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Mechanisms of Cell Volume Regulation in the Proximal Segment of the Malpighian Tubule of *Rhodnius neglectus*

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Abstract. The cell volume regulation of the lower segment cells of the Malpighian tubule of *Rhodnius neglectus* in anisosmotic media was evaluated by using videooptic techniques. When the medium osmolality was increased with addition of 100 mM mannitol the cells shrank to a minimum of 16.84 ± 2.62% and subsequently swelled towards their initial volume undergoing a typical regulatory volume increase (RVI). Replacement of either K^+ or Cl^-or HCO_3^- by Na^+, gluconate and phosphate, respectively, abolished the RVI response. Furthermore, the substitution of Na^+ by tetramethylammonium (TMA^+) in isosmotic conditions led to cellular swelling and death. Addition of either amiloride 10^{-4} M, anthracene-9-COOH 5×10^{-4} M, furosemide 5×10^{-4} M or ethacrynic acid 5×10^{-5} M, also abolished RVI. On the other hand, addition of either Ba^{2+} 10^{-3} M, SITS 5×10^{-4} M, ouabain 10^{-3} M or vanadate 10^{-3} M, did not change the RVI response. When the tubules were incubated in hyperosmotic media with EGTA 2 mM or verapamil 10^{-6} M, the RVI response was abolished. In contrast, a decrease of NaCl concentration from 129 to 79 mM induced a cell swelling to a maximum of 33.11 ± 1.73%, but the cells maintained swollen, only partially regulating their volume. These results show that the proximal cells of Malpighian tubule of *R. neglectus* are able to regulate their volume in hyperosmotic but only partially regulating in hyposmotic solutions. The mechanisms in RVI involve Na^+, K^+, Cl^-, Ca^{2+} and HCO_3^- transport pathways and a ouabain-insensitive ATPase stimulated by Na^+.

Key words: RVD — RVI — Cell volume regulation — Malpighian tubule — Furosemide — Ethacrynic acid

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Introduction

Most cells are able to regulate their volume in hyposmotic medium with a typical regulatory volume decrease (RVD). Only few cell types regulate volume in hyperosmotic conditions by regulatory volume increase (RVI) (Montrose-Rafizadeh & Guggino, 1990; Hoffmann & Ussing, 1992; MacCarty & O'Neil, 1992; Onuchic et al., 1992). Some cells, however, recover their volume when exposed to hyperosmotic medium, with a typical RVI after a pretreatment in hyposmotic medium (Corasanti et al., 1990; Hoffmann & Ussing, 1992; Onuchic et al., 1992). In mouse medullary thick ascending limb, it was shown that the RVI depends on the antidiuretic hormone (ADH) (Hebert, 1986). It has been suggested that this difference between the cell's capacity to regulate it volume in hyposmotic or hyperosmotic media developed early in evolution and allowed animals to live in fresh water (Hoffmann & Ussing, 1992).

In isosmotic conditions, cell volume regulation has been explained by a "pump-leak" hypothesis, in which the $(Na^+ + K^+)$ATPase is the crucial mechanism to maintain Na^+ and K^+ gradients (Leaf, 1959; Tosteson & Hoffmann, 1960). On the other hand, several groups have found that in some cells volume regulation is resistant to ouabain (Russo et al., 1985; Proverbio et al., 1989). These observations led some investigators to propose that a cardiac glycoside-insensitive sodium pump plays an important role in cell volume regulation (Del Castillo et al., 1982; Marín et al., 1985; Proverbio et al., 1986; 1989).

Cell volume regulation involves several steps before the activation of its final effectors (Lewis & Donaldson, 1990; MacCarty & O'Neil, 1992; Hoffmann & Ussing, 1992). After the detection of the volume change, the cell effectors have to be stimulated to start the regulatory response. The activation of effectors proceeds via sec-

ond messengers including Ca^{2+}, cyclic AMP and others (MacCarty & O'Neil, 1992). It has been reported that Ca^{2+} is involved in the RVD response of some cells. In some others, Ca^{2+} does not seem to be involved in the RVI response (Cala et al., 1983; Foskett & Spring, 1985; Bear, 1990; Pierce & Politis, 1990; Lewis & Donaldson, 1990; Montrose-Rafizadeh & Guggino, 1991; MacCarty & O'Neil, 1991; 1992; Hoffmann & Ussing, 1992). Thus, the nature of the second messengers controlling volume regulation varies among different cells.

The first step in insect urine formation is the secretion of an isosmotic fluid in distal segment of the Malpighian tubule. In following segments including the proximal segment of Malpighian tubule, hindgut and rectum, solutes are reabsorbed selectively. During diuresis, transcellular fluid transport across these insect epithelial cells is very fast (Phillips, 1981, Maddrell & O'Donnell, 1992). Thus, because the content of these cell exchanges every few minutes, it is important that these cells regulate their cell volume accurately.

In this paper, we study whether the proximal segment cells of the Malpighian tubule of *R. neglectus* regulate their volume in anisosmotic solutions and investigate the mechanisms involved in this regulation. Most of the previous studies concerning transport mechanisms in the Malpighian tubule have focused on distal cells (Maddrell, 1969, Phillips, 1981, Maddrell & O'Donnell, 1992, Nicolson, 1993). Thus, in this paper, we compare the results obtained in proximal cells with those obtained in distal segment.

Materials and Methods

Preparation of Malpighian Tubules

Male *Rhodnius neglectus,* fasted for 5–8 days, were used in the experiments. After decapitation, the animals were kept in isosmotic solution and the tubules were dissected out with thin tweezers (Dumont #5) under a stereoscopic microscope. For the microperfusion experiments, 1 mm long lower segments were cut with microsurgical needles (Circon, Micro Surgical, USA) and transferred to the microperfusion chamber. The tubule was viewed through an inverted microscope (Diaphot—TMD, Nikon, Tokyo, Japan). The condenser lens was removed and replaced by a revolving nosepiece containing a 40× water immersion objective (40/0,75 W, 160/-, Carl Zeiss, Germany). This lens was important both to allow a larger amount of light and to help to maintain a laminar flow in the perfusion chamber, which is essential to avoid vibration and to keep the tubule in focus. The condenser was changed to a 10× lens by rotating the nosepiece when a large light beam was required in order to position the pipettes and perfuse the tubules under a lower magnification. The tubules were cannulated and perfused according to techniques used in a previous work adapted from the original ones described by Burg et al., 1966) to permit exchange of luminal and peritubular solutions (Lopes et al., 1988). These modifications made it possible to exchange the solutions of the bath or the lumen perfusate in less than 5 sec.

The holding pipettes were drawn in a microforge in order to obtain a 800 μm long parallel section with diameter of 75 μm at the tip and a constriction of 45 μm. The perfusion pipettes were 1,200 μm long with a external diameter of 35 μm.

To allow the best exchange of luminal solution and to avoid vibration, cell volume was always analyzed in cells placed close to the tip of the perfusion pipette.

Solutions

The control solution contained (in mM): 129 NaCl, 8.6 KCl, 8.5 $MgCl_2$, 2 $CaCl_2$, 4.3 Na_2HPO_4/NaH_2PO_4, 10.2 HCO_3^-, 34 glucose and 3 alanine, with final osmolality of 320 ± 5 mOsm/Kg. The solution was gassed with a mixture of 95% O_2-5% CO_2, and the pH was adjusted to 7.0. The hyposmotic solution had the same ionic composition as the control, except that NaCl concentration was decreased to 79 mM, leading to a final osmolality of 220 ± 5 mOsm/Kg. Hyperosmotic solutions were prepared by adding 100 mM mannitol to the control solution, to obtain a final osmolality of 420 ± 5 mOsm/Kg. Solutions free of Na^+, K^+, Cl^- and HCO_3^- were prepared by replacement of Na^+ by with tetramethylammonium (TMA^+), K^+ by Na^+, Cl^- by gluconate and HCO_3^- by HEPES. Ion substitutions were always made in both perfusate and bath, simultaneously after the tubules had been equilibrated for 20 min in the chosen isosmotic ion-depleted solution prior to the hyperosmotic shock. To block the role of K^+ and of Cl^- channels, 10^{-3} M Ba^{2+} and 5×10^{-4} M anthracene-9-COOH were used, respectively. To determine if the Na^+/H^+, Cl^-/HCO_3^- and $Na^+/K^+/2Cl^-$ cotransporters were involved in the RVI response the follows specific inhibitors were added: 10^{-4} M amiloride, 5×10^{-4} M SITS and 5×10^{-4} M furosemide, respectively. Ouabain and vanadate were added to the bath at concentrations of 10^{-3} M and used to detect a possible role of basolateral ($Na^+ + K^+$)ATPase in RVI. Ethacrynic acid 5×10^{-4} M was used to determine if the ouabain-insensitive Na^+-ATPase was involved in RVI response. In the experiments where these drugs were added, tubules were incubated for 20 min in isosmotic solutions containing the drug prior to the hyperosmotic shock. In all experiments, each condition was studied during a period of 20 min.

Cell Volume Measurements

Cell volume measurements were carried out by video-optical techniques similar to those used in previous publications (Guggino et al., 1985; Lopes et al., 1988). Briefly, to evaluate cell volume, the level of focus of a 32× objective was adjusted near the center of the tubule lumen to get a longitudinal side view, which was observed in a television monitor. Besides the use of an air table to hold the microperfusion setup, two basic procedures were employed to avoid undesirable movements of the tubule during the experiments: (1) the tubule length between the pipette tips was kept very short, and (2) the tubule was positioned between the bottom of the chamber and the water-immersion lens used as a condenser. With this care, it was possible to obtain a laminar flow and a very stable preparation. By this means, the cells were kept stable at the same focal plane during the whole procedure, which was essential for the reliability of the results. Images of the tubule were recorded during all the experiments. The frozen display of the video image on the television screen was used for the direct measurement of tubule diameter and cell height. The volume of a constant tubule segment was calculated by means of a mathematical model based on the difference between its external and internal cylinders. The temporal resolution for cell volume measurements was limited by the system to a frame-by-frame sampling rate. However, due to the slow rate of phenomenon, we took one measurement every minute.

The total cell volume obtained under each experimental condition was normalized to the total cell volume of the same segment under

Table 1. Cell volume regulation during hyposmotic and hyperosmotic shocks, expressed in % of control volume

	Hyperosmotic	Hyposmotic
V_1	−16.84 ± 2.62	33.11 ± 1.73
V_2	−2.05 ± 1.53	16.11 ± 3.18
V_4	19.14 ± 2.74	−7.06 ± 1.10
n	6	6
P values		
V_2 *vs.* V_0	NS	<0.05
V_2 *vs.* V_1	<0.05	<0.05
V_4 *vs.* V_0	<0.05	<0.05

Values are means ± SE expressed as % change of cell volume obtained under each experimental condition normalized to the total cell volume of the same segment under control conditions. n = number of experiments. NS = not statistically significant. V_0 = control volume in isosmotic solution; V_1 = maximal % change during hyperosmotic or hyposmotic stress; V_2 = final % change during hyposmotic or hyperosmotic stress; V_4 = % change after reintroduction of isosmotic solution. Positive and negative values represent swelling and shrinking, respectively.

control conditions, and the results are shown as percent cell volume variation calculated by the following equation:

$$\% \text{ cell volume change} = (100V/V_o) - 100,$$

where V_o = control cell volume and V = cell volume under a given experimental condition, as indicated in the table legends.

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 least significant difference statistical contrast test (Montgomery, 1976). The considered level of significance was 0.05. The statistical comparisons for each experimental group are shown in the tables.

Results

Volume Regulation During Osmotic Shock

To verify if the cells of Malpighian tubule were able to regulate their volume in hyposmotic or in hyperosmotic media, the osmolality of both intraluminal and bath fluids was decreased or increased, respectively. Table 1 shows that the cells' shrinkage occurred when the osmolality was increased by the addition of 100 mM mannitol. Following shrinkage, the cell volume returned to the initial value. This pattern is a typical response to cells during RVI. In contrast, when the osmolality was decreased by reduction of NaCl from 129 to 79 mM the cells swelled, but only partially recovered. They remained partially swollen after 15 min of the osmotic shock. In addition, upon return to normal osmolality, after a hyperosmotic or hyposmotic shock, the cells swelled or shrank. This observed "undershoot" or "overshoot" indicates that during the RVI or the partial RVD, there occurs either an increase or a decrease of intracellular

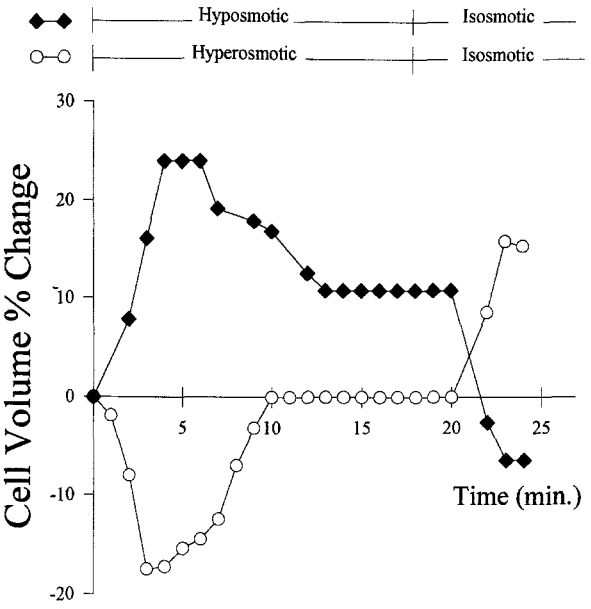

Fig. 1. Cell volume regulation during hyposmotic and hyperosmotic shocks expressed in % of initial volume. Experimental conditions are shown at the top.

solutes, respectively. One representative experiment is illustrated in Fig. 1. These results indicate that the proximal cells of Malpighian tubule of *R. neglectus* are able to fully regulate their volume during hyperosmotic shock. On the other hand, the regulation of cell volume in hyposmotic is incomplete.

Ionic Dependence of RVI Response

The dependence of RVI on K^+, Cl^- or HCO_3^- is shown in Table 2. In separate experiments, tubules were first incubated for 20 min in an isosmotic medium in which K^+ was replaced by Na^+, Cl^- by gluconate, or HCO_3^- by HEPES. Substitution of these ions in both luminal and bath solutions did not result in a significant change in cellular volume during this period. When these tubules were exposed to hyperosmolality, the cells showed no RVI. Re-addition of K^+, Cl^- or HCO_3^-, in the hyperosmotic solution, restored RVI. Upon return to the isosmotic solution the cell swelled, in a way similar to that observed in Fig. 1. One of these experiments is shown in Fig. 2. When Na^+ was replaced by TMA^+, even in isosmotic solution, the cell swelled and died (*data not shown*). Based on these results, we may conclude that the RVI depends upon Na^+, K^+, Cl^- and HCO_3^-.

Ions Transport Mechanisms Involved in RVI Response

To determine the ion transport mechanisms involved in RVI, we utilized inhibitors of several transport process,

Table 2. Cell volume variations during hyperosmotic shock in the absence of K^+, Cl^- or HCO_3^-, expressed in % of control volume

	0 K^+	0 Cl^-	0 HCO_3^-
V_1	−24.60 ± 1.26	−20.94 ± 1.39	−22.26 ± 1.25
V_2	−18.89 ± 2.90	−20.94 ± 1.39	−22.26 ± 1.25
V_3	−1.09 ± 0.40	0	−1.26 ± 0.69
V_4	17.93 ± 6.38	23.80 ± 1.56	17.89 ± 3.84
n	6	6	6
P value			
V_2 *vs.* V_0	<0.05	<0.05	<0.05
V_2 *vs.* V_1	NS	NS	NS
V_3 *vs.* V_2	<0.05	<0.05	<0.05
V_4 *vs.* V_0	<0.05	<0.05	<0.05

Values are means ± SE expressed as % change of cell volume obtained under each experimental condition normalized to the total cell volume of the same segment under control conditions. n = number of experiments. NS = not statistically significant. V_0 = control volume in isosmotic solution; V_1 = maximal % change during hyperosmotic stress in the absence of the ions shown at the top of each column; V_2 = final % change during hyperosmotic stress in the absence of the ions shown at the top of each column; V_3 = % change after re-addition of the ions shown at the top of each column, still in hyperosmolality; V_4 = % change after re-introduction of isosmotic solution containing all the ions. Positive and negative values represent swelling and shrinking, respectively.

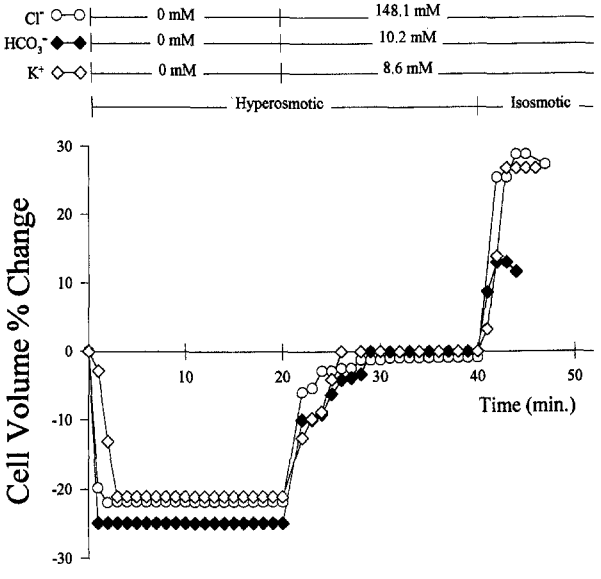

Fig. 2. Cell volume variations during hyperosmotic shock in the absence of K^+, Cl^- or HCO_3^- expressed in % of initial volume. Experimental conditions are shown at the top.

as described in Material and Methods. To study the possible involvement of Na^+-dependent volume regulatory mechanisms (either Na channels or Na^+-H^+ exchange or both), another group of tubules was exposed to hyperosmotic shock in the presence of 10^{-4} M amiloride in both luminal and bath fluids. Table 3 shows the effect of

Table 3. Cell volume variations during hyperosmotic shock in the presence of amiloride 10^{-4} M, expressed in % of control volume

	Amiloride
V_1	−17.04 ± 3.30
V_2	−17.04 ± 3.30
V_3	−17.04 ± 3.30
V_4	−7.19 ± 2.98
n	4
P value	
V_2 *vs.* V_0	<0.05
V_2 *vs.* V_1	NS
V_3 *vs.* V_2	NS
V_4 *vs.* V_0	NS

Values are means ± SE expressed as % change of cell volume obtained under each experimental condition normalized to the total cell volume of the same segment under control conditions. n = number of experiments. NS = not statistically significant. V_0 = control volume in isosmotic solution; V_1 = maximal % change during hyperosmotic stress in the presence of amiloride; V_2 = final % change during hyperosmotic stress in the presence of amiloride; V_3 = % change after amiloride removal, still in hyperosmolality; V_4 = % change after re-introduction of isosmotic solution, in the absence of amiloride. Positive and negative values represent swelling and shrinking, respectively.

amiloride on RVI. The preincubation of the tubule for 20 min in the isosmotic medium with amiloride, did not alter cell volume significantly. The tubule was then exposed to hyperosmolality in the presence of this drug. The cells shrank without any RVI, even after the drug had been washed out under hyperosmotic medium. The return to isosmotic solution led to some RVI, but to a value that was still lower than the control volume. One of these experiments is represented in Fig. 3. These data are consistent with the conclusion that an amiloride-sensitive pathway involving either Na^+ channels or Na^+/H^+ cotransporters or both is involved in RVI.

In the following group of experiments, cells were initially incubated in isosmotic solutions in the presence of 10^{-3} M Ba^{2+} or 5×10^{-4} M furosemide in both luminal and bath fluids (Table 4). Both drugs had no effect on cell volume under isosmotic conditions. When the osmolality was increased in the presence of Ba^{2+}, the cells shrank and recovered their volume completely. Thus, RVI was not inhibited by Ba^{2+}. On the other hand, when the osmolality was increased in the presence of furosemide the cells shrank without RVI. After washout of the cells with a furosemide-free hyperosmotic solution, they swelled to their initial volume. The return to isosmotic solution, in both cases, promoted cell swelling above their initial volume, as shown in Table 4. One representative experiment is shown in Fig. 4. These data demonstrate that K^+ participates in RVI probably through $Na^+/K^+/2Cl^-$ cotransporters.

To investigate Cl^- pathways involved in the RVI response, a group of experiments were conducted with addition of 5×10^{-4} M anthracene-9-COOH or 5×10^{-4} M

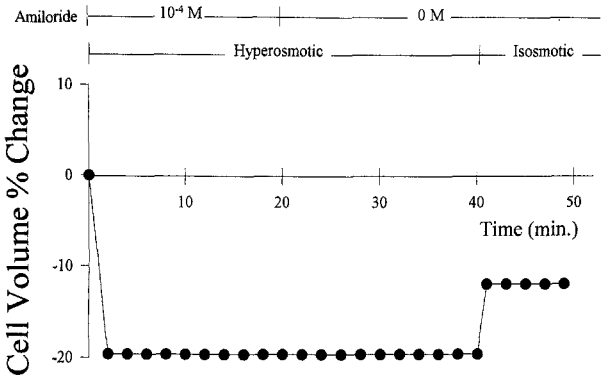

Fig. 3. Cell volume variations during hyperosmotic shock in the presence of amiloride expressed in % of intial volume. Experimental conditions are shown at the top.

Table 4. Cell volume variations during hyperosmotic shock in the presence of furosemide 5×10^{-4} M or Ba^{2+} 10^{-3} M, expressed in % of control volume

	Furosemide	Ba^{2+}
V_1	−19.45 ± 2.12	−16.54 ± 2.16
V_2	−19.45 ± 2.12	−1.42 ± 1.34
V_3	−1.10 ± 0.50	−0.41 ± 0.20
V_4	25.15 ± 4.03	19.79 ± 4.20
n	6	6
P values		
V_2 *vs.* V_0	<0.05	NS
V_2 *vs.* V_1	NS	<0.05
V_3 *vs.* V_2	<0.05	NS
V_4 *vs.* V_0	<0.05	<0.05

Values are means ± SE expressed as % change of cell volume obtained under each experimental condition normalized to the total cell volume of the same segment under control conditions. n = number of experiments. NS = not statistically significant. V_0 = control volume in isosmotic solution; V_1 = maximal % change during hyperosmotic stress in the presence of furosemide or barium; V_2 = final % change during hyperosmotic stress in the presence of furosemide or barium; V_3 = % change after furosemide or barium removal, still in hyperosmolality; V_4 = % change after re-introduction or isosmotic solution, in the absence of furosemide or barium. Positive and negative values represent swelling and shrinking, respectively.

SITS (Table 5). These agents did not cause any significant changes in volume during the isosmotic incubation period. Hyperosmotic shock in the presence of anthracene-9-COOH or SITS, however, was followed by a significant cell shrinkage (*see* Table 5). These cells were able to recover their volume in the presence of SITS (3.58 ± 1.64%), but were unable to recover their volume with anthracene-9-COOH even after a washout with a blocker-free, hyperosmotic solution. Re-addition of the isosmotic solution led to a cellular swelling only in the presence of SITS, indicating that the participation of Cl^- in the RVI response is mediated by an anthracene-9-COOH-sensitive and not a SITS-sensitive pathway

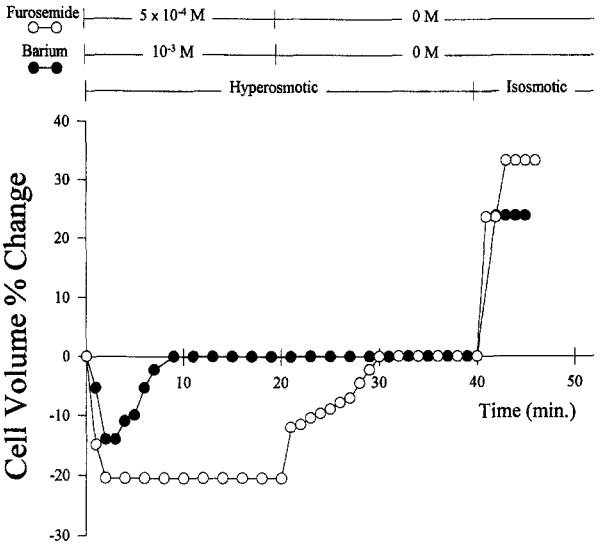

Fig. 4. Cell volume variations during hyperosmotic shock in the presence of furosemide or barium expressed in % of initial volume. Experimental conditions are shown at the top.

Table 5. Cell volume variations during hyperosmotic shock in the presence of anthracene-9-COOH 5×10^{-4} M or SITS 5×10^{-4} M, expressed in % of control volume

	Anthracene-9-COOH	SITS
V_1	−21.01 ± 2.45	−21.79 ± 1.51
V_2	−19.63 ± 3.00	−3.58 ± 1.64
V_3	−12.98 ± 4.92	−2.59 ± 1.43
V_4	11.75 ± 6.75	12.17 ± 2.86
n	6	6
P values		
V_2 *vs.* V_0	<0.05	NS
V_2 *vs.* V_1	NS	<0.05
V_3 *vs.* V_2	NS	NS
V_4 *vs.* V_0	<0.05	<0.05

Values are means ± SE expressed as % change of cell volume obtained under each experimental condition normalized to the total cell volume of the same segment under control conditions. n = number of experiments. NS = not statistically significant. V_0 = control volume in isosmotic solution; V_1 = maximal % change during hyperosmotic stress in the presence of anthracene-9-COOH or SITS; V_2 = final % change during hyperosmotic stress in the presence of anthracene-9-COOH or SITS; V_3 = % change after anthracene-9-COOH or SITS removal, still in hyperosmolality; V_4 = % change after re-introduction of isosmotic solution, in the absence of anthracene-9-COOH or SITS. Positive and negative values represent swelling and shrinking, respectively.

mostly likely involving Cl^- channels. One representative experiment is illustrated in Fig. 5. It is important to stress that some Cl^-/HCO_3^- cotransporters are insensitive to SITS, as in turtle bladder (Kohn et al., 1990) and collecting duct intercalated cells (Furuya et al., 1991; Schuster, 1993). Therefore, it cannot be inferred from the SITS insensitivity of RVI that the anion cotransport-

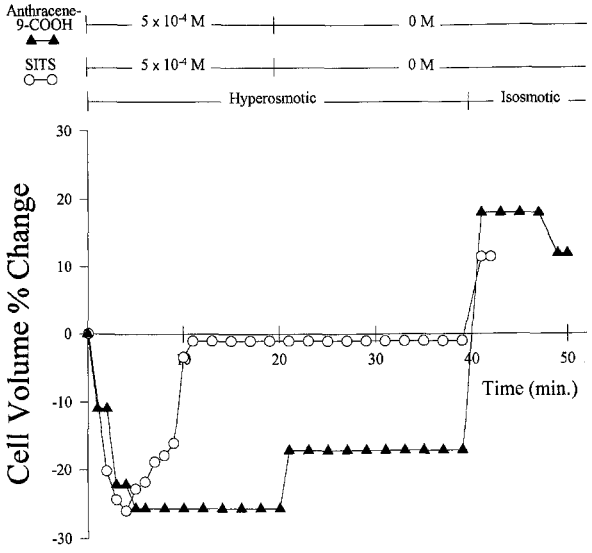

Fig. 5. Cell volume variations during hyperosmotic shock in the presence of anthracene-9-COOH or SITS expressed in % of initial volume. Experimental conditions are shown at the top.

ers do not play a role in this process. This is especially true because the dependence of RVI on HCO_3^- in the medium is consistent with the participation of a HCO_3^--dependent anion cotransporter.

To determine whether primary active transport mechanisms generate the ionic gradients responsible for volume regulation, hyperosmotic shock was induced in the presence of either 10^{-3} M ouabain, 10^{-3} M vanadate or 5×10^{-5} M ethacrynic acid (Table 6). The incubation of tubules in an isosmotic solution with these drugs did not significantly change cell volume. When the hyperosmotic shock was induced in the presence of ouabain, vanadate or ethacrynic acid the cells shrank. RVI was abolished by ethacrynic acid but not by ouabain or vanadate. When the cells were washed with hyperosmotic solution in absence of ethacrynic acid, full RVI was observed. In three experimental conditions, return to isosmotic solutions led to cellular swelling. One representative experiment is shown in Fig. 6.

Signaling Mechanisms in RVI

It was reported that, in several cells, RVI response is independent of the intracellular Ca^{2+} concentration (Hoffmann & Ussing, 1992; MacCarty & O'Neil, 1992). Therefore, to verify if the RVI response is dependent on extracellular Ca^{2+}, the Malpighian tubule was exposed to hyperosmotic medium in the presence of 2 mM EGTA or 10^{-6} M verapamil (blocker of voltage dependent Ca^{2+} channels). Initially, the cells shrank to a minimum in the presence of EGTA and verapamil, respectively. In both cases, RVI response was abolished. When Ca^{2+} was re-

Table 6. Cell volume variations during hyperosmotic shock in the presence of ouabain 10^{-3} M, vanadate 10^{-3} M or ethacrynic acid 5×10^{-5} M, expressed in % of control volume

	Ouabain	Vanadate	Ethacrynic acid
V_1	−16.75 ± 2.93	−19.18 ± 0.50	−15.43 ± 3.09
V_2	−2.12 ± 0.92	−2.60 ± 2.05	−15.43 ± 3.09
V_3	−1.16 ± 0.60	−2.45 ± 2.11	−0.37 ± 0.30
V_4	22.06 ± 2.97	12.70 ± 9.25	20.70 ± 3.67
n	3	6	6
P value			
V_2 *vs.* V_0	NS	NS	<0.05
V_2 *vs.* V_1	<0.05	<0.05	NS
V_3 *vs.* V_2	NS	NS	<0.05
V_4 *vs.* V_0	<0.05	<0.05	<0.05

Values are means ± SE expressed as % change of cell volume obtained under each experimental condition normalized to the total cell volume of the same segment under control conditions. n = number of experiments. NS = not statistically significant. V_0 = control volume in isosmotic solution; V_1 = maximal % change during hyperosmotic stress in the presence of ouabain, vanadate or ethacrynic acid; V_2 = final % change during hyperosmotic stress in the presence of ouabain, vanadate or ethacrynic acid; V_3 = % change after ouabain, vanadate or ethacrynic acid removal, still in hyperosmolality; V_4 = % change after reintroduction or isosmotic solution, in the absence of ouabain, vanadate or ethacrynic acid. Positive and negative values represent swelling and shrinking, respectively.

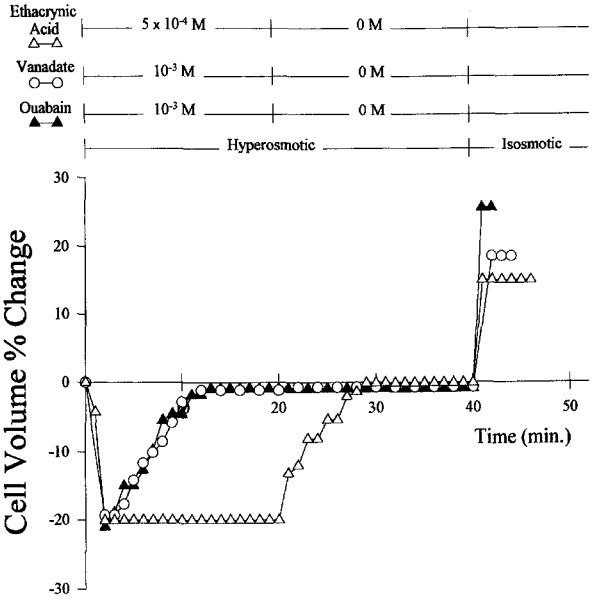

Fig. 6. Cell volume variations during hyperosmotic shock in the presence of ouabain, vanadate or ethacrynic acid expressed in % of initial volume. Experimental conditions are shown at the top.

added or verapamil was removed the cells showed a typical RVI response. The return to isosmotic solution promoted the increase of cell volume (Table 7). A typical experiment is shown in Fig. 7.

Table 7. Cell volume variations during hyperosmotic shock in the presence of EGTA 2 mM or verapamil 10^{-3} M

	EGTA	Verapamil
V_1	−22.00 ± 1.70	−15.39 ± 1.84
V_2	−19.57 ± 1.23	−15.39 ± 1.84
V_3	−1.79 ± 0.45	−3.14 ± 1.20
V_4	11.66 ± 4.05	16.00 ± 6.60
n	6	6
P values		
V_2 *vs.* V_0	<0.05	>0.05
V_2 *vs.* V_1	NS	NS
V_3 *vs.* V_2	<0.05	<0.05
V_4 vs. V_0	<0.05	<0.05

Values are means ± SE expressed as % change of cell volume obtained under each experimental condition normalized to the total cell volume of the same segment under control conditions. n = number of experiments. NS = not statistically significant. V_0 = control volume in isosmotic solution; V_1 = maximal % change during hyperosmotic stress in the presence of EGTA or verapamil; V_2 = final % change during hyperosmotic stress in the presence of EGTA or verapamil; V_3 = % change after EGTA or verapamil removal, still in hyperosmolality; V_4 = % change after the re-introduction of isosmotic solution, in the absence of EGTA or verapamil. Positive and negative values represent swelling and shrinking, respectively.

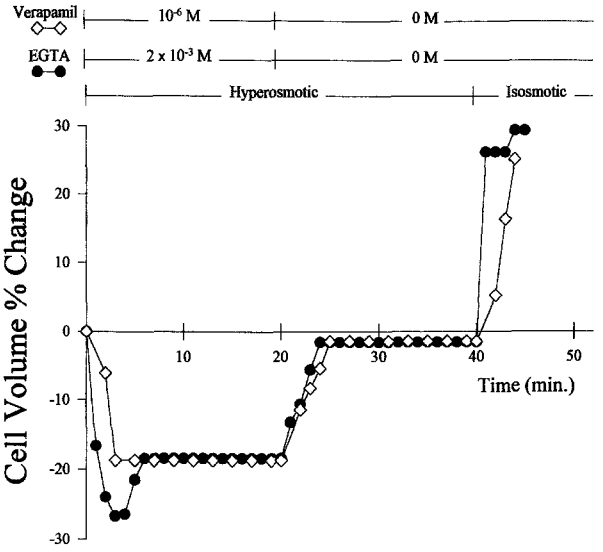

Fig. 7. Cell volume variations during hyperosmotic shock in the presence of EGTA or verapamil expressed in % of initial volume. Experimental conditions are shown at the top.

Discussion

In the present work, we have used video-optical techniques similar to those used previously (Guggino et al., 1985; Lopes et al., 1988; Onuchic et al., 1992) to study the cell volume regulation in proximal cells of Malpighian tubule of *R. neglectus* exposed to hyperosmotic or hyposmotic solutions. These cells regulate their volume in hyperosmotic medium with a typical RVI, but they are not able to regulate completely their volume in hyposmotic medium (i.e., they do not show a full RVD). These results are in contrast with the data obtained in previous studies performed on amphibian and mammalian cells (Ussing, 1982; Hoffmann et al., 1984; Davis & Finn, 1985). Several studies have shown that: (1) many mammalian renal epithelial cells regulate their volume in hyposmolality but fail to completely regulate cell volume when submitted to hyperosmotic stress (Montrose-Rafizadeh & Guggino, 1990; Onuchic et al., 1992); (2) organic osmolyte accumulation may contribute to RVI in renal medullary cells, although no direct evidence has been shown so far. This process may involve uptake from extracellular compartment (Nakanishi et al., 1988) or intracellular synthesis of organic solutes (Bagnasco et al., 1988). While the incorporation of extracellular ions constitutes an acute response (Hebert, 1986), the organic osmolyte mechanisms are slow and progressive, constituting probably a second line of cell volume defense. These processes are more stable and may refine the ability to regulate cell volume, and (3) the electrolyte uptake mechanisms involved in the short term response of RVI of some of these cells require the presence of specific hormones in solution (Hebert, 1986) or specific pre-osmotic exposure to be activated (Montrose-Rafizadeh & Guggino, 1990). In contrast, in proximal cells of the Malpighian tubule: (1) volume regulation in hyperosmotic stress is acutely established by ionic uptake, without the need of pre-activation processes; (2) the RVI response observed was complete, whereas RVD was incomplete; and (3) several ionic mechanisms seem to be involved in the RVI response, giving the cell a handful of possibilities to regulate volume. Therefore, we postulate that, in general, insect Malpighian tubule cells may regulate their volume in hyperosmolality using ionic transporters independent of pre-activation mechanisms, while renal mammalian cells might require organic osmolyte dependent or pre-activated processes to regulate cell volume in hyperosmotic stress. On the other hand, insect Malpighian tubule cells may require prestimulation either by hormones or previous activation of transport systems for complete RVD. This might be attributed to evolutionary adaptation of animal cells, such as different basal or variations of environmental osmolality to which the cells are submitted, as well as by different functions. However, further experiments are still required to test this hypothesis.

The observation that replacement of Na^+ by TMA^+ promoted cell swelling and death in isosmotic medium indicates that cell volume regulation in basal state requires Na^+. The addition of amiloride abolished RVI, suggesting that Na^+ participates in RVI either through a Na^+/H^+ exchanger or/and Na^+ channels. It has been well known that Na^+ transport pathways inhibited by

amiloride play a central role in fluid secretion in the distal portions of the *Rhodnius* Malpighian tubule (Maddrell, 1969; Gee, 1976; Phillips, 1981; Baldrick et al., 1988; Maddrell & O'Donnell, 1992; Nicolson; 1993). Phillips (1981), proposed that the amiloride-sensitive Na^+ transport is energized by a "second Na^+ pump," located in the basolateral membrane. More recent observations are compatible with the presence of an amiloride-sensitive Na^+/H^+ cotransport in the luminal membrane of the distal portion of the Malpighian tubule, energized by H^+-ATPase located in same membrane (Maddrell & O'Donnell, 1992; Nicolson, 1993). In addition, it has been shown that the basal Na^+ permeability of this segment is low. On the other hand, it has been proposed that there is a Na^+ channel stimulated by cAMP that is present in the same portion of *Aedes* and *Glossima* Malpighian tubules (Nicolson, 1993). Sawyer & Beyenbach (1985) also working with fluid secretion in the distal portion of *Aedes* Malpighian tubules showed that addition of cAMP depolarizes Vb (basolateral voltage) but has no effect no Va (apical voltage), indicating that the Na^+ channel is located in the basolateral membrane. The electrochemical gradient for Na^+ favors the influx across the basolateral membrane (Maddrell, 1969; Phillips, 1981).

Because 5–8 day-fasting animals were used in the present experiments, we expect that the level of diuretic hormone is very low (Beyenbach & Petzel, 1987). Therefore, we postulate that in the antidiuretic state that the Na^+/H^+ antiporters and/or amiloride-sensitive Na^+ channels, which are involved in fluid secretion in the diuretic state, may participate in the RVI response during periods of antidiuresis. Our observation that replacement of Na^+ by TMA^+ in isosmotic solutions induces cell swelling and death suggests that Na^+-dependent transport pathways may also be important in maintaining cell homeostasis in resting conditions. On the other hand, if we consider that the Malpighian tubule plasma membrane is permeable to TMA^+, the favorable gradient could explain the cell swelling caused by TMA^+ influx.

In our experiments, SITS did not change the RVI response, suggesting the independence of this mechanism on a SITS-sensitive Cl^-/HCO_3^- antiport. The participation of this transporter in fluid secretion also had been ruled out (Phillips, 1981, Nicolson, 1993). On the other hand, most of the animal cells studied so far that regulate volume using a Na^+/H^+ antiporter also utilize a Cl^-/HCO_3^- antiporter in this process (Ericson & Spring, 1982; Grinstein et al., 1984; Cala, 1985; Hoffmann et al., 1992). In these cells, both antiporter and exchanger work together to support net Cl^- and Na^+ influx in hyperosmotic solutions. This operates because the exchange of Na^+ for H^+ increases intracellular pH and HCO_3^- concentration. Higher HCO_3^- concentrations enhance the Cl^-/HCO_3^- exchange, resulting in the net movement of Cl^- into the cell and removing excess HCO_3^-. As a consequence, the parallel function of these transporters results in the exchange of osmotically active for osmotically inactive particles, represented by the influx of Na^+ and Cl^- and the outflow of CO_2 and H_2O (Cala, 1980; Hoffmann & Ussing, 1992). Despite the SITS-insensitivity, we did observe that the RVI response requires the presence of HCO_3^- in medium. For these reasons, we postulate that there may be a SITS-insensitive Cl^-/HCO_3^- antiporter involved in RVI response of Malpighian tubule of *R. neglectus*.

The present data clearly indicate that RVI is inhibited by anthracene-9-COOH, a well known Cl^--channel blocker. It has been reported in previous studies the participation of Cl^- channels in RVD response in several cell types, but not in RVI response (Knoblauch et al., 1989; Hoffmann & Ussing, 1992; McCarty & O'Neil, 1992; Onuchic et al., 1992). The presence of Cl^- channels in the apical membrane of the Malpighian tubule cells involved in fluid secretion has been described (Maddrell, 1969; Phillips, 1981; O'Donnell & Maddrell, 1984; Wright & Beyenbach, 1987; Nicolson, 1993). Moreover, the Cl^- electrochemical gradient in apical membrane favors Cl^- secretion. It becomes difficult then to postulate that apical Cl^- channels are involved in RVI response. However, because the Cl^- electrochemical gradient in the basolateral membrane favors the Cl^- uptake, it is possible that there are Cl^- channels in the basolateral membrane that activate only during RVI response (Maddrell, 1969; Phillips, 1981; Hevert, 1984). In agreement with this hypothesis, there is evidence that Cu^{2+}-sensitive anionic channels are indeed present in the basolateral membrane (Phillips, 1981). Following the electroneutrality principle, the participation of Cl^- channel in RVI response may indicate that the mechanism of Na^+ transport during RVI could be through Na^+ channels in addition to a Na^+/H^+ antiporter. However, in this paper, whether the amiloride effect was through the Na^+/H^+ antiporter or through the Na^+ channel was not further investigated.

Since the effects of amiloride on the Na^+/H^+ antiporter or Na^+ channel and anthracene-9-COOH on the Cl^- channel are readily reversible, the present observation of an irreversible action could also indicate that these drugs disrupt cell volume regulation through different amiloride- and anthracene-9-COOH-sensitive pathways.

The inhibition of the RVI promoted by substitution of K^+ by Na^+ could be explained by the K^+ depletion established during the 20-min removal of K^+ during the isosmotic equilibrium period. The intracellular K^+ is necessary to sustain the $(Na^+ + K^+)$ATPase and in its absence would inhibit the enzyme activity. However, this possibility can be ruled out because addition of ouabain and vanadate, blockers of the $(Na^+ + K^+)$ATPase, did not inhibit the RVI response. Therefore, we conclude the involvement of K^+ in RVI could be through

$Na^+/K^+/2Cl^-$ cotransporter, as it has been shown in other cells (Gargus & Slayman, 1980; Mercer & Hoffman, 1985; Montrose-Rafizadeh & Guggino, 1990; Hoffmann & Ussing, 1992). It has been proposed that this transporter is involved in fluid secretion of the Malpighian tubule, and that it is located in the basolateral membrane (Maddrell, 1969; Gee, 1976; Phillips, 1981; O'Donnell & Maddrell, 1984; Nicolson, 1993). This possibility is supported further by the observation that the addition of furosemide, an inhibitor of $Na^+/K^+/2Cl^-$ cotransporter, blocked the RVI response.

Some cells exhibit a volume regulatory response which is insensitive to ouabain (Russo et al., 1985, Proverbio et al., 1989). In our experiments, we observed that even with the addition of very high concentrations of ouabain or vanadate the cells shrank after hyperosmotic shock followed by a typical RVI. On the other hand, the addition of ethacrynic acid abolished RVI. Ethacrynic acid can be associated with the possible inhibition of the $Na^+/K^+/2Cl^-$ cotransporter, as described in other cells (O'Grady et al., 1987). But, it is also known that this drug blocks a ouabain-insensitive pump (Proverbio et al., 1989). These observations could be interpreted to indicate that the primary active transport involved in RVI in the Malpighian proximal tubules after hyperosmotic stress is via a ouabain-insensitive, ethacrynic acid-sensitive Na^+ pump (Proverbio et al., 1989). Similar pumps have been proposed to be present in other cells, where after swelling, they regulate volume by a ouabain-insensitive mechanism (Whittembury & Proverbio, 1970; Del Castillo et al., 1982; Marín et al., 1985; Proverbio et al., 1986; Proverbio et al., 1989). It has also been proposed that that the "primary" energy source for fluid secretion in the Malpighian tubule is a Na^+ stimulated, ouabain-insensitive ATPase, located in the apical membrane (Maddrel, 1969; Gee, 1976; Phillips, 1981; O'Donnell & Maddrell, 1984; Pannabecker et al., 1992). Thus, we postulate that the primary active transport involved in RVI could be this ouabain-insensitive, Na^+-stimulated pump. However, it has also been proposed that the "prime mover" for fluid secretion in Malpighian tubule is a H^+-ATPase in parallel with K^+/H^+ or Na^+/H^+ exchangers (Wieczorek et al., 1991; Bertram et al., 1991; Nicolson, 1993). Thus, further experiments will be needed to prove our hypothesis.

Finally, this paper shows that RVI of the cells in the Malpighian tubule is dependent on extracellular Ca^{2+} and is mediated by verapamil-sensitive transport pathways. Several papers have shown that Ca^{2+} is involved in RVD in some cells. Whereas, the role of the Ca^{2+} in RVI response has been ruled out in some others (Bear, 1990; Pierce & Politis, 1990; Montrose-Rafizadeh & Guggino, 1991; Hoffmann & Ussing, 1992; Lewis & Donaldson, 1990; MacCarty & O'Neil, 1991, 1992). MacCarty & O'Neil (1990, 1991, 1992) showed that verapamil-sensitive Ca^{2+} channels play a role in RVD in rabbit proximal tubule cells. It seems that these channels are inactive in isosmotic medium and would activate in hyperosmotic solutions increasing the intracellular concentration of Ca^{2+}. Thus, increases in intracellular Ca^{2+} concentration would stimulate the volume regulatory mechanisms either directly or indirectly (MacCarty & O'Neil, 1992). Because RVI is Ca^{2+}-and verapamil-sensitive, it is possible that a similar mechanism participates in RVI in our insect tubules. Figure 8 illustrates the possible membrane ion transport systems involved in RVI as discussed above: (1) Na^+/H^+ antiporter and/or Na^+ channel; (2) Cl^- channels; (3) Cl^-/HCO_3^- antiporter; (4) $Na^+/K^+/2Cl^-$ cotransporter; (5) ouabain-insensitive, Na^+-stimulated ATPase, and (6) verapamil-sensitive Ca^{2+} channels.

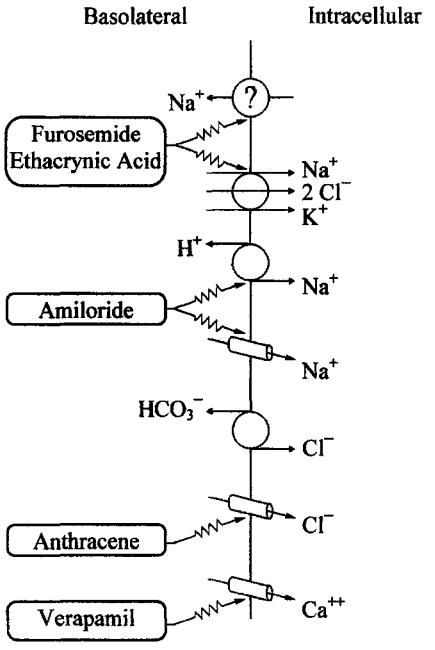

Fig. 8. Working model showing postulated transporters involved in cell volume regulation in the Malpighian tubule during hyperosmotic shock. Inhibitors of the different pathways shown in boxes.

Thus, we conclude that proximal segment cells of Malpighian tubules of *R. neglectus* regulate their volume effectively in hyperosmotic solutions but only partially in hyposmotic solutions.

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