

Ouabain-Insensitive Na⁺-ATPase Activity in *Trypanosoma cruzi* Epimastigotes

Celso Caruso-Neves^a, Marcelo Einicker-Lamas^b, Carlos Chagas^a,
Mecia Maria Oliveira^b, Adalberto Vieyra^c and Aníbal Gil Lopes^a

^a Laboratório de Fisiologia Renal

^b Laboratório de Biomembranas, Instituto de Biofísica Carlos Chagas Filho

^c Departamento de Bioquímica Médica, Instituto de Ciências Biomédicas,
Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Z. Naturforsch. **54c**, 100–104 (1999); received September 22/October 27, 1998

Na⁺-ATPase, *T. cruzi*, ATPase, Epimastigote, Furosemide

In the present paper, the presence of a ouabain-insensitive Na⁺-stimulated, Mg²⁺-dependent ATPase activity in *T. cruzi* epimastigotes CL14 clone and Y strain was investigated. The increase in Na⁺ concentration (from 5 to 170 mM), in the presence of 2 mM ouabain, increases the ATPase activity in a saturable manner along a rectangular hyperbola. The V_{\max} was 18.0 ± 1.0 and 21.1 ± 1.1 nmoles Pi x mg⁻¹ x min⁻¹ and the half-activation value (K_{50}) for Na⁺ was 34.3 ± 5.8 mM and 37.7 ± 5.3 in CL14 clone and in Y strain, respectively. The Na⁺-stimulated ATPase activity was inhibited by 5-[aminosulfonyl]-4-chloro-2-[(2-furanylmethyl)-amino] benzoic acid (furosemide) in a dose-dependent manner. The half-inhibition value (I_{50}) was 0.22 ± 0.03 and 0.24 ± 0.07 mM, and the Hill number (n) was 0.99 ± 0.2 and 2.16 ± 0.29 for CL14 clone and Y strain, respectively. These data indicate that both cell types express the ouabain-insensitive Na⁺-ATPase activity, which might be considered the biochemical expression of the second Na⁺ pump.

Introduction

In spite of the description of several ATPases in *T. cruzi* epimastigote, little is known about the enzyme involved in the genesis of a Na⁺ gradient across cell plasma membrane. Recently, we observed that the *T. cruzi* epimastigote CL14 clone and Y strain express a ouabain-sensitive, Na⁺ plus K⁺ stimulated ATPase activity (Caruso-Neves *et al.*, 1998a). However, the Na⁺ stimulated ATPase activity was not completely abolished by ouabain raising the possibility that there is a second Na⁺ pump insensitive to ouabain in *T. cruzi* epimastigotes of both cell lines tested.

In several tissues two Na⁺ pumps have been described: 1) the classic ouabain-sensitive

(Na⁺+K⁺)ATPase, and 2) the ouabain-insensitive, furosemide-sensitive Na⁺-ATPase. The ouabain-insensitive Na⁺-ATPase activity was initially described in aged microsomal fractions from guinea-pig kidney cortex and was so-called the second sodium pump (Proverbio *et al.*, 1989). Later, the presence of this enzyme was demonstrated in plasma membranes from several different tissues of different species (Moretti *et al.*, 1991; Caruso-Neves *et al.*, 1998b). The Na⁺-ATPase transports Na⁺ against an electrochemical gradient and is not stimulated by K⁺ (Proverbio *et al.*, 1989). This pump also has a parallel distribution with the (Na⁺+K⁺)ATPase, and it has been observed only in the plasma membrane (Proverbio *et al.*, 1989; Caruso-Neves *et al.*, 1997).

In the present study we investigated the possible existence of the ouabain-insensitive, furosemide-sensitive Na⁺-ATPase activity in *T. cruzi* epimastigote CL14 clone and Y strain.

Material and Methods

Chemicals

ATP (magnesium salt), ouabain, sodium orthovanadate, Hepes, Tris, oligomycin and sodium deoxycholate were purchased from Sigma Chemi-

Abbreviations: EDTA, (ethylenediaminetetraacetic acid); Hepes, (N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid); Pi, (orthophosphate); Tris, (tris(trishydroxymethyl)-aminomethane); ATP, (adenosine triphosphate); furosemide, 5-[aminosulfonyl]-4-chloro-2-[(2-furanylmethyl)amino] benzoic acid.

Reprint requests to Aníbal Gil Lopes.

Instituto de Biofísica Carlos Chagas Filho – Universidade Federal do Rio de Janeiro, CCS Bloco G, 21949–900 – Rio de Janeiro, RJ, Brazil.

Fax: 55(21)280–8193.

E-mail: agilopes@chagas.biof.ufrj.br

0939–5075/99/0100–0100 \$ 06.00 © 1999 Verlag der Zeitschrift für Naturforschung, Tübingen · www.znaturforsch.com · D



Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung“) beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition “no derivative works”). This is to allow reuse in the area of future scientific usage.

cal Co. (St. Louis, MO). All chemical reagents were of the highest purity available. [³²P]Pi was obtained from the Instituto de Pesquisas Energéticas e Nucleares (São Paulo, Brazil). [γ -³²P]ATP was prepared as described by Maia *et al.* (1983).

Cell cultures

T. cruzi (epimastigotes) from NIH NTY (a gift from Dr. G. Cross, Rockefeller University, NY) and CL strain CL14 clone (a gift from Dr. Egler Chiari, Federal University of Minas Gerais, MG, Brazil), were cultivated in LIT medium as previously described (Rondinelli *et al.*, 1988).

Cell Preparations

The cells were counted in a hemocytometer and washed four times in 0.5 M Hepes-Tris (pH 7.0) in the absence of Na⁺ and K⁺. The cell lysates were prepared by pre-incubation for 30 minutes in a solution containing: EDTA 1 mM, deoxycholate (DOC) 0.1% (w/v) and sufficient cells to give 6 mg protein/ml (3 × 10⁹ cells/ml). After this solubilization step the hydrolytic activity was assayed by addition of cell lysates (0.2 ml; 5% of the total volume) to the reaction medium to give a final protein concentration of 0.3 mg/ml. The Na⁺ contamination from the sodium deoxycholate and EDTA tetrasodium salt was 0.12 mM and 0.2 mM, respectively.

Measurement of ATPase activity

Except when noted, standard assay medium (0.2 ml) contained: 10 mM MgCl₂, 5 mM [γ -³²P]ATP, 20 mM Hepes-Tris (pH 7.0), 2 μ g/ml oligomycin and specified amounts of Na⁺ and/or K⁺. In all cases, the total concentration of Na⁺ plus K⁺ was always kept at 150 mM in order to maintain constant the ionic strength.

ATPase activity was measured using the method described by Grubmeyer and Penefsky (1981). The reaction was started by the addition of cell lysates (to the reaction medium), and stopped after 30 min by the addition of 2 volumes of activated charcoal in 0.1 N HCl. The [³²P]Pi released was measured in an aliquot of the supernatant obtained after centrifugation of the charcoal suspension for 20 min at 1,500xg in a clinical centrifuge.

Spontaneous hydrolysis of [γ -³²P]ATP was measured in tubes run in parallel in which the enzyme was added after the acid. Protein concentrations were determined by the Bradford method (Bradford, 1958) using bovine serum albumin as a standard.

Statistical analysis

The data were analyzed by two-way analysis of variance (ANOVA), considering as factors the treatments. The magnitude of the differences were verified "a posteriori" by the Bonferroni's test. In all cases the considered level of significance was less than 0.05. Statistical comparisons for each experimental group are indicated in the legends of the Table. The experiments were carried out in duplicate. When the data were expressed as percentage of the control values, the statistical test was applied to the absolute results.

Results and Discussion

Determination of the ouabain-insensitive

Na⁺-ATPase activity in the T. cruzi epimastigotes CL14 clone and Y strain

It has been observed that the second sodium pump is a ouabain- and K⁺-insensitive Na⁺ stimulated ATPase activity. To investigate the presence of this enzyme in *T. cruzi* epimastigotes CL14 clone and Y strain the parasites, previously treated with deoxycholate, were assayed for Na⁺-ATPase activity by determining the effect of 120 mM Na⁺ on the ATPase activity in the presence of 2 mM ouabain. The results are shown in Table I. It is clear that Na⁺ stimulates the ATPase activity in both strains (14.2 ± 4.1 and 17.6 ± 1.2 nmol Pi × mg⁻¹ × min⁻¹ in CL14 clone and Y strain, respectively). Furthermore, it can be seen that 30 mM K⁺ has no effect on the Na⁺ stimulated ATPase activity in the presence of 2 mM ouabain. This ouabain-insensitive Na⁺-stimulated ATPase activity of the *T. cruzi* epimastigote CL14 clone and Y strain is Mg²⁺-dependent (data not shown). The addition of 2 mM ouabain in the absence of Na⁺ and K⁺ does not change the basal Mg²⁺-ATPase activity.

Several papers have shown that the principal characteristic of the Na⁺-ATPase is its insensitivity to ouabain and K⁺ (Proverbio *et al.*, 1989). We observed that 2 mM ouabain, in the absence of K⁺,

Table I. ATPase activities in *T. cruzi* epimastigotes in the presence of ouabain.

Condition	ATPase activity nmol Pi x mg ⁻¹ x min ⁻¹	
	CL14 clone	NIH NTY
a. Mg ²⁺	34.4 ± 4.9	42.4 ± 4.2
b. Mg ²⁺ + Na ⁺	48.6 ± 7.6*	61.1 ± 6.9*
c. Mg ²⁺ + Na ⁺ + K ⁺	43.5 ± 6.0*‡	53.7 ± 6.7*‡
d. (b - a)	14.2 ± 4.1	17.6 ± 1.2

Prior to ATPase assays, the cells were treated with 0.1% (w/v) deoxycholate and 1 mM EDTA for 30 minutes at room temperature (see Material and Methods). All assays were carried out in duplicate in the presence of 10 mM MgCl₂, 5 mM ATP (as magnesium salt), 20 mM Hepes-Tris (pH 7.0), 2 µg/ml oligomycin, 2 mM ouabain and when indicated 120 mM Na⁺ (as NaCl) and 30 mM K⁺ (as KCl). The differences were calculated by paired data. The data are expressed as means ± SEM (*n* = 20). *d* is the difference between the ATPase activity in the presence and in the absence of Na⁺.

* Statistically significant when compared to ATPase activity in the presence of Mg²⁺ alone.

‡ Not statistically significant when compared to ATPase activity in the presence of Mg²⁺ plus Na⁺.

did not change the Na⁺-stimulated ATPase activity in either CL14 clone or Y strain *T. cruzi* epimastigotes. In most tissues the affinity for ouabain is in the micromolar range, but other preparations such as that of the rat proximal tubule membranes show a millimolar affinity for this drug (Sweadner, 1989; Blanco *et al.*, 1995). The sensitivity of the (Na⁺+K⁺)ATPase for ouabain depends on the isoform expressed by the cells and is also tissue specific (Akeru *et al.*, 1985; Blanco *et al.*, 1995). However, in all tissues which express the (Na⁺+K⁺)ATPase activity the addition of 2 mM ouabain is sufficient to achieve complete inhibition of this ATPase activity, indicating that the insensitivity to ouabain is not due to the concentration of ouabain used in this work to measure Na⁺-ATPase activity.

Main characteristics of the ouabain-insensitive Na⁺-ATPase

The effect of varying concentrations of Na⁺ on the Mg²⁺-ATPase activity of *T. cruzi* epimastigotes CL14 clone and Y strain is presented in Fig. 1. The ATPase increases its activity concomitantly with the rise in the concentration of Na⁺ in the medium, reaching maximal values at approximately 100 mM

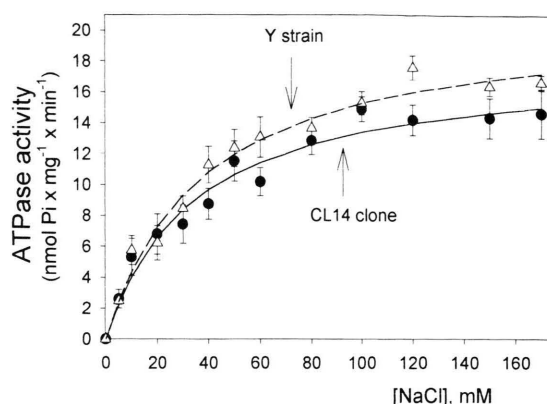


Fig. 1. Na⁺ concentration dependence of the ATPase activity in *T. cruzi* epimastigotes in the presence of 2 mM ouabain.

ATPase activity was measured as described in Material and Methods. Prior to the ATPase assays, the cells were treated with 0.1% deoxycholate (w/v) and 1 mM EDTA for 30 minutes at room temperature. All assays were carried out in the presence of 10 mM MgCl₂, 5 mM ATP (as magnesium salt), 20 mM Hepes-Tris (pH 7.0), 2 µg/ml oligomycin and the indicated Na⁺ (as NaCl) concentrations. The data (mean ± SE) correspond to the difference in assays performed in parallel in the absence or presence of 2 mM ouabain. All experiments were done in duplicate (*n* = 8).

Na⁺ in both strains. The kinetic parameters were calculated with the use of a Michaelian-like equation:

$$v = (V_{\max} \times [\text{Na}^+]) / (K_{50} + [\text{Na}^+])$$

The *K*₅₀ is 34.3 ± 5.8 and 37.7 ± 5.3 mM and the maximal rate (*V*_{max}) is 18.0 ± 1.0 and 21.1 ± 1.1 nmol Pi x mg⁻¹ x min⁻¹ for CL14 clone and Y strain, respectively.

It is known that the Na⁺-ATPase is inhibited specifically by furosemide (Proverbio *et al.*, 1989; Caruso-Neves *et al.*, 1998a; 1998b). Fig. 2 shows the effect of furosemide on the Na⁺-stimulated ATPase activity in the presence of 2 mM ouabain. The increase in furosemide concentration from 0.1 to 2.0 mM completely inhibited the Na⁺-stimulated ATPase activity in a dose-dependent manner, with maximal effect observed in the presence of 2 mM furosemide in both cell types. The kinetics parameters were calculated by the following equation:

$$v_i = (v_o \times K_i) / (K_i + I^n)$$

The *I*₅₀ was calculated as *K*_i^{1/*n*} (0.22 ± 0.03 and 0.24 ± 0.07 mM for CL14 clone and Y strain,

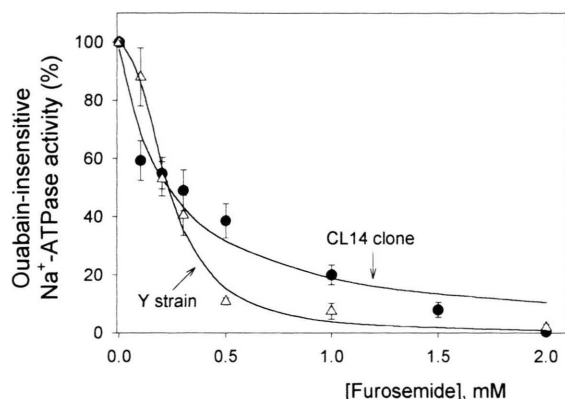


Fig. 2. Furosemide concentration dependence of the ouabain-insensitive Na⁺-stimulated ATPase activity in *T. cruzi* epimastigotes.

ATPase activity was measured as described in Material and Methods. Prior to the ATPase assays, the cells were treated with 0.1% deoxycholate (w/v) and 1 mM EDTA for 30 minutes at room temperature. All assays were carried out in the presence of 10 mM Mg²⁺ (as MgCl₂), 5 mM ATP (as magnesium salt), 20 mM Hepes-Tris (pH 7.0) and 2 µg/ml oligomycin. The ouabain-insensitive Na⁺-stimulated ATPase activity was calculated as the difference between the ATPase activities measured in the presence and in the absence of 120 mM Na⁺, both in the presence of 2 mM ouabain. The furosemide concentration was increased from 0.1 to 2 mM. The data are expressed as percentage of the control. All the experiments were carried out in duplicate ($n = 6$).

respectively). The Hill number (n) was 0.99 ± 0.2 and 2.16 ± 0.29 for CL14 clone and Y strain, respectively. The addition of 2 mM furosemide does not change the ouabain-sensitive (Na⁺+K⁺)-ATPase activity (data not shown). The differences found in the n coefficient indicate that the furosemide binding site is different for both cell types.

This effect of furosemide can not be correlated to the Mg²⁺-ATPase activity or the (Na⁺+K⁺)-ATPase activity since we observed that these

activities did not change upon addition of furosemide (data not shown). Therefore, these data indicate that the cells used in this work express the so-called second Na⁺ pump. This hypothesis is also supported by the observation that K⁺, a classical stimulator of the (Na⁺+K⁺)-ATPase activity, did not change the Na⁺-stimulated ATPase activity in the presence of 2 mM ouabain (Table I).

Finally, we must consider another possible physiological role for the ouabain-insensitive, furosemide-sensitive Na⁺-ATPase of *T. cruzi*. In all tissues studied, the (Na⁺+K⁺)-ATPase activity is 10 fold higher than the Na⁺-ATPase activity (Proverbio *et al.*, 1989), while the CL14 clone and Y strain present a Na⁺-ATPase activity of about 3 fold higher than the (Na⁺+K⁺)-ATPase activity (compare Table I this paper with Caruso-Neves *et al.*, 1998^a). Therefore, it is plausible to postulate that the main enzyme responsible for the generation of a Na⁺ gradient across the cell plasma membrane in *T. cruzi* is the ouabain-insensitive Na⁺-ATPase described in this study.

Acknowledgements

The authors would like to thank Dr. Maria Christina Fialho de Mello for critically reviewing the manuscript and Ms. Rosilane Taveira da Silva for helpful technical assistance.

This work was supported by grants from Programa de Apoio ao Desenvolvimento Científico e Tecnológico (PADCT), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Fundação Universitária José Bonifácio (FUJB/UFRJ).

- Akera T., Yuk-Chow N. G., Shieh I. S., Bero E., Brody T. M. and Braselton W. E. (1985), Effects of K⁺ on the interaction between cardiac glycosides and Na⁺, K⁺ - ATPase. *Eur. J. Pharmacol.* **111**, 147–157.
- Blanco G., Sánchez G. and Mercer R. W. (1995), Comparison of the enzymatic properties of the Na, K-ATPase $\alpha 3\beta 1$ and $\alpha 3\beta 2$ isozymes. *Biochemistry* **34**, 9897–9903.
- Bradford M. M. (1958), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.* **72**, 248–254.
- Caruso-Neves C., Francisco-Pedro L. G., Souza L. P., Chagas C. and Lopes A. G. (1997), Effect of adenosine on the ouabain-insensitive Na⁺-ATPase activity from basolateral membrane of the proximal tubule. *Biochim. Biophys. Acta* **1329**, 336–344.
- Caruso-Neves C., Einicker-Lamas M., Chagas C., Oliveira M. M., Vieyra A. and Lopes A. G. (1998a), *Trypanosoma cruzi* epimastigotes express the ouabain- and vanadate-sensitive (Na⁺+K⁺)ATPase activity. *Z. Naturforsch.*, in press.
- Caruso-Neves C., Meyer-Fernandes J. R., Saad-Nehme J., Proverbio F., Marín, R., Lopes A. G. (1998b), Ouabain-insensitive Na⁺-ATPase activity of malpighian tubules from *Rhodnius prolixus*. *Comp. Biochem. Physiol.*, **119(B)**, 807–811.
- Felibertt P., Bermúdez R., Caryino V., Dawidowicz K., Dagger F., Proverbio T., Marín R. and Benaim G. (1995), Ouabain-sensitive Na⁺, K⁺- ATPase in the plasma membrane of *Leishmania mexicana*. *Mol. Biochem. Parasitol* **74**, 179–187.
- Grubmeyer C. and Penefsky H. S. (1981), The presence of two hydrolytic sites on beef heart mitochondrial adenosine triphosphatase. *J. Biol. Chem.* **256**, 3718–3727.
- Maia J. C. C., Gomes S. L. and Juliani M. H. (1983), Preparation of (gamma-³²P)- and (alfa-³²P)-nucleoside triphosphates with high specific activity. In: C. M. Morel (ed.) *Genes of antigens of parasites: a laboratory manual*. Editora Fundação Oswaldo Cruz, Rio de Janeiro, 146–167.
- Moretti R., Martín M., Proverbio T., Proverbio F. and Marín R. (1991), Ouabain-insensitive Na-ATPase activity in homogenates from different animal tissues. *Comp. Biochem. Physiol.* **98B** (4), 623–626.
- Proverbio F., Marín R. and Proverbio T. (1989), The 'second' sodium pump and cell volume, *Curr. Topics Membranes Transports* **34**, 105–120.
- Rondinelli E., Silva R., Carvalho J. F. O., Soares C. M. A., Carvalho E. P. and Castro F. T. (1988), *Trypanosoma cruzi*: an *in vitro* cycle of cell differentiation in axenic culture. *Exp. Parasitol.* **66**, 197–203.
- Sweadner K. J. (1989), Isozymes of the Na⁺/K⁺-ATPase. *Biochim. Biophys. Acta* **988**, 185–220.