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A calmodulin-activated (Ca^{2+}–Mg^{2+})-ATPase is involved in Ca^{2+} transport by plasma membrane vesicles from *Trypanosoma cruzi*

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High-affinity Ca^{2+}-activated ATPases that do not show any demonstrable dependence on Mg^{2+} have been reported in the plasma membranes of different trypanosomatids, and it has been suggested [McLaughlin (1985) Mol. Biochem. Parasitol. **15**, 189–201; Ghosh, Ray, Sarkar & Bhaduri (1990) J. Biol. Chem. **265**, 11345–11351] that these enzymes may have a role in Ca^{2+} transport by the plasma membrane and in the regulation of intracellular Ca^{2+} in these parasites. In this report we investigated Ca^{2+} transport by *Trypanosoma cruzi* plasma membrane vesicles using Arsenazo III as a Ca^{2+} indicator. These vesicles accumulated Ca^{2+} upon addition of ATP only when Mg^{2+} was present and released it in response to the Ca^{2+} ionophore A23187, but were insensitive to inositol 1,4,5-trisphosphate. Ca^{2+} transport was insensitive to antimycin A, oligomycin and carbonyl cyanide *p*-trifluorophenylhydrazone, ruling out any mitochondrial contamination. Staurosporine and phorbol myristate acetate had no effect on this activity, while low concentrations of vanadate (10 μM) completely inhibited it. In addition, we describe a high-affinity vanadate-sensitive (Ca^{2+}–Mg^{2+})-ATPase in the highly enriched plasma membrane fraction of *T. cruzi*. Kinetic studies indicated that the apparent K_m for free Ca^{2+} was 0.3 μM. On the other hand, Ca^{2+}-ATPase activity and Ca^{2+} transport were both stimulated by bovine brain calmodulin and by endogenous calmodulin purified from these cells. In addition, trifluoperazine and calmidazolium, at concentrations in the range in which they normally exert anti-calmodulin effects, inhibited the calmodulin-stimulated Ca^{2+}-ATPase activity. These observations support the notion that a Mg^{2+}-dependent plasma membrane Ca^{2+} pump is present in these parasites.

INTRODUCTION

The free cytosolic Ca^{2+} concentration is the key variable governing the intracellular actions of Ca^{2+}. In most eukaryotic cells Ca^{2+} homeostasis is achieved by the concerted operation of several Ca^{2+}-transporting systems located in the plasma membrane, endoplasmic reticulum and mitochondria [1,2]. The Ca^{2+}-transporting systems of eukaryotic cells have different kinetic properties, designed to satisfy the different requirements of the cells during their functional cycle [1]. In general, whenever the need arises to transport Ca^{2+} with high interaction affinity, ATPases are present, since this appears to be the only transport mode that confers to the system high Ca^{2+} affinity. As a result, cells rely solely on ATPases for the fine tuning of their Ca^{2+} concentration [1].

Evidence is accumulating in support of an important role for Ca^{2+} in trypanosomatids. Thus microtubule assembly in *Trypanosoma brucei* [3], flagellar movement in *Crithidia oncopelti* [4], variant surface glycoprotein release in African trypanosomes [5] and cellular differentiation in *Leishmania* [6] appear to be regulated by Ca^{2+}. In addition, the enzymes responsible for cyclic nucleotide metabolism [7,8] and for the inositol phosphate/diacylglycerol signalling pathway [9] in these parasites are also activated by Ca^{2+}. Furthermore, the main Ca^{2+}-binding protein of eukaryotic cells, calmodulin, has been detected and isolated from American [8,10] and African [11] trypanosomes, and from *Leishmania* [12].

By using fluorescent Ca^{2+} indicators, submicromolar intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) have been detected in different trypanosomatids [13–16]. In addition, two intracellular Ca^{2+} transport systems have been detected in trypanosomatids, in the mitochondria and the endoplasmic reticulum [16–19]. However, little is known about the role of the plasma membranes of these parasites in the regulation of $[Ca^{2+}]_i$. In this regard, high-affinity Ca^{2+}-ATPases that do not show any demonstrable dependence on Mg^{2+} have been reported in the plasma membranes of *T. brucei* [20] and *Leishmania donovani* [21], and it has been suggested [20,21] that these enzymes may have a role in the regulation of intracellular Ca^{2+} homeostasis in these trypanosomatids. In this report we demonstrate a Mg^{2+}-dependent Ca^{2+} uptake by plasma membrane vesicles from *T. cruzi*. In addition, we describe a high-affinity (Ca^{2+}–Mg^{2+})-ATPase present in these membrane fractions having general characteristics common to plasma membrane ATPases involved in Ca^{2+} transport, such as stimulation by calmodulin and inhibition by low concentrations of vanadate and anti-calmodulin agents.

MATERIALS AND METHODS

Culture methods

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 5% fetal bovine serum [22]. At 5 days after inoculation, cells were collected by centrifugation and washed twice in a medium containing 11 mM-KCl, 140 mM-NaCl and 75 mM-Tris/HCl, pH 7.6. The final concentration of cells was determined using a Neubauer chamber. The protein concentration was determined by the biuret assay in the presence of 0.2% deoxycholate [23].

Isolation of plasma membrane vesicles

Plasma membrane vesicles were prepared essentially as reported for the isolation of vesicles from *Leishmania mexicana* [24], *L. braziliensis* [25], and *T. cruzi* [26]. Briefly, after a final

Abbreviations used: FCCP, carbonyl cyanide *p*-trifluorophenylhydrazone; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentrations; PMSF, phenylmethanesulphonyl fluoride.

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wash in a medium containing 400 mM-mannitol, 10 mM-KCl, 2 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride (PMSF), soy bean trypsin inhibitor (0.15 mg/ml), leupeptin (10 μg/ml) and 20 mM-Hepes/KOH (pH 7.6), the cell pellet was mixed with acid-washed glass beads (75–120 μm 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 centrifugation 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-β-mercaptoethanol and 75 mM-Hepes (pH 6.8). The suspension was then gently passed three times through a Dounce homogenizer (AA; Thomas Scientific, Swedesboro, NJ, U.S.A.) immersed in an ice-cold water-bath. As reported previously [24–26], this preparation was highly enriched in plasma membrane vesicles, as inferred from its specific binding of ^{125}I-labelled concanavalin, which was 12–14-fold enriched with respect to the entire homogenate. This fraction was also devoid of succinate:cytochrome *c* oxidoreductase activity, thus indicating the absence of mitochondrial contamination. In addition, the specific activity of Ca^{2+}-ATPase was 13–14-fold higher with respect to the homogenate, and co-purified with the oligomycin-insensitive Mg^{2+}-ATPase, which is considered to be another marker enzyme for the plasma membrane of these parasites [24–27].

Purification of calmodulin and assay of its biological activity

Purification of calmodulin was achieved essentially as described previously [12,28] with the following modifications. After washing the cells as described above, the pellet was resuspended in a medium containing 10 mM-KCl, 10 mM-KOH/Hepes, pH 7.2, 25 mM-EDTA, 0.15 mg of soy bean trypsin inhibitor/ml, 0.15 mg/ of PMSF/ml and 10 μg of leupeptin/ml, and sonicated with a Branson Sonic Power sonifier for 20 s at 10 A (d.c.). The disrupted cells were then centrifuged at 105000 *g* for 1 h at 4 °C. The step using DEAE cellulose [12,28] was omitted in order to increase the efficiency (or yield) and the supernatant was precipitated with 55% $(NH_4)_2SO_4$. The extract was centrifuged at 25000 *g* for 30 min at 4 °C and the supernatant was precipitated with 93% $(NH_4)_2SO_4$ and centrifuged again as described before. The final pellet, which contained the calmodulin, was then dialysed for 24 h against 10 mM-NH_4HCO_3 and 0.1 mM-$CaCl_2$, and for another 24 h against 10 mM-NH_4HCO_3. After lyophilization the extract was suspended in a medium containing 50 mM-NaCl, 1 mM-$CaCl_2$, 2 mM-β-mercaptoethanol and 20 mM-KOH/Hepes buffer, pH 7.2, and layered on top of a phenyl-Sepharose column previously washed and equilibrated with the same buffer. The column was then washed with the same solution, but with 500 mM- instead of 50 mM-NaCl and 0.1 mM- instead of 1 mM-$CaCl_2$. After no detectable absorption was observed in a u.v. recorder, calmodulin was eluted with the same final solution but with 1.2 mM-EGTA instead of $CaCl_2$. (Ca^{2+}–Mg^{2+})-ATPase was isolated from human red blood cells as previously reported [29], and stimulation of the (Ca^{2+}–Mg^{2+})-ATPase activity by calmodulin was assayed with a coupled enzyme system in an SLM Aminco DW2000 spectrophotometer as described previously [29]. Calmodulin from bovine brain was isolated according to the method of Guerini *et al.* [28].

Chemicals

ATP, GTP, CTP, UTP, ITP, oligomycin, antimycin A, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), EGTA, Arsenazo III, β-mercaptoethanol, sodium vanadate, staurosporine, inositol 1,4,5-triphosphate, trifluoperazine, calmidazolium, leupeptin, soy bean trypsin inhibitor, PMSF, Hepes, phenyl-Sepharose and Ca^{2+} ionophore A23187 were from Sigma. All other reagents were analytical grade.

Determination of Ca^{2+} movement

Variations in free Ca^{2+} concentrations were monitored by measuring the changes in the absorbance spectrum of Arsenazo III, using the SLM Aminco DW2000 spectrophotometer at the wavelength pair 675–685 nm [30] to avoid interference by Mg^{2+} [31]. No free radical formation from Arsenazo III occurred under the conditions used [32–34].

Determination of ATPase activity in the plasma membrane vesicles

Aliquots of 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-β-mercaptoethanol, 1 mM-EGTA, 1 μg A23187/ml and the appropriate concentration of $CaCl_2$ to obtain the desired free Ca^{2+} concentration. Concentrations of the ionic species and complexes at equilibrium were calculated by employing an iterative computer program [35], modified from that described by Fabiato & Fabiato [36], taking into account the dissociation constants reported by Schwarzenbach *et al.* [37]. After 45 min incubation at 24 °C, the reaction was arrested by the addition of 8% (final concentration) trichloroacetic acid. The mixture was centrifuged and the supernatant was kept for determination of P_i. The latter was carried out according to the method of Fiske & Subbarow [38], modified by the use of $FeSO_4$ as reducing agent.

RESULTS

A time course of ATP-driven Ca^{2+} uptake by *T. cruzi* plasma membrane vesicles is shown in Fig. 1. In the presence of Mg^{2+}, and after a short lag period, addition of vesicles to the reaction mixture caused a decrease in absorbance (at 675–685 nm) which was caused by the net decrease in Ca^{2+} concentration owing to sequestration into the vesicles. No ATP-dependent uptake occurred when Mg^{2+} was omitted from the reaction medium. The finding that the accumulated Ca^{2+} could be readily released into the medium upon addition of the Ca^{2+} ionophore A23187

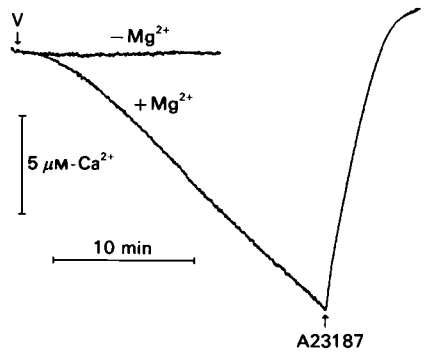

Fig. 1. Effect of Mg^{2+} on Ca^{2+} transport by plasma membrane vesicles from epimastigotes

The reaction medium (1 ml, 24 °C) contained 150 mM-KCl, 75 mM-Hepes (pH 6.8), 2 mM-β-mercaptoethanol, 1 mM-ATP, 1 mM-$MgCl_2$ and 40 μM-Arsenazo III where indicated. The arrows indicate the addition of vesicles (V, 0.2 mg of protein/ml) or 10 μM Ca^{2+} ionophore A23178.

Table 1. Effects of calmodulin and different inhibitors on the (Ca^{2+}–Mg^{2+})-ATPase activity in and Ca^{2+} uptake by plasma membrane vesicles from *T. cruzi*

(Ca^{2+}–Mg^{2+})-ATPase activity was determined as described in the Materials and methods section. The control activities (100%) of the Mg^{2+}-ATPase and the (Ca^{2+}–Mg^{2+})-ATPase were 38.6±5.2 and 5.1±0.2 nmol of P_i/min per mg of protein respectively. The control activity (100%) of Ca^{2+} uptake was 5.5 nmol of Ca^{2+}/min per mg of protein. The Ca^{2+} concentration was 10 μM. Bovine brain calmodulin, when present, was added at a final concentration of 10 μg/ml. A similar concentration of *T. cruzi* calmodulin have similar effects on both Ca^{2+} uptake and (Ca^{2+}–Mg^{2+})-ATPase activity. Vanadate (10 μM), antimycin A (1 μg/ml), FCCP (2 μM), oligomycin (2 μg/ml), trifluoperazine (TFP, 30 μM) and calmidazolium (CMZ, 10 μM) were added where indicated. Each value represents the mean±S.D. of at least three determinations.

Inhibitor or activator	Ca^{2+}-ATPase (% of control)	Ca^{2+} uptake (% of control)
Calmodulin	167±4	165±16
TFP	83±1	101±2
Calmodulin+TFP	85±4	100±13
CMZ	82±2	96±1
Calmodulin+CMZ	99±1	111±4
Vanadate	16±4	0
Antimycin A	103±5	93±1
FCCP	95±2	88±7
Oligomycin	100±2	100±3

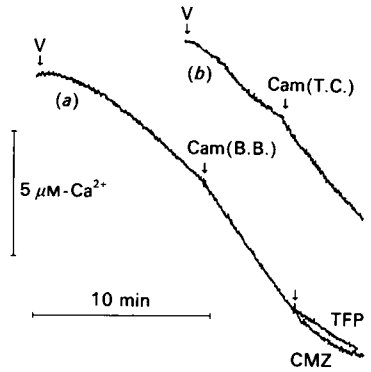

Fig. 2. Effect of calmodulin on Ca^{2+} transport by plasma membrane vesicles from epimastigotes

Experimental conditions were as in Fig. 1. Where indicated by the arrows, 20 μg of bovine brain calmodulin [Cam (B.B.)]/ml, 1 μg of *T. cruzi* calmodulin [Cam (T.C)]/ml, 30 μM-trifluoperazine (TFP) or 10 μM-calmidazolium (CMZ) were added. V, vesicles.

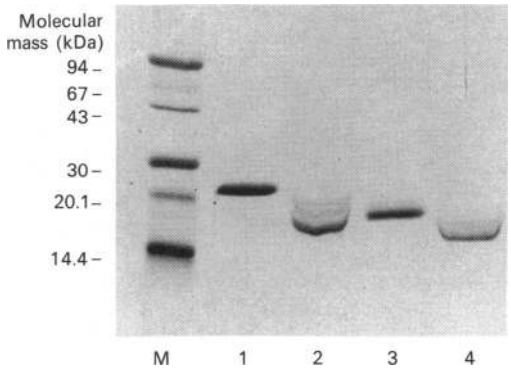

Fig. 3. Determination of the molecular mass of calmodulin from epimastigotes

Electrophoresis was a 7.5–15% gradient polyacrylamide gel with SDS stained with Coomassie Blue. Lane 1, bovine brain calmodulin plus 1 mM-EGTA; lane 2, bovine brain calmodulin; lane 3, epimastigote calmodulin plus 1 mM-EGTA; lane 4, epimastigote calmodulin. The molecular mass markers (lane M) were phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

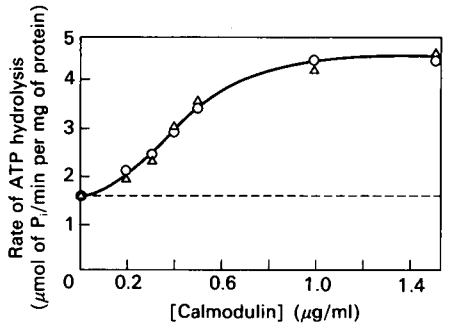

Fig. 4. Stimulation of the Ca^{2+}-ATPase from human red blood cells by different concentrations of calmodulin from bovine brain (○) or from epimastigotes (△)

The broken line represents basal activity. Other experimental conditions were as described in the Materials and methods section.

strongly suggested that Ca^{2+} was being actively transported against a concentration gradient. The Ca^{2+} overshoot observed after adding A23187 probably arose from endogenous Ca^{2+} which was trapped during the fractionation procedure (see the Materials and methods section).

When these experiment were carried out in the presence of sodium orthovanadate (10 μM), which inhibits (Ca^{2+}–Mg^{2+})-ATPases [39], the addition of ATP resulted in no stimulation of Ca^{2+} uptake by the plasma membrane vesicles (Table 1). The Ca^{2+} uptake was specific for ATP and other nucleotides (GTP, UTP, ITP or CTP) could not replace ATP (results not shown). Ca^{2+} uptake was not significantly inhibited by mitochondrial ATPase inhibitors such as oligomycin (2 μg/ml), or by antimycin A (1 μg/ml) or FCCP (2 μM) (Table 1). Other compounds, such as phorbol myristate acetate (2 μg/ml), arachidonic acid (3 μM) and staurosporine (2 μg/ml), did not show any discernible effect.

If the vesicles were loaded with Ca^{2+} by incubation with ATP and 100 mM-NaCl was added to the assay medium, no Ca^{2+} release occurred (results not shown), thus ruling out the presence of a Na^+/Ca^{2+} exchanger in the plasma membrane of these parasites. In addition, no Ca^{2+} release occurred when inositol 1,4,5-trisphosphate (10 μM) was added to these preparations.

When bovine brain calmodulin was added to the incubation medium (Fig. 2*a*), Ca^{2+} uptake was stimulated. Although the degree of simulation varied among different preparations, it was consistent within each preparation. On the other hand, the calmodulin-sensitivity was higher when the membrane fraction had been previously treated with EDTA during the preparation procedure in order to remove endogenous calmodulin (stimulation by bovine brain calmodulin was about 70% when EDTA was used and 30% when it was not). The anti-calmodulin agents trifluoperazine (30 μM) and calmidazolium (10 μM) inhibited the

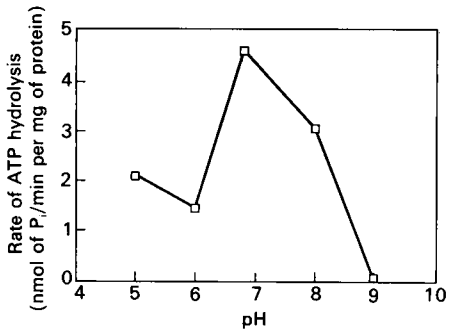

Fig. 5. Effect of pH on (Ca^{2+}–Mg^{2+})-ATPase activity of plasma membrane vesicles from epimastigotes

The assay medium composition and other conditions are described in the Materials and methods section. Mg^{2+}-ATPase activity was substracted. The free Ca^{2+} concentration was 1 μM.

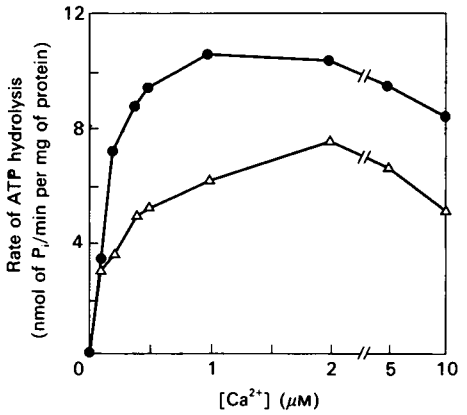

Fig. 6. Activation by calmodulin of the (Ca^{2+}–Mg^{2+})-ATPase activity of plasma membrane vesicles from epimastigotes

The assay medium composition was 150 mM-KCl, 75 mM-Hepes (pH 6.8), 2 mM-β-mercaptoethanol, 1 mM-ATP, 1 mM-$MgCl_2$, 1 mM-EGTA, 1 μg of Ca^{2+} ionophore A23187/ml and the final concentration of Ca^{2+} indicated. Mg^{2+}-ATPase activity was substracted. ●, With 10 μg of bovine brain calmodulin/ml; △, without addition of calmodulin.

stimulation of Ca^{2+} transport by bovine brain calmodulin (Fig. 2*a*).

In order to ascertain whether endogenous calmodulin could stimulate Ca^{2+} uptake by these preparations, *T. cruzi* calmodulin was purified from epimastigotes as described in the Materials and methods section. To confirm the purity of the calmodulin isolated from *T. cruzi*, it was run in parallel on SDS/PAGE with a sample of bovine brain calmodulin and suitable standards. Fig. 3 shows that calmodulin from epimastigotes migrated faster than that from bovine brain. The determination of the apparent molecular mass using appropriate standards indicated a value of 15.8 kDa, close to that of bovine brain calmodulin (16.7 kDa, [12,28]). As occurs with bovine brain calmodulin [40], the addition of EGTA to *T. cruzi* calmodulin caused a shift in its electrophoretic mobility.

The ability of *T. cruzi* calmodulin to stimulate the purified (Ca^{2+}–Mg^{2+})-ATPase from human red blood cells was also investigated (Fig. 4). The calmodulin concentration required to obtain half-maximal stimulation was about 0.4 μg/ml, and maximal stimulation (3-fold) was obtained with 1.0 μg/ml. The stimulation observed was indistinguishable from that obtained with bovine brain calmodulin. Similar results have been reported with *L. braziliensis* and *L. mexicana* calmodulins [12].

As expected, *T. cruzi* calmodulin could stimulate Ca^{2+} transport by plasma membrane vesicles from the same parasites (Fig. 2*b*).

T. cruzi plasma membrane vesicles hydrolysed an appreciable amount of ATP in the absence of Ca^{2+} (Table 1). In the presence of saturating Mg^{2+} concentrations, ATP hydrolysis was further increased by about 20–25% upon addition of micromolar amounts of Ca^{2+} (Table 1). As in the case of Mg^{2+}-ATPase, maximal Ca^{2+}-ATPase activity was attained when equimolar amounts of MgATP were present in the medium (results not shown). This suggested that the Mg–ATP complex was the substrate for the Ca^{2+}-ATPase, as shown for the same enzyme from higher eukaryotic systems [39,41]. The optimal pH of this enzyme was in the physiological range (Fig. 5). The Ca^{2+} affinity of the ATPase was very high (Fig. 6), and comparable with those other plasma membrane Ca^{2+}-ATPases. Thus the enzyme became saturated with micromolar amounts of Ca^{2+}, reaching a $V_{max.}$ of 8 nmol of ATP/min per mg of apparent protein. The K_m was approx. 0.3 μM at optimal Mg^{2+} and ATP concentrations. The apparent K_m was decreased to 0.12 μM and the $V_{max.}$ was increased to 12 nmol ATP/min per mg of protein when calmodulin was present. Similar results were observed independently of the source of the calmodulin used. At Ca^{2+} concentrations higher than 5 μM an inhibition of the rate of ATP hydrolysis was detected (Fig. 6). In contrast, Ca^{2+} transport by the plasma membrane vesicles was not affected by these high Ca^{2+} concentrations, thus indicating that this effect was probably due to inhibition of the Mg^{2+}-ATPase activity by Ca^{2+}. Since there is no specific inhibitor of the (Ca^{2+}–Mg^{2+})-ATPase, we could not investigate the characteristics of this inhibitory effect.

When the ATPase inhibitor vanadate was added to the medium, a significant inhibition of the (Ca^{2+}–Mg^{2+})-ATPase activity was detected (Table 1). In contrast, when the calmodulin antagonists trifluoperazine (30 μM) and calmidazolium (10 μM) were added, only the stimulation by calmodulin of the (Ca^{2+}–Mg^{2+})-ATPase activity was inhibited (Table 1). At higher concentrations of the drugs (100 μM-calmidazolium or -trifluoperazine) complete inhibition of the basal activity was obtained, thus indicating non-specific effects similar to those reported previously [16,42].

DISCUSSION

In this study an ATP-dependent Ca^{2+}-transporting system has been characterized in plasma membrane vesicles derived from *T. cruzi* epimastigotes. These vesicles were able to accumulate Ca^{2+} against a concentration gradient, as indicated by the ability of the Ca^{2+} ionophore A23187 to rapidly release the vesicle-associated Ca^{2+} and by the requirement for MgATP. The activity of ATP-dependent Ca^{2+} uptake is considered to be principally localized in the plasma membrane, since the preparation used here was not contaminated by other membrane organelles such as mitochondria or endoplasmic reticulum, as shown by lack of marker enzymes. In addition, the sensitivity to calmodulin of the Ca^{2+} uptake and the insensitivity to inositol 1,4,5-trisphosphate of the Ca^{2+} pool accumulated by these preparations also suggests that the plasma membrane and not the endoplasmic reticulum is the origin of the preparation. In this regard, although the (Ca^{2+}–Mg^{2+})-ATPase from the endoplasmic reticulum shares some general characteristics common to all plasma membrane Ca^{2+}-ATPases, it is not stimulated by calmodulin [1]. In addition, it has been reported that inositol 1,4,5-trisphosphate releases

Ca^{2+} from the endoplasmic reticulum of *Leishmania donovani* promastigotes [43].

It has been reported that protein kinase C is able to stimulate Ca^{2+} transport by inside-out vesicles from human erythrocytes [44]. Although protein kinase C is mainly associated with membrane fractions in *T. cruzi* epimastigotes [45], Ca^{2+} transport by plasma membrane vesicles from *T. cruzi* was not affected by the protein kinase C inhibitor staurosporine or by the protein kinase C activator phorbol myristate acetate.

In addition to these results, we describe a new two-step high-yield method for the purification of calmodulin from trypanosomatids. The yield of this method is about 4–5 times greater than with the method reported previously [12]. This method is also useful for the purification of calmodulin from *L. mexicana* and *L. braziliensis* (G. Benaim, unpublished work).

The ATP-dependent Ca^{2+} uptake by the plasma membrane vesicles was observed to exhibit similar properties to those of the (Ca^{2+}–Mg^{2+})-ATPase. Both activities were stimulated by calmodulin and inhibited by vanadate, in a similar concentration range. The stimulation by calmodulin was inhibited by trifluoperazine and calmidazolium with a concentration range in which these drugs normally exert anti-calmodulin-dependent effects. Vanadate inhibited both activities at a very low concentration (10 μM). These findings suggest compellingly 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. Although the degree of stimulation by calmodulin was relatively low, it is well established that various treatments can substitute for calmodulin in stimulating plasma membrane Ca^{2+}-ATPases. Thus partial proteolytic degradation [29,46], acidic phospholipids [47], enzyme self-association [48] and modification of the water structure surrounding the enzyme can mimic calmodulin [49]. Any of these effects might be partially masking the action of calmodulin on *T. cruzi* vesicles. Alternatively, the plasma membrane fraction may not have been totally depleted of endogenous calmodulin, even after pretreatment with EDTA. As shown for other systems, total removal of this protein is not easily achieved [40].

The general characteristics of the enzyme reported in this study, including both Mg^{2+} and Ca^{2+} requirements, optimal pH and vanadate sensitivity, are essentially identical to those described for Ca^{2+} transport ATPases in higher eukaryotic systems. This suggests an E1–E2 type ATPase, which is not different from the plasma membrane Ca^{2+}-ATPase from the host cells [39,41,50].

The presence of a Na^+/Ca^{2+} exchanger, which seems relevant in some mammalian systems [1,51] is not supported by the experiments reported here. However, this mechanism seems to be less ubiquitous than the plasma membrane Ca^{2+} pump [1,51]. Since other mechanisms reponsible for Ca^{2+} regulation that are present in these parasites [15,16–18] also appear to be similar to those described for higher eukaryotes [1], it can be suggested that intracellular Ca^{2+} homeostasis is well conserved during evolution from lower to higher eukaryotic organisms.

High-affinity Ca^{2+}-activated ATPases that do not show any demonstrable dependence on Mg^{2+} have been reported in the plasma membranes of *T. brucei* [20] and *L. donovani* [21], and it has been suggested that these enzymes may have a role in the regulation of intracellular Ca^{2+} in these parasites [20,21]. However, plasma membrane ATPases involved in Ca^{2+} transport in most eukaryotic organisms investigated thus far are Mg^{2+}-dependent [1,25]. Our results, as well as the results recently reported using *Leishmania braziliensis* promastigotes [25] do not support the hypothesis [20,21] that plasma membrane Mg^{2+}-independent Ca^{2+}-ATPases are involved in Ca^{2+} transport in trypanosomatids.

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