Osmotic Modulation of the Ouabain-Sensitive (Na⁺+K⁺)ATPase from Malpighian Tubules of *Rhodnius prolixus*

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The presence and regulation by hyperosmotic medium of the ouabain-sensitive (Na⁺+K⁺)ATPase of the Malpighian tubule cells of *Rhodnius prolixus* was investigated. The ouabain-sensitive (Na⁺+K⁺)ATPase activity was 5.4 ± 0.5 nmol Pi x mg^{-1} x min^{-1}. Vanadate 100 μM completely abolished this ATPase activity. In hyperosmotic medium, obtained by addition of 180 mM mannitol, the (Na⁺+K⁺)ATPase activity was inhibited by 60%. When the cell lysates were preincubated in hyperosmotic medium for 30 minutes and the ATPase activity was assayed in isosmotic medium, the (Na⁺+K⁺)ATPase activity was not modified. Addition of 50 ng/ml sphingosine, a protein kinase C inhibitor, abolished the inhibition of (Na⁺+K⁺)ATPase activity in hyperosmotic medium. Furthermore, phorbol ester (TPA), an activator of protein kinase C, mimicked the effect of hyperosmotic shock on (Na⁺+K⁺)ATPase activity. The increase in Ca^{2+} concentration decreased the (Na⁺+K⁺)ATPase activity by 60% in isosmotic medium, with maximal effect obtained in 10^{-6} M Ca^{2+}. No effect was observed in hyperosmotic medium. The inhibitory effect of Ca^{2+} on the (Na⁺+K⁺)ATPase was not reversed by sphingosine. These results indicate that the ouabain-sensitive (Na⁺+K⁺)ATPase activity of the Malpighian tubule is regulated by both increasing Ca^{2+} concentration and by the osmolality of the medium by different and integrative ways.

Introduction

(Na⁺+K⁺)ATPase is a crucial mechanism to the survival of most cells (Sweadner, 1989). The enzyme is an integral plasma membrane protein which actively transports three Na⁺ to the outside of the cell and two K⁺ to the inside, maintaining the electrochemical gradient across the cell membrane (Sweadner, 1989). This enzyme consists of two noncovalently linked subunits in an equimolar ratio: α and β (Xie and Morimoto, 1995). Based on the observation that ouabain, on the basolateral side, increases fluid secretion in Malpighian tubule of *Rhodnius*, it has been proposed that the (Na⁺+K⁺)ATPase is located in the basolateral membrane (Maddrell and Overton, 1988; Nicolson, 1993; Pannabecker, 1995). This hypothesis was confirmed by Lebovitz *et al.* (1989) who cloned the cDNA of the α-subunit in basolateral membrane of Malpighian tubule of the *Drosophila melanogaster.*

The Malpighian tubule cells of the bloodsucking insects are exposed to different osmolalities that depend on the feed state of the animal (Beyenbach and Petzel, 1987; Nicolson, 1993). After a blood meal the osmolality of the hemolymph is decreased because the osmolality of the blood is lower than that of the hemolymph. On the other hand, during starvation the osmolality of the hemolymph is increased. So cell volume regulation is a crucial mechanism for the survival of the Malpighian tubule cells. In isosmotic conditions, cell volume regulation is explained by a "pump-leak" hypothesis in which the (Na⁺+K⁺)ATPase is crucial for maintaining Na⁺ and K⁺ gradients (Leaf,

1959; Tosteson and Hoffmann, 1960). Furthermore, (Na$^+$+K$^+$)ATPase is also involved in cell volume regulation during anisosmotic shock (Hoffmann and Dunham, 1995).

During cell volume regulation there is a variation in the amount of the osmotic active solute inside the cell (Hoffmann and Duhnan, 1995). It has been described that the variation of the osmolality of the medium regulates several transport proteins (Yancey *et al.*, 1982). In this paper, we show that the increase of the osmolality of the medium regulates the ouabain-sensitive (Na$^+$+K$^+$)ATPase activity of Malpighian tubule cells.

Material and Methods

Reagents and solutions

ATP (sodium salt), oligomycin, ouabain, EGTA, EDTA, Tris, Hepes, CaCl$_2$ and MgCl$_2$ were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of the highest quality commercially available. [^{32}Pi]P was obtained from the Institute of Energetic and Nuclear Research (São Paulo, SP,Brazil). All solutions were prepared with deionized glass-distilled water. [γ-^{32}P]ATP was prepared as described by Maia *et al.* (1983).

Insects

Rhodnius prolixus Stal, 1859 (Hemiptera:Reduviidae), a bloodsucking insect, the vector of Chagas's disease, was obtained from a colony maintained in the Biochemistry Department by Dr. Hatisaburo Masuda. The insects were maintained at 28 °C and 70–80% relative humidity.

Preparation of Malpighian tubule cell lysates

Adult male *Rhodnius prolixus*, fasted for 5 weeks, were used. Malpighian tubules were dissected with thin tweezers (Dumont # 5) under a stereomicroscope. After dissection, the cells were lysed by homogenization of the tubules in cold isosmotic solution (pH 7.0) employing a Teflon and glass homogenizer and used immediately. As detected by dye exclusion tests all of Malpighian tubule cells were disrupted by the homogenization procedure. The isosmotic solution for dissection and homogenization contained (in mM): sucrose 280, DTT 0.5, PMSF 0.2 and Hepes-Tris 20 (pH = 7.0).

Measurement of ATPase activity

Except as noted under "Results", standard assay medium (0.2 ml) contained: 10 mM MgCl$_2$; 5 mM [γ-^{32}P]ATP (specific activity of approximately about 10^4 Bq/nmol ATP); 20 mM Hepes-Tris (pH = 7.0); 2 μg/ml oligomycin; 1 mM EGTA, 90 mM NaCl and 20 mM KCl. The final osmolality was adjusted to 320 mOsm/kg for the isosmotic solution or to 500 mOsm/kg for the hyperosmotic solution by addition of mannitol. A 30-min preincubation hyperosmotic medium was used when indicated. The hyperosmotic medium of preincubation was the same as used to dissect Malpighian tubules plus mannitol to a final osmolality of 500 mOsm/kg.

ATPase activity was measured by the method described by Grubmeyer and Penefsky (1981). The reaction was started by the addition of Malpighian tubule cell lysates (final protein concentration 0.2 mg/ml) and was stopped after 40 min by the addition of 2 volumes of activated charcoal in 0.1 N HCl. The [^{32}P]Pi released was measured in an aliquot of the supernatant obtained after centrifugation of the charcoal suspension for 20 min at 1,500 x g. Spontaneous hydrolysis of [γ-^{32}P]ATP was measured in tubes run in parallel in which the enzyme was added after the acid. (Na$^+$+K$^+$)ATPase activity was calculated as the difference between the [^{32}P]Pi released in the absence and in the presence of 1 mM ouabain (Jorgensen and Skou, 1971). Protein concentrations were determined using the Folin phenol reagent (Lowry *et al.*, 1951) and bovine serum albumin as a standard.

The data were analyzed by two-way analysis of variance (ANOVA), considering the treatments as factors. The magnitude of the differences were verified "a posteriori" by the Bonferroni t-test. The accepted level of significance was 0.05. The statistical comparisons for each experimental group are shown in the legend of the figures. The statistical test was performed in absolute values and the results were expressed in percentage of the control.

Results

Determination of the (Na$^+$+K$^+$)ATPase activity in Malpighian tubule

The first group of experiments was performed to determine the (Na$^+$+K$^+$)ATPase activity in the Malpighian tubule cells. One of the main characteristics of the (Na$^+$+K$^+$)ATPase is its inhibition by ouabain (Sweadner, 1989). Table I, shows the effect of 1 mM ouabain on the ATPase activity in cell lysates of the Malpighian tubule. The ouabain-sensitive ATPase activity was 5.4 ± 0.5 nmol Pi x mg^{-1} x min^{-1}, which represents 30% of the total ATPase activity. The (Na$^+$+K$^+$)ATPase is a P-type enzyme since it is able to form a phosphorylated intermediary during the catalytic cycle (Blanco *et al.*, 1995). It has been described that the P-type enzymes are inhibited by vanadate (Cunha *et al.*, 1992). We observed that the (Na$^+$+K$^+$)ATPase activity was completely abolished by vanadate 100 μM (data not shown). These data indicate that the Malpighian tubule cells express ouabain-sensitive (Na$^+$+K$^+$)ATPase activity.

Regulation of (Na$^+$+K$^+$)ATPase activity by hyperosmotic medium

In a previous paper we showed that ouabain did not change the cell volume regulation of the Malpighian tubule of *Rhodnius neglectus* during hyperosmotic shock (Arenstein *et al.*, 1995). Because (Na$^+$+K$^+$)ATPase was inhibited during hyperosmotic shock, this could explain the absence of the effect of ouabain. We observed that the increase

Table I. Effect of ouabain 1 mM on the ATPase activity of cell lysates from Malpighian tubules of *Rhodnius prolixus.*

	ATPase activity (nmol Pi x mg^{-1} x min^{-1})
a. Na$^+$ + K$^+$	18.0 ± 1.9
b. Na$^+$ + K$^+$ + ouabain	12.1 ± 1.5*
(b−a)	5.4 ± 0.5

All assays were carried out in the presence of MgCl$_2$ 10 mM, ATP (as sodium salt) 5 mM, NaCl 90 mM, KCl 20 mM, Hepes-Tris (pH 7.0) 20 mM, oligomycin 2 μg/ml and EGTA 1 mM. The ouabain-sensitive ATPase activity was calculated as the difference between the ATPase activity in the absence and in the presence of 1 mM ouabain. The difference was calculated by paired data. The data are expressed as means ± SE (*n* = 19). *P < 0.01.

in osmolality, by addition of mannitol 180 mM, inhibited 60% of the (Na$^+$+K$^+$)ATPase activity of the Malpighian tubule of *Rhodnius prolixus*. Similar results were obtained with Malpighian tubules isolated and incubated in hyperosmotic medium (data not shown). These data indicate that the effect of the increase of osmolality does not depend on the cellular integrity.

To verify the reversibility of this effect, the cell lysates were preincubated in hyperosmotic medium and (Na$^+$+K$^+$)ATPase activity was assayed in isosmotic medium. In this condition the ATPase activity did not change.

Signaling pathway

Several studies have suggested the involvement of protein kinases during cell volume regulation (McCarty and O'Neil, 1992; Hoffmann and Dunham, 1995). Larsen and coworkers (1994) observed that protein kinase C activity is increased by 174% during hyperosmotic shock in Ehrlich ascites tumor cells. Furthermore, it was observed that the activation of protein kinase C inhibits the (Na$^+$+K$^+$)ATPase of rat renal proximal tubule cells (Bertorello and Aperia, 1989). To ascertain whether protein kinase C has a role in modulating the (Na$^+$+K$^+$)ATPase activity of Malpighian tubule of *Rhodnius*, in medium of increased osmolality, experiments were performed in the presence of 50 ng/ml sphingosine, a protein kinase C inhibitor (Hannun *et al.*, 1991; Vannier-Santos *et al.*, 1995), or phorbol ester (TPA), a protein kinase C activator (Newton, 1995; Vannier-Santos *et al.*, 1995).

Figure 1A shows that sphingosine 50 ng/ml not only suppressed the inhibition of (Na$^+$+K$^+$)-ATPase activity by hyperosmotic shock but also promoted a 33% increase in its activity. To confirm that PKC is involved in the inhibition of the (Na$^+$+K$^+$)ATPase activity, we tested the effect of TPA on enzyme activity (Fig. 1B). (Na$^+$+K$^+$)-ATPase activity was 50% inhibited in isosmotic solutions when TPA 20 ng/ml was added. The inhibition of the (Na$^+$+K$^+$)ATPase activity was similar to that obtained by hyperosmotic shock. On the other hand, in hyperosmotic medium TPA 20 ng/ml did not change the enzyme activity.

Recently, we observed in Malpighian tubule cells of *Rhodnius* that cell volume regulation in-
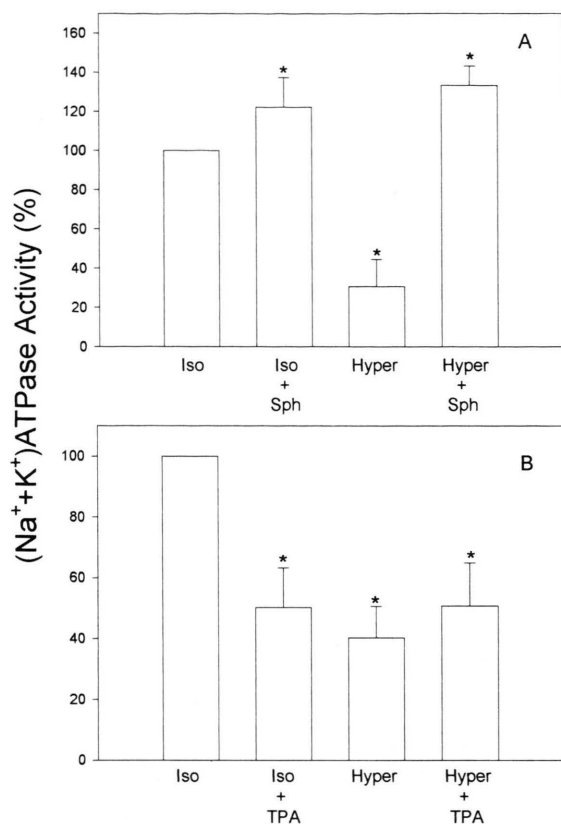
Fig. 1. Effect of sphingosine 50 ng/ml (A) and of phorbol ester (TPA) 20 ng/ml (B) on the (Na⁺+K⁺)ATPase activity in isosmotic or hyperosmotic medium.
ATPase activity was measured as described in Materials and Methods. The reaction was run in isosmotic or hyperosmotic medium made by addition of mannitol to a final osmolality of 500 mOsm/Kg. Sphingosine (Sph) 50 ng/ml or TPA 20 ng/ml was added when indicated. The data are expressed as means ± SE and percentage of the control ($n=5$). *Statistical significance in relation to the control ($p < 0.05$). The (Na⁺+K⁺)ATPase activity in isosmotic condition was 5.9 ± 0.6 nmol Pi x mg^{-1} x min^{-1}.

volves an increase in the intracellular Ca^{2+} (Arenstein *et al.*, 1995). Since, it has also been described that Ca^{2+} inhibits the (Na⁺+K⁺)ATPase from different tissues of several animals, it is possible that Ca^{2+} could be involved in the inhibition of the (Na⁺+K⁺)ATPase during osmotic shock (Rodrigo and Novoa, 1992). An increase in the Ca^{2+} concentration from 10^{-9} to 10^{-4} M, in isosmotic medium, inhibited the (Na⁺+K⁺)ATPase activity by 60%. The maximal effect was obtained in the presence of Ca^{2+} 10^{-6} M (Fig. 2). The (Na⁺+K⁺)ATPase activity in hyperosmotic me-
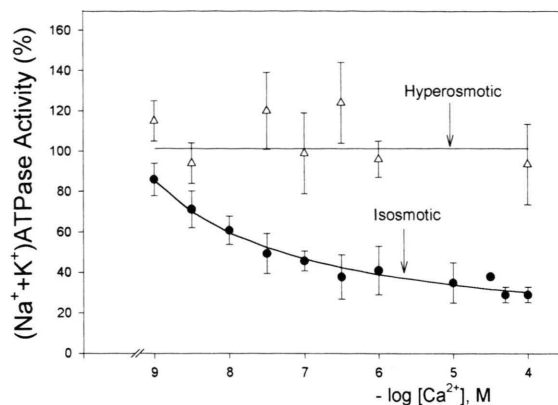

Fig. 2. Modulation of the (Na⁺+K⁺)ATPase activity by Ca^{2+} in isosmotic or hyperosmotic medium.
ATPase activity was measured as described in Materials and Methods. $CaCl_2$ was added to achieve the free Ca^{2+} concentrations indicated in the Figure. The reaction was run in isosmotic or hyperosmotic medium made by addition of mannitol to a final osmolality of 500 mOsm/Kg. The data are expressed as means ± SE and percentage of the control ($n=5$). *Statistical significance in relation to the control ($p < 0.05$). The (Na⁺+K⁺)ATPase activity in isosmotic condition was 5.4 ± 0.5 nmol Pi x mg^{-1} x min^{-1}.

dium (2.19 ± 0.34 nmol Pi x min^{-1} x mg^{-1}) was lower than in isosmotic medium (5.84 ± 0.53 nmol Pi x min^{-1} x mg^{-1}). In hyperosmotic medium, the addition of Ca^{2+} in the same range of concentration did not change the (Na⁺+K⁺)ATPase activity (Fig. 2). So, in hyperosmotic medium, Ca^{2+} was not able to promote additional inhibition of the (Na⁺+K⁺)ATPase activity.

To determine if the effect of Ca^{2+} on the (Na⁺+K⁺)ATPase activity could be due to the activation of protein kinase C, we performed experiments in the presence of either sphingosine or TPA. In Fig. 3, it can be seen that the simultaneous addition of Ca^{2+} 1 μM and TPA 20 ng/ml did not have additive effects on the (Na⁺+K⁺)ATPase activity by isosmotic and hyperosmotic media. Fig. 3 shows that sphingosine did not change the effect of Ca^{2+} 1μM in isosmotic medium. On the other hand, the inhibition of the (Na⁺+K⁺)ATPase activity observed in hyperosmotic medium in the presence of Ca^{2+}, was completely abolished by sphingosine.

Discussion

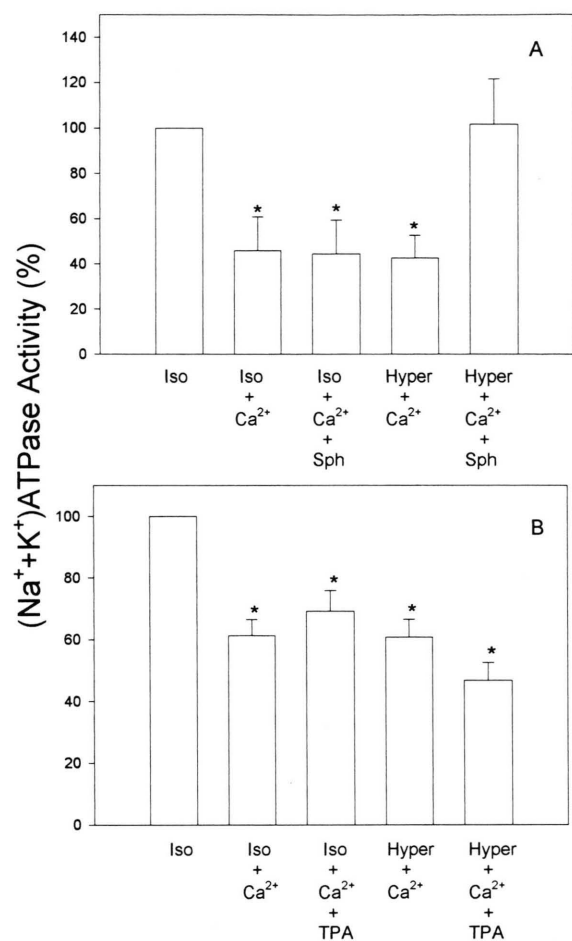In this paper we examine the regulation of (Na⁺+K⁺)ATPase activity of Malpighian tubules

Fig. 3. Modulation of the Ca^{2+} effect on the (Na⁺+K⁺)ATPase activity by sphingosine 50 ng/ml (Sph) (A) and phorbol ester 20 ng/ml (TPA) (B) in hyperosmotic medium.

ATPase activity was measured as described in Materials and Methods. $CaCl_2$ was added to a final free Ca^{2+} concentration of 1 μM when indicated in the Figure. The medium in the absence of Ca^{2+} was made by addition of EGTA 1 mM. The reaction was run in isosmotic or hyperosmotic medium made by addition of mannitol to a final osmolality of 500 mOsm/Kg. The data are expressed as means ± SE and percentage of the control ($n=5$). *Statistical significance in relation to the control ($p < 0.05$). The (Na⁺+K⁺)ATPase activity in isosmotic condition was 5.6 ± 0.4 nmol Pi x mg⁻¹ x min⁻¹.

of *Rhodnius prolixus* by hyperosmotic medium and the involvement of protein kinase C. We show that lysates of Malpighian tubule cells of *Rhodnius prolixus* present a vanadate- and ouabain-sensitive (Na⁺+K⁺)ATPase with activity of 5.4 ± 0.5 nmol Pi x mg⁻¹ x min⁻¹. We used ouabain 1 mM since it

has been described that this concentration is able to completely inhibit all isoforms of the (Na⁺+K⁺)ATPase. (Sweadner, 1989; Blanco *et al.*, 1995).

The role of the (Na⁺+K⁺)ATPase in Malpighian tubule cells is still unclear. Maddrell and Overton (1988) observed that ouabain increased unstimulated fluid secretion in Malpighian tubule of *Rhodnius* suggesting that this enzyme could be involved in fluid reabsortion (Lebovitz *et al.*, 1989; Nicolson, 1993). However, in many insect species it was observed that ouabain did not change fluid secretion (Wenning te al., 1991, Nicolson, 1993, Pannabecker, 1995). These results suggest that the (Na⁺+K⁺)ATPase could be involved in a house keeping function such as cell volume regulation. Recently, we observed that ouabain did not change cell volume regulation of Malpighian tubule of *Rhodnius* during hyperosmotic shock, indicating that this enzyme was inhibited during the shock (Arenstein *et al.*, 1995). This hypothesis agrees with our observation that an increase in the osmolality of the medium decreases the (Na⁺+K⁺)ATPase activity. Inhibition of the enzyme could promote the accumulation of actively osmotic solute inside the cell, leading to the influx of water and to the return of the normal cell volume.

The effect of the osmolality on the (Na⁺+K⁺)ATPase could be due to a direct effect on the structure of the enzyme (Yancey *et al.*, 1982) or through the activation of the signaling pathway involving protein kinase C. The involvement of protein kinase C in cell volume regulation during hyperosmotic shock was demonstrated by Larsen *et al.* (1994) in Ehrlich mouse ascites tumor cells. In lymphocytes, it has been shown that shrinkage-induced stimulation of the Na⁺/H⁺ exchanger activity can be mimicked by treatment with 12-O-tetradecanoylphorbol 13-acetate, a protein kinase C activator (Grinstein *et al.*, 1986; Weinman and Shenolikar, 1986). However, in shrunken lymphocytes, it was observed that neither diacylglycerol (DAG) nor inositol 1,4,5-trisphosphate (IP₃) was released, indicating that the increase of protein kinase C activity during hyperosmotic shock did not involve the mobilization of DAG or IP₃. We showed that TPA 20 ng/ml, an activator of PKC, mimicked the effect of increased osmolality in inhibiting the (Na⁺+K⁺)ATPase ac-

tivity, and this effect was abolished by sphingosine 50 ng/ml, an inhibitor of PKC. These data are compatible with the involvement of PKC in the regulation of the (Na^++K^+)ATPase in an increased osmolality environment. In renal proximal tubule cells of the rat, it was observed that the activation of PKC by phorbol ester inhibited the (Na^++K^+)ATPase activity (Bertorello and Aperia, 1989). This effect of PKC could be due to direct phosphorylation of the enzyme. In agreement with this hypothesis it was observed that the α-subunit of the (Na^++K^+)ATPase can be phosphorylated in vitro by PKC in homogenates of Xenopus oocytes and rat kidney cortical tubules (Bertorello *et al.*, 1991; Carranza *et al.*, 1996).

Recently, we have proposed that the Ca^{2+} concentration in the cytosol is increased in Malpighian tubule cells of *Rhodnius* during hyperosmotic shock (Arenstein *et al.*, 1995). In this paper, we showed that an increase in Ca^{2+} concentration inhibited the (Na^++K^+)ATPase activity. On the basis of the results, we suggest that during the hyperosmotic shock, an increase in the Ca^{2+} concentration in Malpighian tubule cells promotes the inhibition of the (Na^++K^+)ATPase activity leading to the accumulation of the active osmotic solute in the cell. Similar inhibition of the (Na^++K^+)ATPase activity by Ca^{2+} was observed in different cells (Rodrigo and Novoa, 1992; Rayson, 1993). It has been described that the effect of Ca^{2+} could be correlated to stimulation of PKC (Newton, 1995). This hypothesis is favored by the observation that Ca^{2+} did not change the ATPase activity in hyperosmotic medium and did not have an additive effect with TPA in either isosmotic or hyperosmotic medium. However, the possibility that inhibition of the (Na^++K^+)ATPase promoted by increasing the medium osmolality could be correlated to the increase in Ca^{2+} concentration seems not to be true since our experiments were performed in the presence of EGTA 1 mM and sphingosine did not change the Ca^{2+} effect on the ATPase activity in isosmotic medium.

Taken together these data suggest that the (Na^++K^+)ATPase of the Malpighian tubule of *Rhodnius* is inhibited by increasing the osmolality and also by Ca^{2+} in different and integrated ways.

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