ELSEVIER

Molecular and Biochemical Parasitology 74 (1995) 179–187

MOLECULAR AND BIOCHEMICAL PARASITOLOGY

Ouabain-sensitive Na^+,K^+-ATPase in the plasma membrane of *Leishmania mexicana*

Pimali Felibertt[a], Roldán Bermúdez[a], Vincenza Cervino[a], Karl Dawidowicz[a]†, Fracehuli Dagger[a], Teresa Proverbio[b], Reinaldo Marín[b], Gustavo Benaim*[a]

[a]*Centro de Biología Celular, Facultad de Ciencias, Universidad Central de Venezuela, Apartado 47114, Caracas, Venezuela*
[b]*Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela*

Received 10 July 1995; revision received 11 September 1995; accepted 11 September 1995

Abstract

The mechanism responsible for the regulation of intracellular Na^+ and K^+ concentrations in trypanosomatids is unknown. In higher eukaryotes a ouabain-sensitive Na^+,K^+-ATPase located in the plasma membrane is the main mechanism for the regulation of the intracellular concentrations of Na^+ and K^+, while in trypanosomatids there are conflicting evidences about the existence of this type of ATPase. By the use of a highly enriched plasma membrane fraction, we showed that an ouabain-sensitive Na^+,K^+-ATPase is present in *L. mexicana*. The affinity of the enzyme for Na^+ and K^+ is similar to that reported for the mammalian Na^+,K^+-ATPase, showing also the same kinetic parameters regarding the relative concentration of those cations that give the optimal activity. Vanadate (10 μM) fully inhibits the ATPase activity, suggesting that the enzyme belongs to the P-type family of ionic pumps. The enzyme is sensitive to ouabain and other cardiac glycosides. These cardiac glycosides do not show any appreciable effect on the higher Mg^{2+}-ATPase activity present in the same preparation. By the use of [^{3}H]ouabain, we also show in this report that the binding of the inhibitor to the enzyme was specific. Taken together, these results demonstrate that an ouabain-sensitive Na^+,K^+-ATPase is present in the plasma membrane of *Leishmania mexicana*. Therefore, this Na^+,K^+-ATPase should participate in the intracellular regulation of these cations in *Leishmania*.

Keywords: Na^+,K^+-ATPase; Ouabain; Plasma membrane; Ionic regulation; Trypanosomatids; *Leishmania mexicana*

1. Introduction

Intracellular cation regulation is achieved in eukaryotic cells by several mechanisms. Among them, ionic pumps are the most widely distributed because of their high affinity and ion selectivity. In

Abbreviations: FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; DTT, dithiothreitol; PMSF, phenylmethylsulphonyl fluoride.

* Corresponding author. Tel.: +58 2 7510111; Fax: +58 2 7525897; Email: gbenaim@dino.conicit.ve.

† Deceased.

SSDI 0166-6851(95)02497-Z

the case of trypanosomatids, little is known about the mechanism of maintenance of intracellular ion concentration. A plasma membrane H^+-ATPase has been implicated in the generation of an H^+ gradient across the plasma membrane in *Leishmania donovani* [1] and has been recently characterized [2], and demonstrated that it belongs to the P-type family of the ionic ATPases, since it forms a phosphorylated intermediate and is inhibited by the micromolar concentrations of vanadate [3]. An Mg^{2+}-dependent ATPase activity, which probably corresponds to the same H^+-translocating ATPase, has been identified in *Trypanosoma cruzi* [4–6] epimastigotes. Also, a plasma membrane Ca^{2+}-ATPase has been recently reported to be involved in the intracellular homeostasis of this divalent cation in *Leishmania braziliensis* [7], *L. donovani* [8], *L. mexicana* [9], *Trypanosoma cruzi* [5,6,10] and *T. brucei* [11]. On the other hand, despite that the most ubiquitous mechanism for the regulation of Na^+ and K^+ in higher eukaryotic cells is an ouabain-sensitive Na^+,K^+-ATPase, its presence in different trypanosomatids has been the subject of controversy. Thus, Voorheis et al. [12] and Mancini et al. [13] have suggested the presence of a Na^+,K^+-ATPase located in the plasma membrane from *T. brucei*, based on its selective inhibition by ouabain. By contrast, Frasch et al. [14], in subcellular fractions of epimastigotes from *T. cruzi*, have reported insensitivity of the ATPase activity to the same inhibitor. More recently, Oz et al. [15], on whole *T. cruzi* epimastigotes, have postulated the presence of an Na^+,K^+-ATPase on the basis of results indicating an effect of the addition of ouabain to the extracellular medium, while Blum [16] did not observe any change in the osmolality-induced $^{86}Rb^+$ transport in *L. donovani* in the presence of ouabain. On the other hand, in these parasites, Zilberstein and Dwyer [1], and Glaser et al. [17], have reported the absence of a Na^+,K^+-ATPase, based on its insensitivity to ouabain. The aim of the present work was to clarify the discrepancies about the presence of a plasma membrane ouabain-sensitive Na^+,K^+-ATPase in these parasites, by studying the effect of ouabain on the ATPase activity in highly enriched plasma membrane fractions from these parasites, as well as by determining the specific binding of labeled ouabain to the plasma membranes from *L. mexicana*. We show in the present report that an ouabain-sensitive Na^+,K^+-ATPase is present in *L. mexicana* promastigotes.

2. Materials and methods

2.1. Chemicals

ATP, EGTA, ouabain, K-strophanthin, strophanthidin, dithiothreitol (DTT), sodium orthovanadate, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), soybean trypsin inhibitor, leupeptin and phenylmethylsulphonyl fluoride (PMSF) were from Sigma. [^{3}H]Ouabain was purchased from New England Nuclear. All other reagents were analytical grade.

2.2. Culture methods

Leishmania mexicana promastigotes (NR strain) were grown at 22°C in a liquid medium consisting of brain-heart infusion (37 g l^{-1}) and 8% fetal bovine serum. Five days after inoculation (exponential growth, 25×10^6 cells ml^{-1}), cells were collected by centrifugation and washed twice in a medium containing 11 mM KCl, 140 mM NaCl and 75 mM Tris–HCl (pH 7.6). The protein concentration was determined by the biuret assay in the presence of 0.2% deoxycholate [18].

2.3. Determination of alkaline cations

The promastigotes of *L. mexicana* in culture medium (2×10^7 cells ml^{-1}) were used as control samples or exposed to diverse inhibitors. At the end of the incubation periods, promastigotes were harvested and each cell pellet (3×10^8 cells) was washed twice in 200 ml of 450 mM mannitol. Then, each pellet was suspended in 2 ml bidistilled water and the cell suspension was exposed to sonic disruption with a Branson Sonic Power sonifier at 10 D.C. amperes, by means of six pulses of 20 s each, at 4°C. Na^+ and K^+ levels were determined from proper dilutions of the lysates by flame emission at 588.8 and 766.3 nm, respectively. Measurements were done with a Varian Techtron AA-6 flame spectrophotometer.

2.4. Isolation of the plasma membrane fraction

Plasma membrane vesicles were prepared essentially as reported for the isolation of vesicles from *L. mexicana* [9,19], *L. braziliensis* [7], *T. cruzi* [5,10,20] and *T. brucei* [11]. As discussed in previous reports [5–7,9–11,19,20], this method has been proved to be very efficient for the isolation of a highly enriched plasma membrane fraction from different trypanosomatids. Briefly, after a final wash of the cells in a medium containing 400 mM mannitol, 10 mM KCl, 2 mM EDTA, 1 mM PMSF, soybean trypsin inhibitor (0.15 mg ml^{-1}), leupeptin (10 μg ml^{-1}) and 20 mM MOPS-Tris (pH 7.0), the cell pellet was mixed with acid-washed glass beads (75–120 μm in diameter) at a ratio of 1:4 (wet weight:weight of beads). The cells were disrupted by abrasion in a chilled mortar until 90% disruption was achieved as determined under optical microscope. This generally took about 5–7 min. The glass beads, unbroken cells and large debris were removed by centrifuging at 1000 × *g* for 15 min at 4°C. The supernatant was subjected to differential centrifugation, first at 16 000 × *g* for 30 min at 4°C and then at 105 000 × *g* for 1 h at the same temperature. The resulting pellet was resuspended in about 3 ml of a medium containing 2 mM DTT and 75 mM MOPS-Tris (pH 7.0). The suspension was then gently passed three times through a Dounce homogenizer (AA, Arthur Thomas) immersed in an ice-cold water bath and stored at −70°C. As previously reported [5–7,9,19,20], this preparation consisted of plasma membrane vesicles with a diameter varying between 0.2 and 0.9 μm. This fraction was also devoid of succinate-cytochrome *c* oxidoreductase activity, thus indicating the absence of mitochondrial contamination. In addition, the specific activity of the calmodulin-dependent Ca^{2+}-ATPase, a typical enzyme marker for plasma membranes, was 13- to 14-times higher when compared with its activity in the homogenate, and copurified with the oligomycin-insensitive Mg^{2+}-ATPase, which is also considered to be another marker enzyme for the plasma membrane of these parasites [5–7,9,11].

2.5. Determination of ATPase activity

Aliquots of plasma membrane vesicles (about 0.5 mg protein ml^{-1}) were incubated in a medium containing 3 mM ATP, 4 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 50 mM MOPS-Tris (pH 7.0), 10 μg ml^{-1} leupeptin, 1 mM PMSF, 10 μg ml^{-1} soybean trypsin inhibitor and requisite amounts of Na^+ and/or K^+. In any case, the total concentration of Na^+ plus K^+ was always kept at 150 mM in order to maintain the ionic strength constant. The final volume was 0.5 ml. After 45 min of incubation at 28°C, the reaction was arrested by the addition of 8% (final concentration) trichloroacetic acid. The mixture was centrifuged and the supernatant was kept for inorganic phosphate determination, according to the method of Fiske and SubbaRow [21], modified by the use of a ferrous sulfate-reducing agent.

2.6. Ouabain binding

The experiments on ouabain binding were carried out by a modification of the method of Hoffman for human red blood cell ghosts [22]. Two hundred μl of the plasma membrane fraction from *Leishmania mexicana* promastigotes (3–4 mg protein ml^{-1}) were incubated for 30 min at 30°C in a medium containing (10 ml final volume) 50 mM MOPS-Tris (pH 7.0), 1 mM EGTA, 2 mM DTT, 130 mM NaCl, 4 mM $MgCl_2$, 3 mM ATP and 1×10^{-7} M [^{3}H]ouabain. Then, the whole suspension was centrifuged at 48 000 × *g* for 30 min at 4°C. The supernantant was carefully discarded and the pellet was resuspended with the same incubation medium but without radioactive ouabain, and centrifuged again at the same speed. The final pellet was dissolved with 500 μl of Triton X-100, resuspended in 5 ml of scintillation liquid and counted in a Rack-Beta LKB-Wallac counter. The nonspecific binding was determined in the same way but with the addition of 1.5 mM of non-radioactive ouabain to the incubation medium. The specific binding was calculated as the difference between the radioactivity bound to the membrane in the absence and in the presence of non-radioactive ouabain.

3. Results

K^+/Na^+ ratio of the culture medium employed for growth and maintenance of *L. mexicana* promastigotes is approximately 0.036 (5 mM K^+ and

Table 1
Effect of inhibitors on the intracellular contents of K^+ and Na^+ in *L. mexicana* promastigotes

Inhibitor	Time (h)	K^+ (mmol mg protein^{-1})	Na^+ (mmol mg protein^{-1})	K^+/Na^+	(*n*)
None	0	0.310 ± 0.008	0.076 ± 0.008	4.08 ± 0.47	10
	4	0.317 ± 0.006	0.081 ± 0.009	3.91 ± 0.58	5
	8	0.307 ± 0.014	0.078 ± 0.010	3.94 ± 0.55	9
Oligomycin (6 μg ml^{-1})	8	0.265 ± 0.008	0.093 ± 0.004	2.85 ± 0.21	5
KCN (1 mM)	8	0.176 ± 0.014	0.112 ± 0.010	1.57 ± 0.41	3
FCCP (1 μM)	8	0.281 ± 0.014	0.093 ± 0.008	3.02 ± 0.27	5
Ouabain (0.4 mM)	4	0.301 ± 0.004	0.096 ± 0.002	3.14 ± 0.10	5
	8	0.234 ± 0.005	0.105 ± 0.003	2.23 ± 0.13	6
Amphotericin B (5 μM)	0.5	0.103 ± 0.012	0.209 ± 0.013	0.49 ± 0.01	3

The results are expressed as mean ± SD. *n* represents the number of independent determinations carried out in triplicate. Changes in Na^+,K^+ and K^+/Na^+ were statistically significant with respect to the controls ($P \leq 0.05$, Student's *t*-test).

140 mM Na^+). Na^+ and K^+ measurements by flame spectrophotometry from promastigote lysates show that intracellular K^+/Na^+ ratio is 111 times greater than the present in the culture medium (Table 1, controls). Thus, results of Table 1 indicate that Na^+ and K^+ ions are asymmetrically distributed between the intracellular medium of the promastigotes and their extracellular space. In the presence of amphotericin B, whose effects on ion permeability are known in many systems [23], the changes in the Na^+ and K^+ contents of *L. mexicana* promastigotes were fast and large. This result shows that K^+, predominantly, is not bound within the cell, which is an indication that an actual K^+ gradient exists across the plasma membrane of *L. mexicana*. Table 1 also shows that upon exposure of promastigotes to different mitochondrial inhibitors as KCN (a respiratory inhibitor), oligomycin (a mitochondrial ATPase inhibitor) and FCCP (an uncoupler of oxidative phosphorylation), there are significant changes on the intracellular Na^+ and K^+. Thus, the maintaining of a stationary intracellular K^+/Na^+ ratio depends on mitochondrial oxidative metabolism. Accordingly, it is admissible to consider the presence of an active mechanism for Na^+ extrusion and K^+ uptake in these parasites.

Ouabain and K-strophanthin are very well-known specific inhibitors of the Na^+,K^+-ATPase from higher eukaryotes [24,25]. It is also known that these inhibitors bind to this ionic pump at its external side, specifically on the K^+ binding site [24,25]. Incubations of promastigotes with ouabain for 4 h produced significant changes in the cell contents of Na^+ and K^+ (Table 1). At 8 h of incubation with ouabain these changes were more pronounced. Quantitatively, the observed changes were only surpassed by those obtained upon amphotericin B and KCN additions. These results are consistent with the presence of an Na^+,K^+-ATPase in the plasma membrane of *L. mexicana* promastigotes.

In order to evaluate the Na^+,K^+-ATPase activity, we used a highly enriched fraction of plasma membranes of *L. mexicana*. This fraction contains a large ATPase activity in the presence of Mg^{2+} [9], which is likely to correspond to an H^+-ATPase, as described for other species of *Leishmania* [1,2,7]. This ATPase activity could be observed in the preparation used in the present work when the assay was carried out in the presence of 4 mM $MgCl_2$ and 150 mM NaCl, in the absence of K^+. In order to study the possibility of the presence of a Na^+,K^+-ATPase activity in our preparation, in previous experiments we determined the ATPase activity in the presence of different concentrations of Na^+ and K^+ in the assay medium, keeping the total ionic strength constant, as described in Materials and methods. This experimental approach has been previously used for the identification and characterization of the Na^+,K^+-ATPase from bovine brain and from

other sources [24]. In bovine brain, Skou [24] reported that with an Na^+/K^+ ratio of 6.5/1 (130 mM/20 mM), all the cation sites of the enzyme became saturated, giving the maximal ATPase activity. When this Na^+/K^+ ratio was used on the plasma membrane fraction from *L. mexicana*, in the absence of ouabain, a noticeable increase in the ATPase activity was obtained, corresponding to about 30% of the overall ATPase activity. Thus, the ATPase activity in the presence of Mg^{2+} and Na^+ was 86 ± 8 nmol Pi mg protein^{-1} min^{-1}, while in the presence of Mg^{2+}, Na^+ and K^+ (130 mM Na^+/20 mM K^+) this activity increased to 126 ± 10 nmol Pi mg protein^{-1} min^{-1}. In order to ascertain if the observed increase in the ATPase activity is indeed due to the presence of a Na^+,K^+-ATPase in this preparation, the well-known specific inhibitor of this ATPase, ouabain, was added to the assay medium. It could be observed that 1 mM ouabain had a strong inhibitory effect in the presence of Mg^{2+}, Na^+ and K^+, fully abolishing the increase in ATPase activity induced by the addition of K^+ (not shown). This cardiac glycoside at the same concentration, had no effect on the ATPase activity when K^+ was omitted from the assay medium (not shown). The activity of the ouabain-sensitive Na^+,K^+-ATPase was about 40 nmol Pi mg protein^{-1} min^{-1}.

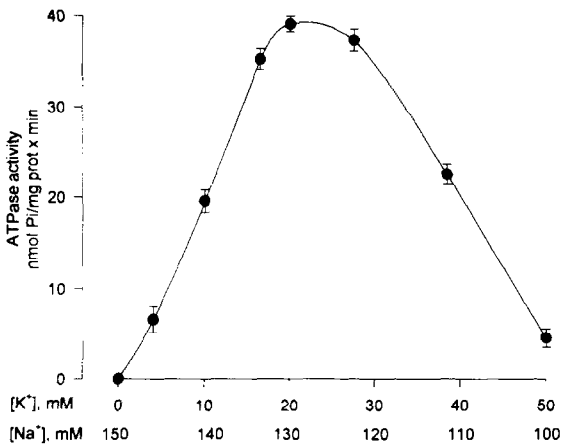

Fig. 1. Effect of different concentrations of Na^+ and K^+ on the ATPase activity of plasma membranes from *Leishmania mexicana*. The assay medium (0.5 mg protein ml^{-1} in a final volume of 0.5 ml, 30°C) contained 3 mM ATP, 4 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 50 mM MOPS-Tris (pH 7.0), 10 μg ml^{-1} leupeptin, 1 mM PMSF, 10 μg ml^{-1} soybean trypsin inhibitor. The required amounts of NaCl and KCl were added to give (150 mM final concentration) the final desired Na^+/K^+ ratio as indicated. The value corresponding to the Mg^{2+}-ATPase (i.e., the ATPase activity obtained in the presence of 4 mM $MgCl_2$ and 150 mM NaCl, in the absence of K^+) was subtracted to each experimental point. The reaction was carried out for 45 min and arrested by the addition of cold trichloroacetic acid (8% final concentration), and inorganic P_i was determined as explained in Materials and methods. Other conditions were as indicated in Materials and methods. Each point represents the mean value ± SD of four independent experiments.

The effect of different concentrations of Na^+ and K^+ on the ATPase activity is shown in Fig. 1. In this figure, the value corresponding to the Mg^{2+}-ATPase (i.e., the ATPase activity obtained in the presence of 4 mM $MgCl_2$ and 150 mM NaCl, in the absence of K^+) was subtracted to each experimental point. It can be observed that the ATPase activity increased concomitantly as the proportion of Na^+/K^+ ratio became lower, reaching a narrow *plateau* around 130 mM NaCl/20 mM KCl (Fig. 1). As mentioned above, this is the Na^+/K^+ ratio reported by Skou [24], for the optimal activity of the bovine brain Na^+,K^+-ATPase. It can also be seen that when the concentration of K^+ was further increased, the ATPase activity began to decrease, reaching at 100 mM NaCl/50 mM KCl the same activity observed in the absence of K^+ or in the presence of ouabain.

The effect of different concentrations of ouabain was then tested, using the optimal Na^+/K^+ ratio obtained in the experiments shown in Fig. 1. It can be observed (Fig. 2) that ouabain had a stepped inhibitory effect on the Na^+,K^+-ATPase activity at a concentration reported to inhibit the same enzyme from other sources [25–27]. It was also observed that when higher K^+ concentrations were used, the effect of ouabain was significantly reduced (not shown). Other cardiac glycosides that also inhibit the Na^+,K^+-ATPase of higher eukaryotes, were then tested on the plasma membrane fraction from *L. mexicana*. As can be seen in Fig. 2, strophanthidin and K-strophanthin fully abolished the Na^+,K^+-ATPase activity, but showing a different inhibitory potency than the one shown by ouabain (not shown). Thus, the concen-

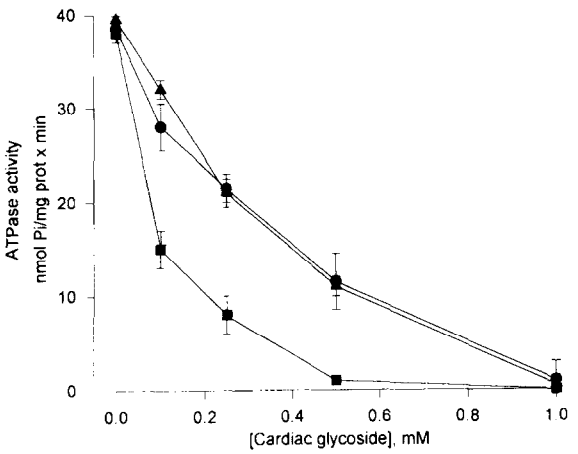

Fig. 2. Effect of different concentrations of cardiac glycosides on the Na^+,K^+-ATPase activity of plasma membranes from *Leishmania mexicana*. Experimental conditions were as in Fig. 1. The optimal ratio of Na^+/K^+ obtained in the experiments shown in Fig. 1 was used (130 mM NaCl and 20 mM KCl, final concentrations). (●) ouabain; (▲) strophanthidin; (■) K-strophanthin. Data points represent the mean value ± SD of six independent experiments.

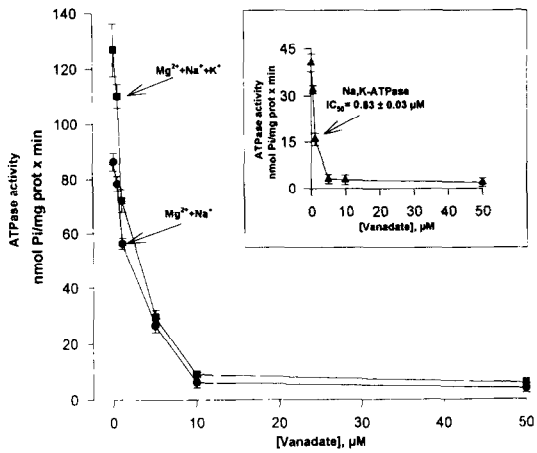

Fig. 3. Effect of vanadate on the basal and K^+-stimulated ATPase activity of plasma membranes from *Leishmania mexicana*. Experimental conditions were as in Fig. 1. Basal activity (●) corresponds to the assay medium in the absence of K^+ (150 mM NaCl) and K^+-stimulated ATPase activity (■) corresponds to the assay medium in the presence of 130 mM NaCl and 20 mM KCl. Sodium orthovanadate was added as indicated. Inset: effect of vanadate on the net Na^+,K^+-ATPase activity (▲). Each point was obtained subtracting the ATPase activity in the presence of K^+ minus the corresponding value obtained in the absence of K^+, and plotted versus the vanadate concentration. Data points represent the mean value ± SD of four independent experiments.

trations of the different cardiac glycosides to cause half inhibition of the Na^+,K^+-ATPase activity were 0.10 ± 0.06, 0.21 ± 0.04 and 0.28 ± 0.03 mM for K-strophanthin, ouabain and strophanthidin, respectively.

Since Na^+,K^+-ATPase belongs to the P-type family of ionic pumps, and since vanadate is the classical inhibitor of these ATPases [28], it was of interest to test the sensitivity to vanadate of the ATPase activity present in the plasma membrane from *Leishmania*. However, the Mg^{2+}-ATPase activity present in this preparation should also be inhibited by vanadate, since this could also be a P-type ionic pump [2,3,6]. For this reason, we studied the effect of vanadate on the basal ($Mg^{2+} + Na^+$) and on the K^+-stimulated ($Mg^{2+} + Na^+ + K^+$) ATPase activity. The results of this experiment are shown in Fig. 3. When the above mentioned activities were subtracted at each concentration of vanadate, and the resulting values were plotted as a function of the vanadate concentration, as shown in the inset, a clear inhibition of the remaining ATPase activity can be observed. Since this activity corresponds to the Na^+,K^+-ATPase, it can be concluded that this enzyme is inhibitable by vanadate. The IC_{50} for this inhibitor for the Mg^{2+}-ATPase was 1.75 ± 0.25 µM, while for the Na^+,K^+-ATPase was 0.83 ± 0.03 µM. Both values are comparable to the IC_{50} for this compound reported for other P-type ionic pumps [28].

The binding of ouabain to the Na^+,K^+-ATPase is very specific and it has been largely used before as a criterion for the identification and characterization of this ATPase [24–27]. Therefore, next we studied the binding of ouabain to the plasma membrane fraction from *L. mexicana* by the use of radiolabeled cardiac glycoside. As described in Materials and methods, the specific ouabain-binding was calculated as the difference between the radioactivity obtained in the presence and absence of cold ouabain. It was found that the plasma membranes from *Leishmania mexicana* showed a specific ouabain-binding of 1.6 ± 0.2 pmol

[^{3}H]ouabain per mg of membrane protein (mean ± S.E. of determinations with three different membrane preparations). If we assume that the stoichiometry of the bound ouabain to the Na^+,K^+-ATPase of *Leishmania* is 1:1 (which is well known for the Na^+,K^+-ATPase of higher eukaryotes, see Ref. [29]), the specific ouabain-binding allows us to calculate the apparent turnover rate of the pump. This value, which represents the time for one catalytic cycle of the enzyme to complete, was calculated by dividing the Na,K-ATPase activity (37.96 ± 2.6 nmol Pi mg protein^{-1} min^{-1}) over the specific ouabain-binding. It is assumed that there is one molecule of bound ouabain per ATPase, and each cycle consumes a molecule of ATP. Thus, the apparent turnover rate was 23 725 min^{-1}

4. Discussion

The identification of the mechanisms responsible for ion regulation in trypanosomatids has been a difficult task. One of the main reasons is undoubtedly the difficulties inherent to the isolation of a highly enriched plasma membrane fraction from these parasites specially because of the presence of only one mitochondrion per cell, which occupies about 10–12% of the total cell volume [30], and which contaminates the microsomal fraction after standard disruption procedures. This fact has delayed progress in the investigation of the function of the plasma membrane components of this group of parasites. The development of a method based on cell disruption by abrasion in a chilled mortar followed by differential centrifugation [19,20] has proved to be ideal to obtain a highly enriched plasma membrane fraction from these parasites. This preparation has also proved to be suitable for the determination of different ATPase activities associated with the function of ionic pumps [6,7,9–11] and also to study ionic movements associated with these ATPases, such as Ca^{2+} transport [5,7,11,31]. Another difficulty inherent to the study of ionic ATPases in plasma membrane preparations is due to the presence of a very high Mg^{2+}-ATPase activity associated with a proton pump [1–3,5–7,9]. The activity of this ATPase is higher than those of other ionic ATPases present in the same preparations, and thus could have masked the presence of the Na^+,K^+-ATPase in some of the previous studies. Another fact that could have made difficult the identification of the Na^+,K^+-ATPase is the narrow range of the Na^+/K^+ ratio required to get the optimum Na^+,K^+-ATPase activity. As shown in Fig. 1, when the Na^+/K^+ ratio is slightly moved aside from the narrow optimal *plateau*, the ATPase activity begins to decline until it reaches the basal level corresponding to the (Mg^{2+} + Na^+)-ATPase activity. Another cation that may potentially alter the Na^+,K^+-ATPase determinations is Ca^{2+}. This cation inhibits the Na^+,K^+-pump by competing with Na^+ at the inner side of the plasma membrane and reducing the rate of its phosphorylation [24]. Albeit this inhibition is not significant at submicromolar levels of free cytoplasmic Ca^{2+}, which is the level of this cation present in *Leishmania* promastigotes [32], the concentration of Ca^{2+} in the assay medium could easily surpass the micromolar concentration, mainly due to the contamination originating from the distilled water and other reagents. For this reason we used excess of EGTA, a calcium-quelating agent, in all the experimental conditions during the present work, in order to keep the free calcium concentration well below the micromolar concentration. This could have helped to reveal in our preparation the Na^+,K^+-ATPase activity. When all the above mentioned effects are considered together it is not difficult to conceive the discrepancies in the literature about the existence of a Na^+,K^+-ATPase in trypanosomatids.

Concerning the effect of ouabain, it has been extensively reported that the binding and consequent inhibition by different cardiac glycosides of the Na^+,K^+-ATPase is greatly influenced by the microenvironment surrounding the enzyme [25,29]. These factors include ionic composition, mainly the monovalent cation concentration, phosphate ligands, temperature, pH, mechanical shaking and the source of enzyme [25,29]. We have carefully chosen, for the experiments carried out in order to evaluate the effect of cardiac glycosides on the ATPase activity, the optimal conditions reported in works from other eukaryotic cells where successful inhibition of the Na^+,K^+-

ATPase activity has been obtained [22,24–26]. This optimal conditions concern mainly with the Na^+/K^+ ratio since, as mentioned above, the percentile inhibition of the Na^+,K^+-ATPase activity by ouabain is lower when the K^+ concentration is increased over 20 mM. In this regard, a competitive inhibition of the ouabain-binding to the enzyme by low concentrations of K^+ has been extensively reported [26]. Under the experimental conditions used in this work, all the cardiac glycosides studied exerted a full inhibitory effect on the Na^+,K^+-ATPase activity. Interestingly, K-strophanthin, which is a mixture of four cardiac glycosides, was the most potent of these inhibitors. However, this is not surprising since it has been reported [26,29] that different cells and tissues respond with distinct sensitivity to specific cardiac glycosides, depending on their chemical structure.

Ionic ATPases of the P-type are characterized [28] by a high affinity and high selectivity for the ions they transport upon hydrolysis of ATP and by the formation of a phosphorylated intermediate during its catalytic cycle, through a change in the conformation of the enzyme from the so-called E_1-state to the E_2-state [28]. Vanadate, at micromolar concentrations, is able to substitute the phosphate at the catalytic site, stabilizing the E_2 conformation [33]. This reaction is highly specific for the P-type class ionic ATPases, and thus it is considered as a distinctive characteristic of this family. In the present work we showed that the Na,K-ATPase activity from *L. mexicana* plasma membranes is fully inhibited by very low concentrations of vanadate, in the same range reported for other P-type enzymes [28], including the Na,K-ATPase from higher eukaryotes [24]. These results strongly suggest that the enzyme described in this study belongs to the P-type class of ionic ATPases. /Finally, the apparent turnover rate of the Na^+,K^+-ATPase from *Leishmania mexicana* found in this work, deserves some comments. This number came out to be around 23 725 min^{-1}, which is in the same order of magnitude but somewhat higher than the turnover rate reported previously [34] for the Na^+,K^+-ATPase of membranes isolated from kidney cells (7000 min^{-1}), or nerve cells (around 10 000 min^{-1}) [35], skeletal and cardiac muscle (between 8000 and 10 000 min^{-1}) [36,37] and eel electroplax (15 000 min^{-1}) [38]. However, it is possible that the ouabain-binding reported in this work might be somehow underestimated because of the presence of a large glycocalix in the plasma membrane of this parasite, which could interfere with the binding of the cardiac glycoside to the ATPase.

In conclusion, our studies indicate that a P-type ouabain-sensitive Na^+,K^+-ATPase is present in the plasma membrane of *Leishmania mexicana*. This enzyme should participate in the intracellular regulation of Na^+ and K^+ in these parasites.

Acknowledgments

We wish to thank Dr. Fulgencio Proverbio for fruitful discussions and Ms. Eva Nieto for her excellent technical assistance during this work. This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Tecnológicas (RP-IV 110034) to G.B. and the Consejo de Desarrollo Científico y Humanístico from the Universidad Central de Venezuela U.C.V. CDCH-C-03-10-2832-95 to R.B and CDCH-C-03-10-3351/94 to G.B.

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