# A calmodulin-stimulated $Ca^{2+}$ pump in plasma-membrane vesicles from *Trypanosoma brucei*; selective inhibition by pentamidine

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Despite previous reports [McLaughlin (1985) Mol. Biochem. Parasitol. **15**, 189–201; Ghosh, Ray, Sarkar and Bhaduri (1990) J. Biol. Chem. **265**, 11345–11351; Mazumder, Mukherjee, Ghosh, Ray and Bhaduri (1992) J. Biol. Chem. **267**, 18440–18446] that the plasma membrane of different trypanosomatids only contains $Ca^{2+}$-ATPase that does not show any demonstrable dependence on $Mg^{2+}$, a high-affinity ($Ca^{2+}$–$Mg^{2+}$)-ATPase was demonstrated in the plasma membrane of *Trypanosoma brucei*. The enzyme became saturated with micromolar amounts of $Ca^{2+}$, reaching a $V_{max.}$ of $3.45 \pm 0.66$ nmol of ATP/min per mg of protein. The $K_{m,app.}$ for $Ca^{2+}$ was $0.52 \pm 0.03$ $\mu$M. This was decreased to $0.23 \pm 0.05$ $\mu$M, and the $V_{max.}$ was increased to $6.36 \pm 0.22$ nmol of ATP/min per mg of protein (about 85%), when calmodulin was present. *T. brucei* plasma-membrane vesicles accumulated $Ca^{2+}$ on addition of ATP only when $Mg^{2+}$ was present, and released it on addition of the $Ca^{2+}$ ionophore A23187. In addition, this $Ca^{2+}$ transport was stimulated by calmodulin. Addition of NaCl to $Ca^{2+}$-loaded *T. brucei* plasma-membrane vesicles did not result in $Ca^{2+}$ release, thus suggesting the absence of a $Na^+/Ca^{2+}$ exchanger in these parasites. Therefore the ($Ca^{2+}$–$Mg^{2+}$)-ATPase would be the only mechanism so far described that is responsible for the long-term fine tuning of the intracellular $Ca^{2+}$ concentration of these parasites. The trypanocidal drug pentamidine inhibited the *T. brucei* plasma-membrane ($Ca^{2+}$–$Mg^{2+}$)-ATPase and $Ca^{2+}$ transport at concentrations that had no effect on the $Ca^{2+}$-ATPase activity of human or pig erythrocytes. In this latter case, pentamidine behaved as a weak calmodulin antagonist, since it inhibited the stimulation of the erythrocyte $Ca^{2+}$-ATPase by calmodulin.

## INTRODUCTION

In mammalian cells, $Ca^{2+}$-stimulated $Mg^{2+}$-dependent ATPases are part of the $Ca^{2+}$-transport system present in the plasma membrane [1]. In contrast with extensive studies in mammalian cells, the information available on $Ca^{2+}$-ATPase activity in *Trypanosoma brucei* plasma membrane is limited to a few contradictory reports. Although a ($Ca^{2+}$–$Mg^{2+}$)-ATPase activity was initially reported in membrane-rich fractions of *T. brucei* [2], a further communication by the same authors described its absence [3]. In addition, a high-affinity $Ca^{2+}$-ATPase that does not show any demonstrable dependence on $Mg^{2+}$ was also reported in the plasma membrane of *T. brucei* [4], and it was suggested [4] that this enzyme might have a role in the regulation of intracellular $Ca^{2+}$ homoeostasis in this trypanosomatid. The relative importance of this protein, however, has not been determined, nor has it been demonstrated that this protein functions as a $Ca^{2+}$ pump. In addition, calmodulin-sensitivity of this *T. brucei* $Ca^{2+}$-ATPase was not investigated [4]. Similarly contradictory reports have been published concerning plasma-membrane $Ca^{2+}$-ATPases of other trypanosomatids. For example, Ghosh et al. [5] reported the presence of a $Mg^{2+}$-independent $Ca^{2+}$-ATPase in the plasma membrane of *Leishmania donovani*. The same authors later reported [6] that when this $Ca^{2+}$-ATPase was studied in a membrane-associated form it showed positive modulation with $Mg^{2+}$ or with calmodulin. This would be in contrast with what occurs with the $Ca^{2+}$-ATPase of higher eukaryotes, since, on the basis of studies with the purified enzyme [7–9], it was concluded that the enzyme has the same kinetic and regulatory characteristics when it is studied either *in situ* or in pure form. On the other hand, $Mg^{2+}$-dependent plasma-membrane $Ca^{2+}$-ATPases have been demonstrated in *L. braziliensis* [10], *L. mexicana* [11] and *T. cruzi* [12].

Pentamidine is a cationic drug that has been used for more than 50 years in the therapy and prophylaxis of African trypanosomiasis and leishmaniasis. During the past several years the drug has also been used in the prophylaxis and treatment of *Pneumocistis carinii* infections, especially in patients with AIDS (acquired immunodeficiency syndrome). However, its mode of action against these parasites has not been completely elucidated [13]. It has been reported recently that pentamidine can cause membrane damage in *L. donovani* promastigotes [14]. In this regard, other cationic trypanocidal drugs, such as Crystal Violet, have also been shown to cause membrane damage and inhibit the $Ca^{2+}$-ATPase activity of plasma-membrane preparations of *T. cruzi* [15].

We have used a method that has been demonstrated previously to be very suitable for the preparation of highly enriched plasma-membrane fractions of different trypanosomatids [10–12] to identify a high-affinity ($Ca^{2+}$–$Mg^{2+}$)-ATPase present in *T. brucei* plasma membrane. In addition, we demonstrate a $Mg^{2+}$-dependent $Ca^{2+}$ uptake by plasma-membrane vesicles from *T. brucei*. Both $Ca^{2+}$ transport and the ($Ca^{2+}$–$Mg^{2+}$)-ATPase activities are stimulated by calmodulin, and inhibited by pentamidine at concentrations that do not inhibit the $Ca^{2+}$-ATPase activity of mammalian erythrocytes. In addition, we present evidence of the absence of a $Na^+/Ca^{2+}$ exchanger in the plasma membrane of these parasites.

Abbreviation used: PMSF, phenylmethanesulphonyl fluoride.

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## MATERIALS AND METHODS

### Culture methods and preparation of plasma-membrane vesicles

*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 [16]. Plasma-membrane vesicles were prepared as reported for the isolation of vesicles from *L. mexicana* [11,17], *L. braziliensis* [10] and *T. cruzi* [12,15,18]. Briefly, after a final wash in a medium containing 400 mM mannitol, 10 mM KCl, 2 mM EDTA, 1 mM phenylmethanesulphonyl fluoride (PMSF), soybean trypsin inhibitor (0.15 mg/ml), leupeptin (10 $\mu$g/ml) and 20 mM Hepes/KOH (pH 7.6), the cell pellet was mixed with acid-washed glass beads (75–120 $\mu$m in diameter) at a ratio of 1:4 (wet wt./wt. of beads). The cells were disrupted by abrasion in a chilled mortar until 90% disruption was achieved, as determined under an optical microscope. This generally took about 5–7 min. The glass beads, unbroken cells and large debris were removed by centrifuging at 1000 *g* for 15 min at 4 °C. The supernatant was subjected to differential centrifugation, first at 16000 *g* for 30 min at 4 °C and then at 105000 *g* for 1 h at the same temperature. The resulting pellet was resuspended in about 3 ml of a medium containing 150 mM KCl, 2 mM $\beta$-mercaptoethanol and 75 mM Hepes (pH 6.8). The suspension was then gently passed three times through a Dounce homogenizer (AA; Arthur Thomas) immersed in an ice-cold water bath.

### Preparation of erythrocyte inside-out plasma-membrane vesicles, purified $Ca^{2+}$-ATPase and bovine brain calmodulin

Inside-out plasma-membrane vesicles from human erythrocytes were obtained by the method of Sarkadi et al. [19], with some modifications in order to increase its calmodulin-dependence. Freshly drawn human red blood cells (20–40 ml; 90% haematocrit) were centrifuged at 1000 *g* for 10 min. White blood cells were eliminated by suction, and the pH of the red-blood-cell suspension was adjusted to pH 7.6 with 1 M Tris. The red blood cells were washed twice with 150 mM NaCl/10 mM Tris/HCl (pH 7.6) by centrifugation for 5 min at 1000 *g* and once with the same solution by centrifugation for 20 min at 1000 *g*. The red blood cells were haemolysed in 8 vol. of 20 mM KCl/20 mM sucrose/40 $\mu$M EDTA/Tris (pH 7.0)/10 mM Tris/HCl, pH 7.6, for 5 min at 0 °C. After centrifugation at 30000 *g* for 20 min, the sediment was washed four times with 2 mM KCl/2 mM sucrose/1 mM Tris/HCl, pH 8.0 (centrifugations at 20000 *g*, 20 min); 1 mM PMSF dissolved in dimethyl sulphoxide was added to the first wash. The haemoglobin-free membranes obtained were immediately diluted in 40 vol. of 0.5 mM Tris/HCl/50 $\mu$M $\beta$-mercaptoethanol/20 $\mu$M Tris/EDTA (pH 8.5), incubated for 30 min at 0 °C and for 15 min at 37 °C in the presence of 5 $\mu$g/ml leupeptin, sedimented at 40000 *g* for 20 min and washed twice with the same volume of the above 0.5 mM Tris/HCl buffer containing $\beta$-mercaptoethanol and EDTA/Tris, by centrifugation at 40000 *g* for 20 min. The sediment was homogenized by passing it five times through a 25-gauge needle, and washed twice with 10 vol. of 10 mM Tris/HCl (pH 7.4; 20 min centrifugation at 40000 *g*). The sediment was resuspended to obtain a 4–5 mg of protein/ml solution in 0.16 M KCl buffered with 5 mM Tris/HCl to pH 7.4. Either the inside-out vesicles obtained were used for the transport experiments within 24 h after the preparation, or 0.3 ml batches of the concentrated vesicle suspension were stored at −70 °C. Purified $Ca^{2+}$-ATPase from pig or human erythrocytes was isolated as described by Benaim et al. [20]. Bovine brain calmodulin was obtained as described by Guerini et al. [21] with minor modifications described in [12]. The protein concentration was determined by the biuret assay [22] in the presence of 0.2% deoxycholate, or by the method of Bradford [23].

### Chemicals

ATP, EGTA, arsenazo III, $\beta$-mercaptoethanol, phosphatidylcholine, pentamidine, leupeptin, PMSF, soybean trypsin inhibitor and calcium ionophore A23187 were from Sigma. All other reagents were analytical grade.

### Determination of ATPase activity

In order to measure the $Ca^{2+}$-ATPase activity, portions of *T. brucei* plasma-membrane vesicles (about 0.5 mg of protein/ml) were incubated in a medium containing 150 mM KCl, 75 mM Hepes/KOH (pH 6.8), 1 mM ATP, 1 mM $MgCl_2$, 2 mM $\beta$-mercaptoethanol, 1 mM EGTA, 1 $\mu$g/ml calcium ionophore A23187, 1 $\mu$g/ml oligomycin, and the appropriate concentrations of $CaCl_2$ to obtain the desired free $Ca^{2+}$ concentration. Oligomycin inhibited the basal $Mg^{2+}$-ATPase activity by less than 5% (result not shown). The final concentration of $Ca^{2+}$ was calculated by using an iterative computer program as described previously [12]. Since the rates of ATPase activity were linear over 45 min incubation at 30 °C, the reaction was arrested at 45 min by addition of cold 8% trichloroacetic acid (final concn.). The mixture was centrifuged and the supernatant was kept for $P_i$ determination. The latter was carried out by the colorimetric method of Fiske and Subbarow [24], modified by the use of $FeSO_4$ as reducing agent, and with an SLM Aminco DW2000 spectrophotometer. The $Ca^{2+}$-ATPase activity of the purified enzyme from erythrocytes was measured as described previously [20].

### Determination of $Ca^{2+}$ movements

Variations in free $Ca^{2+}$ concentration were monitored by measuring the changes in the absorbance spectrum of arsenazo III, by using the SLM Aminco DW2000 spectrophotometer at the wavelength pair 675−685 nm [25]. No free-radical formation from arsenazo III occurred under the conditions used [26,27]. The calibrations were performed by addition of known concentrations of EGTA. The initial $Ca^{2+}$ concentration in the solution was obtained by atomic-absorption spectroscopy, and the $Ca^{2+}$ concentration after EGTA addition was calculated by employing an iterative computer program as described previously [12]. Each experiment was repeated at least three times and the values shown are from representative experiments.

## RESULTS

*T. brucei* plasma-membrane vesicles hydrolysed an appreciable amount of ATP in the absence of $Ca^{2+}$ (Figure 1a), thus indicating the presence of a $Mg^{2+}$-ATPase in the plasma membrane of these parasites. In the presence of $Mg^{2+}$, ATP hydrolysis was further increased by about 10–15% upon addition of micromolar amounts of $Ca^{2+}$ (Figure 1a). This $Ca^{2+}$-ATPase activity became saturated with micromolar amounts of $Ca^{2+}$, reaching a $V_{max.}$ of $3.45 \pm 0.66$ nmol of ATP/min per mg of protein ($n = 5$). The $K_{m,app.}$ was $0.52 \pm 0.03$ $\mu$M. It was decreased to $0.23 \pm 0.05$ $\mu$M and the $V_{max.}$ was increased to $6.36 \pm 0.22$ nmol of ATP/min per mg of protein ($n = 5$) (about 85%) when calmodulin was present.

Similarly to what occurs with plasma-membrane vesicles preparations obtained from other trypanosomatids [10–12,15, 17,18], those obtained from *T. brucei* bloodstream trypomasti-

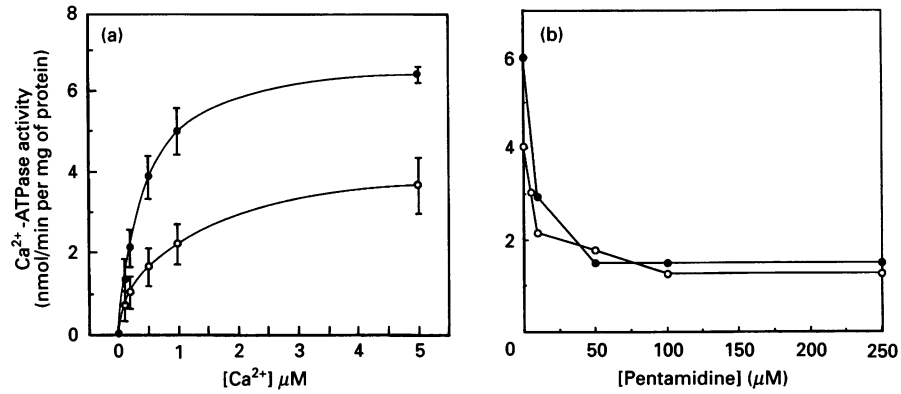


**Figure 1 ($Ca^{2+}$–$Mg^{2+}$)-ATPase activity of plasma-membrane vesicles from bloodstream trypomastigotes**

(**a**) Activation by calmodulin. The assay medium composition was as described in the Materials and methods section. The final concentration of $Ca^{2+}$ is indicated. $Mg^{2+}$-ATPase activity was subtracted. The control activity of the $Mg^{2+}$-ATPase was 35.04 ± 2.25 nmol/min per mg of protein ($n = 5$). Symbols: ●, with 5 μg/ml calmodulin; ○, without addition of calmodulin. The Figure shows the mean values of five experiments and the associated error values. (**b**) Inhibition by different concentrations of pentamidine: ○, basal activity; ●, activity in the presence of 5 μg/ml calmodulin. Final $[Ca^{2+}]$ was 2 μM. Other experimental conditions were as described in (**a**) and in the Materials and methods section. The experiment shown is typical of three repeats.

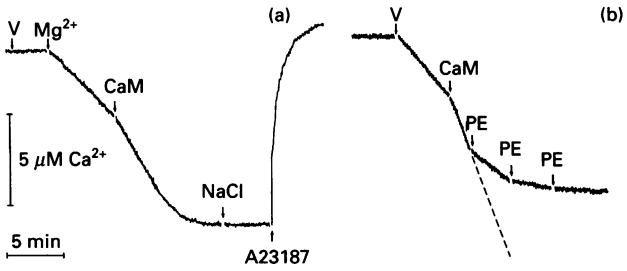


**Figure 2 $Ca^{2+}$ transport by plasma-membrane vesicles from *T. brucei* bloodstream trypomastigotes**

(**a**) Activation by calmodulin. The reaction medium (1 ml, 30 °C) contained 10 μM $Ca^{2+}$, 150 mM KCl, 75 mM Hepes (pH 6.8), 2 mM β-mercaptoethanol, 1 mM ATP and 40 μM arsenazo III. The arrows indicate the addition of vesicles (V; 0.2 mg of protein/ml), 1 mM $MgCl_2$, calmodulin (CaM; 5 μg/ml), 100 mM NaCl, or 1 μM calcium ionophore A23187. The scale bar is linear. (**b**) Inhibition by pentamidine. Experimental conditions were as in (**a**). The arrows indicate the addition of vesicles (V; 0.5 mg of protein/ml), calmodulin (CaM; 5 μg/ml) and pentamidine (PE; 25, 50 and 100 μM final concns.). Dashed line shows the result in the absence of pentamidine.

gotes are mainly composed of tightly closed vesicles, as observed by electron microscopy (G. Benaim, unpublished work). A time course of ATP-driven $Ca^{2+}$ uptake by *T. brucei* plasma-membrane vesicles is shown in Figure 2(a). In the presence of $Mg^{2+}$, addition of vesicles to the reaction mixture caused a decrease in $\Delta A$ (at 675−685 nm), which was due to the net decrease in $Ca^{2+}$ concentration by sequestration into the vesicles. When $Mg^{2+}$ was omitted, no $Ca^{2+}$ transport was observed. When calmodulin was added to the incubation medium, $Ca^{2+}$ uptake was stimulated about 2-fold (Figure 2a). Although the degree of stimulation varied among different preparations, it was self-consistent within each preparation.

In order to investigate the presence of a $Na^+/Ca^{2+}$ exchanger in the plasma membrane of these parasites, the vesicles were loaded with $Ca^{2+}$ by incubation with ATP, and 100 mM NaCl was added to the assay medium under conditions where $Ca^{2+}$ was expected to be released if such an exchanger were present [28]. However, no $Ca^{2+}$ release occurred (Figure 2a), thus suggesting the absence of a $Na^+/Ca^{2+}$ exchanger in the plasma membrane of these parasites, in agreement with previous reports in other trypanosomatids [10,12]. In addition, the finding that the accumulated $Ca^{2+}$ could be rapidly released into the medium upon addition of the $Ca^{2+}$ ionophore A23187 strongly suggested that $Ca^{2+}$ was being actively transported against a concentration gradient. Furthermore, the releasing ability of the $Ca^{2+}$ ionophore, which could not function if the store lumen were acidic [29], does not support the presence of a $Ca^{2+}/H^+$ exchanger energized by a $H^+$ pump. Taken together, these results support the notion that a calmodulin-activated $Mg^{2+}$-dependent plasma-membrane $Ca^{2+}$ pump is present in these parasites as their main $Ca^{2+}$-efflux pathway.

We have demonstrated previously [15] that cationic drugs, such as Crystal Violet, are able to inhibit the $Ca^{2+}$-ATPase present in the plasma membrane of trypanosomatids. Since the trypanocidal drug pentamidine has a p$K$ of 11.4 and thus is fully ionized as the cation at physiological pH [13], we investigated its effect on the *T. brucei* plasma-membrane $Ca^{2+}$ pump. Figure 1(b) shows that pentamidine inhibited the ($Ca^{2+}$–$Mg^{2+}$)-ATPase activity of these plasma-membrane preparations in either the presence or the absence of calmodulin. In contrast, the $Mg^{2+}$-ATPase activity of the plasma-membrane fraction was not affected by similar concentrations of pentamidine (results not shown). The pentamidine concentration needed to obtain half-maximal inhibition of the $Ca^{2+}$-ATPase was about 20 μM, and maximal inhibition was obtained with 100 μM pentamidine. In agreement with these results, pentamidine caused a concentration-dependent inhibition of $Ca^{2+}$ uptake by the *T. brucei* plasma-membrane vesicles at similar concentrations to those that affected the ($Ca^{2+}$–$Mg^{2+}$)-ATPase (Figure 2b).

To verify if this inhibitory effect of pentamidine was specific for *T. brucei* ($Ca^{2+}$–$Mg^{2+}$)-ATPase, we investigated the ability of this drug to inhibit the purified ($Ca^{2+}$–$Mg^{2+}$)-ATPase from human (results not shown) or pig (Figure 3a) erythrocytes. Although at high concentrations pentamidine inhibited the stimulation by calmodulin of this ATPase activity, it did not modify the non-stimulated ATPase activity. The pentamidine concentration needed to obtain half-maximal inhibition of the calmodulin-stimulated $Ca^{2+}$-ATPase was about 200 μM. In agreement with these results, pentamidine caused a concentration-dependent

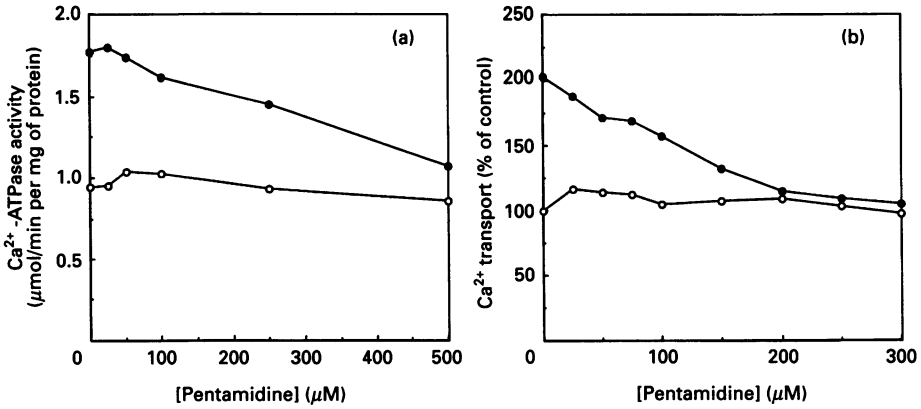


**Figure 3 Inhibition of the purified $Ca^{2+}$-ATPase from pig erythrocytes (a) and of $Ca^{2+}$ transport by human erythrocyte inside-out plasma-membrane vesicles (b) by different concentrations of pentamidine**

(a) Effect on $Ca^{2+}$-ATPase activity: ○, basal activity; ●, activity in the presence of 5 μg/ml calmodulin. Other experimental conditions were as described in the Materials and methods section. Final [$Ca^{2+}$] was 2 μM. The experiment shown is typical of three repeats. (b) Effect on $Ca^{2+}$ transport: ○, basal activity; ●, activity in the presence of 5 μg/ml calmodulin. The starting concentration of $Ca^{2+}$ was 10 μM. Other experimental conditions were as described in the Materials and methods section.

inhibition of the calmodulin-stimulated $Ca^{2+}$ uptake by human erythrocyte inside-out vesicles at concentrations similar to those that affected the calmodulin-activated purified ($Ca^{2+}$–$Mg^{2+}$)-ATPase, without any discernible effect on the basal $Ca^{2+}$-transport activity (Figure 3b).

## DISCUSSION

In this study an ATP-dependent $Ca^{2+}$-transporting system has been characterized in plasma-membrane vesicles derived from *T. brucei* bloodstream trypomastigotes. These vesicles were able to accumulate $Ca^{2+}$ against a concentration gradient, as indicated by the ability of the $Ca^{2+}$ ionophore A23187 to release rapidly the vesicle-associated $Ca^{2+}$ and by the requirement for MgATP.

The ATP-dependent $Ca^{2+}$ uptake of the plasma-membrane vesicles had similar properties to those of the ($Ca^{2+}$–$Mg^{2+}$)-ATPase. Both activities exhibited the same $Ca^{2+}$- and $Mg^{2+}$-dependence and were stimulated by calmodulin in a similar concentration range. This finding suggests that the $Ca^{2+}$-stimulated ATP hydrolysis and the ATP-stimulated $Ca^{2+}$ uptake are catalysed by the same enzyme, i.e. the ($Ca^{2+}$–$Mg^{2+}$)-ATPase of the plasma membrane. The rate of $Ca^{2+}$ transport by these vesicles was approx. 3 nmol of $Ca^{2+}$/min per mg of protein. Therefore the coupling ratio, i.e. the amount of $Ca^{2+}$ ions transported per mol of ATP hydrolysed, was about 0.8. This value is close to the stoichiometry reported [30,31] for the purified erythrocyte $Ca^{2+}$ pump reconstituted in artificial liposomes (1:1). The difference from the optimal value could be explained by taking into account that some vesicles could be 'leaky'. Although the degree of stimulation by calmodulin varied among different preparations, it is well established that various treatments can substitute for calmodulin in stimulating plasma-membrane $Ca^{2+}$-ATPase. Thus, partial proteolytic degradation [20,32], acidic phospholipids [33], enzyme self-association [34] and modification of the water structure surrounding the enzyme can mimic calmodulin [35,36]. Any of these effects might be partially masking the action of calmodulin on *T. brucei* vesicles. Alternatively, in some preparations the plasma-membrane fraction may not have been totally depleted of endogenous calmodulin. As shown for other systems, total removal of this protein is not easily achieved [37]. In this regard, the presence of calmodulin in *T. brucei* has been well documented [38].

Our results, as well as the results recently obtained using *L. braziliensis* [10] and *L. mexicana* promastigotes [11] and *T. cruzi* epimastigotes [12], do not support the hypothesis [4,5] that plasma-membrane $Mg^{2+}$-independent $Ca^{2+}$-ATPases are involved in $Ca^{2+}$ transport in trypanosomatids. The difficulties in measuring a $Ca^{2+}$-ATPase activity in the presence of a much higher $Mg^{2+}$-ATPase activity probably accounts for some of the contradictory reports about this enzyme that have been published previously [2,3]. The $Mg^{2+}$-ATPase present in the plasma membrane of different trypanosomatids is associated with a $H^+$ pump and is apparently used for the transport of glucose and amino acids [39–41]. To discriminate between these two activities, the use of highly purified plasma-membrane fractions of trypanosomatids ([10–12], and the present work) is essential. In this regard, Frasch et al. [42] isolated a $Ca^{2+}$-dependent ATPase from *T. cruzi* epimastigotes disrupted by freeze–thawing, homogenization in a Sorvall–Ribi disintegrator or homogenization in a Sorvall Omni-Mixer, whereas Letelier et al. [43] obtained a microsomal $Ca^{2+}$-ATPase from *T. cruzi* after epimastigote disruption in a French press. On the other hand, we reported [12] obtaining ($Ca^{2+}$–$Mg^{2+}$)-ATPase from *T. cruzi* after epimastigote disruption by abrasion with glass beads. Differences between these ATPase preparations were remarkable, since the enzymes isolated by Frasch et al. [42] and Letelier et al. [43] required no $Mg^{2+}$ for $Ca^{2+}$ stimulation of activity, which, however, was essential for our preparations [12]. On the other hand, after cell disruption of *T. cruzi* epimastigotes by the abrasion method, Cataldi de Flombaum and Stoppani [44] could reproduce our results. Similarly, Ghosh et al. [5] reported the presence of a $Mg^{2+}$-independent $Ca^{2+}$-ATPase in the plasma membrane of *L. donovani*. The same authors later reported [6] that when this $Ca^{2+}$-ATPase was studied in a membrane-associated form it showed positive modulation with $Mg^{2+}$ or with calmodulin.

In most mammalian cells, the $Ca^{2+}$ pump and the $Na^+/Ca^{2+}$ exchanger are the main pathways for $Ca^{2+}$ efflux present in the plasma membrane [1]. Although a number of intracellular organelles can contribute to the regulation of intracellular [$Ca^{2+}$], the $Ca^{2+}$-uptake and -efflux pathways at the plasma membrane

are the key determinants of cellular $Ca^{2+}$ content in the steady state. This is because each of the intracellular $Ca^{2+}$ pools has a finite capacity for $Ca^{2+}$ sequestration and release, whereas the plasma-membrane $Ca^{2+}$-transport mechanisms maintain a dynamic balance between the cytosol and the effectively infinite $Ca^{2+}$ pool of the extracellular medium [45]. The use of digitonin to permeabilize the plasma membrane of *T. brucei* [46–48] has allowed the identification of two intracellular $Ca^{2+}$ pools. $Ca^{2+}$ uptake by the first pool is inhibited by FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) and antimycin A in procyclic trypomastigotes, or by FCCP and oligomycin in bloodstream trypomastigotes, and is stimulated by respiratory substrates in procyclic trypomastigotes or by ATP in bloodstream trypomastigotes [46,47]. This pool has a high capacity and low affinity for $Ca^{2+}$ and is able to buffer external $Ca^{2+}$ at concentrations in the range 0.6–0.7 $\mu$M [46,47]. These are characteristics typical of mitochondria [49]. $Ca^{2+}$ uptake by the other intracellular pool is inhibited by sodium orthovanadate, and stimulated by ATP [46,47]. This pool has a low capacity and a high affinity for $Ca^{2+}$ and is able to buffer external $Ca^{2+}$ at concentrations in the range 0.05–0.1 $\mu$M [46,47]. These are characteristics typical of the endoplasmic reticulum [1], although in these parasites this pool is insensitive to inositol 1,4,5-trisphosphate and thapsigargin [47]. The presence of a third intracellular $Ca^{2+}$ pool sensitive to changes in intracellular pH has been suggested in *T. brucei* bloodstream trypomastigotes [50] on the basis of the changes observed in the fluorescence of fura-2-loaded cells when nigericin was included in the incubation medium. The present work completes the characterization of the most important mechanisms involved in the regulation of $Ca^{2+}$ homoeostasis in *T. brucei*. Our results indicate that a $Ca^{2+}$ pump is apparently the only mechanism for $Ca^{2+}$ efflux operating in *T. brucei* bloodstream forms. In this regard, the $Na^+/Ca^{2+}$ exchanger seems to be less ubiquitous than the plasma-membrane $Ca^{2+}$ pump [28], and we have been unable to detect its presence in other trypanosomatids [10,12]. Although Oz et al. [51] recently suggested the presence of a $Na^+/Ca^{2+}$ exchanger in *T. cruzi* epimastigotes on the basis of experiments in which fluorescence of fura-2-loaded cells increased when $Na^+$ was decreased in the incubation medium, their evidence was indirect.

Pentamidine was shown to have a potent inhibitory effect on *T. brucei* $Ca^{2+}$-ATPase and a weak anti-calmodulin action on the erythrocyte plasma-membrane $Ca^{2+}$ pump. Concerning the latter effect, its chemical structure closely resembles those of some of the best-known anti-calmodulin agents [52]. Pentamidine has been shown to collapse the mitochondrial membrane potential of *L. donovani* promastigotes at concentrations 10 times higher (200 $\mu$M) than those that could inhibit the plasma-membrane $Ca^{2+}$-ATPase of *T. brucei* (Figure 3). Our results suggest that inhibition of the plasma-membrane $Ca^{2+}$-ATPase of *T. brucei* could be involved in its selective trypanocidal action, and that this $Ca^{2+}$-ATPase could be an important target for other trypanocidal drugs.

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