Ca²⁺ storage in *Trypanosoma brucei*: the influence of cytoplasmic pH and importance of vacuolar acidity

David A. SCOTT, Silvia N. J. MORENO and Roberto DOCAMPO*

Department of Veterinary Pathobiology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, U.S.A.

The hypothesis that changes in cytosolic pH effect the release from intracellular compartments of stored calcium in *Trypanosoma brucei* was addressed by the use of procyclic and bloodstream trypomastigotes of *T. brucei* loaded with the fluorescent reagents 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF) to measure intracellular pH (pH_i), or fura 2 to measure intracellular free calcium ([Ca²⁺]_i). Experiments were performed in EGTA-containing buffers, so increases in [Ca²⁺]_i reflected release of stored calcium rather than Ca²⁺ entry. Nigericin reduced pH_i and increased [Ca²⁺]_i in loaded cells, whilst propionate reduced pH_i, but did not affect [Ca²⁺]_i, and NH₄Cl increased both variables, so there appears to be no correlation between pH_i and [Ca²⁺]_i. Treatment of the cells with

the calcium ionophore ionomycin under similar conditions (nominal absence of extracellular Ca²⁺) resulted in an increase of [Ca²⁺]_i which was greatly increased by addition of either NH₄Cl, nigericin or the vacuolar H⁺-ATPase inhibitor bafilomycin A₁. Similar results were obtained when the order of additions was reversed or when digitonin-permeabilized cells were used with the Ca²⁺ indicator arsenazo III. The results suggest that more Ca²⁺ is stored in this acidic compartment in procyclic than in bloodstream forms. Taking into account the relative importance of the ionomycin-releasable and the ionomycin-plus-NH₄Cl-releasable Ca²⁺ pools, it is apparent that a significant amount of the Ca²⁺ stored in *T. brucei* trypomastigotes is present in the acidic compartment thus identified.

INTRODUCTION

Various findings suggest the potential chemotherapeutic importance of acidic compartments in parasitic protozoa of the genus *Trypanosoma*. The weak base chloroquine accumulates in the acidic vacuoles of *T. brucei*, decreases the growth rate of procyclic trypomastigotes *in vitro* and slows down the increase in parasitaemia, prolonging the survival time of infected mice [1]. An intracellular acidic compartment has also been implicated in the lysis of *T. brucei* by normal human serum [2,3]. Acidic vacuoles have been postulated to rupture upon accumulation of a minor subclass of human serum high-density lipoproteins called 'trypanosome lytic factor' [3]. Also 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 proteinases [1,4]. LDL receptors are apparently also present in procyclic trypomastigotes [1]. Transferrin is similarly taken up by receptor-mediated endocytosis [5–8] and subsequently degraded in lysosomes [9]. Epimastigote forms of *T. cruzi* also take up LDL and transferrin, which are transported to a somewhat acidic proteinase-containing compartment called the 'reservosome' [10,11].

It was recently reported 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 [12]. Ca²⁺ uptake by this acidic compartment, which was named the 'acidocalcisome', is apparently due to the presence of a Ca²⁺/H⁺-ATPase [12]. The presence of a H⁺-countertransporting Ca²⁺-ATPase involved in Ca²⁺ sequestration that resides in acidic vacuoles has also been described in

Dictyostelium discoideum [13], and a Ca²⁺-ATPase has been localized to the yeast vacuole membrane [14]. In mammalian cells there are isolated reports of calcium storage in acidic compartments [15–17], though this is disputed by others [18].

We report here experiments using intact fura 2-loaded cells or digitonin-permeabilized cells that demonstrate that a considerable portion of the releasable Ca²⁺ in both *T. brucei* procyclic and bloodstream trypomastigotes is located in acidocalcisomes, and that the release of stored Ca²⁺ in the cell is not dependent upon the intracellular pH, as has previously been asserted [19].

MATERIALS AND METHODS

Culture methods

T. brucei procyclic forms (ILTar 1 procyclics) were grown at 28 °C in medium SDM-79 [20] supplemented with haemin (7.5 mg/litre) and 10 % heat-inactivated foetal-calf serum. At 2 or 3 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 [21]. The final concentration of cells was determined using a Neubauer chamber. The protein concentration was determined by the biuret assay [22] in the presence of 0.2 % deoxycholate.

Chemicals

ATP, sodium propionate, arsenazo III, EGTA, antimycin A, oligomycin, NH₄Cl, nigericin, ionomycin, and digitonin were

Abbreviations used: BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (fura 2 is defined in the text); /AM, acetoxymethyl ester; LDL, low-density lipoprotein; [Ca²⁺]_i, intracellular free calcium; pH_i, intracellular pH.

* To whom correspondence should be addressed.

purchased from Sigma. 1-[2-(5-Carboxyoxazol-2-yl)-6-amino-benzofuran-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, Eugene, OR, U.S.A. Bafilomycin A₁ was obtained from Dr. K. Altendorf (University of Osnabrück, Osnabrück, Germany).

Spectrofluorometric determinations

Fura 2 determinations were performed essentially as described previously [23,24]. After harvesting, cells 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 MgSO₄, 5.5 mM D-glucose and 50 mM Hepes at pH 7.4 [23]. Cells were resuspended to a final density of 1×10^9 /ml in loading buffer, which contained 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM glucose, 1.5% sucrose, 50 mM Hepes at pH 7.4, and 6 μ M fura 2/AM or 9 μ M BCECF/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 (procyclics) or resuspension buffer [21] (bloodstream trypomastigotes) to remove extracellular dye. Cells were resuspended to a final density of 1×10^9 cells/ml in buffer A (procyclics) or 1×10^8 cells/ml in resuspension buffer (bloodstream trypomastigotes) [21] and were kept in ice [23,24]. For fluorescence measurements, 5×10^7 cells/ml (final density) were suspended in a cuvette containing buffer A placed in a thermostated (30 °C, for procyclics, 37 °C for bloodstream trypomastigotes) Hitachi F-2000 spectrofluorimeter. Excitation was at 340 and 380 nm and emission was measured at 510 nm. The fura 2 fluorescence response to the intracellular calcium concentration ($[Ca^{2+}]_i$) 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 Grynkiewicz et al. [25]. $[Ca^{2+}]_i$ was calculated by titration with different concentrations of Ca-EGTA buffers [23, 24]. Concentrations of the ionic species and complexes at equilibrium were calculated by employing an iterative computer program as described previously [23,24]. For intracellular-pH measurements, 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 [26]. Other experimental conditions and calibrations were as described previously [26]. Representative traces from experiments conducted on three separate cell preparations are shown in the Figures.

Ca²⁺ uptake and release by permeabilized cells.

For experiments using permeabilized cells, variations in free (external) Ca²⁺ concentrations were monitored by measuring the changes in the absorbance spectrum of arsenazo III [27], using a SLM Aminco DW2000 spectrophotometer at the wavelength pair 675–685 nm. No free-radical formation from arsenazo III occurred under the conditions used [28,29]. Each experiment was repeated at least twice with different cell preparations, and the Figures show representative experiments. Experimental mixtures were maintained at a temperature of 37 °C (bloodstream forms) or 30 °C (procyclic forms). The standard assay buffer used was as described previously [12], and routinely included 2 mg/ml oligomycin, 1 mg/ml antimycin A (procyclic forms only) and 50 μ M EGTA to deplete mitochondria of Ca²⁺ and prevent any re-uptake [12]. Cells were permeabilized 3 min prior to each experimental run by the addition of 8 μ M or 20 μ M digitonin for bloodstream and procyclic forms, respectively. CaCl₂ (70 μ M)

was added 1 min prior to runs to give an initial free $[Ca^{2+}]$ of 9 μ M [23].

RESULTS

Effect of altering the cytosolic pH on cytosolic Ca²⁺ concentration

Changes in cytosolic pH (pH_i) have been postulated to regulate $[Ca^{2+}]_i$ in *T. brucei* bloodstream trypomastigotes. Acidification of the cytosol was proposed as the reason for $[Ca^{2+}]_i$ increase after addition of the ionophore nigericin, a K⁺/H⁺ exchanger, to these cells [19]. Here, we have investigated further if there is any connection between pH_i and $[Ca^{2+}]_i$ in bloodstream and procyclic trypomastigotes of *T. brucei*. The effect of reagents on pH_i was monitored using BCECF-loaded cells. Experiments were performed in the effective absence of extracellular Ca²⁺ (in the presence of 1 mM EGTA) in order to prevent refilling of intracellular Ca²⁺ pools by Ca²⁺ entry, and in the nominal absence of bicarbonate. With an extracellular pH (pH_e) of 7.4, the mean baseline pH_i of *T. brucei* procyclic and bloodstream trypomastigotes was 7.05 ± 0.08 ($n = 4$) and 7.40 ± 0.06 ($n = 3$) respectively. Addition of nigericin caused a significant decrease in pH_i in procyclic (Figure 1b, trace b) and bloodstream (Figure 1d, trace b) forms and an increase in $[Ca^{2+}]_i$ in both forms (Figures 1a and 1c, traces b), in agreement with previous results [19]. However, pH_i recovery from nigericin-induced acidification was faster (Figure 1d, trace b) than $[Ca^{2+}]_i$ recovery to steady-state levels (Figure 1c, trace b) in bloodstream trypomastigotes. The possible correlation between cytosolic acidification and $[Ca^{2+}]_i$ was tested further with a weak organic acid that readily enters the cytoplasm but is excluded from acidic compartments. Addition of 20 mM sodium propionate to either procyclic or bloodstream trypomastigotes resulted in a 0.2–0.4 unit decrease in pH (Figures 1b and 1d, traces c), but no changes in $[Ca^{2+}]_i$ were detected (Figures 1a and 1c, traces c). Recovery from propionate-induced acidification was faster with bloodstream than with procyclic trypomastigotes. A different effect was observed with exposure of *T. brucei* procyclic or bloodstream trypomastigotes to 20 mM NH₄Cl. This caused a rapid increase in pH_i (Figures 1b and 1d, traces a). In the continued presence of NH₄Cl, pH_i gradually returned to basal levels over a 2–3 min period, the recovery being faster with bloodstream forms (Figure 1d, trace a). As shown in Figures 1(a) and 1(c), 20 mM NH₄Cl also induced a concomitant increase in $[Ca^{2+}]_i$ in both forms of the parasite. Addition of equimolar NaCl or choline chloride (20 mM) in the absence of an iso-osmotic correction neither increased pH_i nor increased $[Ca^{2+}]_i$ in either procyclic or bloodstream trypomastigotes (results not shown), thus indicating that the effect was not due to changes in osmotic pressure. The effect of NH₄Cl on $[Ca^{2+}]_i$ and pH_i was dose (5, 10 or 20 mM)-dependent, and NH₄⁺ was concluded to be the active ion, since (NH₄)₂SO₄ produced identical results (results not shown). These results indicate that the NH₄Cl-induced rise in $[Ca^{2+}]_i$ is probably due to release of Ca²⁺ from intracellular stores. Therefore, a decrease in cytosolic pH was not associated with an increase in $[Ca^{2+}]_i$ as had been proposed in bloodstream forms [19].

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 NH₄Cl and the Ca²⁺ ionophore ionomycin. Adding ionomycin (1 μ M) to *T. brucei* procyclics previously exposed to NH₄Cl (20 mM) caused a second, higher, rise in $[Ca^{2+}]_i$ (Figure 2a, trace a) without any coincident change or with only a small decrease

Figure 1 Effect of NH_4Cl , nigericin and sodium propionate on intracellular pH and Ca^{2+} concentration

Procyclic (a and b) and bloodstream (c and d) trypomastigotes were loaded with either fura 2/AM (a and c) or BCECF/AM (b and d) as described in the Materials and methods section and suspended in buffer containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO_4 , 5.5 mM glucose, 50 mM Hepes, at pH 7.4, and 1 mM EGTA. NH_4Cl (20 mM, traces a), nigericin (NIG, 1 μM , traces b) or sodium propionate (PRO, 20 mM, traces c) were added where indicated.

Figure 2 Synergistic effect of NH_4Cl and ionomycin on Ca^{2+} release from intact procyclic and bloodstream trypomastigotes

Procyclic (a) and bloodstream (b) trypomastigotes were loaded with fura 2/AM and suspended in the same buffer described in Figure 1. NH_4Cl (20 mM) or ionomycin (ION, 1 μM) were added where indicated (traces a, NH_4Cl added first, then ionomycin; traces b, order of additions reversed).

Figure 3 Combined effect of bafilomycin A_1 or nigericin and ionomycin on Ca^{2+} release from intact procyclic and bloodstream trypanomastigotes

Procyclic (a and b) and bloodstream (c and d) trypanomastigotes were loaded with fura 2/AM and suspended in the same buffer described in Figure 1. Bafilomycin (BAF, 5 μ M), nigericin (NIG, 1 μ M) or ionomycin (ION, 1 μ M) were added where indicated (in a and b: traces a, ionomycin added first, then nigericin or bafilomycin; traces b, order of additions reversed. In (c) and (d): traces a, nigericin or bafilomycin added first, then ionomycin; traces b, order of additions reversed).

in the pH_i (results not shown). If the order of additions was reversed (Figure 2a, trace b), ionomycin caused a small increase in $[Ca^{2+}]_i$ accompanied by a small decrease in pH_i (results not shown). Addition of NH_4Cl , which elevated pH_i (results not shown; see Figure 1b), caused a secondary increase in $[Ca^{2+}]_i$ which was significantly higher than in the absence of ionomycin. Ionomycin or NH_4Cl alone did not affect $[Ca^{2+}]_i$ markedly in bloodstream trypanomastigotes, and the simultaneous presence of both compounds was necessary to detect much of an increase in $[Ca^{2+}]_i$ (Figure 2b). The Ca^{2+} mobilized by ionomycin in the absence of NH_4Cl probably comes from internal pool(s) different 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 move Ca^{2+} out of acidic compartments because of competition from protons at the inside face of the membrane [30]. In the absence of NH_4Cl , it releases a relatively small amount of Ca^{2+} only from neutral or alkaline compartments, but much more Ca^{2+} is released after addition of NH_4Cl . This reagent crosses membrane barriers as NH_3 , and combines with protons in acidic compartments, thereby raising the pH [31]. NH_4Cl has been shown to alkalinize acidic vesicles in *T. brucei* by measurement of Acridine Orange absorbance [12].

Effects of nigericin and bafilomycin A_1 on $[Ca^{2+}]_i$ in the presence of ionomycin

To show that the above combined effects of ionomycin and NH_4Cl were not dependent on NH_4^+ , these experiments were repeated with addition of ionomycin to fura 2-loaded procyclics incubated in the presence of nigericin (Figure 3a, trace b) or bafilomycin A_1 (Figure 3b, trace b) in a Ca^{2+} -free medium (with addition of 1 mM EGTA). Nigericin and the vacuolar H^+ -ATPase inhibitor bafilomycin A_1 [32] were also previously shown to diminish vesicular acidity in *T. brucei* [12]. In all cases there was a significant increase in $[Ca^{2+}]_i$, and similar results were observed when the order of additions was reversed (traces a). In bloodstream trypanomastigotes, results were qualitatively identical, but quantitatively less (Figures 3c and 3d).

Permeabilized-cell experiments

Confirmation of the storage of Ca^{2+} in an acidic compartment was obtained by use of the combination of ionomycin and the above pH-gradient-disrupting reagents in permeabilized cell experiments. Addition of ATP to cells rendered permeable with

Figure 4 Effects of ionomycin with nigericin or bafilomycin A₁ on Ca²⁺ uptake and release in permeabilized trypanastigotes

Procyclic (a, 1.0 mg of protein/ml; b, 1.2 mg of protein/ml) or bloodstream (2.1 mg of protein/ml, c and d) trypanastigotes were permeabilized with digitonin as described in the Materials and methods section. ATP (1 mM) was added as indicated, followed variously by ionomycin (ION, 4 μ M), nigericin (NIG, 5 μ M) and bafilomycin A₁ (BAF, 2 μ M). Lines represent actual spectrophotometer traces (4675–685), except that lines subsequent to the addition of ionomycin (as the calcium salt) have been adjusted down to discount the increase in absorbance due to Ca²⁺ added with the ionomycin. The dotted line in (b) is included to indicate the linear course of Ca²⁺ release in the absence of bafilomycin A₁.

digitonin led to the uptake of Ca²⁺, recorded as a decrease in the absorbance at 675–685 nm of the dye arsenazo III (Figure 4). In procyclic forms, ionomycin effected a slow release of Ca²⁺, which was accelerated by the addition of nigericin (Figure 4a) or bafilomycin A₁ (Figure 4a). Treatment with either of these reagents prior to ionomycin did not affect the Ca²⁺-uptake rate, but once ionomycin was added, Ca²⁺ was rapidly released, at the same cumulative rate as when reagents were added in reverse order (Figures 4a and 4b). NH₄Cl was not used, since it interferes with arsenazo III measurements (R. Docampo, unpublished work). Note that the amount of Ca²⁺ released by the ionophores was greater than that taken up following ATP addition. The endogenous Ca²⁺ was demonstrated previously, by the use of nigericin alone, to be stored in an acidic environment, [12]. In bloodstream forms the presence of Ca²⁺ storage in an acidic compartment was not always evident. In some experiments, synergistic release by ionomycin and nigericin was observed, as

for procyclics (Figure 4c). More generally, ionomycin alone induced a rapid release of stored Ca²⁺, and the release rate was not enhanced by the addition of nigericin (Figure 4d). Overall, though, these results are compatible with the pH of the most important Ca²⁺-containing intracellular compartment in different stages of *T. brucei* being acidic.

DISCUSSION

In the present paper we have shown, first, that the pH of the cytosol is not a factor in determining release of stored intracellular Ca²⁺ in *T. brucei* (Figure 1). It was previously suggested that decreases in cytoplasmic pH were responsible for Ca²⁺ release in bloodstream *T. brucei* trypanastigotes [19]. This conclusion was drawn on the basis of results (i) using the proton ionophores nigericin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, which, as in the present study, reduced pH_i whilst releasing Ca²⁺; (ii) treatment of cells with NH₄Cl, which was claimed to initially reduce [Ca²⁺]_i (as pH_i increased) and then increase [Ca²⁺]_i whilst pH_i declined until it was below initial values. The illustrated effects in the latter instance are slight, though, and use of propionate in the present work shows that cytosolic acidification is not a requirement for release of stored Ca²⁺.

Our data show, secondly, that most Ca²⁺ within these cells is located in an acidic compartment (Figures 2–4). These latter results extend the previous findings in permeabilized-cell experiments [12] that nigericin (alone) could release a proportion of stored Ca²⁺ and that bafilomycin A₁ inhibited ATP-driven Ca²⁺ uptake but stimulated nigericin-induced Ca²⁺ release. Use of the Ca²⁺ ionophore ionomycin here, in combination with these reagents, allowed much more Ca²⁺ to be released, whilst in most instances ionomycin alone was relatively ineffective, which strongly suggests that the principal Ca²⁺-storage compartment in *T. brucei* is acidic. The exception was in some experiments with digitonin-permeabilized bloodstream forms (Figure 4d), where Ca²⁺ was rapidly released by ionomycin and nigericin did not accelerate this release. This may have been due to digitonin rendering acidic compartments leaky to protons, and thereby allowing them to equilibrate to the assay pH (7.2) before the addition of ionomycin.

The mechanism by which Ca²⁺ is released from the acidic compartment following nigericin, NH₄Cl or bafilomycin A₁ treatment is unclear. It is apparently not due to Ca²⁺/H⁺-ATPase activity, as nigericin effects release from permeabilized cells in the absence of ATP and the presence of the ATPase inhibitor *o*-vanadate [12]. Instead, a rise in intravacuolar pH caused by these reagents may activate a Ca²⁺ channel in the vacuolar membrane, leading to Ca²⁺ efflux. This correlates with the generally faster action of nigericin and NH₄Cl compared with bafilomycin A₁ (Figures 2–4), as the former reagents act directly on vacuolar pH, whereas the latter only raises pH via proton-pump inhibition.

The nature of the acidic Ca²⁺-storage compartment remains unknown. A uniquely trypanosomatid organelle, which appears to be acidic on the basis of chloroquine-induced swelling [1], is the polyphosphate granule. X-ray microanalysis of this organelle suggested it contained calcium as well as phosphorus [33].

Other possible candidates include the 'classic' acidic vacuole, the lysosome, as well as endosomes and the *trans*-Golgi, which have been shown to be acidic in other systems [34]. The possible role of endosomes/lysosomes in Ca²⁺ storage could, in many cell types, be investigated by loading cells with fluorescein-labelled dextran. These conjugates are pH-sensitive [35], and the effects of Ca²⁺ uptake and release on pH could be quantified, rather than just being shown qualitatively, as was previously achieved with Acridine Orange [12]. Unfortunately, in *T. brucei*, procyclic

trypomastigotes do not readily take up fluid-phase endocytic markers [36]. Bloodstream forms will endocytose material more readily [36], but, as shown here, acidic Ca^{2+} storage is not always observable in permeabilized bloodstream forms. Permeabilization is necessary for detection of the effects of varied $[\text{Ca}^{2+}]$ on vacuolar acidity and to show the requirement for ATP, as previously demonstrated using Acridine Orange [12].

Another possible site for acidic Ca^{2+} storage is in the contractile vacuole or associated membranes. The 'acidosomes' of the cellular slime mould *D. discoideum*, initially identified as a low-density vesicular fraction containing vacuolar H^+ -ATPase activity [37] and which also have a $\text{Ca}^{2+}/\text{H}^+$ -ATPase [13], are now thought to represent the contractile-vacuole-associated reticular network, the 'spongione' [38,39]. A similar membrane system is present in trypanosomatids [40].

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