ELSEVIER

FEBS Letters 337 (1994) 293–297

FEBS 13545

FEBS LETTERS

Trypanosoma cruzi epimastigote forms possess a Ca^{2+}–calmodulin dependent protein kinase

S.B. Ogueta[a], A. Solari[b], M.T. Téllez-Iñón[a,*]

[a]*Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI) and Facultad de Ciencias Exactas y Naturales, UBA, Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina*

[b]*Facultad de Ciencias Médicas, UBA, Paraguay 2155, 1121 Buenos Aires, Argentina*

Received 1 November 1993; revised version received 7 December 1993

Abstract

Trypanosoma cruzi epimastigote forms showed a tightly bound Ca^{2+}–calmodulin-dependent protein kinase activity, which could be partially extracted from membranes and axonemes. The enzyme is constituted by subunits which were autophosphorylated in the absence of exogenous substrates. An antibody against CaM kinase II recognized a Ca^{2+}- or Ca^{2+}–CaM-dependent conformational epitope in these fractions. The detected bands were of molecular weights similar to the α and β subunits of the corresponding bovine brain enzyme (60 and 50 kDa).

Studies using [^{125}I]CaM revealed the presence of a CaM-binding domain. These experiments confirm that the parasite possesses a particulate CaM kinase with characteristics similar to the bovine brain enzyme.

Key words: Ca^{2+}–calmodulin protein kinase; Cytoskeleton; Flagella; *Trypanosoma cruzi;* Trypanosomatid

1. Introduction

Trypanosoma cruzi, the protozoan parasite responsible for American trypanosomiasis, Chageas' disease [1], displays different morphological and functional changes during its life-cycle. Evidence is accumulating in support of an important role for Ca^{2+}-mediated processes in trypanosomatids. Several signaling pathways regulated by this ion have been studied in *T. cruzi,* including a calmodulin and calcium-dependent cAMP phosphodiesterase [2], Ca^{2+} transport coupled to mitochondria [3] and Ca^{2+}–phospholipid-dependent protein kinase (PKC) [4], as well as a calmodulin-activated (Ca^{2+}-Mg^{2+})-ATPase [5]. Calmodulin-binding proteins have been detected in *T. cruzi* and are differentially expressed during specific stages of parasite development [6]. Most calcium effects are mediated by calmodulin through the activation of a multifunctional CaM kinase II that phosphorylates diverse target substrates [7]. This enzyme has been identified in other lower eucaryotes, such as *Saccharomyces cerevisiae* [8,9], *Aspergillus nidulans* [10] and *Neurospora crassa* [11].

A special characteristic of Trypanosomatids is their cytoskeleton, which possesses a structure unique among lower eucaryotic cells [12–14]. Ultrastructural studies revealed the presence of intermicrotubule cross-bridges and microtubule-membrane linkages due to the participation of microtubule-associated proteins (MAP's).

In this paper, we describe a particulate calcium–calmodulin-dependent protein kinase in epimastigotes of *T. cruzi,* that might be associated to the parasite cytoskeleton and flagella, and exhibits calmodulin-binding properties. In addition, the protein possesses structural homology with the CaM kinase II found in mammalian systems.

2. Materials and methods

2.1. Parasites

Epimastigotes of *Trypanosoma cruzi* of the Tulahuen strain, Tul2 stock were cultured, harvested and washed as previously described [15] and the pellet was resuspended in 3 vols. of 0.25 M sucrose and 5 mM potassium chloride. Epimastigotes were ruptured by three cycles of freeze-thawing at −20°C and the homogenate was centrifuged 15 min at 121 × *g*, at 4°C (nuclear fraction). The supernatant was centrifuged 15 min at 1,000 × *g* to obtain the flagellar fraction and again centrifuged 45 min at 105,000 × *g* to separate the membrane fraction from the cytosolic one.

* Corresponding author. Fax: (54) (1) 786 8578.

Abbreviations: ATP, adenosine 5′-triphosphate; βME, β-mercaptoethanol; cAMP, cyclic adenosine monophosphate; CaM, calmodulin; CaM kinase II, Ca^{2+}–calmodulin-dependent protein kinase type II; DTT, D,L-dithiothreitol; E_{64}, trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane; EDTA, ethylendiaminetetraacetic acid; EGTA, ethylene glycol-bis(β-amino-ethylether); PMSF, phenylmethyl-sulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLCK, Nα-*p*-tosyl-L-lysine chloro-methyl ketone.

SSDI 0014-5793(93)E1462-U

2.2. Cytoplasmic membrane purification

Membrane fractions were resuspended in buffer A (20 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM EGTA, and a cocktail of protease inhibitors; 0.1 mM PMSF, 0.5 mM TLCK, 0.1 mM E_{64}, 100 μg/ml leupeptin, 2 μg/ml soybean trypsin inhibitor, 1 mM benzamidine and 25 U/ml Trasylol), loaded in a discontinuous sucrose gradient (1.3, 1.6, 1.9, 2.2 M) and centrifuged for 2 h at 105,000 × *g* in an SW 28 rotor (Sorvall) at 4°C. The membranes, which buoyed in the 1.3 M sucrose gradient layer, were recovered and submitted to electron microscopy or determination of enzymatic activity

2.3. Purification of flagella

Flagellar fractions were resuspended in buffer A supplemented with 0.1 M sodium chloride. Aliquots were loaded on a discontinuous sucrose gradient as detailed above. Purified flagellar preparations were collected at the 1.6/1.9 M interface and their enzymatic activity determined.

2.3.1. Axoneme and flagellar membrane purification. The purified flagellar preparations were disrupted according to the method of Cunha e Silva et al. [16], with some modifications, to obtain microtubular axonemes and flagellar membranes. Briefly, the purified flagellar fractions (1.9 M) were extracted with 20 mM buffer Tris-HCl, pH 7.5, 150 mM potassium chloride, 5 mM magnesium sulfate, 1 mM EDTA, 1 mM DTT, and 2% Triton X-100. After 15 min of homogenization, the suspensions were loaded on a discontinuous sucrose gradient and centrifuged for 3 h at 105,000 × *g* in a SW40 rotor at 4°C.

Membranes recovered from the 1.3 M sucrose phase and axonemes recovered from the 1.9/2.2 M sucrose interface, were submitted to electron microscopy and determination of enzymatic activity.

2.4. Particulate enzyme solubilization

Pure membranes (total protein 34 mg) were treated with buffer A supplemented with 1% sodium cholate, 2 mM EDTA, 0.3 M sodium chloride, 10 mM βME and 50 mM potassium chloride. After Dounce homogenization every 5 min during 75 min at 4°C, the preparation was centrifuged for 30 min at 100,000 × *g* in a Ti65 rotor and the supernatant was collected and extensively dialyzed as described below.

Purified flagella (total protein 1.2 mg) were submitted to extraction in buffer A with the addition of 8 M urea, 40 mM DTT and 2 mM EDTA for 30 min at room temperature in a Dounce homogenizer, with 5 min between homogenizations. Supernatants obtained after 30 min were centrifuged at 100,000 × *g* in a Ti65 rotor, dialyzed overnight against buffer A and again centrifuged for 1 h at 130,000 × *g*. Pellets were resuspended in buffer A for enzyme assays and SDS-PAGE.

2.5. Protein kinase activity and autophosphorylation assays

The incubation mixtures contained 20 mM buffer Tris-HCl, pH 8.0, 10 mM magnesium chloride, 1 mg/ml histone IIA, 1 mg/ml bovine serum albumin, 50 μM [γ-^{32}P]ATP (1000 cpm/pmol) with or without 1 mM EGTA or 0.1 mM calcium chloride and 7 μg/ml bovine brain calmodulin in a final volume of 0.1 ml. The enzymatic assay was performed as previously described [4,17].

Fractions from the different purification steps were assayed for autophosphorylation in the absence of exogenous substrate. Aliquots of the enzymes were incubated with 5 μM [γ-^{32}P]ATP, 1 mM EGTA or 0.1 mM calcium chloride and 7 μg/ml calmodulin in the protein kinase activity assay medium. The reaction was incubated 3 min at 30°C, and stopped with cracking buffer. After heating for 2 min at 100°C samples were analyzed by 10% SDS-PAGE. Proteins were stained with Coomassie blue or silver stain. Dried gels were then exposed to film and proteins were visualized by autoradiography.

2.6. Western blot

Both of the particulate fractions obtained from *T. cruzi*, as well as bovine brain CaM kinase II, were submitted to Western blot analysis, according to the method of LeVine et al. [18]. A mouse monoclonal IgG1 antibody against the soluble form of the calmodulin-dependent protein kinase type II (CaM kinase II) was used for the immunological probing. Immune-complexes were detected with the alkaline phosphatase standard reaction using the Vectastain ABC-AP kit, according to the manufacturer's instructions.

2.7. [^{125}I]Calmodulin binding

Calmodulin-binding protein detection was performed by direct gel overlay according to the method of W.M. Wasco et al. [19], with some modifications. Particulate fraction extracts (membranes and flagella) and the bovine brain cytoskeletal fraction were submitted to 10% SDS-PAGE and blotted onto nitrocellulose sheets. Transblots were blocked overnight with 20 mM buffer Tris-HCl, pH 7.5, 200 mM sodium chloride, 1 mg/ml bovine serum albumin, 10 mM magnesium chloride and 1 mM EGTA or 2 mM calcium chloride at 4°C. [^{125}I]CaM (0.15 μCi/5 ml) was then added in the absence of albumin and incubated for 5 h at room temperature. After several washes, nitrocellulose sheets were dried and autoradiographed.

2.8. Materials

Histone HIIA and protease inhibitors (TLCK, PMSF, E_{64}, benzamidine, soybean trypsin inhibitor and leupeptin) were from Sigma. Trasylol (aprotinin) was a gift from Gador Lab., Argentina. Bovine brain calmodulin was prepared according to Téllez-Iñón et al. [2]. Purified CaM kinase II was prepared from fresh bovine brain tissue, according to the method of Kelly and Shenolikar [20]. [^{125}I]CaM and [γ-^{32}P]ATP were purchased from NEN, Dupont (USA). A mouse monoclonal antibody against CaM kinase II was a gift of Naji Sahjoun, Burroughs Welcome Lab. (USA).

3. Results and discussion

No contaminating organelles could be detected in the membrane fractions by electron microscopy (Fig. 1A). Adenylate cyclase, a tightly bound membrane enzyme in *T. cruzi* [21], was used as an enzymatic marker for this fraction. High specific activity of this enzyme was present in the membranes (data not shown).

The purified flagella from the sucrose gradients were disrupted to obtain the microtubular axonemes (Fig. 1B) and the flagellar membranes. Electron microscopy of axoneme fractions revealed only the classical 9+2 axoneme along with the paraflagellar rod structure typical of Trypanosomatids [22], indicating a high degree of purification (Fig. 1B).

Particulate fractions purified through sucrose gradients were analyzed for protein kinase activity. When histone IIA was used as substrate, both membranes and flagella exhibited a Ca^{2+}-activated protein kinase activity. Calmodulin was able to stimulate the phosphorylation in the absence of the modulator (Fig. 2A and B). The activity observed in the presence of only Ca^{2+}, could be due to an endogenous calmodulin, which detected in both fractions using calmodulin-dependent cyclic-AMP phosphodiesterase from bovine brain [2]. The isolated axonemes presented CaM kinase II activity, while flagellar membrane activity was not stimulated by the addition of the modulator (data not shown).

Trypanosomatid microtubules are cross-linked to the membrane in a special form not found in mammalian cells [11,12]. This property adds difficulty to the extraction of associated proteins. Diverse methods such as salt extraction with 1 M NaCl, detergents (e.g. sodium cholate or Triton X-100) or 8 M urea, with or without EGTA or EDTA and DTT or βME, resulted in a very poor enzyme extraction from membranes or axonemes. In contrast, the extraction of the brain enzyme yielded

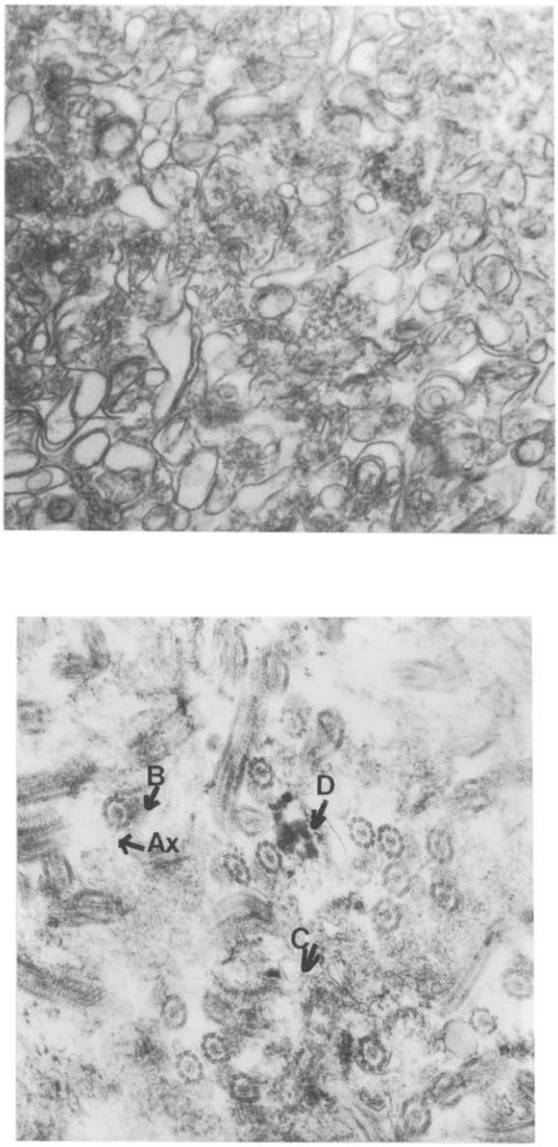

Fig. 1. Electron microscopy of *T. cruzi* fractions purified on sucrose gradients. Fractions were fixed in 25% glutaraldehyde and processed as described [25]. Purified membranes (A) and axonemes (B): Ax, axonemes; B, paraxial cylinder; C, vesicles; D, dense granules.

enough solubilized protein to allow its detection by Western blotting (data not shown) and binding of [^{125}I]CaM (Fig. 2D, lanes 4,8).

The *T. cruzi* enzyme could be partially extracted from membranes using cholate–EDTA–βME (Fig. 2A). Enzymatic activity was highly activated (over 10-fold) by the Ca^{2+}/CaM complex, and a residual protein kinase stimulated by the modulator could be observed (not shown). Enzyme extracts submitted to the autophosphorylation assay showed 3 bands of molecular weights 50, 60 and less than 26.5 kDa (Fig. 2C). The first two bands could be correlated with the α and β subunits of CaM KII. Axoneme enzyme extracted by sonication in a urea–DTT–EDTA mixture also presented protein kinase activity stimulated by CaM (4-fold).

Binding of [^{125}I]CaM was studied using the overlay technique. In the bovine brain cytoskeletal fraction as well as the enzyme extracted from that fraction, calmodulin bound specifically to both the 50 and 60 kDa bands, though with much less intensity to the latter (Fig. 2D). Direct interaction between the 50 kDa band from total membranes or flagellar extract of *T. cruzi* and [^{125}I]CaM could be detected after 30 days of exposure. In membranes, also was observed the 60 kDa band. Other calmodulin-binding proteins could be observed in *T. cruzi* fractions (Fig. 2D). No band could be detected on membrane and axoneme extracts by Western blotting. Silver stained SDS-PAGE revealed bands of 60, 58 and 50 kDa (data not shown).

The particulate fractions from *T. cruzi* were analyzed by Western blotting using the monoclonal antibody against rat brain CaM kinase II. The antibody reacted with a Ca^{2+} and calmodulin dependent epitope of the kinase subunit, different from the catalytic and calmodulin-binding sites as described by LeVine et al. [18]. Western blot analysis of the cytoskeletal fraction CaM kinase II, was used as a positive control, and revealed the 50 and 60 kDa enzyme subunits when Ca^{2+} or Ca^{2+}–CaM were present in the incubation mixture (Fig. 3, lanes 6,9). The antibody interacted with the *T. cruzi* membrane (Fig. 3, Lanes 4,7) and flagellar particulate fractions (Fig. 3, lanes 5,8) in the presence of Ca^{2+} or Ca^{2+}–CaM, even though cross-reactivity was low. In the absence of Ca^{2+} or Ca^{2+}–CaM the antibody did not recognize the CaM kinase enzyme in any of the fractions assayed (Fig. 3).

The multifunctional CaM kinase II is an enzyme that regulates many functions in mammalian cells. The enzyme is a 550,000–650,000 oligomer composed of a major 50 kDa polypeptide (α) and minor 58 and 60 kDa polypeptides (β,β'), with a subunit ratio which depends on the cellular type. Each subunit has catalytic activity and is able to bind calmodulin. Despite similar structural and immunological properties, they are not identical [23].

The presence of this enzyme in Trypanosomatids was detected in homogenous fractions of membranes and flagella. The *T. cruzi* enzyme showed the 50 and 60 kDa subunits corresponding to those of the bovine brain cCytoskeleton-associated CaM KII. These bands are Palso capable of autophosphorylation. In addition, the fractions possess Ca^{2+}–CaM-stimulated protein kinase activity and specifically bind [^{125}I]CaM, demonstrating Cthe presence of a CaM-binding domain within the enzyme.

The ratio between the α and β subunits is variable, as mentioned above. In the case of the *T. cruzi* enzyme, the antibody and [^{125}I]CaM preferentially recognized the 50 kDa peptide indicating that the ratio favored the α-subunit.

CaM kinase has not been reported previously in axonemes. There exists evidence supporting the presence of

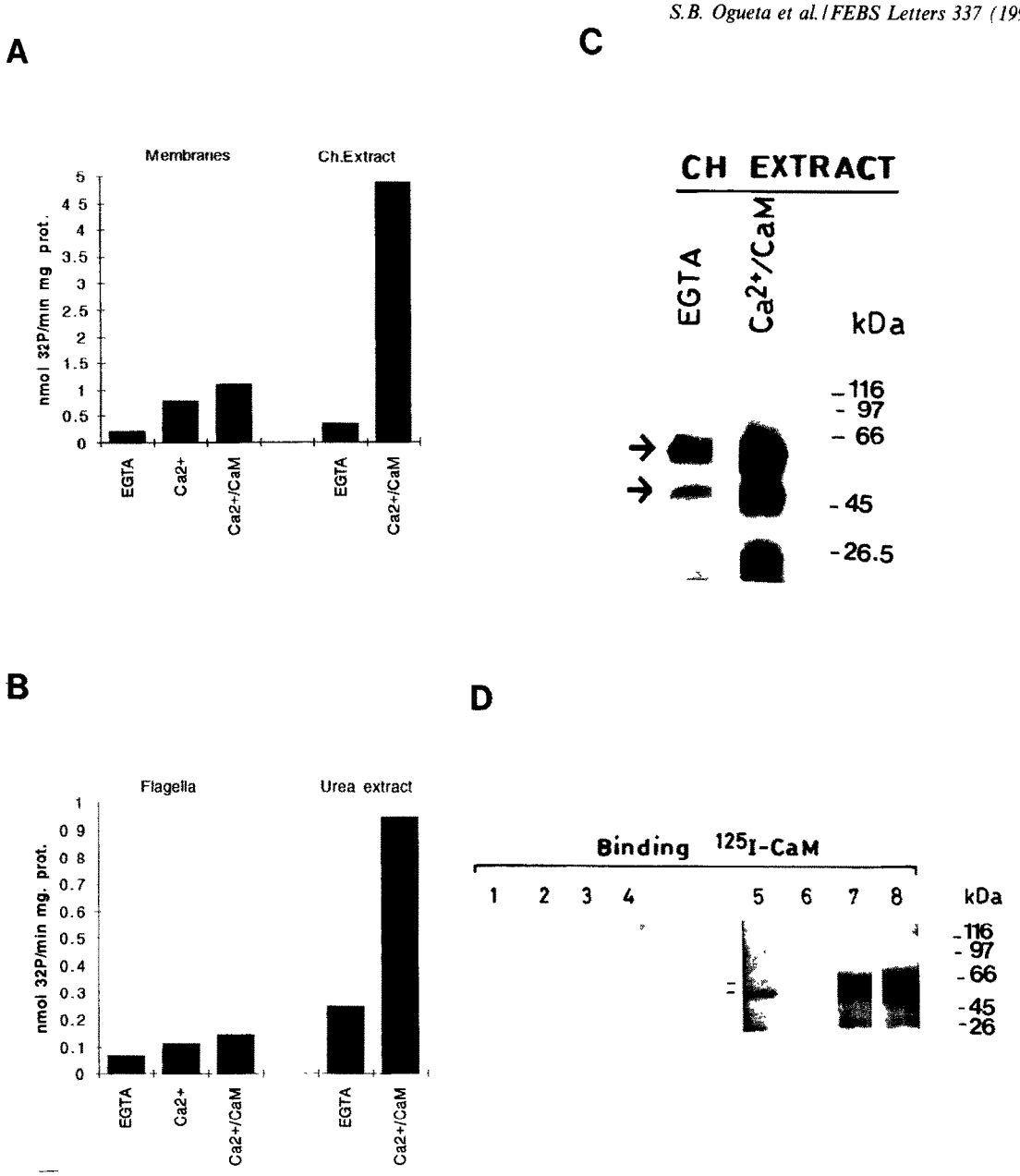

Fig. 2. Protein kinase activity of *T. cruzi* fractions. Purified membranes and the corresponding cholate extract (A); purified and urea-extracted flagella (B). PK assays were carried out in the presence of 1 mM EGTA or 0.1 mM Ca^{2+} or 0.1 mM Ca^{2+} and 7 μg/ml CaM. Values are representative of eight separate experiments. (C) Autophosphorylation of membrane cholate extract in the presence of 1 mM EGTA or 0.1 mM Ca^{2+}/7 μg/ml CaM. Arrows indicate the autophosphorylated kinase subunits. (D) Binding of [^{125}I]CaM. Nitrocellulose sheets were incubated with 1 mM EGTA (lanes 1–4) or 2 mM Ca^{2+} (lanes 5–8) as described in section 2. Lanes 1,5 membrane cholate extract; lanes 2,6, axoneme urea extract; lanes 3,7, cytoskeletal extract fraction and lane 4,8, non-extracted cytoskeletal fraction.

protein kinase A and Ca^{2+}-dependent phosphorylation of proteins associated with mammalian spermatozoa axonemes [24]. However, axoneme fractions of *T. cruzi* contain CaM kinase II, suggesting that the enzyme might play an important role in calcium-dependent events such as parasite morphogenetic differentiation, in particular in those involving flagellar movement. Future efforts will be directed towards elucidating the mechanism by which CaM kinase II controls flagellar movement in *T. cruzi*.

Acknowledgements: This work was supported by the UNDP/World Bank (WHO) Special Program for Research and Training in Tropical Diseases (TDR) and the National Research Council of Argentina (CONICET). We are indebted to Mrs. Berta Franke de Cazzulo and Dr. J.J. Cazzulo for providing *T. cruzi* epimastigotes. MTT-I and AS are Career Members of the CONICET. SBO is a fellow of University of Buenos Aires. We also wish to thank Daniela Conesa for revision of the manuscript.

References

[1] Chagas, C. (1909) Memórias do Instituto Oswaldo Cruz 1, 159–218.
[2] Téllez-Iñón, M.T., Ulloa, R.M., Torruella, M. and Torres, H.N. (1985) Mol. Biochem. Parasitol. 17, 143–153.

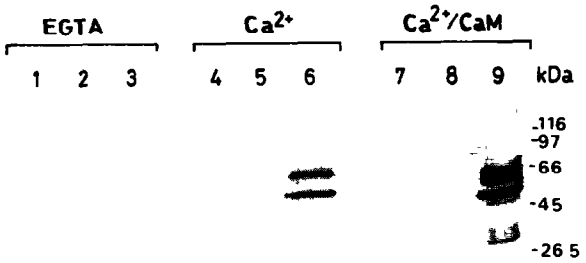

Fig. 3. Western blots of *T. cruzi* membrane and flagella fractions and the bovine brain cytoskeletal fractions. Incubations with the antibody were performed in the presence of 1 mM EGTA or 0.1 mM Ca^{2+} or 0.1 mM Ca^{2+}/5 μg/ml calmodulin. Immunecomplexes were revealed as described in section 2. Lanes 1,4,7, purified membrane; lanes 2,5,8, flagella and lanes 3,6,9, bovine brain cytoskeletal fraction. Molecular weight markers are indicated.

[3] Docampo, R. and Vercesi, A.E. (1989) J. Biol. Chem. 264, 108–111.
[4] Gómez, M.L., Erijman, L., Arauzo, S., Torres, H.N. and Téllez-Iñón, M.T. (1989) Mol. Biochem. Parasitol. 36, 101–108.
[5] Benaim, G., Losada, S., Gadelha F.R. and Docampo, R. (1991) Biochem. J. 280, 715–720.
[6] Orr, G.A., Tanowitz, H.B. and Wittner, M. (1992) Exp. Parasitol. 74, 127–133.
[7] Schulman, H. (1993) Curr. Opin. Cell Biol. 5, 247–253.
[8] Londesborough, J. and Nuutinen, M. (1987) FEBS Lett. 219, 249–253.
[9] Pausch, M.H., Kaim, D., Kunisawa, R., Admon. A. and Thorneer, J. (1991) EMBO J. 10, 1511–1522.
[10] Bartelt, D.C., Fidel, S., Farber, L.H., Wolff, D.J. and Hammel, R.L. (1988) Proc. Natl. Acad. Sci. USA 88, 3279–3283.
[11] Ulloa, R.M., Torres, H.N., Ochatt, C.M. and Téllez-Iñón, M.T. (1991) Mol. Cell. Biochem. 102, 155–163.
[12] Seebeck, T., Hemphill, A. and Lawson, D. (1990) Parasitol. Today 6, 49–52.
[13] Robinson, D., Beattie, P., Sherwin, T. and Gull, K. (1991) Methods Enzymol. 196, 285–299.
[14] Blair, D.F. and Dutcher, S.K. (1992) Curr. Opin. Genet. Dev. 2, 756–767.
[15] Cazzulo, J.J., Franke de Cazzulo, B.M., Engel, J.C. and Cannata, J.J.B. (1985) Mol. Biochem. Parasitol. 16, 329–343.
[16] Cunha e Silva, N.L., Hassan-Voloch, A. and de Souza, W. (1989) Mol. Biochem. Parasitol. 37, 129–136.
[17] Roskoski, R.Jr. (1983) Methods Enzymol. 99, 3–6.
[18] LeVine, H., Su, J.L. and Sahyoun, N.E. (1988) Biochemistry 27, 6612–6617.
[19] Wasco, W.M., Kincaid, R.L. and Orr, G.A. (1989) J. Biol. Chem. 264, 5104–5111.
[20] Kelly, P.T. and Shenolikar, S. (1987) Methods Enzymol. 139, 690–714.
[21] Torruella, M., Flawiá, M.M., Eisenschlos, C., Molina y Vedia, L.M., Rubinstein, C. and Torres, H.N. (1986) Biochem. J. 234, 145–150.
[22] Saborio, J.L., Hernandez, J.M., Narayanswami, S., Wrightsman, R., Palmer, E. and Manning, J. (1989) J. Biol. Chem. 264, 4071–4075.
[23] Sahyoun, N., LeVine, H., Bronson, D., Siegel-Greenstein, F. and Cuatrecasas, P. (1985) J. Biol. Chem. 260, 1230–1237.
[24] Horowitz, J.A., Wasco, W., Leiser, M. and Orr, G.A. (1988) J. Biol. Chem. 263, 2098–2104.
[25] Solari, A. (1980) J. Chromosoma (Berl.) 78, 239–255.