin (D, 40 µM) and 1 µM calcium ionophore (A23187) [traces (a) and (c) respectively] were added where indicated.

Figure 2 Effect of preincubation with bafilomycin A₁ on the nigericin-sensitive non-mitochondrial Ca²⁺ compartment of procyclic trypomastigotes

After preincubation of the intact cells (0.36 mg of protein/ml) for 90 min, at 30 °C, in a buffer containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM glucose, and 50 mM Hepes, pH 7.0, in the presence or absence of 14 μ M bafilomycin A₁, the cell suspension was centrifuged and the pellet was resuspended in the standard reaction medium containing 2 μ g/ml oligomycin, 1 μ g/ml antimycin A, 200 μ M sodium orthovanadate, and 40 μ M arsenazo III, as described in Figure 1. Digitonin (D; 40 μ M), nigericin (NIG; 2.6 μ M) [traces (a) and (b) respectively], or 1 μ M calcium ionophore A23187 were added where indicated.

Figure 3 Effect of bafilomycin A₁ on Ca²⁺ uptake and release from non-mitochondrial Ca²⁺ compartments of procyclic trypomastigotes

The cells (0.3 mg of protein/ml) were added to the standard reaction medium containing 2 μ g/ml oligomycin, 1 μ g/ml antimycin A, 40 μ M arsenazo III, and 0.5 mM ATP, in the presence (dashed trace) or absence (solid trace) of 10 μ M bafilomycin A₁. Digitonin (DIG, 40 μ M), vanadate [VAN, 200 μ M, trace (b)], trace (a) after steady state, and dashed line], 2 μ M nigericin [NIG, trace (a) and dashed line] or 1 μ M calcium ionophore A23187 were added where indicated.

reduced by the bafilomycin treatment, while under the control conditions this Ca²⁺ pool was increased (Figure 2) relative to the same pool before the preincubation (see Figure 1). These results indicated the involvement of an H⁺-ATPase in this mechanism of Ca²⁺ compartmentation and prompted us to study the effect of bafilomycin A₁ on the initial rates of non-mitochondrial Ca²⁺ uptake and release by digitonin-permeabilized *T. brucei* procyclics. Figure 3 shows changes in medium Ca²⁺ concentration when the cells were incubated in medium containing ATP, antimycin A and oligomycin in the absence [trace (a)] or presence

Figure 4 ATP-driven Acridine Orange accumulation by digitonin-permeabilized procyclic trypomastigotes

The cells (0.19 mg of protein/ml) were added to the standard reaction medium containing 2 μ g/ml oligomycin, 1 μ g/ml antimycin A, and 40 μ M digitonin. Acridine Orange (3.3 μ M) was added 3 min after the cells. Panel A: ATP (0.5 mM), 10 μ M bafilomycin A₁, and 1.3 μ M nigericin were added where indicated. [trace (a)] control; [trace (b)] in the presence of 400 μ M vanadate; [trace (c)] in the presence of 10 μ M bafilomycin A₁ or 20 μ M NBD-Cl, or in the absence of ATP (traces were superimposable). Panel B: 1 mM EGTA was present in the reaction medium. ATP (0.5 mM) and 20 mM NH₄Cl or 2 μ M FCCP were added where indicated.

(dashed trace) of bafilomycin A₁. Vanadate [trace (b)] totally inhibited Ca²⁺ uptake and slowly released the accumulated Ca²⁺. A significant inhibition in the initial rate of ATP-dependent Ca²⁺ uptake (control: 100 $\pm$ 20.5%; bafilomycin A₁-treated: 55 $\pm$ 2.5%; n = 3) and stimulation in the initial rate of nigericin-induced Ca²⁺ release bafilomycin A₁ (control: 100 $\pm$ 15.8%; bafilomycin A₁-treated: 215 $\pm$ 42%; n = 3) was observed (dashed trace). Although these results appear to contradict the results of Figure 2, where it was shown that in the presence of bafilomycin A₁ a smaller Ca²⁺ pool was available for nigericin-induced release, it is interesting to note that only the initial rate of nigericin-induced Ca²⁺ release was increased and not the total amount of Ca²⁺ released during this short incubation period (10 min) in the presence of bafilomycin A₁. Addition of vanadate [which itself caused a slow Ca²⁺ release; Figure 3, trace (b)] before nigericin, to prevent Ca²⁺ re-uptake by the endoplasmic reticulum during the period of nigericin-induced Ca²⁺ efflux, was required to see the effect of bafilomycin A₁ [trace (a)]. In agreement with the experiment of Figure 2, these results indicate that Ca²⁺ flux across the putative acidic compartment of *T. brucei* procyclic trypomastigotes is affected by the activity of a bafilomycin A₁-sensitive H⁺-ATPase. On the other hand, bafilomycin A₁ had no effect on mitochondrial Ca²⁺ transport as indicated by its lack of effect on succinate-dependent Ca²⁺ uptake [7] by permeabilized *T. brucei* procyclic trypomastigotes (results not shown).

ATP-dependent vacuolar acidification in permeabilized procyclic trypomastigotes is stimulated by sodium orthovanadate and EGTA and inhibited by bafilomycin A₁, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) and Ca²⁺

Acridine Orange is a tertiary amine that becomes concentrated in the acidic compartments of different cells [23]. This dye changes both its absorbance and fluorescence properties as a consequence of its accumulation in membrane vesicles and subsequent dimerization [23]. Addition of ATP to a reaction medium containing antimycin A, oligomycin and permeabilized procyclics caused a significant time-dependent decrease in the absorbance of Acridine Orange [Figure 4, panel (A), trace (a)]. This uptake was completely prevented by 10 μ M bafilomycin A₁ or by 20 μ M NBD-

Figure 5 Accumulation of Acridine Orange in acidic vacuoles in permeabilized procyclic trypanomastigotes

Cells were treated as in [Figure 4; panel A, trace (a)], without the addition of inhibitors (control; a) or after treatment with 1.3 μM nigericin for 5 min (b). Note the vacuoles swelled in (a). Cells were photographed using a Zeiss Standard fluorescence microscope (D-7082, Oberkochen, West Germany) fitted with a fluorescein filter set and a Kodak Ektachrome 64T professional film. Bar, 5 μm .

Cl, another relatively specific inhibitor of H^+ -ATPases [24], or in the absence of ATP (dashed trace). In contrast, vanadate, an inhibitor of P-type ATPases [24], caused a significant increase in the rate of Acridine Orange absorbance decrease. The addition of bafilomycin A_1 , on top of vanadate, completely reversed the decrease in Acridine Orange absorbance. The experiment depicted in (B) of Figure 4 shows the presence of 1 mM EGTA, which decreased the concentration of contaminant Ca^{2+} in the medium from about 4.5 μM to less than 1 nM, increased both the rate and extent of Acridine Orange absorbance decrease. Under all experimental conditions tested the decrease in Acridine Orange absorbance induced by ATP was reversed by nigericin, FCCP or NH_4Cl . This confirms that the decrease in Acridine Orange absorbance was caused by its accumulation into an acidic compartment. This could also be directly visualized by using a

Figure 6 Effect of calcium on ATP-driven Acridine Orange accumulation by digitonin-permeabilized procyclic trypanomastigotes

The cells (0.22 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, 20 μM EGTA and 40 μM digitonin. Acridine Orange (3.3 μM) was added 3 min after the cells. ATP (0.5 mM), 40 μM CaCl_2 , 20 mM NH_4Cl or 200 μM vanadate (VAN) were added where indicated. Trace (a): in the presence of 200 μM vanadate from the beginning of the experiment; trace (b): 200 μM vanadate was added where indicated; trace (c): no vanadate added.

fluorescence microscope. Under the conditions of Figure 4 [panel (A), trace (a)], *T. brucei* procyclic trypanomastigotes accumulated Acridine Orange in round vacuoles dispersed in large numbers throughout the cytoplasm (Figure 5a). The vacuoles were stained from bright yellow to deep orange, suggesting variation in their pH [26]; the cytoplasm was colourless. That these vacuoles were acidified by an H^+ -ATPase was suggested in Figure 5(b), which shows the disappearance of their fluorescence upon treatment of permeabilized cells with 1.3 μM nigericin. Similar results were observed after treatment with 5 μM bafilomycin A_1 (results not shown).

The sensitivity of this ATP-dependent compartment acidification to bafilomycin A_1 was studied by measuring the rates of Acridine Orange absorbance decrease in the presence of different concentrations of the inhibitor. The inhibition curve was hyperbolic (not shown); half-inhibition was observed at about 0.5 μM and complete inhibition was attained at about 3 μM bafilomycin A_1 (results not shown). The vacuolar H^+ -ATPases described in other cell types [25] have a similar high sensitivity to bafilomycin A_1 . This seems to confirm the existence of such an H^+ -ATPase in the procyclic forms of *T. brucei*.

The effect of Ca^{2+} on the ATP-dependent compartment acidification by permeabilized procyclics incubated in a reaction medium containing antimycin A, and oligomycin, was also investigated. Figure 6 shows that addition of 40 μM CaCl_2 [trace (c)] reversed the decrease in Acridine Orange absorbance by the preparation. Addition of 20 mM NH_4Cl completed this reversal, indicating release of Acridine Orange. This release was decreased if vanadate was included in the incubation medium after calcium [trace (b)] and did not occur when vanadate was included in the

Figure 7 ATP-driven Acridine Orange accumulation and non-mitochondrial nigericin-sensitive Ca²⁺ pool in digitonin-permeabilized bloodstream trypomastigotes

Panel A: the cells (2.9 mg of protein/ml) were added to the standard reaction medium containing 2 µg/ml oligomycin and 30 µM digitonin. Acridine Orange (AO, 3.3 µM) was added 5 min after the cells. ATP (0.5 mM), 10 µM bafilomycin A₁ (BAF, dashed line) or 20 mM NH₄Cl were added where indicated. Trace (a), control; trace (b), 10 µM bafilomycin A₁ was present from the beginning of the experiment. Panel B: the cells (2.9 mg of protein/ml) were added to the standard reaction medium containing 2 µg/ml oligomycin, 200 µM vanadate and 40 µM arsenazo III. Digitonin (D, 30 µM), 2.6 µM nigericin (NIG) or 1 µM calcium ionophore A23187 were added where indicated.

incubation medium from the beginning of the experiment [trace (a)].

The stimulation of Acridine Orange uptake by EGTA (Figure 4) and its release by Ca²⁺ (Figure 6) indicate that this dye and Ca²⁺ are, in fact, being accumulated in the same compartment. In addition, the stimulation of Acridine Orange uptake by vanadate (Figure 4) and the inhibition by vanadate of Acridine Orange release caused by Ca²⁺ (Figure 6) confirm that Ca²⁺ exchange for H⁺ depends on a vanadate-sensitive mechanism.

ATP-dependent vacuolar acidification in permeabilized bloodstream trypomastigotes

Figure 7 (panel A) shows that the addition of Acridine Orange to permeabilized bloodstream trypomastigotes was followed by a time-dependent decrease in absorbance of the dye [trace (a)]. This decrease in absorbance attained a plateau after 2 min and underwent an additional decrease by the addition of ATP. The inclusion of bafilomycin A₁ (dashed trace), instead of ATP, or NH₄Cl after ATP, caused a complete reversal of the Acridine Orange absorbance decrease. When Acridine Orange was added to a reaction medium containing permeabilized bloodstream trypomastigotes preincubated for 5 min in the presence of 5 µM bafilomycin A₁, the absorbance of the dye was not decreased either before or after ATP addition [trace (b)]. On the other hand, bafilomycin A₁ solvent (dimethyl sulphoxide) had no effect at the concentration used (results not shown). The uptake of Acridine Orange that precedes ATP addition in the absence of bafilomycin A₁ indicates that this acidic membrane compartment is able to maintain its pH gradient for, at least, 5 min after the onset of the plasma membrane permeabilization by digitonin.

Non-mitochondrial nigericin-sensitive Ca²⁺ pool in bloodstream trypomastigotes

The identification of an acidic vacuole prompted us to reinvestigate [16] the existence of a nigericin-sensitive Ca²⁺ pool in these

T. brucei stages using a high cell protein concentration and a protocol similar to that of Figure 1. It can be observed (Figure 7, panel B) that when these cells were permeabilized in the presence of oligomycin to prevent Ca²⁺ uptake by mitochondria [7,8] and vanadate to prevent Ca²⁺ uptake by non-mitochondrial Ca²⁺ pools, no significant Ca²⁺ release occurred before the addition of the Ca²⁺ ionophore A23187. However, the addition of nigericin was followed by the release of about 2.8 nmol of Ca²⁺/mg of protein.

DISCUSSION

In this study, we have identified and characterized a Ca²⁺-transport system in *T. brucei* that is associated with an intracellular acidic compartment. As reported previously [7,16] all the ATP-dependent non-mitochondrial Ca²⁺ uptake undertaken by these cells is completely abolished by sodium orthovanadate (Figure 3). The vanadate sensitivity of all the non-mitochondrial Ca²⁺ uptake leads us to believe that a P-type ATPase, presumably a Ca²⁺-ATPase, is responsible for the Ca²⁺ uptake associated with the acidic compartment. Although P-type ATPases are usually inhibited by lower concentrations of vanadate than we used in this work, vanadate does not pass easily through membranes and high concentrations of this inhibitor are usually required when detergent-permeabilized preparations are used [27,28]. The sensitivity to vanadate argues against the involvement of the type of Ca²⁺/H⁺ antiporter believed to exist in the vacuoles of fungi and higher plants [29,30]. The inhibition of the initial rate of Ca²⁺ uptake and the stimulation of the initial rate of nigericin-dependent Ca²⁺ release by the vacuolar H⁺-ATPase inhibitor bafilomycin A₁ (Figure 3) suggests that the inside acid pH gradient due to the vacuolar proton pump facilitates Ca²⁺ uptake and retention. An alternative explanation, such as direct inhibition of the vanadate-sensitive Ca²⁺ uptake by bafilomycin A₁, seems unlikely because although bafilomycin A₁ has been shown to inhibit the Ca²⁺-ATPase of skeletal muscle sarcoplasmic reticulum [25], it did so only at concentrations several orders of magnitude higher than those demonstrated here for inhibition of proton pump activity. Furthermore, addition of sodium orthovanadate, which inhibits Ca²⁺-ATPases, or EGTA, which decreases medium Ca²⁺ concentration (Figure 4), increased the rate of ATP-dependent vacuolar acidification in permeabilized cells, thus indicating a close relationship between Ca²⁺ uptake and vacuolar acidification. Accordingly, addition of vanadate prevents vacuolar alkalinization caused by Ca²⁺ addition (Figure 6). Similar vanadate-sensitive Ca²⁺ uptake systems associated with acidic compartments have been found in the cellular slime mould *Dictyostellium discoideum* [31], and in rat parotid [32] and pancreatic [33] acinar cells. It has been proposed [31–33] that these cells possess an acidified calcium store filled by the action of a Ca²⁺/H⁺ exchanger, which may be an ATPase.

An interesting observation was the detection of Acridine Orange uptake by permeabilized bloodstream trypomastigotes in the absence of exogenously added ATP. This indicated that this acidic membrane compartment was able to maintain its pH gradient, for at least 5 min, after the onset of the plasma membrane permeabilization by digitonin. *T. brucei* bloodstream trypomastigotes are very rich in glycosomes, microbody-like organelles that contain several glycolytic enzymes [34]. It has been calculated that they possess about 230 glycosomes per cell [34]. Glycosomes remain intact after permeabilization of trypanosomatids with low concentrations of digitonin [3,35] and they could have provided the endogenous substrates required for ATP generation during the initial period of permeabilization. Another possible explanation is the presence of ATP inside the

acidic vacuoles. In this regard, it is known that several acidic compartments, such as secretory granules [24], contain a high concentration of ATP.

The presence of multiple non-mitochondrial Ca^{2+} pools has been proposed in the case of pancreatic acinar cells [33]. Our results favour this hypothesis in *T. brucei*. For example, Figure 2 shows that preincubation of the cells with bafilomycin A_1 to inhibit the vacuolar H^+ -ATPase resulted in a decrease in the nigericin-sensitive Ca^{2+} pool without changes in the total Ca^{2+} release by the calcium ionophore A23187 (Figure 2). This indicates that during the preincubation period Ca^{2+} was possibly transferred from a bafilomycin A_1 -sensitive to a bafilomycin A_1 -insensitive store (probably the endoplasmic reticulum). Furthermore, addition of vanadate, and therefore inhibition of all vanadate-sensitive Ca^{2+} uptake mechanisms, was necessary to demonstrate a difference in the initial rate of nigericin-induced Ca^{2+} release in the presence and absence of bafilomycin A_1 (Figure 3). This again indicates the presence of a bafilomycin A_1 -insensitive, but vanadate-sensitive, Ca^{2+} pool.

Digitonin is thought to permeabilize cells by complexing sterols and altering membrane structure [36,37]. Interestingly, the acidic compartments or acidosomes of *D. discoideum* were shown to have the lowest sterol content of any other subcellular fraction tested [38]. This is also apparently true for the acidic compartment of *T. brucei*, since we could easily measure Ca^{2+} transport and acidification in this compartment using the digitonin permeabilization technique.

Acidosomes [38] are specialized organelles, so far identified only in protozoa, that appear to be responsible for acidifying the endocytic circuit by reversible interaction with incoming endosomes [31,38]. These acidosomes were observed to fuse with and acidify emergent phagosomes within 3–4 min of their formation in *Paramecium* [39]. The fusion of these acidified vacuoles with lysosomes occurred a few minutes later [40]. While the phagosome-directed acidosomes in *Paramecium* have been considered to be specialized [41], they appear to have counterparts in other cells. It has been observed that the phagosomes of *Amoeba proteus* became acidified shortly before their fusion with lysosomes [42] and it has been suggested that the plasma membrane was the source of the acidification. In macrophages, newly formed phagosomes were also shown to become acidic before their merger with lysosomes; fusion with unidentified granules was suggested as a mechanism [43]. Given all the data, it has been postulated that nascent phagosomes are generally acidified by fusion with specific organelles, i.e. the prelysosomal vacuole [44]. Similar prelysosomal vacuoles have been widely observed in mammals as well as in lower eukaryotes [45], including *T. brucei* bloodstream forms [46]. In these parasites, spherical vacuoles, not containing endocytosed markers, have been observed by electron microscopy apparently fusing with endosome-like structures [46]. However, the powers of acidification of these prelysosomal vacuoles were not investigated until recently [44].

There are several reports on acidic compartments in trypanosomatids [47–50]. 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 [47,48]. LDL receptors are apparently also present in procyclic trypomastigotes [48]. 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 thus prolonging the survival time of infected mice [48]. These findings indicate the potential chemotherapeutic importance of these acidic organelles. A prelysosomal vacuole has also been identified in *Trypanosoma cruzi* and was designated as reservosome [49,50]. This is also acidic

and rich in cysteine protease, and does not contain acid phosphatase or other lysosomal membrane proteins [50].

The possible presence of a H^+ -countertransporting Ca^{2+} -ATPase involved in Ca^{2+} sequestration that apparently resides in acidic vacuoles has only been described in *D. discoideum* [31], although vanadate-sensitive Ca^{2+} uptake systems associated with acidic compartments have been found in rat parotid [32] and pancreatic [33] acinar cells, and the existence of a lysosomal $\text{Ca}^{2+}/\text{H}^+$ -ATPase pump had been suggested in *T. brucei* bloodstream forms [12]. In this regard, X-ray microanalysis of *T. brucei* vacuoles has already demonstrated that the electron-dense component of the vacuoles, named polyphosphate vacuoles, has a high phosphorus content and appreciable Ca^{2+} content [51]. Their relation with the acidic vacuoles described in this work, and which we tentatively named acidocalcisomes, is presently being investigated.

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