THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1992 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 267, No. 26, Issue of September 15, pp. 18440-18446, 1992
Printed in U.S.A.

Allosteric Modulation of *Leishmania donovani* Plasma Membrane Ca^{2+}-ATPase by Endogenous Calmodulin*

(Received for publication, April 24, 1992)

Sarmila Mazumder, Tanmoy Mukherjee, Jagadananda Ghosh, Manju Ray‡§, and Amar Bhaduri

From the Leishmania Group, Enzyme Division, Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road and the ‡Department of Pharmacy, Division of Biochemistry, Jadavpur University, Calcutta 700032, India

The plasma membrane of the human pathogen *Leishmania donovani* possesses a high-affinity transmembrane Ca^{2+}-ATPase that has its catalytic site oriented toward the cytoplasmic milieu (Ghosh, J., Ray, M., Sarkar, S., and Bhaduri, A. (1990) *J. Biol. Chem.* 265, 11345–11351). When the enzyme is studied in its more authentic, physiologically relevant, membrane-associated form, it exhibits pronounced sigmoidal kinetics with Ca^{2+} ($K_{0.5} \approx 700$ nM) in a *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid buffering system that effectively complexes all available Mg^{2+}. Addition of exogenous Mg^{2+} (60 μM) completely abolishes sigmoidicity and establishes strictly hyperbolic kinetics, and the K_m for Ca^{2+} reduces to 100 nM. Mg^{2+} can be replaced by heterologous calmodulin. The exclusive dependence of the enzyme on only Ca^{2+} for its activity and its positive allosteric modulation by Mg^{2+} distinguish this enzyme from other well-characterized plasma membrane Ca^{2+}-ATPases.

Employing this Ca^{2+}-ATPase as the assay system, a soluble endogenous activating protein factor was purified that, by several criteria, corresponds to authentic calmodulin. The parasite calmodulin shifts the kinetics to hyperbolic kinetics, increases the V_{max} 2-fold, and most important lowers the K_m (≈100 nM) to a physiological level. The interaction with endogenous calmodulin thus converts the enzyme from a totally inactive to a fully active state.

The human pathogen *Leishmania donovani* is the causative agent for visceral leishmaniasis or kala azar, a fatal disease that is endemic in many parts of the tropical world (1). This parasite has a digenic life cycle. The flagellated promastigote or the vector form enters liver macrophages and quickly undergoes morphogenetic transformation into the aflagellated pathogenic form in the phagolysosomal complex of the macrophages (2). Here it overcomes the host defense mechanism, multiplies, and invades neighboring macrophages, thus ensuing pathogenicity. This cellular differentiation that leads to pathogenicity is most probably dependent on some as yet unidentified signal(s) from the host macrophage milieu. The biochemical nature of the signal and its transduction and amplification remain to be elucidated.

Calcium ion is now recognized as a major intracellular signal through which external stimuli evoke a variety of crucial cellular responses in eucaryotic cells. Movement or mobilization of Ca^{2+} leading to the transitory increase in its concentration at the site of action, its interaction with response elements, and the subsequent rapid removal of excess free Ca^{2+} constitute the general scenario for the Ca^{2+} messenger system (3–5). High-affinity membrane-inserted Ca^{2+}-ATPases acting as properly oriented Ca^{2+} pumps are critical components in the overall Ca^{2+} homeostasis of the target cell (5). We have recently isolated a plasma membrane Ca^{2+}-ATPase from *L. donovani* promastigotes that has its catalytic site directed toward the cytoplasmic face. The highly purified enzyme, initially solubilized by extraction with deoxycholate, has extremely high affinity for Ca^{2+}, acts independently of Mg^{2+}, and shows strictly hyperbolic kinetics when complexed with EGTA[1] (6). We now report that the enzyme, when studied in its more authentic plasma membrane-associated form, exhibits properties that are significantly different from the solubilized form of the enzyme. The membrane-associated enzyme in unsealed permeabilized vesicles shows distinct allosteric kinetics with free Ca^{2+} when complexed with CDTA. The kinetics shifts to strictly hyperbolic kinetics in the presence of very low concentrations of free Mg^{2+} when Mg^{2+} is added exogenously to the system. The positive activating effect of Mg^{2+} could be replaced by a cytosolic protein factor that was found on purification to be endogenous calmodulin. The allosteric nature of leishmanial Ca^{2+}-ATPase and its positive modulation by the parasite calmodulin make it unique among plasma membrane Ca^{2+}-ATPases from various sources. More important, since the cytoplasmic concentration of free Ca^{2+} in *L. donovani* promastigotes is known to be ~100 nM (7), the increased affinity for Ca^{2+} on interaction with endogenous calmodulin corresponds to a shift from an inactive to a physiologically active state of the enzyme, where it can act as an extrusion pump for Ca^{2+}. The detection and characterization of endogenous calmodulin coupled with its physiologically significant regulatory role in the activity of this unique plasma membrane Ca^{2+}-ATPase strongly suggest a role for Ca^{2+} in the life cycle of this human pathogen.

MATERIALS AND METHODS[2]

All biochemicals were purchased from Sigma unless otherwise mentioned. Triethanolamine salt of ATP or Tris/ATP was used as substrate for Ca^{2+}-ATPase assay. [γ-^{32}P]ATP (3 mCi/mmol) was purchased from Bhabha Atomic Research Center (India).

Organism—The organism used in this work is a clinical isolate of

* This work was supported in part by a Council of Scientific and Industrial Research grant (to M. R.) and United Nations Development Program Grant IND/87/018. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Reader, Indian Association for the Cultivation of Science, Calcutta 700032, India.

[1] The abbreviations used are: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PME, plasma membrane-enriched; TBS, Tris-buffered sucrose; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid.

[2] Portions of this paper (including part of "Materials and Methods," Fig. 6, and Tables 2 and 3) are included in Miniprint.

L. donovani from a kala azar patient. The strain originally designated as UR6 is presently renamed as MHOM/IN/1978/UR-6 (8). The cells were grown and maintained on a solid-blood-agar medium that has been described elsewhere (6).

Isolation of Plasma Membrane-enriched Fraction—*L. donovani* promastigotes, grown for 72 h on solid blood-agar medium, were collected in Tris-buffered sucrose (TBS) (25 mM Tris-HCl, pH 7.4, and 0.25 M sucrose) and were washed twice at 1000 × *g* for 5 min in TBS. The washed fully motile cells were suspended in a hypotonic medium of 5 mM Tris-HCl, pH 7.4 (50 ml/g of wet cells), for 1 h with occasional vortexing (six to eight times) of 2-min duration each time. This resulted in the effective release of all cytoplasmic kinetoplast-mitochondrial matrix and glycosomal marker enzymes (>90%) from the cells in the hypotonic medium. These osmotically shocked permeabilized *L. donovani* promastigote ghosts fully retained 3′-nucleotidase, the marker enzyme for leishmanial plasma membrane (9), and nearly 30% of the total succinate dehydrogenase representing mitochondrial membrane fragments. We have shown earlier that on removal of the mitochondrial membrane fragment by the procedure of Scarborough (10), the Ca^{2+}-ATPase remains exclusively in the pure plasma membrane fragment (6). The leaky unsealed ghosts obtained after the hypotonic shock were collected after spinning at 1000 × *g* for 15 min. The pellet was suspended in 50 mM Tris-HCl, pH 7.4 (4 ml/g of cells), and homogenized in a Potter-Elvejhem homogenizer. The homogenate was centrifuged at 7000 × *g* for 12 min. The resulting membrane pellet was washed twice with the same buffer and finally resuspended for enzymatic analysis in TBS (4 ml/g of cells) containing ~2 mg of protein/ml of suspension. The prepared plasma membrane-enriched (PME) fraction in suitable aliquots was used as the source of Ca^{2+}-ATPase activity for all subsequent assays. The PME fraction at this stage contained 0.82 μg of Ca^{2+} and 1.3 μg of Mg^{2+}/mg of membrane protein as determined by atomic absorption spectroscopy. The PME fraction was kept frozen at −70 °C for subsequent use. All the different steps of membrane preparation were carried out at 4 °C.

Assay of Ca^{2+}-ATPase—Ca^{2+}-ATPase activity was determined either colorimetrically by measuring the Ca^{2+}-dependent release of inorganic phosphate or by measuring the liberation of ^{32}P from [γ-^{32}P]ATP. In all cases, the concentration of free Ca^{2+} was controlled by complexation with either EGTA or CDTA.

For colorimetric assay, the final assay mixture contained, in a total volume of 1.0 ml, 100 mM Tris-HCl, pH 7.4, 500 μM EGTA or CDTA, with or without $CaCl_2$ and 0.5 mM ATP. The reaction was initiated by addition of ~200 μg of PME fraction protein as the source of enzyme. $CaCl_2$ concentration was varied to give the required free Ca^{2+} concentration as described by Pershadsingh and McDonald (11), based on the experimental values earlier obtained by Sillen and Martell (12). After incubation at 28 °C for 30 min, the reaction was terminated by addition of 50 μl of 20% trichloroacetic acid. After removal of the precipitate, the released inorganic phosphate was measured following the method of Lowry and Lopez (13).

For radioactive assay, the method of Bais (14) was followed with few modifications. In this case, the final volume of the assay mixture was reduced to 0.1 ml. The composition and concentration of all the ingredients of the assay mixture remained the same as before. In this assay, 50 nmol of [γ-^{32}P]ATP (10^5 cpm) was added. After 10 min of incubation at 28 °C, the reaction was stopped by addition of 5 μl of 20% trichloroacetic acid. To this, 10 μl of 100 mM KH_2PO_4 and 0.1 ml of suspension of 50% activated charcoal in water were added consecutively. After mild agitation for 10 min, charcoal was precipitated by centrifugation. The process was repeated once more, and finally, 100 μl of the supernatant was transferred to a scintillation vial.

When the free Ca^{2+} concentration was controlled by complexation with EGTA, the amounts of contaminating Ca^{2+} from the plasma membrane fraction (4 μM) and from other added reagents (8 μM) were included in the calculations. Ca^{2+}-stimulated ATPase activity was determined by subtracting the values with chelator alone from the values with calcium plus chelator.

When complexation was carried out with CDTA in the presence of exogenously added Mg^{2+}, separate calculations were made both for free Ca^{2+} and for Mg^{2+}. This was based on the original work of Pershadsingh and McDonald (11) and is elaborated in the Miniprint.

Assay of Mg^{2+}-ATPase—The Mg^{2+}-ATPase activity was determined colorimetrically. The concentration of free Mg^{2+} was controlled by complexation with CDTA. The assay mixture contained, in a total volume of 1 ml, 100 mM Tris-HCl, pH 7.4, 500 μM CDTA, with or without $MgCl_2$ and 0.5 mM ATP. The reaction was initiated by addition of 200 μg of PME fraction protein as the source of enzyme. $MgCl_2$ concentration was varied to give the required free Mg^{2+} concentration as described previously (11). Incubation was for 30 min at 28 °C. The rest of the procedure was the same as described for Ca^{2+}-ATPase assay.

To determine the free Mg^{2+} concentration, the amounts of contaminating Mg^{2+} from the plasma membrane fraction (10 μM) and from other reagents (4 μM) were included in the calculations. Mg^{2+}-stimulated ATPase activity was determined by subtracting the values with chelator alone from the values with magnesium plus chelator.

Limited Proteolysis of Plasma Membrane—Trypsin treatment of the PME fraction suspended in TBS was carried out at 30 °C. Since the plasma membrane Ca^{2+}-ATPase activity could be partially solubilized by treatment with trypsin, the trypsin:plasma membrane ratio and the time of exposure to the PME fraction had to be determined separately before the effect of calmodulin could be studied on trypsinized Ca^{2+}-ATPase in its membrane-inserted form. In general, digestion was started by addition of trypsin and arrested at selected time intervals by addition of soybean trypsin inhibitor in the trypsin:inhibitor weight ratio of at least 1:10. The digested sample was then centrifuged at 10,000 × *g* for 12 min. The PME pellet was resuspended in TBS in the same proportion present prior to digestion and used for enzymatic analysis. Control enzyme activity was determined by adding inhibitor before trypsin and then incubating for the respective time intervals before assay.

RESULTS

Kinetic Characteristics of Plasma Membrane-associated Ca^{2+}-ATPase—When the Ca^{2+}-ATPase activity in its plasma membrane-associated form was assayed in the presence of varying concentrations of free Ca^{2+} with EGTA as the chelating agent, strictly hyperbolic kinetics was recorded. The K_m for free Ca^{2+} was 30 nM, and the V_{max} was 21.5 nmol/min/mg of membrane protein (Fig. 1). The kinetics obtained with ATP at a saturating free Ca^{2+} concentration was also found to be conventional with a K_m for ATP of 100 μM. The extremely high affinity for Ca^{2+} and the hyperbolic nature of the kinetics are very similar in nature to those obtained earlier by us with the solubilized and highly purified enzyme (6). However, a sharply contrasting picture emerged when the

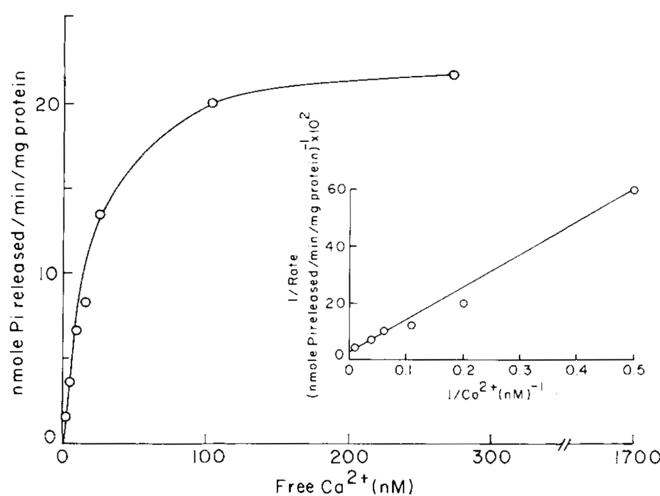

FIG. 1. **Ca^{2+} dependence of Ca^{2+}-ATPase for ATP hydrolysis.** The rate of Ca^{2+}-dependent ATP hydrolysis was estimated colorimetrically by measuring the inorganic phosphate liberated in the presence of Ca^{2+}. An appropriate control was run in the absence of Ca^{2+}. No other cation was added to the assay mixture, and the free Ca^{2+} concentration was maintained by the EGTA (0.5 mM)/Ca^{2+} buffering system. Other compositions of the assay mixture and assay conditions are described under "Materials and Methods." For this experiment, 200 μg of PME fraction was used as enzyme source. *Inset*, Lineweaver-Burk plot of the same data. The same experiment was performed by measuring the liberation of ^{32}P from [γ-^{32}P]ATP, and similar results were obtained.

same membrane-associated enzyme fraction was assayed with CDTA as the complexing agent. In this case, a dramatic shift in the kinetic pattern was observed. With varying free Ca^{2+} concentrations, distinct and highly pronounced sigmoidal kinetics was obtained (Fig. 2). The extent of sigmoidicity increased with increasing CDTA concentrations, but reached a constant value at a 450–500 μM concentration of the complexing agent. The nature of the curve remained the same at significantly higher concentrations of CDTA (1.5 mM). Fig. 2 shows that the enzyme remains catalytically totally inactive at 100–150 nM free Ca^{2+} and that significant catalytic activity could be observed only beyond 300 nM. The $K_{0.5}$ in this case was ~700 nM. The V_{max} for the enzyme was, however, calculated to be 18–19 nmol/min/mg of protein, which is not significantly different from the value obtained with the EGTA buffering system. The degree of cooperativity with free Ca^{2+} is 4, as the Hill coefficient was calculated to be 3.6 (Fig. 2, *inset*).

Effect of Mg^{2+} on Ca^{2+}-ATPase Activity—The differential behavior of Ca^{2+}-ATPase in EGTA and CDTA buffering systems led us to analyze the effect of Mg^{2+} on Ca^{2+}-ATPase activity more critically. The difference in chelating properties of CDTA and EGTA is that CDTA chelates both Ca^{2+} and Mg^{2+} with nearly equal facility, whereas EGTA chelates exclusively Ca^{2+}. The possible role of free Mg^{2+} as an exogenous allosteric activator therefore needed to be investigated in some detail.

The plasma membrane of the parasite is known to have an ATP-hydrolyzing activity that is exclusively dependent on Mg^{2+} (15). This enzyme has been implicated in proton translocation (15) and in active accumulation of metabolites, such as glucose or proline (22). Since ATP hydrolysis due to this Mg^{2+}-ATPase could be interpreted as Ca^{2+}-ATPase activity under situations where both free Ca^{2+} and Mg^{2+} are present, we decided to kinetically characterize this enzyme in its plasma membrane-associated form. Fig. 3 demonstrates the kinetics of this enzyme in the presence of varying concentrations of free Mg^{2+}. The concentration of free Mg^{2+} was controlled by complexation with CDTA. This enzyme shows strictly hyperbolic kinetics and has a fairly high affinity for Mg^{2+}. It is, however, quite clear that at concentrations of free Mg^{2+} below 200 nM, there is no significant ATP hydrolysis due to this enzyme.

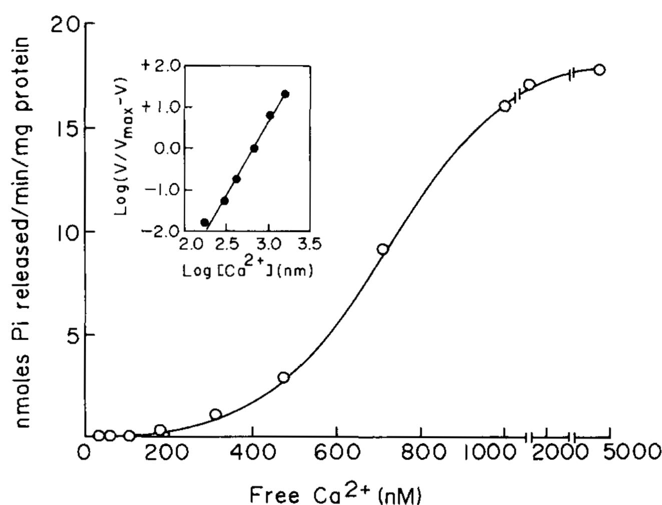

FIG. 2. **Ca^{2+} dependence of Ca^{2+}-ATPase for ATP hydrolysis in CDTA-buffered medium.** Ca^{2+}-ATPase was assayed similarly as described for Fig. 1, except that the free Ca^{2+} concentration was regulated by the CDTA (0.5 mM)/Ca^{2+} buffering system. *Inset,* Hill plot of the data. The same experiment was performed with [γ-^{32}P]ATP following the release of ^{32}P, and similar results were obtained.

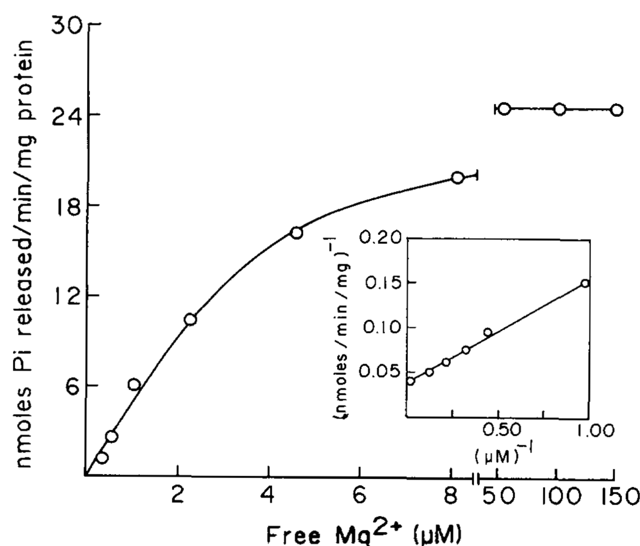

FIG. 3. **Mg^{2+} dependence of Mg^{2+}-ATPase for ATP hydrolysis in CDTA-buffered medium.** The rate of Mg^{2+}-dependent hydrolysis of ATP was estimated colorimetrically by measuring the inorganic phosphate liberated in the presence of Mg^{2+}. An appropriate control was run in the absence of Mg^{2+}. The free Mg^{2+} concentration was maintained by the CDTA (0.5 mM)/Mg^{2+} buffering system. For this experiment, 200 μg of PME fraction was used as enzyme source. *Inset,* Lineweaver-Burk plot of the same data.

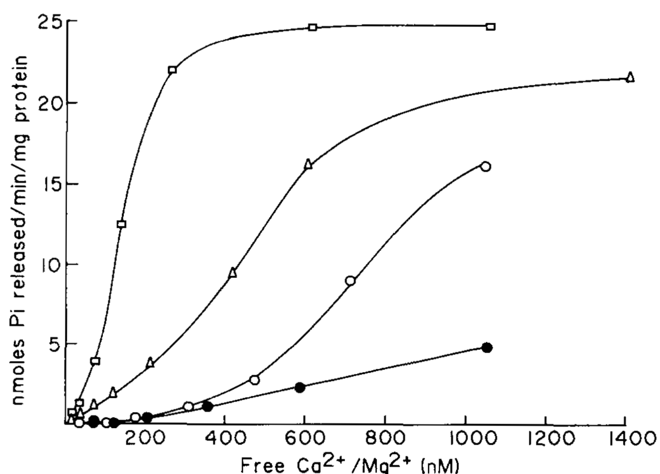

FIG. 4. **Effect of Mg^{2+} on Ca^{2+}-ATPase activity at various concentrations of free Ca^{2+}.** Each assay mixture contained, in a total volume of 1 ml, 100 μmol of Tris-HCl, pH 7.4, 0.5 μmol of ATP, and $MgCl_2$ as indicated, with varying concentrations of Ca^{2+}. The concentrations of Ca^{2+} in the assay mixture were maintained as indicated by the CDTA (0.5 mM)/Ca^{2+}/Mg^{2+} buffering system. The reaction was started by addition of 200 μg of PME fraction. After 30 min of incubation at 25 °C, the reaction was terminated by addition of 50 μl of 20% trichloroacetic acid, and P_i liberated was estimated colorimetrically. ○, Ca^{2+}-ATPase activity without Mg^{2+}; △ and □, Ca^{2+}-ATPase activity in the presence of 16 and 46 μM concentrations of exogenously added $MgCl_2$, respectively, in the assay medium; ●, Mg^{2+}-ATPase activity that was extrapolated from a separate experiment (for comparison) (Fig. 3). Similar results were also obtained by the radioactivity assay method as described under "Materials and Methods."

Addition of exogenous $MgCl_2$ at moderate concentrations had profound effect on the activity of Ca^{2+}-ATPase. In presence of a total Mg^{2+} concentration of 60 μM, the sigmoidal kinetics was effectively abolished (Fig. 4). The total Mg^{2+}

concentration was obtained as the sum of exogenously added Mg^{2+} plus all other available Mg^{2+} from the plasma membrane and from the reagents. The kinetic characteristics recorded in this case broadly resembled the pattern obtained with EGTA as the complexing agent (Fig. 1). The concentration of free Ca^{2+} was varied over a wide range by keeping the concentration of CDTA fixed at 500 μM and varying the concentration of $CaCl_2$ for each of the individual points. The total Ca^{2+} concentration, as in the case of Mg^{2+}, was the sum of all available Ca^{2+}. For each of the individual experimental points, the concentrations of free Ca^{2+} and free Mg^{2+} depended on the relative concentrations of total Ca^{2+} and total Mg^{2+}. The calculations were carried out according to the equations of Pershadsingh and McDonald (11), and the basis of such calculations has been given in detail in the Miniprint. Table 1 also shows specifically the concentrations of free Ca^{2+} and free Mg^{2+} for each of these experimental points. For comparison, the Mg^{2+}-catalyzed ATPase activity at the relevant concentration range of free Mg^{2+}, as obtained from a parallel experiment, is also included in Fig. 4. It is evident from Fig. 4 and Table 1 that the strikingly enhanced ATP hydrolytic activity at low free Ca^{2+} concentrations is due to the activation of Ca^{2+}-ATPase in presence of Mg^{2+}. We have not studied in detail the mechanism of allosteric activation by Mg^{2+}. The anion apparently does not have any role to play in this interaction as $MgCl_2$ could be replaced by $MgSO_4$. Divalent cations like Mn^{2+} (50 μM) or Zn^{2+} (50 μM) could partially mimic the effect of Mg^{2+} (data not shown).

Modulation of Ca^{2+}-ATPase Activity by Heterologous Calmodulin—The potential physiological significance of the sigmoidal behavior of the Ca^{2+}-ATPase in its plasma membrane-associated form led us to search for an endogenous modulator that could abolish the sigmoidicity and increase the affinity of the enzyme for Ca^{2+} to a physiologically relevant level. We have shown earlier that calmodulin from heterologous sources has a moderate but distinct activating effect on the hyperbolic kinetics of the purified Ca^{2+}-ATPase (6). Bovine brain calmodulin was found to have a dramatic effect on the membrane-associated enzyme (Fig. 5). The sigmoidicity was effectively abolished, and the K_m for Ca^{2+} was reduced to 100 nM. Increasing the calmodulin concentration had no further discernible effect on the enzyme. The enzyme once stimulated by heterologous calmodulin could not be further activated by addition of Mg^{2+} (data not shown).

TABLE 1

Total amounts of free Ca^{2+} and Mg^{2+} in 500 μM CDTA-buffered medium in the experiment of Fig. 4

In each assay mixture, 500 μM CDTA and the indicated amounts of $CaCl_2$ and $MgCl_2$ were used. The amounts of free Ca^{2+} and Mg^{2+} were calculated from equations a–e as described under "Materials and Methods."

$CaCl_2$	$MgCl_2$	Free Ca^{2+}	Free Mg^{2+}
μM	μM	nM	nM
62.5	30	16	26
125.0	30	38	31
187.5	30	69	38
250.0	30	119	48
312.5	30	208	68
375.0	30	415	112
400.0	30	601	153
437.5	30	1417	329
62.5	60	17	56
125.0	60	41	68
187.5	60	78	84
250.0	60	138	112
312.5	60	258	168
375.0	60	607	329
400.0	60	1052	535

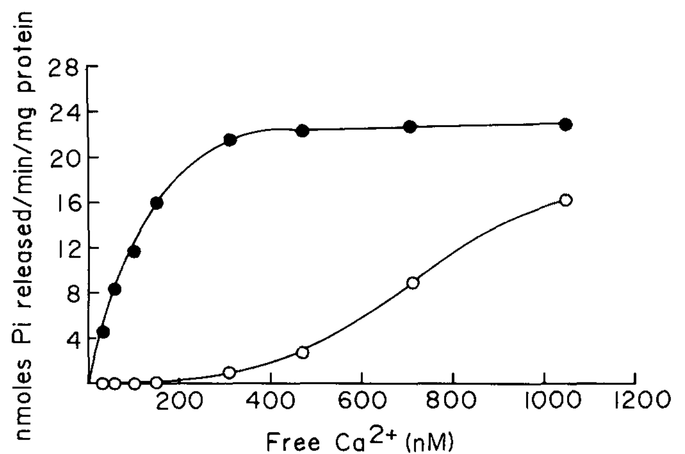

FIG. 5. **Stimulation of Ca^{2+}-ATPase activity of *L. donovani* plasma membrane with heterologous calmodulin.** The assay mixture contained, in a total volume of 1 ml, 100 μmol of Tris-HCl, pH 7.4, 0.5 μmol of ATP, 20 μg of bovine brain calmodulin, and Ca^{2+} as indicated. The Ca^{2+} concentration was varied by the CDTA (0.5 mM)/Ca^{2+} buffering system. The reaction was started by addition of ATP after 10 min of preincubation of the requisite amount of enzyme with calmodulin. After 30 min of incubation at 25 °C, the reaction was terminated by addition of 50 μl of 20% trichloroacetic acid, and liberated P_i was measured colorimetrically. The same experiment was performed by measuring the release of ^{32}P from [γ-^{32}P]ATP, and similar results were obtained. Shown is activity without (○) and with (●) 20 μg of bovine brain calmodulin.

Purification and Characterization of Endogenous Ca^{2+}-ATPase-activating Factor—The striking effect of heterologous calmodulin on the kinetics of plasma membrane Ca^{2+}-ATPase and its potential physiological significance led us to search for a similar protein in the parasite itself. Assuming this to be a soluble protein of the cytoplasm, we could immediately detect an activating protein factor that was released in the medium after hypotonic shock of the promastigote cells. The factor turned out to be a heat-stable protein and was purified to homogeneity by conventional techniques (Table 2). The highly purified protein was obtained in the final step of purification by collecting three to four tubes of the eluents from the Sephadex G-100 column. The pooled fractions that showed maximum stimulating activity were found to be essentially homogeneous in nature and had a mobility on polyacrylamide gel very similar to that of bovine brain calmodulin (Fig. 6). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis studies revealed the protein to be a monomer with a molecular weight of ≈16,000 (Fig. 6). Slight broadening of the protein band is probably due to heterogenous binding of Ca^{2+} to cell calmodulin (23). The monomeric nature of the protein, its apparent molecular weight, and its thermostability strongly suggested that the purified activating protein is the endogenous calmodulin of the parasite. This was confirmed when the effect of the purified protein factor on bovine brain cAMP phosphodiesterase was studied. The enzyme activity was stimulated by >2-fold in the presence of this protein (12 μg), which was abolished by trifluoperazine, a prototype of the phenothiazine group of drugs that are known to act as antagonists for calmodulin (Table 3). We thus conclude that the purified activating protein is the endogenous calmodulin of *L. donovani* promastigotes.

Effect of L. donovani Calmodulin on Ca^{2+}-ATPase Activity—The endogenous calmodulin of the parasite showed a remarkable activating effect on the membrane-associated form of the enzyme (Fig. 7). The sigmoidicity was progressively abolished

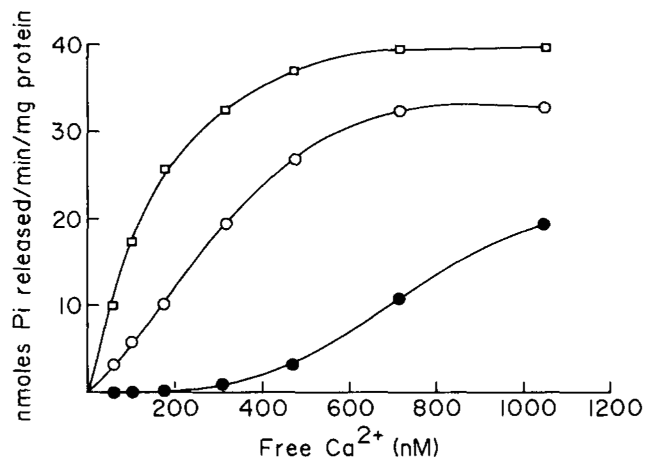

FIG. 7. **Stimulation of Ca^{2+}-ATPase of *L. donovani* plasma membrane by purified endogenous calmodulin.** Calmodulin stimulation was assayed colorimetrically by estimating liberated P_i from ATP and also by following the release of ^{32}P from [γ-^{32}P]ATP. In each assay, 200 μg of enzyme (PME fraction) was preincubated with the concentrations of endogenous purified calmodulin indicated below for 10 min at 25 °C, and the reaction was started by addition of ATP (see legend of Fig. 5). Free Ca^{2+} concentrations as indicated were maintained by the CDTA (0.5 mM)/Ca^{2+} buffering system. ●, activity without calmodulin; ○ and □, activity with 6 and 8 μg of calmodulin, respectively.

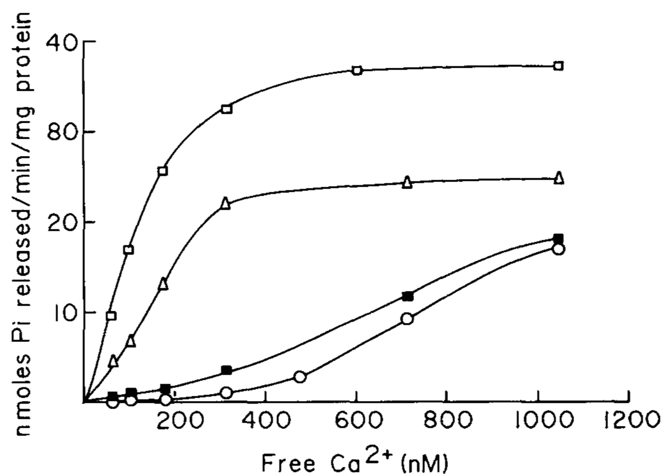

FIG. 8. **Inhibition of endogenous calmodulin-activated Ca^{2+}-ATPase by trifluoperazine.** Each assay mixture contained, in a total volume of 1 ml, 100 μmol of Tris-HCl, pH 7.4, 200 μg of PME fraction protein as enzyme source, 8 μg of pure *L. donovani* calmodulin (where indicated below), and trifluoperazine (at the concentrations indicated below), with varying concentrations of Ca^{2+} maintained by the CDTA (0.5 mM)/Ca^{2+} buffering system. After 10 min of preincubation, the reaction was initiated by addition of 0.5 μmol of ATP. After 30 min of incubation at 25 °C, the reaction was terminated by addition of 50 μl of 20% trichloroacetic acid. P_i liberated was estimated colorimetrically. ○, native Ca^{2+}-ATPase activity; □, in the presence of 8 μg of calmodulin; △, in the presence of 8 μg of calmodulin and 50 μM trifluoperazine; ■, in the presence of 8 μg of calmodulin and 100 μM trifluoperazine.

with increasing concentrations of calmodulin. In the presence of 8 μg or 0.5 μM calmodulin, completely hyperbolic kinetics was observed, and the K_m for Ca^{2+} was reduced to 100 nM. Equally remarkable, the V_{max} was increased by >2-fold. Obviously, the parasite calmodulin had a more pronounced modulatory effect than calmodulin from heterologous sources.

Effect of Calmodulin Antagonists on Activation by Calmodulin—The activating effect of calmodulin on several target systems is known to be inhibited by a wide range of chemically unrelated substances such as phenothiazines, naphthalene sulfonamides, *Vinca* alkaloids, etc. (24). Calmidazolium, a derivative of antimycotic miconazole, is also a powerful inhibitor of the activating effect of calmodulin (25). The effects of some of these potential inhibitors were tested on calmodulin-stimulated Ca^{2+}-ATPase over a wide range of free Ca^{2+} concentrations. Both trifluoperazine and calmidazolium could completely abolish the activating effect of leishmanial calmodulin and could restore the sigmoidicity originally observed in the CDTA buffering system. Fig. 8 shows that trifluoperazine at 50 μM considerably suppressed the activating effect of calmodulin and at 100 μM completely abolished its effect. In the case of calmidazolium, the complete abolition of the stimulatory effect of calmodulin was obtained at 200 μM (data not shown). No other antagonists was tested for this purpose.

Limited Proteolysis of Membrane-associated Ca^{2+}-ATPase—The plasma membrane Ca^{2+}-ATPase appears to have a putative binding domain for calmodulin that is exposed to the hydrophilic milieu. This became evident when the enzyme was subjected to controlled proteolysis. When the *L. donovani* PME fraction was exposed to digestion with very low concentrations of trypsin for a short time, the sigmoidal kinetic pattern for Ca^{2+} dependence was essentially abolished, with significant lowering of the K_m for Ca^{2+} (Fig. 9). No further activation could be demonstrated either with parasite calmodulin or with exogenously added Mg^{2+}. Apparently, mild proteolysis alone can convert the enzyme to a high-affinity state for Ca^{2+} that cannot further be activated either by calmodulin or by Mg^{2+}. We also noted that on increasing the time of trypsinization to 15–20 min, nearly 50% of the enzyme activity could be solubilized. This truncated soluble Ca^{2+}-ATPase did not show any sigmoidal kinetics with varying Ca^{2+} concentrations. The enzyme was also rendered completely insensitive to stimulation by Mg^{2+} or by calmodulin (data not shown).

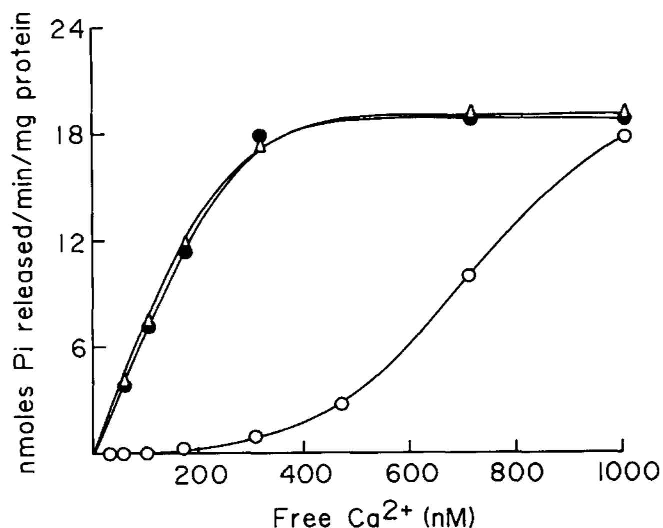

FIG. 9. **Activation of *L. donovani* plasma membrane Ca^{2+}-ATPase by limited proteolysis and effect of calmodulin on it.** 3.0 mg of the membrane preparation in TBS was treated with 2 μg of trypsin for 3 min at 28 °C (1-ml final volume). The digestion was stopped by addition of 50 μg of soybean trypsin inhibitor and centrifuged at 10,000 × *g* for 12 min. The pellet obtained was suspended in 1 ml of TBS, and Ca^{2+}-ATPase activity was assayed immediately at different Ca^{2+} concentrations in the CDTA/Ca^{2+} buffering system. (For assay conditions, see the legend of Fig. 5.) A tube was run for control, where 2 μg of trypsin was added along with soybean trypsin inhibitor. ○, activity of native enzyme; ●, activity of trypsin-treated enzyme; △, activity of trypsin-treated enzyme in the presence of 8 μg of *L. donovani* calmodulin.

DISCUSSION

When studied in its more authentic, physiologically relevant, membrane-associated form, the plasma membrane Ca^{2+}-ATPase from *L. donovani* shows some interesting kinetic and molecular features that set this enzyme apart from other well-studied Ca^{2+}-translocating ATPases. The enzyme, as an integral membrane protein, does not show any dependence on Mg^{2+} for its catalytic activity. Thus, even when all available Mg^{2+} is complexed with CDTA, the enzyme hydrolyzes ATP at a very significant rate at saturating concentrations of Ca^{2+} alone (Fig. 2). This distinguishes this enzyme from other well-characterized Ca^{2+}-ATPases such as red blood cell plasma membrane Ca^{2+}-ATPase and endoplasmic reticulum Ca^{2+}-ATPase, both of which have an obligatory requirement for Mg^{2+} for catalytic activity and both of which have been unambiguously shown to act as extrusion pumps for Ca^{2+} (5, 26). Preliminary reports, on the other hand, are available for several other Ca^{2+}-ATPases (27–30), including one on the plasma membrane of *Trypanosoma rhodesiense* (31), that do not have any apparent requirement for Mg^{2+} for their activity. These enzymes, however, need to be further characterized, and their physiological roles remain to be elucidated.

The unique and most striking feature of the *L. donovani* enzyme is the strong sigmoidal kinetics that it exhibits with varying Ca^{2+} concentrations. Positive modulation with Mg^{2+} (Fig. 4) or with calmodulin (Figs. 5 and 7) and desensitization with trypsin (Fig. 9) clearly demonstrate the allosteric nature of this enzyme. The molecular basis of this enzymatic feature remains to be elucidated. None of the extensively studied Ca^{2+}/Mg^{2+}-ATPases from mammalian sources exhibit such cooperative phenomenon with Ca^{2+} (26). In contrast to these monomeric Ca^{2+}-ATPases, the parasite enzyme appears to be a tetramer of two unequal subunits (6). It is not yet clear as to how this quarternary structure may be related to the cooperative phenomenon observed with this enzyme.

Because of its sigmoidicity, the membrane-integral Ca^{2+}-ATPase remains virtually inactive below 150 nM free Ca^{2+}. Employing fura 2 acetoxymethyl ester as the fluorometric probe, Philosoph and Zilberstein (7) have shown earlier that the cytoplasmic concentration of free Ca^{2+} in the promastigote form of the pathogen is 85 ± 10 nM. We have already shown that the transmembrane enzyme has its catalytic site in the cytoplasmic milieu (6). Obviously, the enzyme can start functioning effectively either when the free Ca^{2+} concentration rises momentarily in response to a physiological signal to a high level (>500 nM) or when it is activated by an endogenous positive modulator that can decrease the K_m of the enzyme for Ca^{2+} to an acceptable physiological level (<150 nM). We suggest that the leishmanial calmodulin acts as a major physiological regulator in modulating the activity of this parasite enzyme. The activating protein that was purified on the basis of its strong positive modulatory effect on allosteric Ca^{2+}-ATPase has all the characteristic properties of calmodulin (see text and Miniprint). Interaction of soluble endogenous calmodulin with plasma membrane-bound Ca^{2+}-ATPase abolishes sigmoidicity, increases the V_{max} significantly, and most important lowers the K_m for free Ca^{2+} to 100 nM (Fig. 7). At the presumed cytoplasmic concentration of 80 nM for free Ca^{2+}, the enzyme shows an activity of ~18 nmol/min/mg of membrane protein. Leishmanial calmodulin therefore has the potential to specifically convert the inactive enzyme to a high-affinity state as it interacts with this target protein. The physiological significance of the nearly identical effect of Mg^{2+} and its interrelationship with endogenous calmodulin remains an unresolved question of this study. The calmodulin gene has been cloned from *Trypanosoma brucei* (32), and calmodulin has been isolated from *Trypanosoma cruzi* on the basis of its activating effect on heterologous cAMP phosphodiesterase (33). Unlike in this report, no possible role of these parasite calmodulins could be assigned in these cases.

The status of Ca^{2+} as a biomodulator in the life cycle of the kinetoplastida group of protozoal pathogens is not clear at the moment. Evidence is slowly accumulating that suggests active Ca^{2+} metabolism in these organisms. Increased cytoplasmic Ca^{2+} concentration has been implicated in the shedding of surface coat protein antigen of *T. cruzi* (34). Calmodulin and Ca^{2+}-activated protein kinase C have been partially characterized from other *Trypanosoma* species (33, 35). Seebeck and Gehr (36) have shown earlier that exposure of resting cells of *T. brucei* to the chlorpromazine group of drugs results in dissociation of pellicular microtubules from the plasma membrane of the organism. In the case of *L. donovani*, using membrane vesicles, Benaim and Romero have demonstrated ATP-dependent Ca^{2+} movement across the plasma membrane (37). ATP-dependent accumulation of Ca^{2+} can also take place in internal organelles of the parasite (7). We already reported the presence of this high-affinity Ca^{2+}-ATPase in *L. donovani*, which can act as a potential extrusion pump for cytoplasmic free Ca^{2+} (6). We now demonstrate that the plasma membrane Ca^{2+}-ATPase is possibly an allosteric enzyme that is under regulatory control of parasite calmodulin and can be activated by it to a physiologically relevant level. We also have evidence suggesting the presence of an active Ca^{2+} entry system that is sensitive to channel blockers for Ca^{2+}.[3] Taken together, these observations clearly show that basic biochemical machinery for Ca^{2+} homeostasis exists in *L. donovani*. The specific physiological role of Ca^{2+} in the life cycle of this or any other parasite, however, remains to be elucidated. We have recently observed that complexation of external Ca^{2+} with EGTA fails to arrest *L. donovani* cell division in culture. It is likely that the biomodulatory role of Ca^{2+} is primarily confined to the morphogenetic transformation of the parasite in the host macrophage environment. We are at present exploring such a possibility employing an *in vitro* macrophage transformation system for the parasite.

Acknowledgment—We gratefully acknowledge the help extended by Badal Bhattacharya (University Science Instrument Center, Jadavpur University) for atomic absorption analysis. We also thank Dr. Chitra Dutta and Dr. Manish Bagchi for their valuable suggestions.

REFERENCES

1. Killick-Kendrick, R. (1979) in *Biology of Kinetoplastida* (Lumsden, W. H. R., and Evans, D. A., eds) Vol. 2, pp. 395–460. Academic Press, New York
2. Alexander, J., and Vickerman, K. (1975) *J. Protozool.* **22,** 502–508
3. Rasmussen, H., and Goodman, D. B. P. (1977) *Physiol. Rev.* **57,** 421–509
4. Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56,** 159–193
5. Carafoli, E. (1987) *Annu. Rev. Biochem.* **56,** 395–433
6. Ghosh, J., Ray, M., Sarkar, S., and Bhaduri, A. (1990) *J. Biol. Chem.* **265,** 11345–11351
7. Philosoph, H., and Zilberstein, D. (1989) *J. Biol. Chem.* **264,** 10420–10424
8. Chakraborty, P., and Das, P. K. (1988) *Mol. Biochem. Parasitol.* **28,** 55–62
9. Dwyer, D. M., and Gottlieb, M. (1984) *Mol. Biochem. Parasitol.* **10,** 139–150
10. Scarborough, G. A. (1975) *J. Biol. Chem.* **250,** 1106–1111
11. Pershadsingh, H. A., and McDonald, J. M. (1980) *J. Biol. Chem.* **255,** 4087–4093
12. Sillen, L. G., and Martell, A. E. (1971) *Stability Constants of Metal Ion Complexes*, Special Publications 17 and 25, Chemical Society, London
13. Lowry, O. H., and Lopez, J. A. (1957) *Methods Enzymol.* **3,** 845–850
14. Bais, R. (1975) *Anal. Biochem.* **63,** 271–273
15. Zilberstein, D., and Dwyer, D. M. (1988) *Biochem. J.* **256,** 13–21
16. Wang, J. H., and Desai, R. (1977) *J. Biol. Chem.* **252,** 4175–4184
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
18. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
19. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121,** 404–427
20. Laemmli, U. K. (1970) *Nature* **227,** 680–685

[3] S. Mazumder, T. Mukherjee, J. Ghosh, M. Ray, and A. Bhaduri, manuscript in preparation.

21. Merril, C. R., Goldman, D., and Keuren, M. L. V. (1984) *Methods Enzymol.* **104,** 441–447
22. Zilberstein, D., and Dwyer, D. M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 1716–1720
23. Vanman, T. C. (1983) *Methods Enzymol.* **102,** 296–310
24. Klee, C. B., Crouch, T. H., and Richman, P. G. (1980) *Annu. Rev. Biochem.* **49,** 489–515
25. Gietzen, K., Wilhrich, A., and Bader, H. (1981) *Biochem. Biophys. Res. Commun.* **101,** 418–425
26. Carafoli, E. (1991) *Annu. Rev. Physiol.* **53,** 531–547
27. Travis, S. M., and Nelson, D. L. (1986) *Biochim. Biophys. Acta* **862,** 39–48
28. O'Neal, S. G., Rhoads, D. B., and Racker, E. (1979) *Biochem. Biophys. Res. Commun.* **89,** 845–850
29. Tuana, B. S., and Dhalla, N. S. (1982) *J. Biol. Chem.* **257,** 14440–14445
30. Iwasa, Y., Iwasa, T., Higashi, K., Matsui, K., and Miyamato, E. (1982) *FEBS Lett.* **142,** 67–71
31. McLaughlin, J. (1985) *Mol. Biochem. Parasitol.* **15,** 189–201
32. Tschudi, C., Young, A. S., Ruben, L., Patton, C. L., and Richards, F. F. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 3998–4002
33. Tellez-Inon, M. T., Ulloa, R. M., Torruella, M., and Torres, H. N. (1985) *Mol. Biochem. Parasitol.* **17,** 143–153
34. Voorheis, H. P., Bowles, D. J., and Smith, G. A. (1982) *J. Biol. Chem.* **257,** 2300–2304
35. Gomez, H. L., Erijman, L., Arauzo, S., Torres, H. N., and Tellez-Inon, M. T. (1989) *Mol. Biochem. Parasitol.* **36,** 101–108
36. Seebeck, T., and Gehr, P. (1983) *Mol. Biochem. Parasitol.* **9,** 197–208
37. Benaim, G., and Romero, P. J. (1990) *Biochim. Biophys. Acta* **1027,** 79–84

SUPPLEMENTAL MATERIAL TO

ALLOSTERIC MODULATION OF LEISHMANIA DONOVANI PLASMA MEMBRANE Ca^{2+}-ATPASE BY ENDOGENOUS CALMODULIN

SARMILA MAZUMDER, TANMOY MUKHERJEE, JAGADANANDA GHOSH, MANJU RAY AND AMAR BHADURI

DETERMINATION OF FREE Ca^{2+} AND Mg^{2+} IONS IN ASSAY MEDIUM : The plasma membrane of L. donovani promastigote contains a Mg^{2+}-ATPase (**15**) and also a high affinity Ca^{2+}-ATPase (**6**). As liberation of inorganic phosphate could be due to either of these two enzymatic activities, the concentration of each of these ions in their free form had to be determined, particularly when CDTA was used as the complexing agent.

Each colorimetric assay medium contained in a total volume of one ml, approximately 200 µg protein from PME fraction. The contaminating Ca^{2+} and Mg^{2+} ions from this fraction was calculated to be 4 µM and 10 µM respectively. Other sources of contaminating Ca^{2+} and Mg^{2+} were water, different chemicals in the assay mixture and the activating factor for Ca^{2+}-ATPase. In these sources the maximum total amount of Ca^{2+} and Mg^{2+} were 8 µM and 4 µM respectively. In determining free Ca^{2+} and Mg^{2+} these values were included in the calculation.

Free Ca^{2+} and Mg^{2+} concentrations in presence of CDTA or EGTA buffering system were calculated by using the following equations as described by Pershadsingh and McDonald (**11**)

$$[Ca^{2+}] + [CaL^{2-}] = [Ca_T] \quad \ldots \quad (a)$$

$$[Mg^{2+}] + [MgL^{2-}] = [Mg_T] \quad \ldots \quad (b)$$

$$[L] + [CaL^{2-}] + [MgL^{2-}] = [L_T] \quad \ldots \quad (c)$$

$$K'_{CaL} = \frac{[CaL^{2-}]}{[Ca^{2+}][L]} \quad \ldots \quad (d)$$

$$K'_{MgL} = \frac{[MgL^{2-}]}{[Mg^{2+}][L]} \quad \ldots \quad (e)$$

In the equation, $[Ca^{2+}]$ and $[Mg^{2+}]$ are free Ca^{2+} and Mg^{2+} concentrations. $[CaL^{2-}]$ and $[MgL^{2-}]$ are Ca^{2+} and Mg^{2+} concentrations bound to chelator (CDTA or EGTA). $[Ca_L]$, $[Mg_T]$ and $[L_T]$ are total calcium, magnesium and chelator concentrations. $[L]$ represents the total free chelator concentration. K'_{CaL} & K'_{MgL} are apparent association constants at pH 7.4 as given by Pershadsingh and McDonald (**11**)

Equations (**a**), (**c**) and (**d**) were used to calculate free $[Ca^{2+}]$ in Ca^{2+}/chelator buffer where $[MgL^{2-}]$ was neglected from the equation (**c**). Equations (**b**), (**c**) and (**e**) were similarly used to calculate free $[Mg^{2+}]$ in Mg^{2+}/chelator buffer where $[CaL^{2-}]$ was neglected from the equation (**c**). All equations (**a**) to (**e**) were required when free $[Ca^{2+}]$ and $[Mg^{2+}]$ had to be calculated in CDTA buffering system.

In presence of CDTA buffering system (500 µM) with contaminating Ca^{2+} (12 µM) and Mg^{2+} (14 µM), the free contaminating Ca^{2+} and Mg^{2+} concentrations from these sources were calculated by equations (**a**) to (**e**) to be approximately 2.6 nM and 10 nM respectively.

ASSAY OF PLASMA-MEMBRANE ASSOCIATED Ca^{2+}-ATPASE ACTIVATING FACTOR : Stimulation of Ca^{2+}-ATPase activity in the PME fraction was used to assay the endogenous Ca^{2+}-ATPase activating factor of L. donovani promastigotes. A 100% stimulation of the Ca^{2+}-ATPase activity of PME fraction (150-200 µg of PME protein) in CDTA complexed assay system, at a free Ca^{2+} ion concentration of 639 nM, was defined as one unit. The reaction mixture in a total volume of 1.0 ml, contained 100 mM Tris-HCl buffer, pH 7.4, 0.5 mM ATP, 200 µg enzyme of the PME fraction, requisite amount of the factor and 500 µM CDTA with or without 430 µM $CaCl_2$. The enzyme was preincubated with the factor for 10 mins and the reaction was initiated by the addition of ATP. The inorganic phosphate liberated was measured according to the method of Lowry and Lopez (**13**).

OTHER ASSAYS AND METHODS : cAMP phosphodiesterase activity was measured by coupling the reaction with excess of 5'-nucleotidase. The method, described by Wang and Desai (**16**) was modified as follows . The 1.0 ml reaction mixture consisted of 100 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, 0.5 mM cAMP and 0.1 mM $CaCl_2$, 0.05 unit of 5'-nucleotidase and requisite amount of phosphodiesterase. Control was run without 5'-nucleotidase. After 30 mins of incubation, the reaction was terminated by addition of 50 µl of 20% Trichloroacetic acid. The pH was raised to 4.0 followed by the addition of 0.2 ml of 1.0% ascorbic acid and 0.2 ml of 1.0% ammonium molybdate in each tube. Colour was read at 700 nm after 20 mins.

Protein was estimated either by the method of Lowry et. al (**17**) or where necessary by the method of Bradford (**18**). In both cases bovine serum albumin was taken as standard.

Polyacrylamide gel electrophoresis and SDS-gel electrophoresis were carried out following the method of Davis (**19**) and Laemmli (**20**) respectively. The gels were stained with Coomassie Brilliant Blue or silver stained according to the method of Merril et. al. (**21**).

PURIFICATION OF Ca^{2+}-ATPASE ACTIVATING FACTOR : All the operations were done at room temperature, 25°C. Eight grams of washed packed cells were suspended in 400 ml of 5 mM Tris-HCl buffer, pH 7.4 and subjected to hypotonic shock for 1 hr as described earlier (**6**). After centrifugation at 4000xg for 20 mins the supernatant obtained from the hypotonic shock was collected. Solid ammonium sulphate (144.0 mg/ml) was now slowly added to the supernatant with constant stirring. After standing for 10 mins, the precipitate was rejected and the supernatant was further treated with ammonium sulphate (436.0 mg/ml) and pH was adjusted to 4.1. After 10 mins, it was centrifuged at 27,000xg for 15 mins. The pellet was then taken in 40 ml of 10 mM Imidazole-HCl buffer, pH 6.1 and dialysed against 2.0 litres of the same buffer. The dialysed sample was placed in a boiling water bath for 2 mins and then centrifuged to remove the precipitated protein. The supernatant was lyophilised to 4.0 ml and dialysed again against 1.0 litre of 10 mM Imidazole-HCl buffer, pH 6.1 to remove excess salt. The dialysed sample was charged on DEAE-cellulose column (20 cm x 0.5 cm), previously equilibrated with 10 mM Imidazole-HCl buffer, pH 6.1. Elution was performed with a linear gradient of NaCl (0-0.5 M). Fractions containing 80-85% of Ca^{2+}-ATPase activating factor that eluted out as unabsorbed fraction were pooled and lyophilised to 1.0 ml. A small activity elutes out at 0.2 M NaCl, but it was not characterised any further. The pooled, lyophilised fraction was then charged on a Sephadex G-100 column (75 cm x 2 cm), equilibrated with 10 mM Imidazole-HCl buffer, pH 6.1 and eluted at a flow rate of 0.15 ml/min. Active fractions containing Ca^{2+}-ATPase activating factor were pooled and stored at -70°C for subsequent use. The factor remains stable at -70°C for over six months.

Table 2 : Purification of calmodulin from L. donovani

Step(s)	Total Protein (mg)	Total activity (Unit)	Specific activity (Unit/mg)	Purification (fold)	Yield (%)
Ammonium sulfate 25-85% cut	78.3	293	3.74	1	100
Heat treatment	9.7	275	28.35	7.58	93.8
DEAE-cellulose	2.5	233.7	93.2	24.9	79.7
Sephadex G-100	0.629	168	267.9	71.39	57.3

One unit of calmodulin represents 100% stimulation of Ca^{2+}-ATPase activity of 200 µg of membrane protein of the PME-fraction at Ca^{2+} concentration 639 nM.

Table 3 : Activation of bovine brain cyclic nucleotide phosphodiesterase by L. donovani calmodulin

For this experiment 5 milliunit of pure bovine brain cycic nucleotide phosphodiesterase in each tube was preincubated with pure calmodulin from Leishmania donovani or from bovine brain at room temperature (25°C) for 10 mins. For inhibition study with trifluoperazine, calmodulin activated enzyme was preincubated further for another 10 min with the drug. Details of assay procedure has been described in Materials and Methods.

Enzyme	Additions	Activity (n mol Pi released)
Phosphodiesterase	-	56
Phosphodiesterase	L. donovani calmodulin (12 µg)	130
Phosphodiesterase	bovine brain calmodulin (2 µg)	145
Phosphodiesterase	L. donovani calmodulin (12 µg) + Trifluoperazine (50 µM)	78

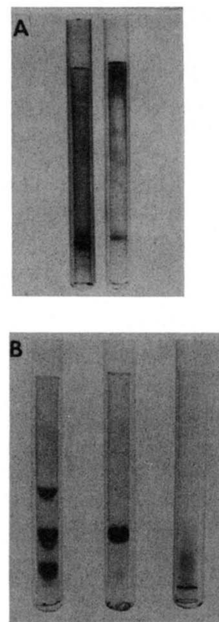

Fig.6 : Polyacrylamide gel electrophoresis and sodium dodecyl sulfate polyacrylamide gel electrophoresis of L. donovani calmodulin.

In A, Polyacrylamide gel electrophoresis in 10.0% gel, tube 1: bovine brain calmodulin (20 µg), tube 2 : calmodulin from Leishmania donovani (15 µg).

In B, Sodium dodecyl sulfate polyacrylamide gel electrophoresis in 10% gel. Tube 1 : lowermost band represents α-lactalbumin, M.W. 14,200, middleone represents trypsin inhibition M.W. 20,100 and uppermost band represents carbonic anhydrase, M.W. 29,000 (2.5 µg each).

Tube 2 : Bovine brain calmodulin (10 µg). Tube 3: calmodulin from L. donovani (25 µg). The gels were stained with Coomassie brilliant blue (0.2%). The migration was from top (cathode) to bottom (anode).