Characterization of the plasma-membrane calcium pump from *Trypanosoma cruzi*

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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] suggesting that the plasma-membrane Ca^{2+} -ATPases of different trypanosomatids differ from the Ca^{2+} pumps present in mammalian cells, *Trypanosoma cruzi* plasma-membrane Ca^{2+} -ATPase shares several characteristics with the Ca^{2+} pumps present in other systems. This enzyme could be partially purified from epimastigote plasma-membrane vesicles using calmodulin–

agarose affinity chromatography. The activity of the partially purified enzyme was stimulated by *T. cruzi* or bovine brain calmodulin. In addition, the enzyme cross-reacted with antiserum and monoclonal antibody 5F10 raised against human red-blood-cell Ca^{2+} -ATPase, has a molecular mass of 140 kDa and forms Ca^{2+} -dependent hydroxylamine-sensitive phosphorylated intermediates. These results, together with its high sensitivity to vanadate, indicate that this enzyme belongs to the P-type class of ionic pumps.

INTRODUCTION

The Ca^{2+} pump of plasma membranes extrudes Ca^{2+} from the cytosol to the extracellular space [1]. This pump also hydrolyses ATP, which provides the energy required for Ca^{2+} movement [2–4] and it belongs to the so called ‘P-type’ ionic pumps, since it forms a phosphorylated intermediate during its catalytic cycle [5]. The Ca^{2+} pump of *Trypanosoma cruzi* epimastigotes has been studied recently [6]. Epimastigote plasma-membrane vesicles transport Ca^{2+} in the presence of Mg^{2+} [6]. A high-affinity vanadate-sensitive Ca^{2+} + Mg^{2+} -ATPase is present in these plasma-membrane vesicles. Kinetic studies indicated that the apparent K_m for free Ca^{2+} is about 0.3 μM . On the other hand, Ca^{2+} -ATPase activity and Ca^{2+} transport are both stimulated by endogenous calmodulin purified from these cells [6]. In addition, trifluoperazine and calmidazolium, at concentrations in the range in which they normally exert anti-calmodulin effects, inhibit the calmodulin-stimulated Ca^{2+} -ATPase activity [6]. These observations support the notion that a Mg^{2+} -dependent plasma-membrane Ca^{2+} pump is present in these parasites [6]. Since no evidence has been found of the presence of a $\text{Na}^+/\text{Ca}^{2+}$ exchanger in these plasma-membrane vesicles [6], these results indicate that a Ca^{2+} pump is apparently the only mechanism for Ca^{2+} efflux operating in these parasites. Similar results were obtained with *T. brucei* bloodstream trypomastigotes [7] and *Leishmania braziliensis* promastigotes [8]. The selective inhibition of the Ca^{2+} pumps of these parasites by trypanocidal agents, such as Crystal Violet [9] and pentamidine [7] indicate the potential chemotherapeutic importance of this enzyme.

Plasma-membrane Ca^{2+} pumps in a number of systems, including those of plants [10] and yeast [11], have been shown to share several characteristics. For example, these enzymes have a molecular mass of about 140 kDa and form a Ca^{2+} -dependent phosphorylated intermediate [2–5]. In addition, these enzymes bind calmodulin and transport Ca^{2+} at a higher rate in the

presence of calmodulin than in its absence [2–4]. When covalently coupled to Sepharose or agarose, calmodulin can be used for purification of this ATPase by affinity chromatography. It not only binds the ATPase tightly in the presence of Ca^{2+} , but it also releases it readily when free Ca^{2+} is reduced by addition of either EDTA or EGTA [12]. At variance with these general characteristics of plasma-membrane Ca^{2+} pumps from different organisms, Ghosh et al. [13] recently reported the purification of a different Ca^{2+} -ATPase apparently located in the plasma membrane of *L. donovani*. The enzyme consisted of two subunits of molecular masses 51 and 57 kDa and had an apparent molecular mass of 215 ± 12 kDa [13,14]. In addition, a high-affinity Ca^{2+} -ATPase that did not show any demonstrable dependence on Mg^{2+} was also reported in the plasma membrane of *T. brucei* [15], and it was suggested that this enzyme might have a role in the regulation of intracellular Ca^{2+} homeostasis in this trypanosomatid.

We now report that calmodulin–agarose affinity chromatography can be used successfully to purify partially the plasma-membrane Ca^{2+} -ATPase of *T. cruzi* epimastigotes, and that a polyclonal and a monoclonal antibody directed against the human red-blood-cell Ca^{2+} pump cross-react with this protein. As occurs with other plasma-membrane Ca^{2+} -ATPases [2–4,10,11], this protein shows an apparent molecular mass of 140 kDa. The formation of a Ca^{2+} -dependent phosphorylated intermediate of this enzyme is also reported.

MATERIALS AND METHODS

Chemicals

ATP, EGTA, β -mercaptoethanol, dithiothreitol, leupeptin, soybean trypsin inhibitor, phenylmethanesulphonyl fluoride, pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, phosphatidylcholine, calmodulin–agarose and phenyl-Sepharose were from Sigma. [γ - ^{32}P]ATP (5000 Ci/mmol) was purchased

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from New England Nuclear. All other reagents were of analytical grade.

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 [6]. 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.

Isolation of plasma-membrane vesicles

Plasma-membrane vesicles were prepared essentially as reported previously [6]. Briefly, after a final wash in a medium containing 400 mM mannitol, 10 mM KCl, 2 mM EDTA, 1 mM phenylmethanesulphonyl fluoride, soybean 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 weight/weight 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 centrifuged at 16000 *g* for 30 min at 4 °C and the resulting supernatant was centrifuged at 105000 *g* for 1 h at the same temperature. The 105000 *g* pellet was resuspended in about 8–10 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; Arthur Thomas) immersed in an ice-cold water bath. As reported previously [6], this preparation is highly enriched in plasma-membrane vesicles, as inferred from its specific binding of ¹²⁵I-labelled concanavalin, which was 12–14-fold enriched with respect to the entire homogenate. This fraction is also devoid of succinate:cytochrome *c* oxidoreductase activity, thus indicating the absence of mitochondrial contamination. In addition, the specific activity of Ca²⁺-ATPase, which is oligomycin-insensitive [6], is 13–14-fold higher with respect to the homogenate, and co-purified with the oligomycin-insensitive Mg²⁺-ATPase, which is also considered to be another marker enzyme for the plasma membrane of these parasites [6].

Purification of calmodulin and Ca²⁺-ATPases

Purification of different calmodulins [6] and of human or pig red-blood-cell Ca²⁺-ATPases [16] was achieved as described previously. Epimastigote Ca²⁺-ATPase was partially purified, by a modification of the procedure described for the purification of the red-blood-cell Ca²⁺-ATPase [16], from 40–50 mg of plasma-membrane vesicle protein. Briefly, plasma-membrane vesicles, suspended at 3 mg/ml in a buffer containing 130 mM KCl, 10 mM Hepes/KOH, pH 7.4, 0.5 mM MgCl₂ and 50 µM CaCl₂, were solubilized on ice for 10 min by adding Triton X-100 to a final concentration of 0.5% (w/v). Non-solubilized material was removed by centrifugation at 105000 *g* at 2 °C for 35 min. Phosphatidylcholine was then added to the supernatant to a final concentration of 0.5 mg/ml, and the mixture was applied to a calmodulin-agarose column (3–4 ml bed volume), which was equilibrated in a buffer containing 0.4% (w/v) Triton X-100, 130 mM KCl, 20 mM Hepes/KOH, pH 7.4, 1 mM MgCl₂, 100 µM CaCl₂, 2 mM dithiothreitol and 0.5 mg/ml of

phosphatidylcholine. The column was loaded and washed at a rate of about 40 ml/h. The column was then washed with 40 ml of the equilibration buffer and with 40 ml of a similar buffer containing 0.05% (w/v) Triton X-100. Finally, the Ca²⁺-ATPase was eluted in a buffer containing 0.05% (w/v) Triton X-100, 130 mM KCl, 20 mM Hepes/KOH, pH 7.4, 1 mM MgCl₂, 2 mM sodium EDTA, 2 mM dithiothreitol, 5% (v/v) glycerol and 0.5 mg/ml of phosphatidylcholine at a rate of 10 ml/h. The Ca²⁺-ATPase activity of the fractions eluted from the column was determined, and the fractions with activity were pooled. A yield of 100 µg of highly enriched *T. cruzi* epimastigote plasma-membrane Ca²⁺-ATPase (concentration about 0.5–0.8 mg/ml) was routinely obtained. These procedures were repeated with at least six different cell preparations. The EDTA contained in the active fractions eluted from the column was neutralized by the addition of MgCl₂ to a final concentration of 2 mM. CaCl₂ was added to a final concentration of 50 µM. The purified ATPase was stored under N₂ at –70 °C.

Antibody preparation

Mouse monoclonal antibody 5F10, which recognizes the Ca²⁺ pump in rat tissues, has been characterized in other studies [17,18] and was a gift from Dr. Ariel Caride and Dr. John T. Penniston (Mayo Clinic Foundation, Rochester, MN, U.S.A.). The preparation of polyclonal antiserum was as described previously [19]. Briefly, 100 µg of purified Ca²⁺-ATPase was dissolved in 1 ml of PBS and mixed with 1 ml of Freund's complete adjuvant by passing the emulsion through a syringe. This mixture was injected intradermally in the back and pads of a rabbit (of about 1.8 kg body weight). After 2 weeks the procedure was repeated, but with Freund's incomplete adjuvant. After 2 more weeks the rabbit serum was used as a source of anti-Ca²⁺-ATPase antibodies.

Electron microscopy

The plasma-membrane vesicles were fixed in 2.5% glutaraldehyde/0.1 M phosphate buffer, pH 7.2, for 60 min at room temperature. After a thorough rinse in phosphate buffer, cells were post-fixed in 1% OsO₄/0.1 M phosphate buffer, pH 7.2, at 4 °C, dehydrated in ethanol, and embedded in Epon. Ultrathin sections were made in a LKB Ultratome III ultramicrotome. Sections were stained with uranyl acetate and lead citrate and observed in a Jeol 100CX electron microscope operating at 80 kV.

Determination of ATPase activity

For the determination of ATPase activity in 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 MgCl₂, 2 mM β-mercaptoethanol, 1 mM EGTA, 1 µg/ml calcium ionophore A23187 and the appropriate concentrations of CaCl₂ to obtain the desired free Ca²⁺ concentration. Concentrations of the ionic species and complexes at equilibrium were calculated by employing an iterative computer program (M-calc, Microsoft QuickBASIC Compiler, version 5.6, 1987) modified from that described by Fabiato and Fabiato [20], taking into account the dissociation constants reported by Schwarzenbach et al. [21]. After 45 min incubation at 28 °C, the reaction was arrested by the addition of 8% (final concentration) cold 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 and SubbaRow [22], modified by the use of FeSO₄ as reducing agent. ATPase activity after purification was

monitored spectrophotometrically [12] in 1 ml of a buffer containing 10 μM free Ca^{2+} , 120 mM KCl, 30 mM Hepes/KOH, pH 7.4, 2.5 mM MgCl_2 , 1 mM ATP, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 1 unit of pyruvate kinase/ml, and 1 unit of lactic dehydrogenase/ml at 37 °C. About 1–2 μg of protein samples were added to start the reaction. Absorbance was monitored at 340 nm with reference at 430 nm using an SLM-Aminco DW2000 dual-wavelength spectrophotometer. The absorption coefficient used for NADH was $6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Phosphorylation of the *T. cruzi* plasma-membrane Ca^{2+} -ATPase

Calcium-pump phosphorylation was performed as described by Schatzmann and Burgin [23]. Accordingly, about 10 mg of plasma-membrane vesicle proteins were incubated for 15–20 s at 0 °C in a medium containing 150 mM KCl, 1 mM EGTA, 0.02 mM MgCl_2 , 0.5 mM LaCl_3 , 75 mM potassium Hepes, pH 7.4, with and without 1.232 mM CaCl_2 , in the presence of 0.019 μM [γ - ^{32}P]ATP (5000 Ci/mmol). La^{3+} was added to prevent dephosphorylation of the pump [2]. The reaction was stopped by 100-fold dilution of the incubation mixture into an ice-cold washing solution consisting of 5% trichloroacetic acid, 50 mM H_3PO_4 and 2 mM ATP. The mixture was centrifuged for 10 s in an Eppendorf centrifuge. The pellet was washed four times by centrifugation and resuspension in 1 ml of ice-cold washing medium and finally dissolved in 40 μl of 50% glycerol/9% SDS/1 mM dithiothreitol. When indicated, 0.8 M hydroxylamine in 0.6 M sodium acetate, pH 5.3, was added to the pellet and incubated for 15 min at room temperature. Then the pellet was washed three times with 150 mM KCl/50 mM KH_2PO_4 , pH 7.4. This experiment was repeated three times and Figure 2(b) shows a representative experiment.

SDS/PAGE and preparation of Western blots

The electrophoretic system used was essentially as described by Laemmli [24]. Aliquots of 10 μl of purified ATPase (about 6–7 μg of protein) were mixed with 10 μl of a buffer containing 125 mM Tris/HCl, pH 7, 10% (w/v) β -mercaptoethanol, 20% (v/v) glycerol, 4.0% (w/v) SDS and 4.0% (w/v) Bromophenol Blue as tracking dye, and were then boiled for 5 min prior to application on the gels. SDS-containing 7.5%- and 10%-polyacrylamide gels were used routinely. Acidic gels for the determination of the phosphorylated intermediate were prepared as described in [25]. Marker proteins of known molecular masses were run on the same gel. When indicated, the gels were stained using Coomassie Blue. Proteins electrophoresed through 7.5% gels were transferred to nitrocellulose by the method of Towbin et al. [26], using a Bio-Rad Transblot apparatus. After immobilization of transferred proteins on nitrocellulose, the nitrocellulose was transferred to a blocking buffer solution of 1% low-fat dried milk in PBS, pH 7.4. A 1:1000 dilution of mouse anti-(human Ca^{2+} pump) antibody 5F10 or polyclonal antiserum in blocking buffer was then applied at room temperature for 90 min. The nitrocellulose was washed four times for 4 min each before adding a 1:500 dilution of peroxidase-conjugated goat anti-mouse IgG also in PBS before adding substrate. The substrate for the peroxidase enzyme consisted of 0.5% diaminobenzidine tetrahydrochloride (grade II) in PBS plus 2% CoCl_2 and 0.1% H_2O_2 . In order to stop the reaction the nitrocellulose was transferred to water. The enhanced-chemiluminescence method (ECL detection kit) was used following the instructions of the manufacturer (Amersham Life Science). Each experiment was repeated at

least four times, and Figures 2–4 (below) show representative experiments.

Protein determination

The protein concentration of the cell suspensions and plasma-membrane fraction was determined by the biuret assay [27] in the presence of 0.2% deoxycholate, using BSA as standard. The method of Bradford [28] was used for the purified proteins.

RESULTS AND DISCUSSION

It is generally agreed that isolation of a highly enriched plasma-membrane fraction from trypanosomatids presents great practical difficulty, especially because of the presence of only one mitochondrion per cell, which occupies about 10–12% of the total cell volume and which contaminates the microsomal fraction after standard disruption procedures. This set of circumstances has for several years hampered progress in the investigation of the plasma-membrane composition of this group of parasites. The development of a method based on cell disruption by abrasion in a chilled mortar followed by differential centrifugation [29,30] has proved to be ideal to obtain a plasma-membrane fraction from these parasites composed of tightly closed vesicles suitable for the determination of Ca^{2+} transport [6–9]. Figure 1 shows a micrograph of the plasma-membrane fraction of *T. cruzi* epimastigotes. The membrane fraction consisted of empty closed vesicles with a diameter varying between 0.2 and 0.9 μm . In some cases smaller vesicles were trapped within larger ones. We did not observe microtubules attached to the membranes.

A Mg^{2+} -dependent Ca^{2+} pump has been shown to be present in *T. cruzi* epimastigote plasma-membrane vesicles [6] with similar properties to the plasma-membrane Ca^{2+} -ATPases of other eukaryotic cells. Accordingly, Figure 2(a) shows the immunological cross-reactivity of a polyclonal antiserum directed against the human red-blood-cell Ca^{2+} -ATPase with a protein of about 140 kDa (lane 1, arrow), typical of the Ca^{2+} pump [12], in the plasma-membrane vesicles of *T. cruzi* epimastigotes. The polyclonal antiserum also cross-reacted with additional bands, at 132 and 204 kDa. The presence of additional bands has been observed by other authors with different cell preparations and antibodies

Figure 1 Electron micrograph showing the general aspect of the *Trypanosoma cruzi* plasma-membrane fraction

Ultrathin sections were stained with uranyl acetate and lead citrate. The membrane fraction consists of empty closed vesicles with a diameter varying between 0.2 and 0.9 μm .

Figure 2 Characterization of the Ca^{2+} pump from *T. cruzi* plasma-membrane vesicles

(a) Western-blot analysis of the binding of a polyclonal antiserum against purified human red-blood-cell Ca^{2+} -ATPase to *T. cruzi* epimastigote plasma-membrane vesicles as detected by the chemiluminescence method. Lane 1, binding of polyclonal antiserum to plasma-membrane vesicles; lane 2, control treated with preimmune serum. The molecular mass of the band indicated by the arrow is 140 kDa. The molecular-mass (M) markers (Kaleidoscope prestained standards from Bio-Rad) were (in kDa) soybean trypsin inhibitor, 29.9; carbonic anhydrase, 42.6; bovine serum albumin, 65.0; β -galactosidase, 132.0 and myosin, 204.0. (b) Phosphorylation of the Ca^{2+} -ATPase of *T. cruzi* plasma-membrane vesicles. The phosphorylation reaction was performed as described in the Materials and methods section. Lane A, [γ - ^{32}P]ATP was added to plasma-membrane vesicles in the absence of Ca^{2+} ; lane B, the phosphorylation was carried out under the same conditions, but in the presence of Ca^{2+} ; lane C, after hydroxylamine addition. The molecular-mass (M) markers were (in kDa) carbonic anhydrase, 29; ovalbumin, 45; BSA, 66; phosphorylase *b*, 97; and human red-blood-cell Ca^{2+} -ATPase, 140.

against the plasma membrane Ca^{2+} pump [31]. These bands appear to be derived from the 140 kDa band by proteolysis and aggregation, and their presence has been discussed previously [31]. No background staining was observed when preimmune serum was used as a control (lane 2).

We have demonstrated previously [6] that the plasma-membrane Ca^{2+} -ATPase from *T. cruzi* is inhibited by low concentrations of sodium orthovanadate, which suggests that this enzyme belongs to the so called 'P-type' ionic pumps [5]. If this classification is correct, the enzyme must contain an aspartate residue which is phosphorylated during the catalytic cycle. Figure 2(b) shows that if [γ - ^{32}P]ATP is added to plasma-membrane vesicles in the absence of free Ca^{2+} (presence of EGTA), the most prominent band which appears in the autoradiogram has a molecular mass of about 100 kDa (lane A). Since the presence of a plasma-membrane H^{+} -ATPase is well documented in these parasites [6], this band could correspond to this enzyme, which in other eukaryotes has a similar molecular mass [32]. When the phosphorylation is carried out under the same conditions but in the presence of Ca^{2+} , another band of about 140 kDa becomes apparent (lane B). It can be also observed that when hydroxylamine was added in order to hydrolyse the putative phosphoanhydrous bond, both the 100 and 140 kDa bands disappear from the autoradiogram (lane C). These results strongly suggest that the phosphorylation observed in these bands corresponds to an acyl phosphate, thus supporting the notion that they correspond to the H^{+} -ATPase and Ca^{2+} -ATPase respectively. The above results also indicate that both enzymes belong to the 'P type' family of ionic pumps [5].

Plasma-membrane vesicles obtained from different fractionations were pooled and solubilized by adding Triton X-100. The solubilized material was supplemented with phosphatidylcholine and CaCl_2 and applied to a calmodulin-agarose column as described in the Materials and methods section. After elution

Figure 3 Western-blot analysis of the binding of polyclonal antiserum against purified human red-blood-cell Ca^{2+} -ATPase to purified pig red-blood-cell Ca^{2+} pump and calmodulin-affinity-purified material from *T. cruzi* plasma-membrane fraction

Lane 1, Coomassie Blue staining of partially purified *T. cruzi* Ca^{2+} pump; lane 2, Coomassie Blue staining of purified pig red-blood-cell Ca^{2+} pump; lane 3, binding of polyclonal antiserum to pig red-blood-cell Ca^{2+} pump as detected by the peroxidase method; lane 4, binding of polyclonal antiserum to *T. cruzi* Ca^{2+} pump as detected by the peroxidase method. The molecular mass of the band indicated by the arrow is 140 kDa. The molecular-mass (M) markers were (in kDa) glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, 36.0; egg albumin, 45.0; BSA, 66.0; phosphorylase *b* from rabbit muscle, 97.4; β -galactosidase from *Escherichia coli*, 116.0; and myosin from rabbit muscle, 205.0.

with a buffer containing EDTA (see the Materials and methods section) the Ca^{2+} -ATPase activity was measured in the absence or presence of calmodulin. *T. cruzi* calmodulin significantly stimulated the Ca^{2+} -ATPase activity recovered in the eluate. The control activity was 277.4 ± 73.2 nmol/min per mg of protein and the activity in the presence of $1 \mu\text{g}/\text{ml}$ of *T. cruzi* calmodulin was 506.4 ± 50.6 nmol/min per mg of protein ($n = 6$). Similar results were obtained using bovine brain calmodulin instead of *T. cruzi* calmodulin (results not shown). On the other hand, the control activity of the plasma-membrane vesicle preparations was 4.8 ± 0.27 nmol/min per mg of protein and the activity in the presence of $1 \mu\text{g}/\text{ml}$ of *T. cruzi* calmodulin was 7.53 ± 0.39 nmol/min per mg of protein ($n = 6$). These results indicate a 58-fold purification of the *T. cruzi* plasma-membrane Ca^{2+} -ATPase from the enriched plasma-membrane vesicle fraction. Since the plasma-membrane vesicle fraction was 14-fold purified with respect to the homogenate [6], the final purification obtained was about 800-fold.

Figure 3 shows the immunological cross-reactivity of the polyclonal antiserum directed against the human red-blood-cell Ca^{2+} -ATPase with a protein of about 140 kDa (lane 4, arrow) in the eluate obtained after passage of *T. cruzi* solubilized plasma-membrane proteins through a calmodulin-agarose column (see above). Lane 3 shows the reaction of the same polyclonal antiserum with purified pig red-blood-cell Ca^{2+} -ATPase. The polyclonal antiserum recognized a band of 140 kDa, and a fainter band at 132 kDa, which is also apparent in lane 4. No cross-reactivity was observed with preimmune serum used as a control (result not shown). Coomassie Blue staining of the eluate (lane 1) showed a weak band of about 132 kDa at the same position as a similar band from the purified pig red-blood-cell Ca^{2+} -ATPase (lane 2), which was also apparent in lane 4. Additional bands, most notably of 104, 101 and 62 kDa, were also observed in the *T. cruzi* eluate, which may correspond to other calmodulin-binding proteins of these parasites.

Monoclonal antibody 5F10 recognizes an epitope between amino acids 724 and 783 of the human red-blood-cell Ca^{2+} pump [33]. This epitope is located in a highly conserved hinge region

Figure 4 Western-blot analysis of monoclonal antibody 5F10 binding to purified pig red-blood-cell Ca^{2+} pump and calmodulin-affinity-purified material from *T. cruzi* plasma-membrane fraction as detected by the chemiluminescence method

Lane 1, binding of 5F10 to pig red-blood-cell Ca^{2+} pump; lane 2, binding of 5F10 to partially purified *T. cruzi* epimastigote Ca^{2+} pump. The molecular mass of the band indicated by the arrow is 140 kDa. The molecular-mass (*M*) markers were (in kDa) carbonic anhydrase, 32.5; ovalbumin, 49.5; BSA, 80.0; phosphorylase *b*, 106.0; and myosin, 205.0.

which is on the intracellular loop of the plasma-membrane Ca^{2+} -ATPase between the putative transmembrane domains M4 and M5. 5F10 reacts with all four known isoforms of the plasma-membrane Ca^{2+} -ATPase from a wide range of species and tissues [32]. Western-blot analysis of partially purified *T. cruzi* Ca^{2+} -ATPase transferred to nitrocellulose from SDS/7.5%-PAGE showed binding of the anti-(Ca^{2+} pump) antibody 5F10 to 140 and 132 kDa polypeptides (Figure 4, lane 2). Antibody 5F10 also cross-reacted with a fainter band at lower molecular mass (105 kDa). These additional bands most probably represent the natural proteolytic products of the pump described previously [31,32]. The reactivity against the purified pig red-blood-cell Ca^{2+} -ATPase is shown in Figure 4, lane 1 (arrow). No cross-reactivity was observed with ascitic fluid that did not contain 5F10 (result not shown). It is noteworthy that the patterns of reactivity of monoclonal antibody 5F10 with both the pig red-blood-cell Ca^{2+} -ATPase and the partially purified Ca^{2+} -ATPase from the *T. cruzi* plasma membrane were identical, while the pattern of reactivity of the polyclonal antibody with each of the proteins was different (Figure 3, lanes 3 and 4). This is easily explained by taking into account the fact that the monoclonal antibody recognizes a very short sequence possibly present in both preparations, while polyclonal antibodies usually recognize multiple domains in a protein. These results also indicate that, although the ATPases are similar, they are not identical.

In conclusion, our study indicates that the plasma-membrane Ca^{2+} pump present in *T. cruzi* epimastigotes is a 140 kDa protein which forms a phosphorylated intermediate. In addition, the enzyme can be partially purified using calmodulin-agarose affinity chromatography, is stimulated by calmodulin and cross-reacts with a monoclonal and polyclonal antibodies directed against epitopes of the human red-blood-cell Ca^{2+} pump. The cross-reactivity of monoclonal antibody 5F10 indicates that

there is a sequence similarity between human and *T. cruzi* plasma-membrane Ca^{2+} pumps in the region corresponding to the residues 724–783 of the human red-blood-cell sequence. The homologous region of the *T. cruzi* Ca^{2+} -ATPase with human red-blood-cell Ca^{2+} -ATPase may contribute to a crucial aspect of the Ca^{2+} -transport mechanism.

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