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# Characterization of a calcium-dependent ATPase in *Entamoeba invadens*

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**A high-affinity calcium-dependent ATPase ($Ca^{2+}$-ATPase) was identified in a crude plasma membrane fraction from *Entamoeba invadens* (IP-1 strain). The $Ca^{2+}$-ATPase activity was solubilized from the membrane by utilizing the non-ionic detergent octylglucoside. The activity had an apparent half maximal saturation constant of $0.4 \pm 0.05$ $\mu$M for free calcium. The calcium activation of ATPase activity followed a cooperative mechanism (Hill number of $2.3 \pm 0.13$) which suggests that two interacting sites were involved. The high-affinity $Ca^{2+}$-ATPase appeared to be magnesium-independent, since by lowering contaminant free magnesium with *trans*-cyclohexane-1,2-diamine-*N,N,N',N'*-tetraacetic acid did not modify the activity observed with $Ca^{2+}$. The apparent $K_m$ of the enzyme for ATP was 31 $\mu$M. The observed activity had an optimum pH of 8.8. The enzyme was insensitive to various agents such as $Na^+$, $K^+$, ouabain, dicyclohexylcarbodiimide, KCN, $NaN_3$, mersalyl, quercetin, ruthenium red and vanadate. Only lanthanum (0.5 mM) inhibited 100% the enzymatic activity. Calmodulin and trifluoperazine at the concentrations tested did not modify the $Ca^{2+}$-ATPase activity.**

## Introduction

It has been shown that variations in the cytoplasmic $Ca^{2+}$ concentration induced by various extracellular stimuli play a critical role in many cellular processes such as metabolism, exocytosis, contraction, vision, cell division, differentiation and fertilization. Inside the cell the role of $Ca^{2+}$ as a trigger is mediated by a set of structurally related, yet functionally distinct, receptor proteins [1]. This implies an accurate control of the basal cytoplasmic $Ca^{2+}$ level by the cell. The $Na^+/Ca^{2+}$ exchange and the ATP-driven $Ca^{2+}$-pump activities are the two known mechanisms for the extrusion of $Ca^{2+}$ through the plasma membranes. The latter system has been extensively studied in several cells, and it is well known that the $Ca^{2+}$-extrusion pump of erythrocyte plasma membrane is a $Ca^{2+}$-ATPase [2]. This enzyme is stimulated by micromolar concentrations of $Ca^{2+}$ and calmodulin [3], and according to some authors the true substrate for the purified enzyme is Ca-ATP [4]. Recently a high-affinity $Ca^{2+}$-dependent ATPase which is fully active in absence of exogenous $Mg^{2+}$, has been reported in the plasma membrane of

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Abbreviations: CDTA, *trans*-cyclohexane-1,2-diamine-*N,N,N',N'*-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DCCD, dicyclohexylcarbodiimide; octylglucoside, octyl-β-D-glucopyranoside; PMSF, phenylmethylsulfonyl fluoride; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; Triton X-100, octylphenoxypolyethoxyethanol.

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various cells: alveolar macrophage [5], adipocyte [6], hepatocyte [7–9], corpus luteum [10], and Ehrlich ascites tumor cell [11]; the above enzymes have been proposed to be $Ca^{2+}$-pumping ATPases.

In *Entamoeba histolytica* a high specific activity membrane $Ca^{2+}$-ATPase without requirement of exogenous $Mg^{2+}$ has been reported [12,13]. In *Entamoeba invadens* an amoeba belonging to the same genus, the presence of a membrane bound $Mg^{2+}$-stimulated ATPase of low specific activity has been described [14,15]. In this communication we report the characterization of an *E. invadens* plasma membrane-associated $Ca^{2+}$-dependent ATPase which does not require $Mg^{2+}$.

## Materials and Methods

*Culture and harvesting of cells*

*Entamoeba invadens*, strain IP-1, was employed throughout this study. Amoebae were cultivated axenically as described previously [16], in Diamond's TYI-S-33 medium [17]. Cultures were inoculated with $3 \cdot 10^4$ cells per ml. Cells were grown for 7–10 days at 22°C, cultures were chilled in ice bath for 5 min and cells collected by centrifugation at $500 \times g$ for 10 min and washed twice with ice-cold 50 mM Tris-HCl (pH 7.4), containing 50 $\mu$M PMSF, 2 mM dithiothreitol (buffer A) and also 10% sucrose.

*Isolation of crude plasma membranes*

The crude plasma membrane fraction was isolated by the method described by Van Vliet et al. [15]. All operations were carried out at 0°C. The cells were resuspended in buffer A plus 10% sucrose and homogenized with 15 strokes in a tight fitting Potter-Elvehjem Teflon-glass homogenizer. The homogenate was centrifuged at $500 \times g$ for 10 min, and the pellet (nuclei, cell debris, and unbroken amoeba cells) was discarded. The supernatant was centrifuged at $4500 \times g$ for 10 min. The top layer (mainly phagolysosomes), was discarded and the remaining supernatant centrifuged at $15\,000 \times g$ for 20 min. The pelleted material was washed twice by resuspending and centrifuging in buffer A plus 10% sucrose. The resulting pellet (crude plasma membrane fraction) was resuspended in buffer A, and was the starting material for further fractionation.

*Preparation of calmodulin-free plasma membranes*

Crude plasma membranes were washed three times with 1 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 1 mM EGTA, followed by two washings with the same buffer minus EGTA. The membranes were then resuspended in buffer A and washed twice. This treatment has been reported to induce depletion of calmodulin from erythrocytes [18].

*Solubilization of $Ca^{2+}$-ATPase*

In order to remove loosely bound membrane proteins from the crude plasma membrane fraction, this fraction was incubated in a solution containing 0.6 M KCl in buffer A for 1 h at 0°C and then centrifuged at $75\,000 \times g$ for 30 min. The supernatant was removed by aspiration, and the pellet was extracted once more with the 0.6 M KCl solution. The resulting pellet was resuspended in buffer A to a final protein concentration of 4 mg per ml. The above fraction was mixed with 2.5% (w/v) octylglucoside and 2 mg protein per ml and the mixture was incubated for 1 h at 0°C. After centrifugation at $175\,000 \times g$ for 90 min, the supernatant fraction was collected by aspiration and the pellet was resuspended in buffer A up to the starting supernatant volume. The resultant protein fractions were divided into small aliquots and stored under liquid $N_2$.

*$Ca^{2+}$-ATPase assay*

The $Ca^{2+}$-ATPase activity was assayed employing a reaction mixture containing 150 $\mu$mol Tris-HCl buffer (pH 7.4), 0.75 $\mu$mol ATP (disodium salt), pH 7.4, 6 $\mu$mol EGTA and $CaCl_2$ to give the indicated concentration of $Ca^{2+}$ [19], and the crude plasma membrane fraction or the solubilized enzyme (10 to 40 $\mu$g protein) in a total reaction volume of 3 ml. The reaction was started by adding ATP, and carried out at 25°C. After 10 min incubation, the reaction was stopped by the addition of 300 $\mu$l of 5 M $H_2SO_4$. The inorganic phosphate ($P_i$) released from ATP was determined colorimetrically [20]. $Ca^{2+}$-ATPase activity was calculated by subtracting the values obtained in the presence of EGTA alone (control) from those obtained with $Ca^{2+}$-EGTA buffer, and are expressed as nmol $P_i$ released from ATP per mg protein per minute at 22°C.

*Calcium and magnesium buffers*

The concentration of free calcium ($Ca^{2+}$) and free magnesium ($Mg^{2+}$) was adjusted in the micromolar range by utilizing the ligands EGTA and CDTA. Free metal ion concentrations were calculated employing a computer program based: on the absolute stability constants for each ligand [19,23–26], on the pH value, and on the competitive effects of $Mg^{2+}$, $Ca^{2+}$, $Na^{+}$ and ATP, as described by Fabiato and Fabiato [19]. The stability constants for the interactions of EGTA, CDTA, and ATP with $H^{+}$, $Ca^{2+}$ and $Mg^{2+}$ employed are given in Table I.

*Gel electrophoresis*

Non-denaturing polyacrylamide gel electrophoresis was carried out according to Laemmli [21] in 6% polyacrylamide rods (0.4 × 10 cm). The gels were prepared with Tris-glycine buffer (pH 8.9) (25 mM Tris/192 mM glycine/20% glycerol) and were prerun for 30 min. The electrode compartments contained the same buffer present in the gel, but without the addition of glycerol. The octylglucoside solubilized preparation (50–100 μg protein) was applied to the gel and electrophoresis was performed at 2 mA per gel at 2–4°C until the bromophenol blue marker reached 1 cm from the end of the gel (about 2 h). The gels were then removed and either stained for protein with Coomassie brilliant blue R, and scanned at 550 nm, or stained for $Ca^{2+}$-ATPase activity using a modification of the method of Abrams and Baron [22]. Gels were incubated in a solution containing 2 mM ATP, 3 mM $CaCl_2$ in 50 mM Tris-HCl (pH 7.4) at 22°C, for 5 min. The gels were then washed with distilled water and incubated in a mixture containing 10% ascorbate solution and 0.42% ammonium molybdate in 0.5 M $H_2SO_4$ (1 : 6, v/v) at 45°C, until the blue band showing the position of the enzyme was visible [20]. Gels were rinsed with distilled water and scanned at 750 nm with an Isco gel scanner.

TABLE I

ABSOLUTE ($K$) AND APPARENT ($K'$) STABILITY CONSTANTS FOR THE LIGANDS EGTA, CDTA, AND ATP

| Ligand | Cation | log $K_1$ ($M^{-1}$) | log $K_2$ ($M^{-1}$) | log $K'$ (pH 7.4) * ($M^{-1}$) |
|---|---|---|---|---|
| EGTA | $H^{+}$ | 9.460 [a] | 8.850 [a] | – |
| | $Ca^{2+}$ | 10.716 [b] | 5.330 [a] | 7.19 |
| | $Mg^{2+}$ | 5.210 [a] | 3.370 [a] | 2.10 |
| CDTA | $H^{+}$ | 11.700 [c] | 6.120 [c] | – |
| | $Ca^{2+}$ | 12.354 [b] | – | 8.032 |
| | $Mg^{2+}$ | 10.320 [d] | – | 5.998 |
| ATP | $H^{+}$ | 6.950 [e] | 4.050 [e] | – |
| | $Ca^{2+}$ | 3.982 [e] | 1.800 [e] | 3.85 |
| | $Mg^{2+}$ | 4.324 [e] | 2.740 [e] | 4.19 |

[a] Values were taken from Schwarzenbach et al. [23].
[b] Values were taken from Allen et al. [26].
[c] Values were taken from Sillén and Martell [25].
[d] Value was taken from Chabere and Martell [24].
[e] Values were taken from Fabiato and Fabiato [19].
* Apparent ($K'$) values were calculated according to Fabiato and Fabiato [19].

*Protein determination*

Protein concentrations were measured by the method of Lowry et al. [27], employing bovine serum albumin as standard.

*Determination of kinetic parameters*

The kinetic constants for the $Ca^{2+}$-ATPase activity were determined by fitting the Hill equation to experimental data according to the iterative method of Crabbe [28].

*Chemicals*

ADP, ATP (disodium salt), bovine serum albumin, CDTA, EGTA, GDP, GTP, *p*-nitrophenyl phosphate, and Triton X-100 were obtained from Sigma. Dithiothreitol, PMSF, octylglycoside and bovine brain calmodulin were purchased from Calbiochem-Behring Corp. Other chemicals used were of the highest purity.

## Results

In order to investigate the presence of $Ca^{2+}$-ATPase activity in *E. invadens* extracts, this enzymatic activity was assayed following the procedure described for *E. histolytica* [13], with some modifications as described in Methods. It was found that the crude homogenate contained a $Ca^{2+}$-ATPase activity with a specific activity of 45 ± 5 nmol $P_i$ per mg protein per min. The $Ca^{2+}$-ATPase activity was found mainly (more

than 90%) in the crude plasma membrane fraction exhibiting an specific activity of 284 ± 20 nmol $P_i$ per mg protein per min (data not shown).

*Solubilization of $Ca^{2+}$-ATPase activity*

Since the $Ca^{2+}$-ATPase activity was found to be associated with the crude plasma membrane fraction, it was of interest to attempt the solubilization of this activity in order to study its properties. When this fraction was incubated with the non ionic detergents Triton X-100 (0.4%(w/v)) or octylglucoside (1.25%(w/v)) in buffer A at pH 7.4, both detergents caused an apparent increase in the $Ca^{2+}$-ATPase activity, which suggested a latency of the enzyme since it required the presence of detergent to reach maximal expression (Table II). The results obtained for the solubilization of the $Ca^{2+}$-ATPase activity with octylglucoside are shown in Table III. Control experiments where crude plasma membrane fraction was treated with 0.6 M KCl indicated that more than 90% of the $Ca^{2+}$-ATPase remained sedimentable. The $Ca^{2+}$-ATPase could be solubilized more than 90% after incubating the KCl-treated fraction with 1.25%(w/v) octylglucoside at 0°C for 60 min, and centrifuged and 175 000 × *g* for 90 min to remove insoluble material as described in Methods. The $Ca^{2+}$-ATPase activity solubilized accounted for more than 90% of the total activity detected in the extract treated with detergent.

TABLE II

EFFECT OF OCTYLGLUCOSIDE AND TRITON X-100 ON $Ca^{2+}$-ATPase

$Ca^{2+}$-ATPase activity in the crude plasma membrane fraction was assayed in the presence of 53 μM $Ca^{2+}$. Final concentrations of protein and detergent were: 2 mg/ml protein and 1.25% octylglucoside or 2 mg/ml protein and 0.4% Triton X-100. Data represent the means ± S.D. of three assays.

| Additions | Activity | | Recovery of activity (%) |
|---|---|---|---|
| | specific (nmol/mg per min) | total (μmol/min) | |
| None | 284 ± 28 | 4.22 | 100 |
| Octylglucoside (1.25%(w/v)) | 513 ± 33 | 7.83 | 186 |
| Triton X-100 (0.4%(w/v)) | 454 ± 31 | 6.75 | 160 |

TABLE III

SOLUBILIZATION OF $Ca^{2+}$-ATPase of *ENTAMOEBA INVADENS*

| Fraction | Total protein (mg) | Activity | |
|---|---|---|---|
| | | total (μmol/min) | specific (nmol/mg per min) |
| Crude plasma membrane | 39.7 | 13.20 | 332 ± 17 |
| KCl-treated crude plasma membrane | 23.4 | 9.85 | 421 ± 24 |
| Octylglucoside supernatant [a] | 16.4 | 12.85 | 784 ± 55 |
| Octylglucoside pellet [a] | 6.02 | 0.48 | 80 ± 7 |

[a] KCl-treated crude plasma membrane fraction (2 mg protein/ml) was incubated with octylglucoside at a final concentration of 1.25%(w/v) for 1 h at 0°C, as described in Methods. After centrifugation at 175 000 × *g* for 90 min, at 0°C, the supernatant fraction was collected and the pellet was resuspended in buffer A up to the same volume of supernatant. Data represent the means ± S.D. of three assays.

*The effect of pH on the $Ca^{2+}$-ATPase activity*

The effect of pH on the soluble $Ca^{2+}$-ATPase activity was assayed in the presence of 150 μM $Ca^{2+}$. The $Ca^{2+}$ concentration was adjusted with EGTA for a given pH, using the method reported by Fabiato and Fabiato [19]. The optimal pH value for the $Ca^{2+}$-ATPase activity was 8.8 (Fig. 1). A broad peak of activity was observed between pH values of 5.0 to 6.0. Nevertheless, the activity observed between pH 5.0 to 6.0 could be inhibited more than 60% by NaF 500 μM, this suggests that in addition to the $Ca^{2+}$-ATPase activity there are acid phosphatase and nucleotide diphosphatase activities present in the preparation, as reported in *E. invadens* and in *E. histolytica* [29,12]. On the other hand the activity between pH 7.0 to 9.6 was not significantly inhibited by NaF (less than 10%) suggesting that the results at alkaline pH reflected only $Ca^{2+}$-ATPase activity.

*The effect of $Ca^{2+}$ on $Ca^{2+}$-ATPase*

The dependence of $Ca^{2+}$ concentration of the enzyme was tested by using the $Ca^{2+}$-EGTA buffer system to control the free calcium ($Ca^{2+}$) concentration in the assay mixture. The absolute stability constants for $Ca^{2+}$-EGTA complex given in

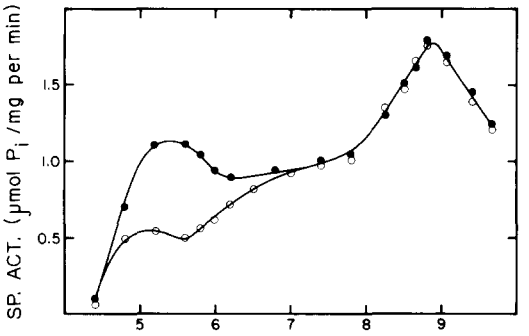


Fig. 1. pH effect on the $Ca^{2+}$-ATPase activity of *E. invadens*. The $Ca^{2+}$-ATPase activity was assayed in the presence of fixed concentrations of $Ca^{2+}$ at 100 $\mu$M adjusted by $Ca^{2+}$-EGTA buffer at various pH values as indicated. Apparent stability constants with ATP and EGTA for a given pH were calculated as reported previously [19]. Activities were assayed in the absence (●———●), or in the presence of 500 $\mu$M NaF (○———○). Each point represents the mean of triplicate assays carried out on two different solubilized preparations.

Table I were employed. Since EGTA cannot be used as effective $Ca^{2+}$ buffer outside the range of pH 6.2 to 7.5 [19], those experiments which required an accurate control of the $Ca^{2+}$ concentration were carried out at pH 7.40. $Ca^{2+}$-ATPase was measured over a wide range of $Ca^{2+}$ concentration from 0.02 to 2000 $\mu$M.

The data shown in Fig. 2 indicated that are

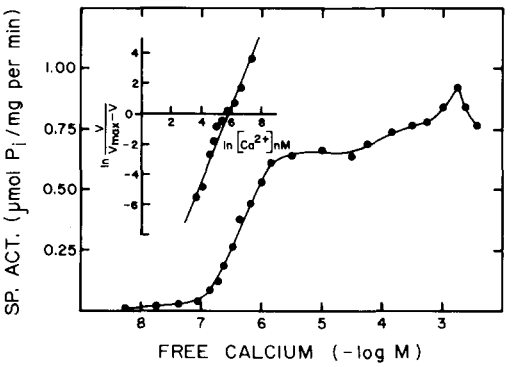


Fig. 2. Effect of $Ca^{2+}$ concentration on ATPase activity. Soluble enzyme was incubated for 10 min in the presence of several amounts of $Ca^{2+}$ using the $Ca^{2+}$-EGTA buffer as desribed in Methods. The inset shows the best-fitting linear form of the Hill equation using data records between $Ca^{2+}$ concentrations of 0.1 and 1.7 $\mu$M. A Hill number of $2.3 \pm 0.13$ and a $K_{0.5}$ of $0.4 \pm 0.03$ $\mu$M were obtained with a correlation coefficient of 0.98. Each point represents the mean of triplicate assays carried out on two different solubilized preparations.

apparently two separate saturable components with respect to $Ca^{2+}$ concentration. One component exhibited an apparent half-maximal saturation constant ($K_{0.5}$) of $0.40 \pm 0.05$ $\mu$M at pH 7.40 and another component with a lower affinity for $Ca^{2+}$ with $K_{0.5}$ about 180 $\mu$M at pH 7.40. The Hill number for the components were $2.3 \pm 0.13$ and 0.9, respectively.

In order to see if the two components observed for $Ca^{2+}$ were due to the presence of more than one enzymatic activity, the solubilized plasma membrane fraction was subjected to polyacrylamide gel electrophoresis under non-denaturing conditions and stained for $Ca^{2+}$-ATPase activity and protein. The gel densitometer traces showed that at saturating $Ca^{2+}$ concentration a single peak of $Ca^{2+}$-ATPase activity could be detected (Fig. 3b), whereas more than 15 protein bands were detected in gel when stained with Coomassie brilliant blue R (Fig. 3a). The same results were

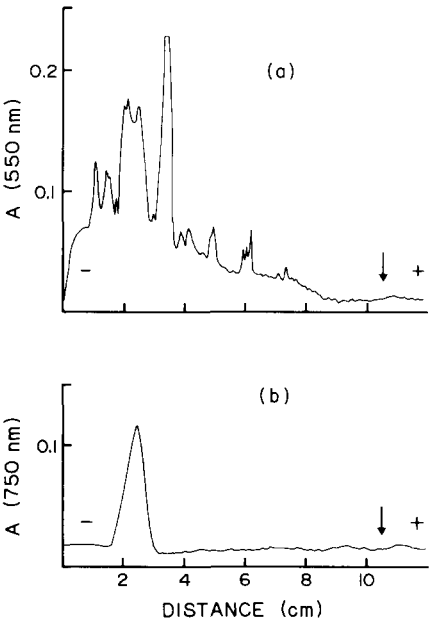


Fig. 3. Gel densitometer traces of octylglucoside solubilized preparation subjected to electrophoresis on 6% non-denaturing polyacrylamide gels. 47 $\mu$g protein were applied to each gel. (a) Gel stained for protein and scanned at 550 nm. (b) Gel stained for $Ca^{2+}$-ATPase activity and scanned at 750 nm. Arrows indicates the position of the bromophenol blue band. Conditions for electrophoresis and detection of protein and enzymatic activity were as described under Methods.

obtained when stained for $Ca^{2+}$-ATPase activity at 10 $\mu$M $Ca^{2+}$ concentration (data not shown).

*Effect of $Mg^{2+}$ on the $Ca^{2+}$-ATPase activity*

To examine the $Mg^{2+}$ requirement of the enzyme, increasing concentrations of $Mg^{2+}$ were tested in the presence of 53 $\mu$M $Ca^{2+}$ adjusted by the $Ca^{2+}$-EGTA buffer system. The addition of increasing concentrations of $Mg^{2+}$ did not show any stimulatory effect on the enzyme activity; in fact it was inhibitory at $Mg^{2+}$ concentrations above 100 $\mu$M, i.e. 50% inhibition was attained at 3 mM $Mg^{2+}$ (Fig. 4). To test the role of the endogenous $Mg^{2+}$ present in the assay mixture on the $Ca^{2+}$-ATPase activity, CDTA was used for controlling more specifically $Mg^{2+}$ and $Ca^{2+}$ concentrations. CDTA has a high affinity for both $Ca^{2+}$ and $Mg^{2+}$ [24–26,30], but it is possible to control the concentrations of the free ions in the media by suitable buffers.

In the conditions of the experiment of Fig. 5 at the highest concentration of free $Ca^{2+}$ (10 $\mu$M), the concentration of free $Mg^{2+}$ was less than 1 $\mu$M. As in these conditions optimal hydrolytic activity was observed, it may be inferred that the activity is independent of $Mg^{2+}$ or that it acts at concentrations below 1 $\mu$M. Fig. 5, also shows that $Ca^{2+}$-CDTA and $Ca^{2+}$-EGTA buffers gave similar results, a slight displacement of the curve to the left reflects a change of the affinity for $Ca^{2+}$ in the

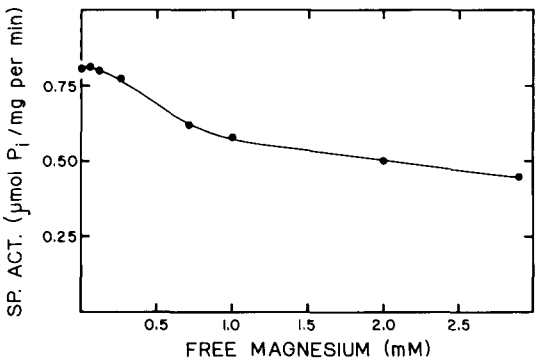


Fig. 4. Effect of exogenous $Mg^{2+}$ on $Ca^{2+}$-ATPase activity. Soluble enzyme was incubated for 10 min in the presence of fixed concentration of $Ca^{2+}$ at 53 $\mu$M adjusted with $Ca^{2+}$-EGTA buffer as described in Methods. Each point represent the mean of triplicate assays carried out on two different solubilized preparations.

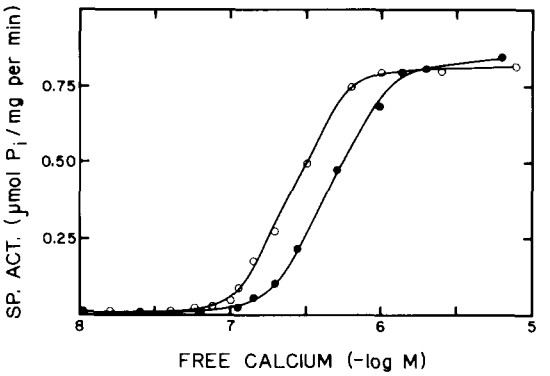


Fig. 5. $Ca^{2+}$ dependence of the ATPase activity in the presence of $Ca^{2+}$-EGTA and $Ca^{2+}$-CDTA buffer systems. The $Ca^{2+}$-ATPase activity was assayed as described in Methods in the presence of various concentrations of $Ca^{2+}$ controlled with EGTA (●———●) or CDTA (○———○). The total concentration for each ligand was 2 mM. Each point represents the mean of triplicate assays carried out on two different solubilized preparations.

$Ca^{2+}$-CDTA buffer system ($K_{0.5}$ of $0.25 \pm 0.05$ $\mu$M), and could be due to the inaccuracy of the values for the absolute binding constants reported for the $Ca^{2+}$-CDTA and $Mg^{2+}$-CDTA complexes [24–26,30].

*Effect of ATP on the $Ca^{+}$-ATPase activity*

The $Ca^{2+}$-ATPase dependence on the ATP concentration in the presence of 150 $\mu$M $Ca^{2+}$ concentration at pH 7.4 was measured (Fig. 6). From a Lineweaver-Burk plot the $K_m$ value for ATP was determined to be $31 \pm 5$ $\mu$M. A Hill number of 0.90 yielded the best-fit linear form of the Hill equation (Fig. 6, inset), giving a correlation coefficient of 0.995. The best substrate for the $Ca^{2+}$-ATPase was ATP, whereas GTP was hydrolyzed 40% as compared to ATP at pH 7.40. GDP, ADP and *p*-nitrophenyl phosphate were not hydrolyzed by the enzyme at pH 7.40 (data not shown).

*Effect of various agents on the $Ca^{2+}$-ATPase activity*

To further characterize the enzymatic activity, several other substances were tested for their effect on the *E. invadens* $Ca^{2+}$-ATPase. As shown in Table IV several inhibitors of the mitochondrial $H^{+}$-ATPase such DCCD, $NaN_3$, did not have any effect on the $Ca^{2+}$-ATPase activity. Ouabain (an

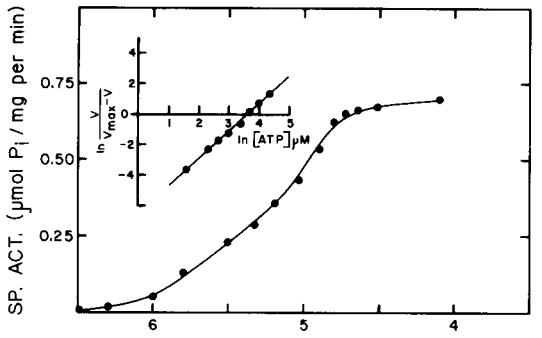


Fig. 6. Dependence of the $Ca^{2+}$-ATPase activity on ATP concentration. $Ca^{2+}$-ATPase activity was assayed for 5 min as described in Methods in the presence of fixed concentrations of $Ca^{2+}$ at 53 μM, adjusted by $Ca^{2+}$-EGTA buffer at various ATP concentrations, as indicated. The inset shows the best-fitting linear form of the Hill equation using data records between ATP concentrations of 1 and 80 μM. A Hill number of $0.9 \pm 0.07$ and a $K_{0.5}$ for ATP of $31 \pm 5$ μM were obtained with a correlation coefficient of 0.995.

inhibitor for the $(Na^+ + K^+)$-ATPase, NaCl and KCl did not shown any effect on the $Ca^{2+}$-ATPase activity. Mersalyl, a sulfhydryl blocking reagent [31], did not alter the enzyme activity. Orthovanadate (0.2–2 mM), a potent inhibitor for many ATPases [1,32–38], and ruthenium red (1 mM) [39], had no effect on either the crude plasma membrane fraction or the solubilized $Ca^{2+}$-ATPase. Quercetin, a flavonoid that inhibits the $Ca^{2+}$-ATPase and $(Na^+ + K^+)$-ATPase [40,47], did not inhibit the *E. invadens* $Ca^{2+}$-ATPase activity. $LaCl_3$ produced near 100% inhibition of the $Ca^{2+}$-ATPase activity at 500 μM concentration, and NaF produced 10% inhibition at 500 μM and 25% inhibition at 2 mM.

TABLE IV

EFFECT OF VARIOUS AGENTS ON $Ca^{2+}$-ATPase ACTIVITY OF *E. INVADENS*

$Ca^{2+}$-ATPase activity was assayed in the presence of 53 μM $Ca^{2+}$ using octylglucoside solubilized enzyme. Data represent the means ± S.D. of three assays.

| Additions | | specific activity | |
|---|---|---|---|
| Agent | mM | nmol/mg per min | % |
| None | | 295 ± 19 | 100 |
| DCCD | 0.1 | 242 ± 34 | 92 |
| KCN | 10.0 | 281 ± 24 | 95 |
| $NaN_3$ | 10.0 | 286 ± 56 | 97 |
| KCl | 100.0 | 287 ± 23 | 97 |
| NaCl | 100.0 | 290 ± 18 | 98 |
| Ouabain | 2.0 | 275 ± 24 | 94 |
| $LaCl_3$ | 0.15 | 155 ± 16 | 53 |
| $LaCl_3$ | 0.50 | 7 ± 9.0 | 2 |
| Mersalyl | 5.0 | 280 ± 19 | 96 |
| NaF | 0.5 | 263 ± 14 | 90 |
| NaF | 2.0 | 219 ± 17 | 75 |
| Quercetin | 0.5 | 288 ± 27 | 98 |
| Ruthenium red | 1.0 | 269 ± 28 | 92 |
| Vanadate | 0.2 | 290 ± 30 | 98 |
| Vanadata | 2.0 | 292 ± 22 | 99 |

Increasing concentrations of calmodulin (10–100 μM) were assayed on plasma membrane fraction previously treated under conditions reported to remove calmodulin from membranes [18], and in the octylglucoside solubilized $Ca^{2+}$-ATPase. The hydrolytic activity was studied at $Ca^{2+}$ concentrations buffered by EGTA (0.1–10 μM $Ca^{2+}$ concentration). No effect was found on the $Ca^{2+}$ affinity ($K_{0.5}$) not on the $V_{max}$ of the enzyme. Since it has been reported that EGTA blocks the calmodulin stimulation of $Ca^{2+}$-ATPase by lowering the dissociation constant of the enzyme for $Ca^{2+}$ [41,42], the effect of calmodulin on the aforementioned membranes was assayed in the absence of EGTA; in this condition the $Ca^{2+}$ concentration was 10 μM. It was observed that the addition of calmodulin (10–100 μM) had no effect on the apparent enzyme activity. The effect of trifluoperazine, a calmodulin antagonist [43] was also tested. The antagonist in a concentration range of 10–100 μM had no effect on any of the preparations employed. The above results suggest that this enzyme may be a calmodulin-insensitive $Ca^{2+}$-ATPase.

## Discussion

The results of this work demostrate the presence of a $Ca^{2+}$-dependent ATPase activity in the plasma membrane of *E. invadens*. The activity could be solubilized from the crude plasma membrane fraction using the non-ionic detergent octylglucoside. From the data of Fig. 5 this enzymic activity appears to be $Mg^{2+}$-independent; apparently in this respect it could resemble the $Mg^{2+}$-independent ATPases described in plasma membranes of alveolar macrophage [5], vascular

smooth muscle cells [44], *Trypanosoma cruzi* [45], hepatocyte [7,8] and *E. histolytica* [12,13], and possibly the $Ca^{2+}$-ATPase of adipocyte [6], Ehrlich ascites tumor cell [11], and hepatocyte [9]. In the last three cases, a $Mg^{2+}$-dependence was described [6,11,9], but the concentrations of free $Ca^{2+}$ and $Mg^{2+}$ were calculated from an abolute binding constant for $Ca^{2+}$-CDTA ($6.92 \cdot 10^{10}$ $M^{-1}$) [6] that seems to be more than two orders of magnitude different from those described by other authors ($2.26 \cdot 10^{12}$ to $1.35 \cdot 10^{13}$ $M^{-1}$), [24–26,30]. Therefore, it would appear that there is a large number of ATPases that are $Ca^{2+}$-dependent and $Mg^{2+}$-independent, which would make them different from the widely studied ($Ca^{2+} + Mg^{2+}$)-ATPases of erythrocyte [1], cardiac cells [35], corpus luteum [10], lymphocyte [46] and sarcoplasmic reticulum [31].

From the report of Klaven et al. [11] the $Ca^{2+}$-ATPase of Ehrlich ascites tumor cells, that is probably $Mg^{2+}$-independent, is involved in $Ca^{2+}$ transport, but the function of the other described $Mg^{2+}$-independent $Ca^{2+}$-ATPases, including the presently described enzyme has not been ascertained; whether these membrane-bound enzymes are involved in a transport process remains to be established.

The kinetics of the hitherto described preparation with respect to $Ca^{2+}$ appears to be biphasic. A similar behavior has been seen in erythrocyte [48], adipocyte [6], corpus luteum [10], hepatocyte [49,8], and Ehrlich ascites tumor cell [11] plasma membranes. In this sense we have detected an apparently single band with ATPase activity either at 10 $\mu$M or 1 mM $Ca^{2+}$ concentration, and no $Mg^{2+}$ added in gels ran under non-denaturing conditions. This is suggestive that a single enzyme activity accounts for the biphasic response to $Ca^{2+}$, although Iwasa et al. [8] have separated the $Ca^{2+}$-ATPase of hepatocytes into two fractions each with a different affinity for $Ca^{2+}$.

It was found that the $Ca^{2+}$-ATPase of *E. invadens* was insensitive to some inhibitors of the mitochondrial $H^+$-ATPase as well as to ouabain and several inhibitors of the plasma membrane $Ca^{2+}$-ATPases. Only $La^{3+}$, similarly to the $Ca^{2+}$-ATPase of erythrocyte, adipocyte, and synaptic membrane preparations [6,50,51] caused near 100% inhibition of the activity at 500 $\mu$M concentration.

It is also noteworthy that under our experimental conditions, calmodulin did not stimulate the $Ca^{2+}$-ATPase activity of *E. invadens*, whether in calmodulin-free plasma membrane fractions or in the solubilized enzyme. In addition the activity was insensitive to trifluoperazine. Again, this would seem to be a behavior similar to that of the $Mg^{2+}$-independent $Ca^{2+}$-ATPase of hepatocytes [49,8,9]. It is of interest that vanadate did not inhibit the $Ca^{2+}$-ATPase activity of *E. invadens*. Since vanadate inhibits the activity of all the ATPases in which a phosphorylated intermediate is formed [1,32–38]; the results would suggest that hydrolysis in our preparation as well as that of hepatocytes [8] occurs through a catalytic mechanism in which covalent phosphorylation of the enzyme is not a part of the catalytic cycle as is the case for the mitochondrial $H^+$-ATPase.

The overall results this work on the catalytic characteristics of the $Ca^{2+}$-dependent ATPase activity of *E. invadens* show that the enzyme is $Mg^{2+}$-independent and that it is insensitive to vanadate and calmodulin. This would indicate that its mechanism of action is different from those enzymes in which phosphorylated intermediates are part of the catalytic cycle, and also that it is not regulated by calmodulin as is the case for other $Ca^{2+}$-dependent membrane bound ATPases.

Moreover it is interesting that the ATPase of *E. invadens* exhibits striking similarities to the $Ca^{2+}$-ATPase of hepatocytes. Therefore future results on its mechanism of action and function of the presently described enzyme would appear to be of interest.

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