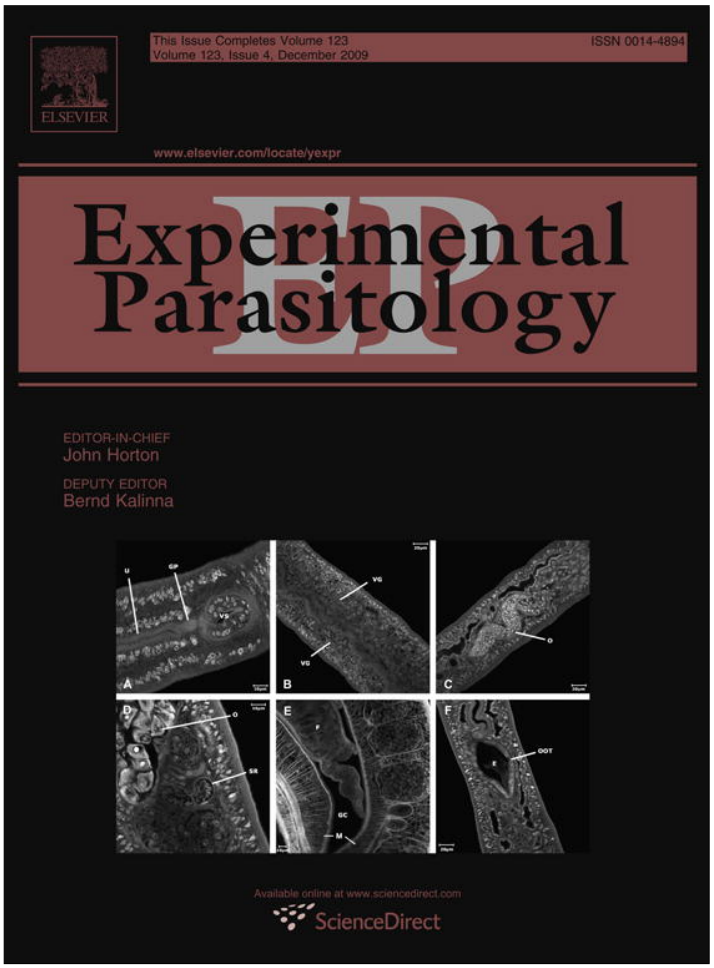

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Trypanosoma cruzi calmodulin: Cloning, expression and characterization

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ABSTRACT

We have cloned and expressed calmodulin (CaM) from *Trypanosoma cruzi,* for the first time, to obtain large amounts of protein. CaM is a very well conserved protein throughout evolution, sharing 100% amino acid sequence identity between different vertebrates and 99% between trypanosomatids. However, there is 89% amino acid sequence identity between *T. cruzi* and vertebrate CaMs. The results demonstrate significant differences between calmodulin from *T. cruzi* and mammals. First, a polyclonal antibody developed in an egg-yolk system to the *T. cruzi* CaM recognizes the autologous CaM but not the CaM from rat. Second, it undergoes a larger increase in the α-helix content upon binding with Ca^{2+}, when compared to CaM from vertebrates. Finally, two classic CaM antagonists, calmidazolium and trifluoperazine, capable of inhibiting the action of CaM in mammals when assayed on the plasma membrane Ca^{2+} pump, showed a significant loss of activity when assayed upon stimulation with the *T. cruzi* CaM.

1. Introduction

Calmodulin is one of the most ubiquitous proteins in the animal kingdom; its function has been associated with the control of many cellular processes, by sensing intracellular Ca^{2+} oscillations (Klee and Vanaman, 1982; Chin and Means, 2000). In mammals, CaM[1] has been described as a polyvalent protein that participates in many and varied cellular pathways. This protein has been implicated in the synthesis and degradation of cyclic nucleotides and phosphoinositides, phosphorylation/desphosphorylation of multiple proteins through several specific protein kinases and phosphatases, gene transcription, and regulation of different transport systems. Also, its role has been studied in the control of metabolism, cytoskeletal organization, cytokinesis, muscle contraction, osmotic cell volume regulation, exocytosis, intracellular communication, cell proliferation, differentiation, and apoptosis (Klee and Vanaman, 1982; Chin and Means, 2000; Benaim and Villalobo, 2002; Carafoli et al., 2001).

The tertiary structure of mammalian CaM was elucidated in the 80-s, using rat testis CaM as a model. CaM is a molecule of 65 Å divided into two similar globular clusters connected by a flexible α-helix. Each cluster contains two Ca^{2+}-binding regions with 12 important residues, principally glutamate and aspartate which coordinate Ca^{2+} association (Babu et al., 1985).

Concerning trypanosomatids, CaM has been found in *Trypanosoma brucei* (Ruben et al., 1983) and *Trypanosoma cruzi* (Benaim et al., 1991), as well as *Leishmania donovani* (Mazmuder et al., 1992), *Leishmania braziliensis*, and *Leishmania mexicana* (Benaim et al., 1987). It has been associated with many different functions in trypanosomatids, such as growth regulation in *T. brucei* (Eid and Sollner-Webb, 1991), cAMP-dependent phosphodiestherase stimulation in *T. cruzi* (Tellez-Iñon et al., 1985), CaM-dependent protein kinase in *T. cruzi* (Ogueta et al., 1994), signal transduction in the cGMP-nitric oxide pathway in *T. cruzi* (Paveto et al., 1995), and stimulation of plasma membrane Ca^{2+}-ATPase from *L. braziliensis* (Benaim and Romero, 1990), *L. mexicana* (Benaim, 1996), *T. cruzi* (Benaim et al., 1995), and *T. brucei* (Benaim et al., 1993).

Significant differences in its amino acid sequence have been found between *T. cruzi* (Chung and Swindel, 1990) and mammalian CaM. A protein sequence analysis showed 15 amino acid substitutions in *T. cruzi* CaM compared to its mammalian counterparts. This is a significant feature since no substitutions have been found

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[1] *Abbreviations used:* CaM, calmodulin; EGTA, [ethylene-bis (oxyethylenenitrilo)] tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HRP, horseradish peroxidase IPTG, isopropyl-1-thio-β-D-galactopiranoside; AP, alkaline phosphatase; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; OPD, *o*-phenylenediamine-peroxidase substrate; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane.

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among CaM sequences from vertebrates, which indicates that this is a very well conserved protein throughout evolution.

Results from our laboratory demonstrated that substitution of Tyr99 by Phe, prevents both phosphorylation and regulation of mammalian CaM (Benaim and Villalobo, 2002; Salas et al., 2005). Accordingly, *T. cruzi* and *L. mexicana* CaMs cannot be phosphorylated by the epidermal growth factor receptor (EGFR) due to the absence of Tyr99 (Benaim et al., 1998). When comparing CaM from different trypanosomatids with the protein from vertebrates, a large difference in electrophoretic mobility was observed by SDS–PAGE (Ruben et al., 1983; Benaim et al., 1991, 1998), despite the fact that both CaMs possess the same molecular mass. This difference was also observed on the typical Ca^{2+}-shift observed when CaMs were run in SDS–PAGE in the presence or absence of Ca^{2+}. These results indicate that, in fact, there are significant differences among these two proteins. Another related result was that a monoclonal antibody designed to the C-terminal region from mammalian CaM was unable to recognize purified CaM from *L. mexicana* and *T. cruzi* (Benaim et al., 1998).

In this work we cloned and expressed CaM from *T. cruzi*, thus allowing a further characterization of this protein. Our results confirmed unique biochemical characteristics for *T. cruzi* CaM, including production of very specific antibodies and singular properties when assayed in the presence of mammalian CaM classic antagonists. These important differences between CaM from trypanosomatids and vertebrates make this protein not only an excellent object of study from the therapeutic point of view, but also it could contribute to the understanding of the Ca^{2+} signaling regulation in these parasites.

2. Experimental procedures

2.1. Reagents

Plasmid pET15b was obtained from Novagen. Restriction enzymes NcoI, XhoI, and EcoRI, were provided by New England BioLabs. Wizard plasmid purification kit was purchased from Promega. Phenyl–Sepharose CL-4B, PMSF, ATP (sodium salt), Triton X-100, Tween 20, Hepes, IPTG, and HRP-goat anti-chicken were all obtained from Sigma. Nitrocellulose membranes were from Millipore. AP-goat anti-chicken was obtained from Santa Cruz Biotechnologies. Other chemicals used in this work were of analytical grade.

2.2. Cloning and expression of T. cruzi calmodulin in Escherichia coli

Cloning and expression of *T. cruzi* CaM in *E. coli* was based on the methodology proposed by Hayashi et al. (1998) and used to clone the rat CaM. The gene encoding *T. cruzi* CaM (GenBank Accession No. P18061 and GeneDB Accession No. $_{Tc00.1047053507483.39}$) was amplified from *T. cruzi* genomic DNA using PCR primers designed by the aid of the computer software DNAMAN®, version 5.2.2. (Lynnon Biosoft). Forward primer was designed to contain in its 5′ end the recognition site for NcoI restriction enzyme (highlighted): CaM FW 5′-CTGGAT**CCATGG**CTGATCAACTGTCC-3′. This primer also contained the initiation codon ATG (underlined) in order to insert the PCR product in frame. For the 3′ end of the protein, we designed a reverse primer containing the recognition sequence for XhoI restriction enzyme (highlighted): CaM RV 5′-GATGATG ATGAGCAAGTGA**CTCGAG**C-3′. The PCR product was digested and cloned into pET15b (Novagen) to yield a construct (pETCaMcruzi) in such a manner that both the poly-His-tag and thrombin cleavage site were excluded from the recombinant polypeptide. By using this approach, the entire coding sequence was free of any foreign fragment resulting in a recombinant polypeptide similar to the *T. cruzi* native protein. Positive recombinant constructs were confirmed by DNA sequencing. pETCM containing the rat CaM, a generous gift from Dr. Antonio Villalobo (Instituto de Investigaciones Biomedicas, Madrid, Spain, who obtained it from Prof. Nobuhiro Hayashi (Fujita Health University, Aichi, Japan).

2.3. DNA sequencing and sequence analysis

Sequencing was performed in an ABI Prism® 310 Genetic Analyzer (Applied Biosystems). The software DNAMAN®, version 5.2.2. (Lynnon Biosoft), was used to assemble DNA sequences and performed alignment analyses.

2.4. Expression and purification of recombinant calmodulin

Escherichia coli BL21(DE3) pLysS cells, transformed with pETCaMcruzi or pETCM, were used to induce the expression of *T. cruzi* and rat CaMs, respectively, following the procedure described by Studier (2005). Briefly, a pre-culture of *E. coli* grown overnight at 37 °C in LB medium under vigorous shaking (250 rpm), supplemented with ampicilin (100 μg/ml) and chloramphenicol (34 μg/ml), was diluted 1:100 in minimal medium (25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 0.5% glucose and 0.25% aspartate). After growing cells for 4 h at 37 °C with vigorous shaking, culture medium was replaced by induction medium (1% tryptone, 0.5% yeast extract, 0.5% glycerol, 0.05% glucose, 0.2% lactose, 2 mM $MgSO_4$, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4) supplemented with ampicilin (100 μg/ml) and chloramphenicol (34 μg/ml), and further incubated for 24 h at 37 °C with vigorous agitation. Recombinant CaM species were purified as described (Hayashi et al., 1998), except that soluble bacterial protein extract was heated at 95 °C for 5 min. Heat resistant proteins remaining in the supernatant were subjected to phenyl–Sepharose chromatography as previously described (Benaim et al., 1991).

2.5. Production of T. cruzi α-calmodulin IgY antibodies in chickens

Isabrown-laying hens (16-weeks-old, 2 kg body mass) were immunized intramuscularly in the breast region at multiple sites with a total amount of 200 μg of CaM from *T. cruzi* emulsified with saline and complete Freund's adjuvant (1:1 v/v). Second and third boost doses were applied with 75 μg of protein emulsified with saline and incomplete Freund's adjuvant (IFA). Booster injections were administered every ten days. Eggs were collected daily, individually identified, and store at 4 °C.

Hens were bred, maintained and cared for in accordance with the guidelines formulated by the European Community for the Use of Experimental Animals (L358-86/609/EEC).

Isolation and purification of IgY from the yolk of preimmune and hyper-immunized eggs was done according to Polson (1990) with little modifications. Briefly, 3.5% (w/v) polyethyleneglycol (PEG 6000) was added to yolk diluted in three volumes of phosphate-saline buffer (PBS) under stirring. Supernatant containing IgY was collected by centrifugation (3000*g*) at 4 °C for 20 min, and filtered through sterile gauze. Filtrate was extracted with 20% chloroform and centrifuged at 2000*g* for 15 min. Finally, an IgY precipitation step with 8.5% PEG was made using the same conditions as above. Purified IgY pellet was resuspended in PBS and stored at −20 °C.

2.6. ELISA evaluation of anti-calmodulin IgY antibody

Microtiter plate wells were coated with 10 μg/ml *T. cruzi* or rat CaM according to Ausubel et al. (1991). Then, wells were blocked with PBS containing 0.05% Tween 20 (PBS-T) plus 5% skim milk

at 37 °C for 2 h in a humidified chamber. After washing away any unbound antibody with PBS-T for 3 times, dilutions of anti-CaMTc IgY antibody (1:10, 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, and 1:5000, in PBS-T containing 1% skim milk powder) were added in triplicate. Wells were then incubated for 90 min at 37 °C in a humidified chamber, washed three times for 5 min, and finally, horseradish peroxidase (HRP)-labeled goat anti-chicken was added to each well (1:10000 in PBS-T). Plates were incubated at room temperature for 1 h at 37 °C, washed as before, and peroxidase activity was followed by adding H_2O_2 in the presence of OPD peroxidase substrate as chromogen. Reaction was measured after 20 min using a microplate reader at 490 nm.

2.7. Immunoblot analysis

Proteins separated by SDS–PAGE as described below were electrotransferred onto a nitrocellulose membrane at 300 mA for 2 h using a buffer containing 48 mM Tris (pH 8.3), 39 mM glycine, 1.3 mM sodium dodecyl sulfate, and 20% (v/v) methanol. Proteins were fixed for 45 min with 0.2% (v/v) glutaraldehyde in TBS (25 mM Tris–HCl at pH 8, 150 mM NaCl, and 2.7 mM KCl) and transiently stained with 0.1% (w/v) Ponceau's red in 50% (v/v) methanol and 10% (v/v) acetic acid. Thereafter, the membrane was blocked with 1.5% (w/v) gelatine in TBS for 2 h at 37 °C, washed extensively with 0.1% (v/v) Tween 20 in TBS (TTBS), assayed sequentially for 90 min at 37 °C with anti-CaM *T. cruzi* polyclonal antibody at 1:100 dilution, and then, goat anti-chicken antibody conjugated to alkaline phosphatase at 1:1000 dilution. Positive bands were detected using 15 mg/ml 5-bromo-4-chloro-3 indolyl phosphate (BCIP) and 30 mg/ml nitroblue tetrazolium (NBT) diluted 1:1000 in buffer containing 0.1 M Tris–HCl pH 9.5 and 0.5 mM $MgCl_2$.

2.8. Plasma membrane Ca^{2+}-ATPase assay

Plasma membranes from human erythrocytes (ghosts) devoid of calmodulin were obtained as described (Benaim et al., 1984), and the Ca^{2+}-ATPase activity determined as previously described (Colina et al., 2002). Briefly, erythrocyte ghosts aliquots (about 1–2 mg protein/ml) were incubated in a medium containing 130 mM KCl, 2 mM Hepes/KOH (pH 7.4), 1 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, and 1 mM $CaCl_2$, to obtain 10 μM free Ca^{2+} concentration. Reaction was carried out for 45 min at 37 °C, and finally, arrested by the addition of 8% (w/v) trichloroacetic acid at 4 °C. The above mixture was centrifuged and the supernatant was kept for inorganic phosphate determination. The phosphate produced by ATP hydrolysis was determined according to the method of Fiske and Subbarow (Fiske and Subbarow, 1925), modified by using $FeSO_4$ as the reducing agent (Benaim and Romero, 1990). The sensitivity of the different CaMs to trifluoperazine and calmidazolium were assessed by increasing the concentration of trifluoperazine (0–200 μM) and calmidazolium (0–100 μM) in the presence of saturating concentration of CaM from *T. cruzi* and from rat (120 nM), using plasma membranes from human erythrocytes (ghosts) devoid of calmodulin and the Ca^{2+}-ATPase activity was determined as described above.

The doses-dependence curve for each inhibitor was considered to be composed of two components: the first, with high affinity and the other with low affinity; each one representing an independent binding site for the inhibitor. This reasoning is plausible taking into account that it has been reported that calmodulin can bind trifluoperazine (TFP) with stoichiometry of 1:2 (Craven et al., 1996), and the same might be occurring here with calmidazolium. The dissection of the inhibition curve into two clear (non-overlapping) components occur if the IC50s differ from each other by a factor higher than two. A simple and reasonable analysis of the biphasic character of the relative inhibition consisted in extrapolating the linear region of each phase toward the *y*-axis, yielding $y_{max,a}$ (equal to 100%) and $y_{max,b}$ for the high and low affinity, respectively. The IC50 for each component would be then the extrapolated concentration of the inhibitor (*x*-value) for which $y = y_{max}/2$.

2.9. Other analytical procedures

Slab gel electrophoresis was performed as described by Laemmli (1970) with minor modifications. Proteins were run at 60 mA for 3 h in a 12 or 15% (w/v) SDS–PAGE gel. To attain the characteristic CaM Ca^{2+}-induced electrophoretic mobility shift (Ruben et al., 1983), we added either 5 mM EGTA or 5 mM $CaCl_2$ to the electrophoresis loading buffer. Gels were stained with Coomassie brilliant blue R-250, and dried under vacuum at 80 °C for 2 h on Whatman 3MM paper as solid support. Final protein concentration was determined as previously described (Lowry et al., 1951) using bovine serum albumin as a standard. The UV-absorption spectra for each purified CaM were made in an Agilent Spectrophotometer in the presence of 2 mM $CaCl_2$. Circular dichroism (CD) spectra were recorded from 200 to 260 nm with a Jobin Yvon Model Mark CD-6 dichrograph, using a 0.2 cm path length quartz cell. Reported spectra were recorded at room temperature and represent the averages of at least five scans. The calmodulins were solubilized in 130 mM KCl, 2 mM Hepes/KOH (pH 7.4). The secondary structure of the calmodulins was quantified using the KD2 software, as reported by Andrade et al. (1993).

3. Results

3.1. Characterization of recombinant CaM from Trypanosoma cruzi

In this work we have developed a procedure to obtain large amounts of *T. cruzi* CaM with the aim to facilitate the study of biochemical and physiological characteristics of this protein. The overexpression in *E. coli* BL21 (DE3) pLysS of recombinant CaMs from *T. cruzi* and rat was obtained by inducing with 0.2% lactose in a pH controlled medium instead of using IPTG, since it has been demonstrated that this medium allows a significant larger growth of *E. coli* (Studier, 2005) with a proportional increase in the expression of the protein of interest. This can be observed in Fig. 1A, where the arrow indicates the expression of CaM in the lane with the construct. It can be also observed in this lane a couple of new bands of 30.000–40.000 MW, probably corresponding to enzymes associated to the Lac-Operon, simultaneously expressed upon induction of the recombinant CaM gen by lactose.

When purifying to homogeneity, both recombinant polypeptides from *T. cruzi* and rat yielded relatively larger amounts of protein (10–20 mg per liter of culture) than results previously reported using IPTG instead of lactose as the inducing agent (Salas et al., 2005).

The purity of recombinant CaMs was assessed by SDS–PAGE (Fig. 1B) where a clear difference in electrophoresis mobility between these two proteins was observed, a result that was previously shown for the native *T. cruzi* CaM (Benaim et al., 1991, 1998).

The purified polypeptides were also evaluated through their UV-absorption spectra, which is very characteristic for this protein, since it possesses no tryptophan residues (Fig. 2, left panel). In our case, both proteins presented the same characteristic spectral profile as the native proteins, including the lower peak at 280 nm for *T. cruzi* CaM, due to the substitution of the Tyr99 residue by Phe in this protein (Benaim et al., 1987).

Fig. 2 (right panel) shows the CD spectra of rat (A) and *T. cruzi* (B) calmodulins in the absence of calcium (1 mM EGTA, grey) or 200 μM $CaCl_2$ (black). The CD spectrum of both calmodulins in

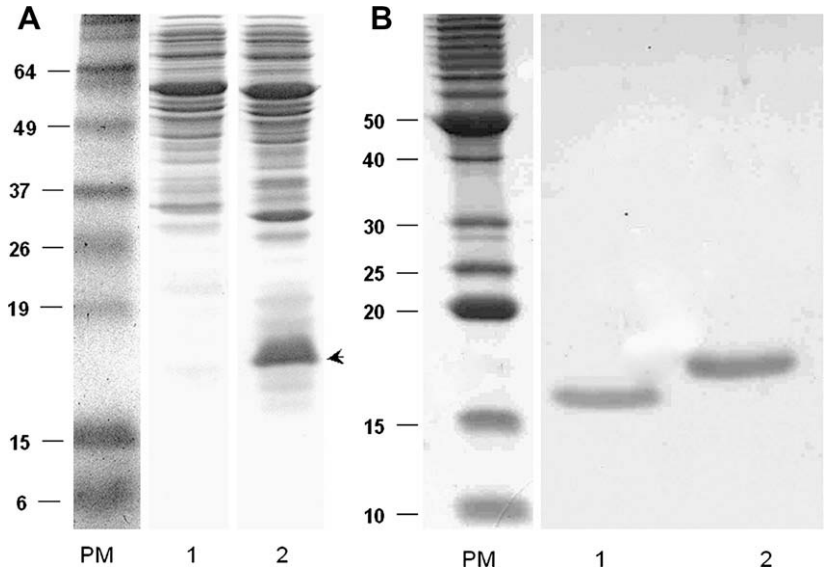

Fig. 1. Expression and purification of recombinant calmodulin from *Trypanosoma cruzi*. (A) Total cell extracts from an identical volume (15 μl) of *E. coli* BL21 (DE3) pLysS cultures, transformed with the pET15b vector (lane 1) or with pETCaMcruzi (lane 2), were incubated in the presence of 0.2% lactose for 24 h at 37 °C and resolved by 12% SDS–PAGE as described in Section 2. The arrow indicates the expression of recombinant *T. cruzi* CaM. (B) Purified *T. cruzi* recombinant CaM (lane 1) and the rat recombinant CaM (lane 2) were separated by 15% SDS–PAGE in the presence of 5 mM EGTA, and stained with Coomassie blue as described in Section 2.

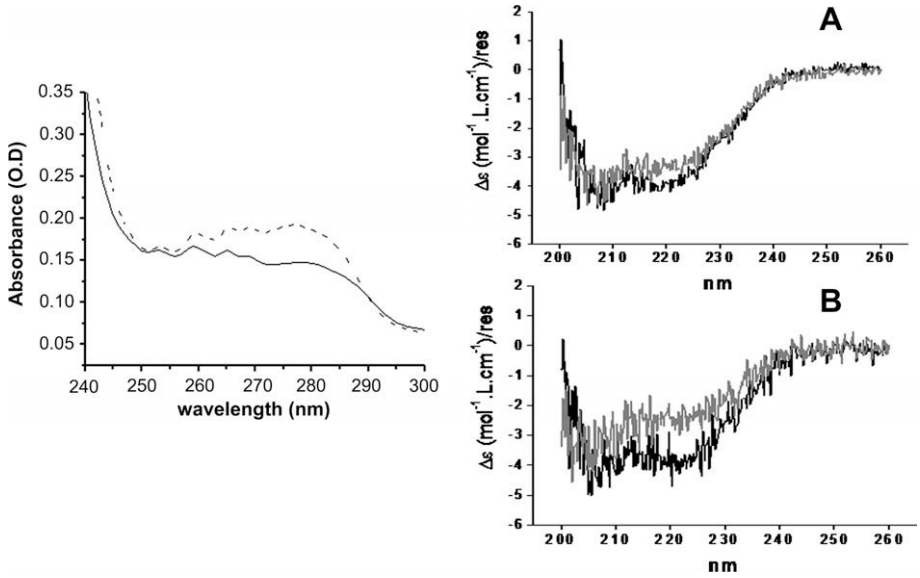

Fig. 2. Absorption spectrum and circular dicroism spectrum of calmodulin from *Trypanosoma cruzi*. The left panel shows the UV-light absorption spectra from different recombinant CaM species that were registered using a 1 mg/ml protein solution as described in Section 2. Straight line: *T. cruzi* CaM, Dotted line: Rat CaM. In the right panel appears Circular dichroism spectra from rat (A) and *T. cruzi* CaM (B), in the presence of 200 μM calcium (black) or 1 mM EGTA (grey).

the presence of Ca^{2+} showed a shape typical of α-helical proteins, with negative minima at 208 and 220 nm. As can be seen in this figure, when 1 mM of EGTA was added to remove the Ca^{2+} from the proteins, both of them yielded a decrease of their α-helical content. The values obtained for the intensity of the CD spectra in the presence of calcium were 39% and 34% α-helix conformation, for rat and *T. cruzi* spectra, respectively, whereas upon addition of EGTA the values fell to 19 for *T. cruzi* and 27% for rat CaM. Thus, there was a larger decrease (15%) in the α-helix conformation of *T. cruzi* CaM, when compared with rat CaM (12%).

3.2. Evaluation of anti-CaM T cruzi IgY antibody

During this work we have developed a specific antibody against *T. cruzi* CaM purified from hen eggs. In order to evaluate the specificity of this anti-CaM*T cruzi* IgY, an ELISA plate was sensitized using 10 μg/ml of CaM from both *T. cruzi* and rat recombinant proteins. When the antibody was tested at different dilutions, we found a specific response to its own antigen. Thus, at a 1:200 dilution, the antibody was not able to detect the rat CaM (Fig. 3, left panel), but still recognize the *T. cruzi* CaM even at 1:1000, when compared with its preimmune antibody response. This is further supported by the experiments shown in the same figure, next panel. Thus, when anti-CaM *T. cruzi* antibodies (1:100) were used in a Western blot (Fig. 3, right panel), even though we observed an evident recognition of *T cruzi* CaM as expected, the antibody did not recognize the rat CaM. Taking these results together, we conclude that, besides the high similarity between both CaM species, there must be important structural differences that might have a significant impact on *T. cruzi* physiological function. For compari-

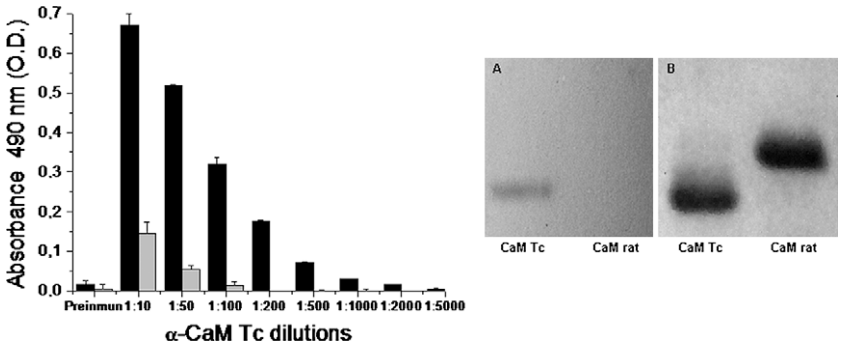

Fig. 3. Evaluation of anti-CaMTc antibodies against different calmodulins. In the left panel the affinity of anti-CaMTc IgY antibodies was determined against 10 μg/ml *T. cruzi* (black) and 10 μg/ml rat CaM (light gray) by an ELISA assay as described in Section 2. In the right panel the specificity of the antibodies was evaluated by Western blot, (A) *T. cruzi* (10 μg) and rat CaMs (10 μg) were fractionated on a SDS–PAGE gel, transferred onto a nitrocellulose membrane and immunodetected with the anti-CaM antibody from *T. cruzi* (dilution 1:100), (B) Corresponding SDS–PAGE gel stained with Coomassie blue.

son purposes, in (B) from right panel Fig. 3 it can be observed the Coomasie blue-stained gel of both purified CaMs at the same concentration used for the blotting shown in (A), where the reported large shift to the anode of *T. cruzi* CaM is readily seen.

3.3. Activation of plasma membrane Ca^{2+}-ATPase by recombinant *T. cruzi* CaM

With the aim to establish the functionality of recombinant CaMs and to compare their relative potency on a well known target, we used a classical functional assay for this protein, namely, activation of plasma membrane Ca^{2+}-ATPase (PMCA) from human erythrocytes (Benaim et al., 1984). This enzyme typically duplicates its activity when assayed in the presence of CaM. As seen in Fig. 4, *T. cruzi* CaM is also capable of stimulating PMCA with a similar kinetic to the one obtained with the mammalian CaM. However, the PMCA displayed a lower affinity for *T. cruzi* CaM (K_a 9.91 ± 0.53) when compared to the stimulation obtained with rat CaM (K_a 7.02 ± 0.76) (Fig. 4). This change is small but statistically significant ($p \leqslant 0.01$). Concerning the maximal velocity of the enzyme, it can be observed that *T. cruzi* CaM is only able to reach 80% (1.70 ± 0.15 μM) of the value obtained with rat CaM (2.13 ± 0.15 μM), even at optimal concentration.

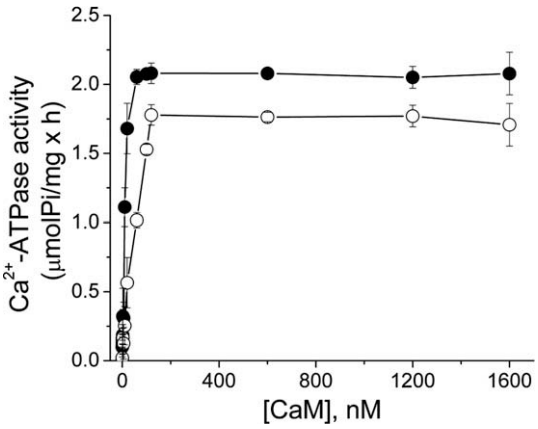

Fig. 4. Stimulation of plasma membrane Ca^{2+}-ATPase activity by different calmodulins. The stimulation of plasmatic membrane Ca^{2+}-ATPase activity was performed at 10 μM Ca^{2+} concentration at the indicated CaM concentrations. The basal value of the plasma membrane Ca^{2+}-ATPase activity (2.1 ± 0.2 μmoles/mg prot × h.) was subtracted from both curves. (●) Rat CaM, (○) *T. cruzi* CaM. Reaction medium and Pi determination are explained in the Section 2.

3.4. Sensitivity of *T. cruzi* CaM to mammal CaM antagonists

To look for other possible differences between *T. cruzi* and human CaM, we carried out experiments with two well known mammal CaM antagonists, namely trifluoperazine and calmidazolium using the stimulation of PMCA by CaM as a tool. The results showed (Fig. 5) that *T. cruzi* CaM is inhibited by these two classical CaM antagonists, but with a significantly smaller sensitivity. As seen in Fig. 5A, the inhibition of CaM stimulated PMCA activity exerted by trifluoperazine appears to present a biphasic kinetic. This result was not unexpected since trifluoperazine binds to CaM with a stoichiometry of 2:1 (Craven et al., 1996). For this reason, we calculated two IC_{50}, one for each slope of the curve, in order to improve the quantification of the possible difference of affinities among both CaMs. Indeed, the lower affinity of trifluoperazine to CaM from *T. cruzi* now became more evident. Thus, the two inhibitory values were 28.48 ± 0.3 μM (IC_{50}a) and 51.05 ± 1.00 μM (IC_{50}b) for *T. cruzi* CaM, at least twice the value obtained for mammal CaM (IC_{50}a 10.62 ± 1.04 μM and IC_{50}b 21.73 ± 2.11 μM).

Calmidazolium showed similar results to those observed with trifluoperazine (Fig. 5B). Accordingly, this antagonist also appears to present a biphasic kinetic. So we proceeded to calculate the IC_{50s} as was done for trifluoperazine. The values obtained also indicated a lower affinity of *T. cruzi* CaM (IC_{50}a 48.07 ± 1.32 μM and IC_{50}b 108.42 ± 6.18 μM) when compared to the values obtained for rat CaM (IC_{50}a 23.18 ± 1.38 μM and IC_{50}b 90.6 ± 6.34 μM). Taken together, the above results demonstrated other significant differences among these CaMs.

4. Discussion

In this work we have made a contribution to the study of *T. cruzi* CaM from two different approaches. Firstly, by developing an expression system in *E. coli* to obtain high amounts of protein, and secondly, by producing an antibody capable of distinguishing *T. cruzi* CaM from the mammalian counterpart, allowing multiple immunoassays (detection, localization, complex formation, etc.) for further studies.

Our cloning strategy, based on Hayashi et al. (1998), allowed the production of *T. cruzi* CaM with the vector pET15b, which confers a tighter regulation of gene expression than pET14b. Our cloning strategy also permitted the insertion of the PCR product containing the full *T. cruzi* CaM coding sequence without the fusion of additional sequences such as the His-tag and thrombin cleavage site, generally expressed as fused polypeptide sequences to facilitate downstream purification of the recombinant protein. By inserting the recognition sites for NcoI and XhoI into the 5′ termi-

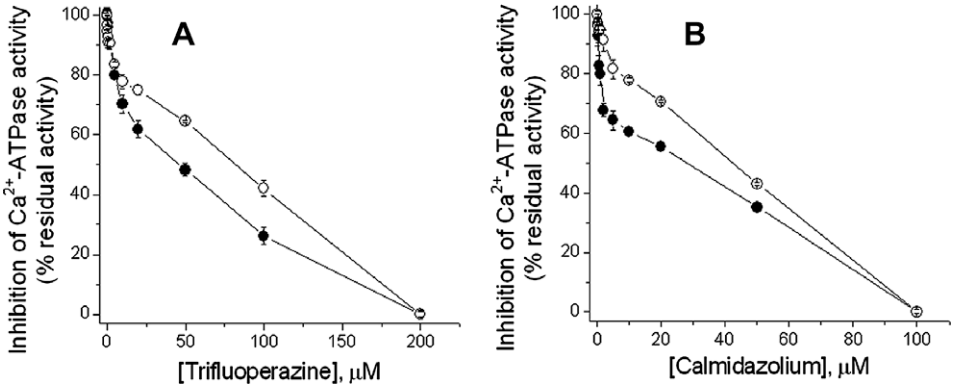

Fig. 5. Inhibition of Ca^{2+}-ATPase activity by trifluoperazine and calmidazolium in the presence of calmodulin from *Trypanosoma cruzi*. (A) Percentage residual activity of PMCA by trifluoperazine inhibition; (B) Percentage residual activity of PMCA by calmidazolium inhibition. In both panels (●) Rat CaM, (○) *T. cruzi* CaM. Values represent the means ± SD from at least five independent experiments. Reaction medium and Pi determination are described at the Section 2.

nal end of the primers we could clone the PCR fragment in frame with the initiation and termination codons from the *T. cruzi* CaM gene. This is an important goal since we intended to obtain a protein as close as possible to the native form. Although the expression construct allowed us to produce the *T. cruzi* CaM with its native primary sequence, it is still important to evaluate whether post-translational modifications, such as those reported for other eukaryotes, could be playing important roles on the enzyme function (Benaim and Villalobo, 2002). However, the obtained protein represents a good beginning point.

The cumulative evidence indicates that CaM from trypanosomatids can be considered a different protein from its mammalian counterpart in terms of its primary sequence, which is probably affecting their secondary or tertiary structure, as revealed by the dissimilar gel electrophoretic mobility (Benaim et al., 1998) and the larger variation in the circular dichorism spectra amplitude induced by the presence of Ca^{2+} (this work, see below). It is known that some proteins can maintain a certain degree of their native structure even under denaturing conditions, present in polyacrylamide gels (Burgess et al., 1980).

Concerning the larger electrophoresis mobility of *T. cruzi* CaM when compared to mammal CaM it is predictable that the 15 amino acidic substitutions will confer to the *T. cruzi* CaM a more compact structure and/or a more hydrophobic behavior so that the protein could bind more SDS per mol generating a more negative net charge and hence, more attraction to the anode. In this context, we have previously noticed that a substitution of Tyr99 by Phe in CaM from mammals is also translated into a faster migration in SDS–PAGE gels (Salas et al., 2005); so does Tyr138 for Phe, which induces either a larger condensation of the molecule or a higher degree of hydrophobicity. Indeed, it is interesting to note that *T. cruzi* CaM presents Phe99 instead of Tyr. Thus, this change in this amino acid could contribute to the faster migration of *T. cruzi* CaM.

Analyzing the hydropathy profile of the two CaMs, we found three regions that involve differences in the hydrophobicity of the related amino acids. The first region is between amino acids 53–65, corresponding to the second Ca^{2+} binding domain of this protein. In this region, the *T. cruzi* CaM reaches smaller values in the hydropathy profile, probably due to an Asn61 substitution by Ser. This substitution is potentially important since asparagine is one of the three amino acids that coordinate the binding of Ca^{2+} to this domain (Cheung, 1982). Moreover, serine is a potential target for phosphotylation by different serine–threonine kinases (Benaim and Villalobo, 2002). The second region in the hydrophathy profile corresponds to the amino acids 78–87, where values from *T. cruzi* CaM are smaller than the mammalian counterpart. This region corresponds to the central core α-helix from the protein, where three amino acids substitutions were observed, Lys77Gln, Thr79Ser, and Lys86Arg. This region is important for the folding of the protein upon interaction with its target proteins. All these modifications could contribute to a more hydrophobic character of the *T. cruzi* CaM as a whole.

The third region which presents differences is located in the C-terminal domain. However, in this case, the *T. cruzi* CaM shows higher values in the respective amino acids than its counterpart in mammals. Accordingly, we had already reported evidence of structural differences using monoclonal antibodies raised against this region from mammal CaM (Benaim et al., 1998).

The UV-light spectra from *T. cruzi* CaM is characterized by the absence of tryptophan, four peaks corresponding to the fine structure of phenylalanine, and a shoulder (instead of a peak) observed in mammal CaM at 276 nm, corresponding to a Tyr (Benaim et al., 1987). The *T. cruzi* CaM obtained in this work by recombinant expression showed the same spectra as the native form.

Taking advantage of the relatively large amounts of CaM obtained, we were able to perform for the first time the CD spectrum of *T. cruzi* CaM, showing that the shape of the CD spectra obtained for rat CaM and this protein were similar. The values obtained for the intensity of the CD spectra of both calmodulins in the presence of Ca^{2+} are comparable to those reported in the literature for mammalian CaM (Martin and Bayley, 1986). However, the variation in the spectra amplitude induced by the presence of Ca^{2+} appears to be larger in the case of *T. cruzi* CaM, when compared to its mammalian counterpart. This effect again points out significant differences among CaMs from parasites and host cells. The larger amplitude of the CD spectra is related to an increase in the α-helix content of the protein under this condition, which in turn is related to an increase in the hydrophobic character of the protein. As mentioned before, since the migration velocity in a SDS–PAGE gel strongly depends on the hydrophobicity of the protein, it is conceivable that the faster migration observed by *T. cruzi* CaM is due to a larger α-helix content of the protein, at least in the presence of Ca^{2+}.

The use of hens for producing antibodies is a methodology described 50 years ago as a reliable alternative to animal bleeding (Russel and Burch, 1995). Even so, it was not until the mid 1990s that it becomes a massively used protocol known as "IgY Technology" (Schade et al., 1997). This methodology introduced great advantages in animal care and manipulation, as well as downstream antibody purification processes. Even more, this procedure can generate higher yield and more specific antibodies than traditional sera obtained from mice or rabbits (Ohinishi et al., 2000; Straumann-Kunz et al., 1991). In this context, it is worthwhile to mention that CaM has been widely recognized as a very poor antigenic protein, probably due to its highly conserved structure

throughout evolution. (Nagai et al., 1996), Thus, in our case, the "IgY Technology" represents a main success in the production of an antibody against CaM, even more if we take into account that the antibody shows a significant specificity to its antigen.

A comparative study of the two CaMs, using two distinct biochemical techniques and using anti-CaMTc, demonstrated a clear difference between these two proteins (Benaim et al., 1998). This kind of study was carried out with a monoclonal antibody that recognized the C-terminal part from mammal CaM, but it did not recognize CaM from *L. mexicana*. However, in this work we produced a polyclonal antibody capable of recognizing its homolog antigen with a higher affinity than its vertebrate counterpart.

Even though the *T. cruzi* CaM has been extensively studied, little is known with respect to its real role in the Ca^{2+} regulation of these parasites. Using the recombinant CaM from *T. cruzi*, we could observe its lesser capacity to stimulate the human plasma membrane Ca^{2+}-ATPase, compared to its analogous CaM from rat.

On the other hand, when we evaluated the inhibitory capacity of two classical vertebrate CaM antagonists (trifluoperazine and calmidazolium) on PMCA stimulation from human erythrocytes, we found that the *T. cruzi* CaM depicted a relatively minor sensitivity. In this sense, it is conceivable to find in the future some CaM antagonist with a much higher affinity for the CaM from the parasites, so that it could be rationally used in therapy against these parasitic infections, supporting the notion that a deeper study on the protein should be done. Since CaMs from other trypanosomatids, like *Leishmania sp*, are identical among them, the above possibility could be extended to other infections caused by these parasites. The evidence above also corroborates the effect that a small number of amino acid substitutions can exert on the physiological behavior of calmodulin.

Trifluoperazine is known to bind to calmodulin from vertebrates with a stoichiometry of 2:1. (Craven et al., 1996). This fact is in very good agreement with our results, where we obtained a biphasic kinetic (two slopes in Fig. 5), when we studied the inhibition by trifluoperazine of the stimulation of Ca^{2+}-ATPase activity by calmodulin from both sources. To our knowledge, there is no information related the stoichiometry of calmidazolium with CaM. However, since the biphasic response observed with trifluopoerazine was also obtained with this antagonist, the data strongly suggests that calmidazolium should bind to CaM with a stoichiometry of 2:1.

In conclusion, in this work we demonstrated that the CaM from parasites is different to the protein from vertebrates in several respects. First it shows a differential antigenic recognition. Second, it undergoes a larger increase in the α-helix content upon binding of Ca^{2+}, when compared to CaM from vertebrates and finally, it shows a different level of inhibition by classical CaM antagonists.

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