Ca^{2+} and calmodulin-dependent protein phosphatase from *Leishmania donovani*

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SUMMARY

A protein phosphatase exclusively dependent upon micromolar amounts of Ca^{2+} and calmodulin has been identified and partially purified from *Leishmania* spp. Complete obliteration of its activity is observed in the presence of calmodulin antagonists such as trifluoperazine, fluphenazine and calmidazolium. Relative insensitivity to okadaic acid and lack of activation in the absence of Ca^{2+} and calmodulin distinguishes this enzyme from PP1, PP2A and PP2C-type protein phosphatases. Cross-reactivity of the enzyme was observed with antibodies that recognize both the A and B chains of calcineurin, a PP2B type Ca^{2+} and calmodulin-dependent phosphatase from brain. FK506, an immunosuppresive drug that inhibits the enzyme from other sources inhbited the enzyme only in the presence of exogenous FK binding protein, whereas Cyclosporin A inhibited the enzyme in crude preparations. Taken together these results reveal the presence of a Ca^{2+} and calmodulin-dependent phosphatase from *Leishmania*. This is the first report of the presence of a PP2B-type protein phosphatase from a pathogenic protozoa.

Key words: *Leishmania* spp., protein phosphatase 2B, promastigote, FK506, visceral leishmaniasis, dephosphorylation, cyclosporin A.

INTRODUCTION

Leishmania donovani is the protozoal pathogen responsible for the fatal human disease, kala-azar or visceral leishmaniasis (WHO, 1987), though *Leishmania tropica* has also been implicated in a few cases (Sacks *et al*. 1995). The parasite displays different morphological and functional changes during its life-cycle, existing in the sandfly as motile promastigotes but transforming into non-motile amastigotes after entry into mononuclear cells. Little is known about the molecular signal(s) that the parasite senses in the host macrophage and the subsequent transduction, processing and amplification of the signal that lead to the crucial morphogenic and pathogenic transformations.

Calcium ion is a major intracellular signal through which external stimuli evoke a variety of short-term and long-term responses in eukaryotic cells (Carafoli, 1987). Although, the physiological role of Ca^{2+} as a second messenger in *Leishmania* species has not yet been established, evidence is rapidly accumulating to suggest the presence of an elaborate machinery for maintaining Ca^{2+} homeostasis in the cytoplasm of the parasite. A plasma membrane high affinity Ca^{2+}-ATPase, acting as an extrusion pump for Ca^{2+}, has been characterized in our laboratory. The activity of the enzyme is regulated by endogenous calmodulin (CaM) (Ghosh *et al*. 1990; Mazumdar *et al*. 1992; Mondal *et al*. 1997). *Leishmania* spp. contain at least 2 intracellular organelles that can store Ca^{2+} (Ruben, Hutchinson & Moshlman, 1991; Vercesi & Docampo, 1992; Sarkar & Bhaduri, 1995; Cohen, 1989). Most interestingly, an upward shift of external temperature as is encountered by the parasite in the host millieu, results in a rapid rise in free cytoplasmic Ca^{2+} i.e. $[Ca^{2+}]_i$ (Sarkar & Bhaduri, 1995). Such transitory rise in $[Ca^{2+}]_i$ is known to modulate activities of target protein kinases or phosphatases that help in further processing of the message.

Ca^{2+}/calmodulin-dependent phosphatases, also known generally as phosphatase 2B, are presumed to be important components of the interlocked cascade systems in many cell types across the eukaryotic phylogenetic scale (Cohen, 1989; Stemmer & Klee, 1991; Mumby & Walter, 1993). In mammals, this enzyme is most abundant in brain (Stemmer & Klee, 1991). It has also been implicated in the activation of T cells (Schreiber, 1992; Liu *et al*. 1992). The enzyme is apparently expressed ubiquitously in eukaryotes, including *Drosophila* (Krinks, Manalan & Klee, 1985) and yeast (Cyert *et al*. 1991). Notwithstanding its wide occurrence, the specific role of this enzyme in performing any defined function of a cellular system remains to be elucidated.

In this paper, we report the presence of a protein phosphatase in the cytosol of *Leishmania* promastigotes that is exclusively dependent on Ca^{2+} and calmodulin for its activity. Initial characterization of this enzyme indicates that it is broadly

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similar in properties to the protein phosphatase 2B as isolated from various sources. This is the first report of the presence of a Ca^{2+} and calmodulin-dependent phosphatase in a protozol pathogen. These results taken together with earlier information (Ghosh *et al*. 1990; Ruben *et al*. 1991; Vercesi & Docampo, 1992; Mazumdar *et al*. 1992; Sarkar & Bhaduri, 1995; Mondal *et al*. 1997) strongly implicate Ca^{2+} as a regulatory component in the life-cycle of *Leishmania* parasite.

MATERIALS AND METHODS

Parasites

The *Leishmania* strains used in this work are clinical isolates from confirmed kala-azar patients. Virulent and pathogenic *Leishmania* strains slowly loose their virulence on repeated subculturing in a suitable medium. The strain MHOM/IN/1978/UR6 which has been used in the majority of the experiments are grown for 72 h (late exponential phase) on solid blood agar plates. Repeated subcultures have resulted in loss of virulence of this strain. Harvested UR6 cells were washed 3 times with Tris-buffered sucrose (25 mM Tris–HCl, pH 7·4, and 0·25 M sucrose) and kept at −20 °C until use. The virulent strain used in some studies is designated as MHOM/IN/83/AG83. This strain is subcultured in liquid medium (Medium199 + 10% FBS) with frequent animal subpassages to maintain its virulence.

Preparation of leishmanial protein kinase

Cytosolic protein kinase from *Leishmania* (UR6), which phosphorylates only the serine residues of protamine and histones was prepared as described earlier (Banerjee & Sarkar, 1990). This enzyme was used for the preparation of phosphorylated histone and protamine as described below. The catalytic and regulatory subunits of bovine heart cAMP-dependent protein kinase were prepared as described (Erlichman *et al*. 1980).

Preparation of partially purified Ca^{2+}/CaM-dependent phosphatase

For the studies of Ca^{2+}/CaM-dependent phosphatase from *Leishmania*, the UR6 strain was routinely used as it can be grown easily. The kinetic properties (data not shown) and subunit molecular weight of this enzyme from strain AG83 were found to be very similar as described later. Frozen packed UR6 cells (1×10^{11} cells) were suspended in 50 ml of 20 mM Tris–HCl buffer, pH 7·5 (RT) containing 2 mM EDTA, 5 mM benzamidine–HCl, 20 mM β-mercaptoethanol, 1 mM phenylmethylsufonyl fluoride, 10 μg/ml leupeptin, 10% glycerol (Buffer A). The cells were stirred for 1 h at 4 °C and extracted as described elsewhere (Banerjee & Sarkar, 1990). Briefly, the cell suspension was homogenized (15 strokes at 1200 rpm in a Potter S homogenizer (Braun Instruments)) and sonicated (45–60 W, 4 times, 20 sec intervals between each sonication in Labsonic 2000 Sonifier (Braun Instruments)). The homogenate was centrifuged at 100000 ***g*** for 60 min. The supernatant fraction was carefully removed and solid ammonium sulfate was added to bring the degree of saturation to 70%, maintaining the pH at 7·4 by adding 1 M ammonium hydroxide. After standing for 30 min, the suspension was centrifuged for 15 min at 13000 ***g***. The supernatant fraction was discarded. The precipitate was resuspended in 3 ml Buffer A and clarified by centrifugation for 10 min at 13000 ***g***. The clear supernatant was layered onto a Sephadex G-100 column ($1{\cdot}3 \times 96$ cm) which was equilibrated with Buffer B (Buffer A containing 150 mM NaCl). The sample was eluted with the same buffer and fractions (about 3·5 ml) were collected. All characterization was done with fraction 18 as it contained the major Ca^{2+}/CaM-dependent phosphatase activity (Fig. 1).

Preparation of phosphorylated substrates

Protamine and histone type II-AS were phosphorylated as described earlier (Banerjee & Sarkar, 1990) with 100 μM [γ-^{32}P]ATP (specific activity 500–700 cpm/pmol). Phosphorylated proteins were isolated according to Meisler & Langan (1966). Phosphorylation by cAMP-dependent protein kinase and isolation of phosphorylated casein was done as described by McGowan & Cohen (1988). [^{32}P]myosin light chains (specific activity 1000 cpm/pmol) were prepared as described (Klee *et al*. 1983). RII peptide, corresponding to a sequence in the RII subunit of cAMP-dependent protein kinase (Blumenthal *et al*. 1986) and regulatory subunit of bovine brain cAMP dependent protein kinase were phosphorylated with the corresponding catalytic subunit in the presence of 100 μM [γ-^{32}P]ATP (specific activity 200–400 cpm/pmol) (Fruman *et al*. 1992). [^{32}P]RII was extensively dialysed against 20 mM MOPS, pH 7·0, containing 20 mM β-mercaptoethanol. [^{32}P]RII peptide was treated with DOWEX AG1X8 (acetate form) in 30% acetic acid. The eluate was lyophilized and washed several times with water to remove residual acetate. The final suspension was passed through a Chelex-100 column to remove any residual Mg^{2+} or Ca^{2+}.

Enzyme assay

The Ca^{2+}/CaM-dependent phosphatase was routinely assayed with [^{32}P]protamine as substrate. The standard reaction mixture contained 50 mM Tris–HCl, pH 7·5, 0·5 mg/ml BSA, 1 mM EGTA, 10 mM $Mg(CH_3COO)_2$, 0·5 mM DTT and 6 μM [^{32}P]protamine (on the basis of the amount of [^{32}P] incorporated) in the absence or presence of 400 μM

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free Ca^{2+} over EGTA (calculated as described by Sillen & Martell (1971)) and 5 μM CaM. Appropriate amounts of enzyme extract were added in a final volume of 50 μl in microfuge tubes. Reactions were carried out for 5 min at 37 °C in the case of protein substrates and 15 min at 30 °C for peptides. With protamine and histone substrates, reactions were terminated by addition of 20 μl of ice-cold 80 mM silicotungstic acid containing 40 mM H_2SO_4 and 0·5 mM inorganic phosphate, followed by 40 μl of 1 mg/ml BSA. The samples were spun for 2 min at 13000 rpm in an Eppendorf centrifuge. The 75 μl of the supernatant (containing released P_i) were spotted onto Whatman 3MM (2 × 2 cm) filter papers, dried and quantitated by liquid scintillation spectrometry. Reactions with casein and RII were stopped by adding 20 μl of 50 % ice-cold TCA (v/v) followed by 40 μl of 1 mg/ml BSA and quantitated as discussed above. Reactions with RII-peptide and myosin light chains were terminated by DOWEX cation-exchange chromatography (Blumenthal *et al*. 1986). Reactions with PNPP were performed as described by Pallen & Wang (1983). Blank assays were performed for all substrates without enzyme. Assays were linear up to 20 % phosphate released and the extent of dephosphorylation was therefore kept within this limit. All the [^{32}P] radioactivity released could be extracted into acid-molybdate. This demonstrated that P_i and not phosphopeptides were released by proteinases. Ca^{2+}/CaM-dependent phosphatase activity was taken as the difference between the activity measured in the presence of Ca^{2+} and CaM and that measured in the presence of EGTA. The finding that trifluoperazine (TFP), a CaM antagonist inhibited this Ca^{2+}/CaM stimulated activity validated such measurements. TFP (150 μM) does not affect the activity of other protein phosphatases. Any variation from the standard assay is described in the appropriate legends.

Immunoblotting

Proteins were resolved by SDS–PAGE (Laemmli, 1970) and the resolved polypeptides were transferred to nitrocellulose as described by Towbin *et al*. (1979) for 3 h at 60 V. Cross-reactive bands were detected using rabbit anti-bovine calcineurin antiserum (1:1000) and alkaline phosphatase-conjugated protein A as described by Blake *et al*. (1984).

Materials

[γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Bhaba Atomic Research Center, Bombay, India, Calmodulin, myosin light chains (smooth muscle) and CaM kinaseII were gifts from Drs T. Soderling and S. Mukherjee (Vollum Institute, Oregon, USA). Polyclonal rabbit antisera against brain calcineurin and RII peptide (DLDVPIPGRFDRRVSVAAE) were generously provided by Dr C. B. Klee (N.I.H., Bethesda, USA). Cyclosporin A (CsA) and FK506 were gifts from Sandoz Pharma and Fujisawa Pharmaceuticals, respectively. Purified calcineurin, histone typeIIAS, protamine sulfate, dephosphorylated casein, PNPP, FK binding protein, Protein A-alkaline phosphatase were all from Sigma Chemical Co., USA. All other reagents were of analytical grade.

RESULTS

Fig. 1A shows the elution profile of the cytosolic extract of strain UR6 from a Sephadex G-100 column. Two peaks of phosphatase activity were obtained. One of the peaks (fractions 17–19) was absolutely dependent on the simultaneous presence of Ca^{2+} and CaM for activation. The other was a widely separated Ca^{2+} and CaM-independent, but Mg^{2+}-dependent phosphatase similar to the eukaryotic PP2C type phosphatase. This activity has been recently partially purified and characterized in our laboratory (Nandi & Sarkar, 1995). Fig. 1B shows the Ca^{2+}/CaM-dependent phosphatase activity profile from the gel filtration column as obtained by subtracting the activity obtained in the absence of Ca^{2+}/CaM from the Ca^{2+} and CaM-dependent activity. TFP, an antipsychotic agent known to antagonize the biological activity of CaM, completely abolished the CaM-dependent activity but did not interfere with the Mg^{2+}-dependent PP2C type phosphatase activity present in the fractions. We had earlier shown that leishmanial PP2C activity remained unaffected by Ca^{2+}, Ca^{2+}/CaM or TFP (Nandi & Sarkar, 1995). The overall purification procedure resulted in a 43-fold purification of the enzyme with a recovery of about 10 %. The partially purified enzyme preparation was stable for at least 3–4 months with less than 5 % loss of activity when stored in final buffer containing 50 % glycerol at −70 °C.

A variety of potential substrates namely protamine, mixed histones, casein, myosin light chains (smooth muscle), RII, RII peptide (a 19 amino acid peptide containing the autophosphorylatable serine residues) and PNPP (Klee, Draetta & Hubbard, 1988) were compared in the standard protein phosphatase assay. Maximum activity was observed with protamine, mixed histones, the next most favourable substrate, was dephosphorylated at 50 % of the protamine rate. Casein and PNPP were dephosphorylated at 30 % and 5 % of the protamine rate, respectively. MLC, RII and RII peptide were poor substrates. This was interesting as the leishmanial cytosolic kinases were known to preferentially phosphorylate protamine and histones whereas acidic proteins were not good substrates (Banerjee & Sarkar, 1990, 1992). RII and RII peptide which were good substrates for calcineurin

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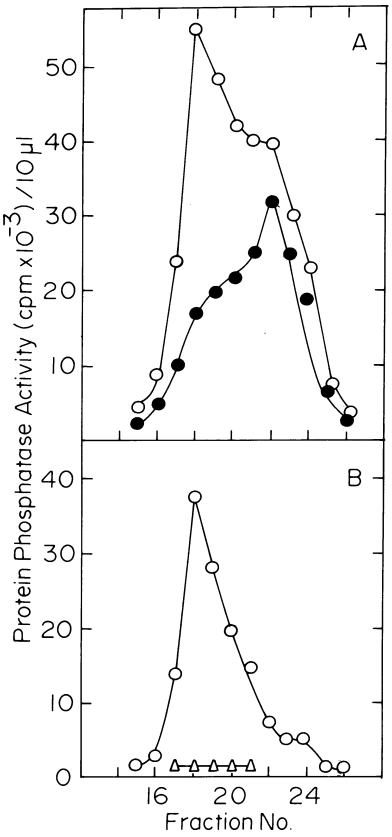

Fig. 1. Gel filtration chromatography of leishmanial Ca^{2+}/CaM-dependent phosphatase on Sephadex G-100. The 0–70 % ammonium sulfate pellet was dissolved in 3 ml of bufferA and applied to (1·3 × 96 m) Sephadex G-100 column and fractions of approximately 3·5 ml were collected. Aliquots of 10 µl were assayed by the standard method. (A) Protein phosphatase activity in the presence of 400 µM free Ca^{2+} and 5 µM CaM (○) or without Ca^{2+}/CaM (●). (B) The fractions with Ca^{2+}/CaM-dependent phosphatase activity (○) (obtained by subtracting the activity obtained in the absence of Ca^{2+}/CaM from the Ca^{2+}/CaM-dependent activity) was assayed with 150 µM TFP + Ca^{2+}/CaM (△).

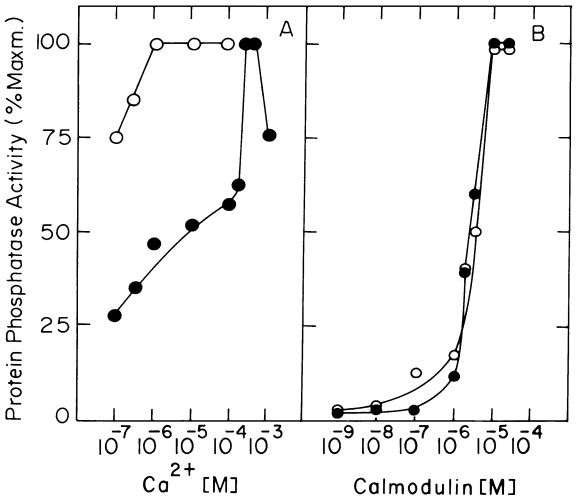

Fig. 2. (A) Activation of protein phosphatase by Ca^{2+}. Experiments were performed with [^{32}P] protamine (●) or [^{32}P] histone (○) and increasing amounts of free Ca^{2+} over EGTA in the presence of 5 µM CaM. Activity is expressed as percentage maximal activity using [^{32}P] protamine (1·1 nmols/min/mg protein) or [^{32}P] histone (0·6 nmols/min/mg protein). (B) Activation of protein phosphatase by calmodulin. Experiments were performed with [^{32}P] protamine (in the presence of 400 µM free Ca^{2+} over EGTA) (●) or [^{32}P] histone (in the presence of 100 µM free Ca^{2+} over EGTA) (○) and increasing amounts of CaM. Activity is expressed as percentage maximal activity with [^{32}P] protamine (2·3 nmols/min/mg protein) and [^{32}P] histone (0·7 nmols/min/mg protein).

(Schreiber, 1992; Blumenthal *et al*. 1986), were not good substrates for the leishmanial enzyme. With our assay conditions RII or RII peptide were found to be very good substrates for the PP2C type leishmanial phosphatase (unpublished observation).

The partially purified Ca^{2+}/CaM-dependent phosphatase activity was totally dependent on the presence of both Ca^{2+} and CaM. Using phosphorylated histones and protamine as substrates and in the presence of 5 µM CaM half maximal activity of the enzyme was recorded at Ca^{2+} concentrations of 100 nM and 10 µM, respectively (Fig. 2A). For these experiments the free Ca^{2+} concentration was varied by adding increasing amounts of Ca^{2+} to the incubation medium containing 1 mM EGTA (Sillen & Martell, 1971). Of the other divalent ions tested, Mn^{2+} (1 mM) and Co^{2+} (1 mM) were found capable of replacing Ca^{2+} whereas Zn^{2+} (1 mM) was found to be ineffective (data not shown). The enzyme was activated at low concentration of CaM. At a fixed concentration of free Ca^{2+} (100 µM for histone and 400 µM for protamine) half maximal activation was obtained with 3 µM CaM and 2 µM CaM, respectively (Fig. 2B).

A number of different agents were tested for their ability to inhibit Ca^{2+}/CaM-dependent dephosphorylation of protamine (Table 1). EGTA (2 mM) completely inhibited the leishmanial Ca^{2+}/CaM-dependent phosphatase activity, indicating the importance of Ca^{2+} in the reaction. Calmidazolium, fluphenazine and TFP, all being CaM antagonists, inhibited the Ca^{2+}/CaM-dependent phosphatase activity. Cypermethrin, a pyrethroid insecticide, has been recently reported to have inhibitory effect on calcineurin. The degree of inhibition was dependent on the the type of substrate chosen (Enan & Matsumara, 1992). No significant inhibition of activity was observed with this compound. Okadaic acid, a well-known inhibitor of PP1 and PP2A type serine/threonine phosphatases failed to inhibit the leishmanial enzyme at low concentrations. However, as observed by other workers the enzyme is slightly inhibited at higher (5 µM) concentration of okadaic acid (Fruman *et al*. 1992).

In higher eukaryotes including plant systems, immunophilin-ligand complexes such as cyclophilin-cyclosporinA (Cyp–CsA) and FKBP–FK506 spe-

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Table 1. Effect of different inhibitors on leishmanial Ca^{2+}/CaM-dependent phosphatase

(Assay was done with 6 μM [^{32}P] protamine as described in the Materials and Methods section with the following inhibitors, in the absence (1 mM EGTA) or presence of 2·5 μM CaM an 400 μM free Ca^{2+} (over 1 mM EGTA). Results are expressed as percentage inhibition of the maximal Ca^{2+}/CaM-dependent activity (330 pmols/min/mg protein) obtained in the absence of any inhibitors.)

Inhibitor	Concentration (μM)	Inhibition (%)
Trifluoperazine	50	50
	100	90
	150	100
Fluphenazine	100	90
Calmidazolium	10	100
EGTA	2000	100
EDTA	5000	100
Okadaic acid	1	0
	5	30
Cypermethrin	10	15

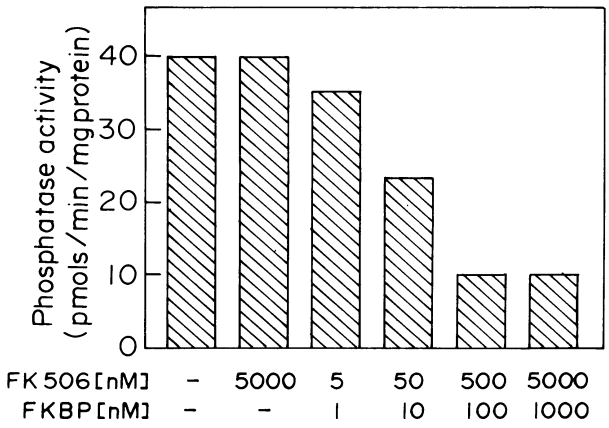

Fig. 3. Effect of FK506–FKBP on leishmanial Ca^{2+}/CaM-dependent phosphatase activity. Ca^{2+}/CaM dependent phosphatase activity is expressed as pmol/min/mg protein. Assay was done using [^{32}P] protamine as substrate. For each assay the immunophilin–ligand complex was pre-incubated with enzyme extract with 400 μM free Ca^{2+} and 2·5 μM CaM or without Ca^{2+}/CaM (with 1 mM EGTA) for 30 min at 4 °C before substrate was added to initiate the reaction. Control tubes received equal amounts of ethanol.

cifically inactivate the Ca^{2+}/CaM-dependent protein phosphatases (Fruman *et al*. 1992; Luan *et al*. 1993). The immunophilins or ligands alone are incapable of inhibiting the enzymes. Addition of FK506 alone did not have any effect on the leishmanial Ca^{2+}/CaM-dependent phosphatase activity in crude cell lysates or partially purified enzymes. Addition of exogenous human recombinant FKBP along with FK506 inhibited the Ca^{2+}/CaM-dependent phosphatase activity of a partially purified preparation (Fig. 3). Presumably, FKBP or its leishmanial homologue is either absent or not present

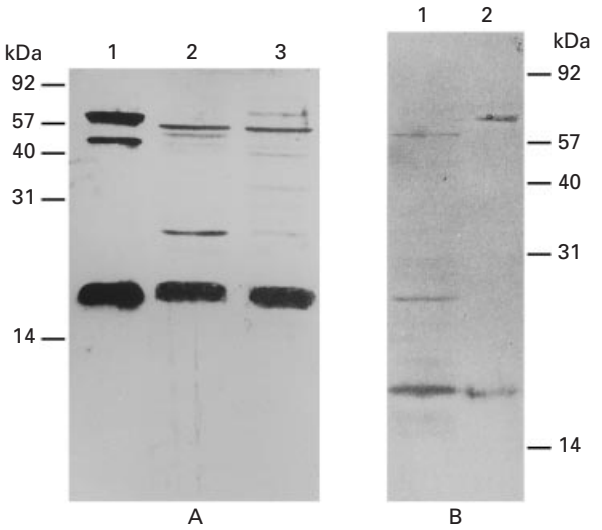

Fig. 4. (A) Immunoblot analysis of *Leishmania* extracts. Proteins were resolved by SDS–PAGE (15% polyacrylamide gel), transferred to nitrocellulose and probed with an antiserum that recognizes both A and B chains of calcineurin. Immunostaining was carried out as described in the Materials and Methods section. Lane 1: pure calcineurin (200 ng), Lane 2: 100000 ***g*** cytosolic extract (150 μg) of non-virulent UR6 strain, Lane 3: 100000 ***g*** cytosolic extract (150 μg) of virulent AG83 strain. (B) Immunoblot analysis of fraction 18 from Sephadex G-100 column and pure calcineurin. Proteins were resolved by SDS–PAGE (15% polyacrylamide gel), transferred to nitrocellulose and probed with anticalcineurin. Lane 1: fraction 18 from Sephadex G-100 column (50 μg), Lane 2: pure calcineurin (50 ng).

in adequate amounts in the enzyme extract to mediate the action of FK506. In contrast, addition of 10 μM CsA alone to the crude cell lysate was followed by 80% inhibition of the enzyme (data not shown).

Immunoblotting experiments (Fig. 4A) with a polyclonal antiserum that recognizes both A and B subunits of calcineurin (titre for B subunit more than A), confirmed the presence of both subunits in the UR6 strain (Lane 2) and the virulent AG83 strain (Lane 3) of *Leishmania*. The 57 kDa polypeptide observed in both the strains was very close to the predominant A band of calcineurin (Lane 1). The B subunit of UR6 and AG83 migrated as a prominent 19 kDa polypeptide similar to that of brain calcineurin. This anti-bovine calcineurin antiserum is known to give a better signal for the B subunit from other organisms than the A subunit (C. B. Klee, personal communications). The 28 kDa polypeptide is probably a proteolytic cleavage product of the higher molecular weight subunit as observed by Fruman *et al*. (1992) in other systems. Fig. 4B shows the immunoblotting pattern of the partially purified fraction (fraction 18 from Sephadex G-100 column) and pure calcineurin using the same polyclonal antiserum. Here also the partially purified fraction (fraction 18) of leishmanial Ca^{2+}/CaM-dependent

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phosphatase (Lane 1) showed a similar banding pattern to the 100000 ***g*** supernatant fraction (Fig. 4A). The intensity of bands at 57 kDa and 19 kDa were lower due to low amount of proteins loaded in each lane compared to the amount of protein loaded in Fig. 4A.

DISCUSSION

It is evident from our results that the parasite displays a Ca^{2+}/CaM-dependent phosphatase activity that is broadly analogous in its properties to the PP2B or calcineurin from higher eukaryotes (Cohen, 1989). The enzyme obligatorily depends on Ca^{2+} and CaM for its activity. Complete obliteration of activity in the presence of trifluoperazine further emphasizes the dependence of this enzyme on CaM for its activity. Relative insensitivity to okadaic acid, specific dose-dependent inhibition by the phenothiazine drugs such as TFP and fluphenazine and lack of activation in the absence of Ca^{2+} and CaM distinguishes this enzyme from PPI, PP2A and PP2C, respectively. The close similarity of this enzyme with other well-characterized PP2B from mammalian sources is further confirmed by the sensitivity of the enyzme to immunophilin-ligand complexes and its specific cross-reactivity with the antibody raised against the brain enzyme.

Taken together, these results leave little doubt that *Leishmania* promastigotes display a protein phosphatase activity that is exclusively dependent on the simultaneous presnce of Ca^{2+} and CaM. This is the first report of the presence of PP2B activity in the kinetoplastid group of protozoan parasites as well as among other protozoal pathogens. Despite the role of Ca^{2+} for the pathogenicity of malarial parasite, no calcineurin activity has yet been demonstrated from this pathogen (Bell, Wernli & Franklin, 1994).

The possible biochemical role of this enzyme in the complex life-cycle of the pathogen remains to be elucidated. The presence of at least 2 internal pools for Ca^{2+} (Ruben *et al*. 1991; Vercesi & Docampo, 1992), the low cytoplasmic free Ca^{2+} that rapidly rises on exposure of the organism to elevated temperature (Sarkar & Bhaduri, 1995) and the presence of an extrusion pump for Ca^{2+} (Mondal *et al*. 1997) are all clear indications of a biomodulatory role for Ca^{2+}. Ca^{2+} signalling, if related to cellular activation or differentiation, is expected to be amplified and processed through protein kinases and phosphatases. So far, there has not been any convincing report of the presence of a kinase in *Leishmania* that is clearly dependent on an effector molecule including Ca^{2+} for its activity. Recently, we have been able to characterize a Ca^{2+}-dependent kinase in the pathogen, the activity of which was masked in the crude fractions due to the presence of this powerful Ca^{2+}/CaM-dependent phosphatase. With the clear demonstration of a Ca^{2+}-dependent protein kinase (manuscript in preparation) and this Ca^{2+}/CaM-dependent protein phosphatase in this parasite, our efforts are now directed towards identifying the target proteins for these enzymes.

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