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Intracellular Activities during Volume Regulation by *Necturus* Gallbladder

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Summary. *Necturus* gallbladder epithelial cells regulate their volume after a change in solution osmolality. We determined the intracellular activities of Na, K and Cl when the mucosal bathing solution osmolality was increased 18% by the addition of mannitol. The gallbladder was mounted in a rapid flow chamber and punctured simultaneously with two single-barrelled microelectrodes. One electrode sensed membrane potential and the other was sensitive to the activity of Na, K or Cl. Cell volume measurements, made in previous studies utilizing quantitative light microscopy, indicated that hypertonicity of the mucosal bath first caused a cell shrinkage of 15% followed by volume readjustment. Some loss of Na, K and Cl was observed during shrinkage; subsequently during volume regulation, the intracellular quantities of all three ions increased. The loss of Na during the initial cell shrinkage could be blocked by ouabain and was therefore due to increased transport. K and Cl losses were probably related to the increase in their concentrations during shrinkage. The gain of Na, K and Cl during volume regulation was similar in magnitude to the loss of these solutes during cell shrinkage. The increase of Na, K and Cl during volume regulation accounted for about 60% of the increase of cell solutes during this period indicating that other solutes also contributed to the volume regulation response.

Key Words cell volume · microelectrodes · ion activities · hypertonicity · ouabain · ion exchange · ion transport · electroneutral · epithelial

Introduction

When *Necturus* gallbladder epithelial cells are exposed to anisotonic bathing solutions they change their volume in accord with the alteration in osmolality. Then, despite continued exposure to the anisotonic solution, cell volume rapidly returns to the original control value (Spring, Hope & Persson, 1981; Persson & Spring, 1982; Spring & Ericson, 1982). Volume regulatory increase after osmotic shrinkage is dependent on the presence of Na and Cl in the mucosal bath (Ericson & Spring, 1982b) as well as HCO_3 in the bathing solutions (Fisher, Persson & Spring, 1981). Volume regulatory increase appears to be due to the transient activation of transport processes in the apical membrane of the epithelial cells. Evidence from inhibitor and ion substitution experiments indicates that Na enters the cell by exchanging for H, and Cl enters by exchanging for HCO_3 (Fisher et al., 1981; Ericson & Spring, 1982b). The influx of NaCl was throught to increase the osmotically active intracellular solutes resulting in the influx of water and the increase in cell volume.

Cell volume regulation is a widespread phenomenon and has been reported in a number of other epithelia such as renal proximal tubule (Grantham et al., 1977), frog gallbladder (Davis & Finn, 1982), and frog skin (MacRobbie & Ussing, 1961). The ionic basis of volume regulation has been more extensively studied in nonepithelial tissues such as erythrocytes (Cala, 1980; Kregenow, 1981), lymphocytes (Cheung et al., 1982), ascites tumor cells (Hoffman, Sjøholm & Simonsen, 1981), heart ventricle (Vislie, 1980), and isolated axon (Kevers, Peguevx & Gilles, 1981). In general, volume regulatory increase results from the uptake of NaCl or KCl followed by water (Macknight & Leaf, 1977; Kregenow, 1981). Our previous studies suggested that the mechanism of volume regulatory increase by *Necturus* gallbladder epithelium was similar to that of the *Amphiuma* red blood cell (Cala, 1980; Kregenow, 1981). In this system, Na enters the cell in exchange for H and the cell becomes more alkaline; Cl enters in exchange for HCO_3. In both the *Amphiuma* red blood cell and *Necturus* gallbladder epithelial cell, the Na/H exchange rate is increased substantially when the osmolality of the bathing solution is increased.

In the present study, we determined the intracellular activities of Na, K and Cl during exposure of the *Necturus* gallbladder epithelium to a hypertonic mucosal bathing solution. The combination

of our previous cell volume measurements with the present ionic activity determinations enabled us to calculate the changes in cell solute content which occurred during volume regulation. In addition, measurements of the ionic activities permitted a definition of the electrochemical driving forces for these ions as a step toward elucidating the transport mechanisms for these substances. It was our expectation based on the results obtained with *Amphiuma* red blood cells that there would be substantial increases in intracellular Na, K and Cl in gallbladder cells as a result of volume regulatory increase. Our results show that the intracellular quantities of Na, K and Cl increase during cell volume regulation, but the gain of these ions was not sufficient to completely account for the increase in osmotically active intracellular solutes required for the volume regulatory response. After volume regulation was complete, the quantity of intracellular Na, K and Cl was virtually identical to the control condition indicating that the increase in cell solute content was due to an elevation in the amount of other intracellular substances.

Materials and Methods

Experimental Protocol

Studies were done using the isolated gallbladder of adult *Necturus maculosus* (Riverside Biological, Oshkosh, Wis.) which were maintained in laboratory aquaria at 15 °C for at least two weeks prior to study. The gallbladder was removed from the anesthetized animal, rinsed and mounted in a modified Ussing chamber designed for cell impalement and rapid solution changes (*see below*). Tissues were equilibrated *in vitro* with the perfusion solutions for at least 30 min prior to puncturing with microelectrodes. The same protocol was used for all experiments. Two cells were punctured simultaneously with single-barrelled microelectrodes. One electrode was sensitive to the intracellular voltage; the other was sensitive to either Na, K or Cl activity. Criteria for acceptable impalements were as previously described (Larson & Spring, 1983). In some experiments voltage divider ratios (VDR) were determined as an additional criterion for acceptability of punctures; experiments were rejected if the values of VDR of the voltage and the ion-sensitive electrode were not identical. The ion-sensitive electrode was calibrated in standard solutions before puncturing cells and intracellular activity was calculated at 5-sec intervals by an on-line computerized data acquisition system. When the calculated activity was stable for at least 30 sec, the mucosal bathing solution was changed to a hyperosmotic Ringer's solution. After 200 to 300 sec of exposure to hypertonicity, the mucosal solution was returned to the isotonic control bathing solution. Previous studies indicated that the cell volume changes in response to hypertonic mucosal bathing solutions were reversible and the gallbladder could be re-exposed to hyperosmotic solutions several times with similar results (Persson & Spring, *unpublished observations*). Therefore, tissues were re-equilibrated in control solution for 30 min after the initial hypertonic exposure, two other cells were punctured, and the experiment was repeated one or more times.

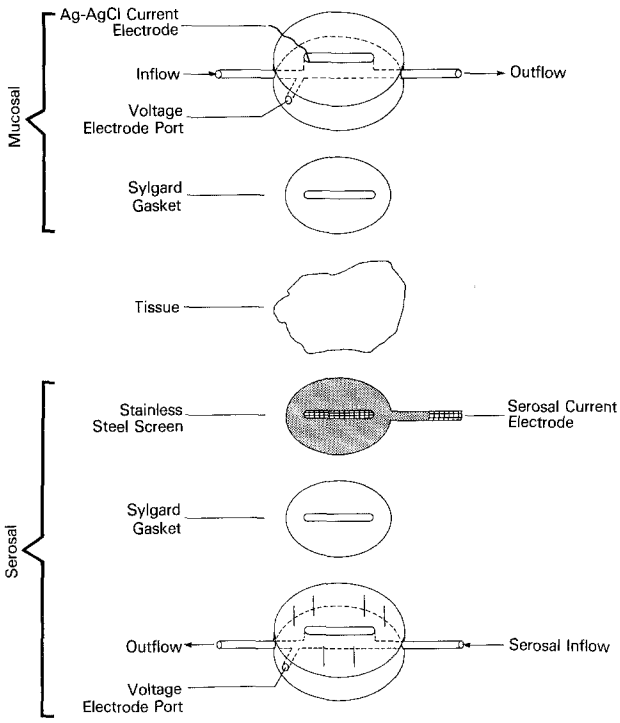

Fig. 1. Schematic view of chamber used for microelectrode studies of gallbladder cells. Area of tissue exposed was 0.08 cm^2. Volume of mucosal and serosal chambers were 8 and 16 µl, respectively. Two Ag-AgCl wires in the mucosal chamber were positioned on each side of the slot along the length of the chamber. *See* text for additional details

Solutions

Two different control solutions were used: The standard control *Necturus* Ringer's solution consisted of (mM) 72 NaCl, 10 $NaHCO_3$, 2.5 KCl, 0.5 NaH_2PO_4, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 36 mannitol, pH = 7.6 gassed with 1% CO_2 and 99% air; 190 to 200 mOsm/liter. As previously shown (Fisher et al., 1981), volume regulation was inhibited when tissues were bathed in a bicarbonate-free Ringers' solution consisting of (mM) 82 NaCl, 1.0 K_2HPO_4, 0.3 KH_2PO_4, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 36 mannitol; pH = 7.6; gassed with air. Mucosal perfusates were rendered 18% hyperosmotic in all studies by the addition of 36 mM mannitol to the control solution. In some studies, 10^{-4} M ouabain (Sigma Chemical, St. Louis, Mo.) was added to the serosal perfusion solution to inhibit the Na,K-ATPase at the basolateral membrane. Ouabain-induced changes in cell volume and intracellular composition were prevented by decreasing the NaCl or Cl concentration in the mucosal perfusate as previously described (Ericson & Spring, 1982*a*). In ouabain experiments the Cl or NaCl concentration of the mucosal perfusate was reduced to 20 mM by equimolar gluconate or mannitol substitution.

Chamber and Perfusion System

The chamber used in these studies is shown schematically in Fig. 1. It was designed to achieve high perfusion rates and rapid solution changes while maintaining cellular impalements. It was critical to minimize the unstirred layer in the mucosal bath to express the full osmotic change at the apical membrane with-

in a few seconds. Solution flow rates in the mucosal chamber ranged from 1.5 to 3 ml/min. Electrophysiologic and dye washout studies of the chamber indicated that these flow rates were sufficient to completely change the mucosal bath in 5 sec. Reservoirs containing the mucosal bathing solutions were positioned 60 to 70 cm above the chamber. Ringer's solution flowed by gravity from each reservoir through glass tubing and an electronic pinch valve (Angar 370, Brunswick Technetics, Cedar Knolls, N.J.) to the chamber. Perfusion rate was monitored with flow meters (Gilmont Instruments Inc., Great Neck, N.Y.) and was adjusted before each experiment to insure identical flows during the control and experimental periods. Three solution lines were connected to a manifold located at the inflow port of the mucosal chamber to minimize the delay in changing the mucosal bath. The serosal compartment consisted of a trough in the lower half of the chamber with ports for solution flow (Fig. 1). A platinized stainless steel screen was used to support the tissue and to pass transepithelial current pulses. Serosal solution flow was maintained by a negative pressure of 10 to 20 cm H_2O at the serosal outflow port. This arrangement also served to stabilize the tissue during impalement and solution changes.

The tissue was visualized with a dissecting microscope (E. Leitz Inc., Rockleigh, N.J.), and the chamber, microscope and perfusion system were positioned on an air suspension table to reduce mechanical vibration (Micro-g, Technical Manufacturing Corp., Woburn, Mass.).

Microelectrodes

All microelectrodes were single-barrelled and were pulled from omega dot glass tubing (1.2 mm OD,W-P Instruments Inc., New Haven, Conn.) on a vertical pipette puller (David Kopf Instruments, Tujunga, Calif.). Voltage-sensitive electrodes were filled immediately before use with 3 M KCl; electrode impedance ranged from 10 to 20 MΩ and was monitored continuously during punctures. Often after immersing 3 M KCl-filled electrodes in the bicarbonate-free Ringer's solution, they became noisy, eventually plugged, and could not be used. This was not observed when electrodes were filled with 0.5 M KCl, and 0.5 M KCl-filled electrodes were used in those studies. Intracellular potentials and experimental results were independent of electrode KCl concentration.

Ion-sensitive electrodes were fabricated as described previously (Kimura & Spring, 1979). In brief, microelectrodes were pulled from glass capillaries which had been cleaned by heating to the glass annealing temperature. Immediately after pulling, electrodes were placed in an environment of low relative humidity (20 to 30%) for 1 to 2 hr. Electrode tips were then silanized in this environment by immersion in pure chlorotrimethylsilane (Pierce Chemical, Rockford, Ill.) for 7 to 10 sec and baked in an oven for 1 hr at 140 °C. Electrodes could be stored dry for several days after silanizing.

In preliminary studies, we found it impossible to adequately silanize electrodes when the ambient relative humidity was above approximately 30%. This was indicated by relatively poor selectivity for the ion of interest and/or slopes less than 50 mV/10-fold change of ion activity. To avoid ambient fluctuations in humidity, we constructed a chamber for incubating and silanizing electrodes. The chamber (50 × 60 × 80 cm) was built from Lucite® and permitted manipulation of electrodes and silane through gloved ports. Chamber humidity was controlled by injecting a nitrogen gas stream of a known relative humidity into the chamber through a small hole. In the present studies, it was always necessary to lower the relative humidity to 30% or less by flowing dry nitrogen gas. However, it was possible to attain any desired humidity by mixing a water-saturated gas stream with a dry stream in appropriate proportions. The humidity of the resultant gas mixture was measured with a humidity sensor near the chamber inlet (Kaymont Inst., Huntington Station, N.Y.). A second hygrometer (Brooklyn, Marksson Science Inc., Del Mar, Calif.) was used to measure the chamber humidity.

Ion-sensitive microelectrodes were filled with ion-exchange resin and calibrated by the method of separate solutions as previously described (Kimuara & Spring, 1979). When electrode responsiveness was poor or the tip was plugged, the electrodes were polished by immersion for about 3 min in a stirred suspension of 0.5 μm alumina particles (Buehler Ltd., Evanston, Ill.) in 100 mM NaCl (Corson, Goodman & Fein, 1979).

Cl-sensitive electrodes, were filled with Cl-exchange resin (Orion Research Inc., Cambridge, Mass.), and stored for 1 to 3 days in 0.1 to 1.0 M NaCl to improve electrode sensitivity. An Ag-AgCl wire contacted the resin directly and was connected to the input probe of the electrometer. Electrodes were calibrated by placing electrode tips in solutions of 10, 50 and 100 mM NaCl. Standard calibration solutions were connected via agar bridges of identical composition to the mucosal bath ground. Linear regression of electrode potential on log Cl activity yielded an average slope of 55.5 ± 0.4 (30) mV/decade change of Cl activity. The Orion Cl ion exchanger is about ten times more selective for Cl over HCO_3 (Spring & Kimura, 1978). However, in the same preparation of gallbladder the Corning Cl ion exchanger produces readings which are about 9 mM lower than those obtained with the Orion exchanger suggesting that the Orion exchanger is less selective (Larson & Spring, *unpublished observations*). K-sensitive electrodes were filled with K exchanger resin (Kimura & Spring, 1979) and were used within 24 hr of filling. Electrodes were calibrated in standard solutions of 1, 10 and 100 mM KCl. The K electrode slopes averaged 56.2 ± 0.3 (36) mV/decade change of K activity. Selectivity for K over Na was determined by placing electrode tips in 100 mM NaCl and averaged 75.3 ± 3.4 (36). Na-sensitive exchange resin (ETH227, Fluka Chemical Corp., Hauppauge, N.Y.) was mixed with 0.5% tetraphenylborate in (*o*-nitrophenyl) octyl ether. Electrode tips were filled with resin in the same manner as Cl and K electrodes but the shanks were backfilled with 1 M NaCl for electrical connection with the chlorided silver wire. Na-sensitive electrodes were calibrated in solutions of 1, 10 and 100 mM NaCl and slopes averaged 55.6 ± 0.3 (65) mV/decade change of Na activity. Na activity calculations were corrected for K interference; electrode selectivity for Na over K was assessed by measuring the electrode potential in 100 mM KCl and averaged 40.7 ± 3.2 (65). Some Ca interference was also detected when electrodes were immersed in Ringers' solution. Selectivity for Na^+ over Ca^{++} ranged between 0.6 and 2.0. This Ca sensitivity is in part due to the addition of tetraphenylborate to the Na-exchange resin (Bers & Ellis, 1982). However, since intracellular Ca activity is presumably about 4 orders of magnitude lower than that of the bathing solution (Lee, Taylor & Windhager, 1980; O'Doherty, Youmans & Armstrong, 1980), the Ca interference probably altered the Na electrode reading by less than 10% and the effect was ignored.

Electrical Connections and Data Acquisition

PD microelectrodes were connected to a dual probe high input impedance electrometer (Model 750, W-P Instruments, New Haven, Conn.) with Ag-AgCl pellet electrode holders. Ion-sensitive electrodes were connected via a chlorided silver wire to a very high impedance electrometer (Model 223, W-P Instruments). The outputs of the electrometers were connected to

separate channels of an 8-channel analog-to-digital converter module (Tecmar Inc., Cleveland, Ohio) of a laboratory microcomputer. Analog signals were digitized and immediately stored in computer memory for analysis.

In some studies, transepithelial electrical parameters of gallbladder were measured. Transepithelial PD (V_t) was recorded with Ringers'-agar bridges in the mucosal and serosal bath through calomel half-cells to the input stage of an electrometer. The output was also digitized and stored in computer memory.

To determine transepithelial resistance (R_t) and the voltage divider ratio (VDR), transepithelial current pulses were applied to the tissue at 5-sec intervals. A current clamp was used to pass 250-msec current pulses sufficient to change the transepithelial PD by about 10 mV. R_t and VDR were calculated as follows:

$$R_t = \Delta V_t / \Delta I_t \qquad \text{VDR} = \Delta V_c / \Delta V_t$$

where ΔV_t and ΔI_t are the changes in transepithelial potential difference and current, respectively. ΔV_c is the change in the apical membrane PD measured with microelectrodes during current passage.

The mucosal bath current electrode was a chlorided Ag-AgCl wire positioned on each side of the mucosal chamber slot (*see* Fig. 1). The stainless steel screen in the serosal chamber served as the current-passing electrode. Platinizing was accomplished by placing the screen as an anode in R-1153 platinizing solution (Hellige, Inc., Garden City, N.Y.). The remainder of the screen was coated with a nonconducting wax to localize the current source directly below the area of exposed tissue. This electrode arrangement provided uniform current density through the tissue. The mucosal bath was held at virtual ground by a current-to-voltage converter in the current clamp. A low-resistance microelectrode in the muocsal bath near the tissue was connected to the second input of the ion-sensitive electrode amplifier and served as the indifferent electrode for the intracellular measurements.

Calculations and Statistics

Activity was calculated using the Nicolsky equation as described elsewhere (Lev & Armstrong, 1975). Average changes of cell volume in response to mucosal hypertonicity were determined previously in our laboratory using quantitative light microscopy (Persson & Spring, 1982). Changes in the intracellular quantity (concentration × cell volume) of Na, K and Cl were calculated for each experiment assuming that the volume data were free of error. Ion concentrations were calculated from the measured activities with the assumption that the activity coefficient was the same as that in free solution (0.78). Thus, the variability of intracellular quantity (Q) reflects only the errors of the activity measurements unless otherwise stated. All values are presented as mean ± SEM (No. of observations) with level of significance determined from the Student's t-test.

Results

Cell Volume

Previous studies in our laboratory demonstrated that gallbladder epithelial cells exposed to hyperosmotic solutions undergo rapid cell shrinkage followed by recovery of cell volume back toward control values (Ericson & Spring, 1982b; Persson & Spring, 1982; Spring and Ericson, 1982). A typical example of this is reproduced in Fig. 2 (upper trace). The osmolality of the mucosal bathing solution was increased rapidly by 18%; cell volume decreased to a minimum within about 40 sec. Cell shrinkage resulted from loss of intracellular water to the mucosal bathing solution by osmosis; intracellular osmolarity must have increased to near that of the mucosal perfusate because the osmotic response of the cell was similar to that expected for an ideal osmometer with respect to the mucosal bath. Subsequently, cell volume returned to control in about 90 sec despite the continued presence of a hypertonic mucosal bathing solution. Also shown in this figure is the response observed when the mucosal bath was returned to the control os-

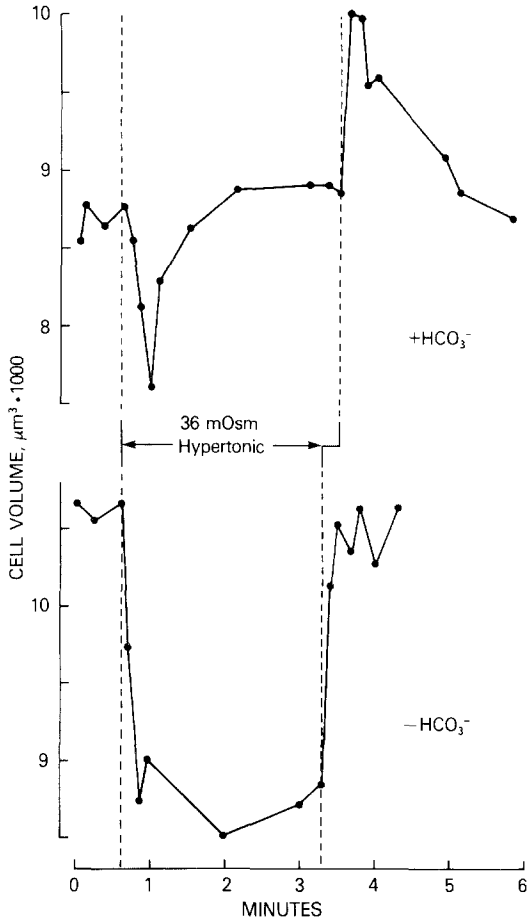

Fig. 2. Cell volume is plotted as a function of time. At the first dotted line, 36 mM mannitol was added to the control mucosal bathing solution in order to increase mucosal osmolarity by 18%. Studies were done in the presence ($+HCO_3$) or absence ($-HCO_3$) of bicarbonate in the perfusion solutions. Tissues were returned to control conditions at the second dotted line. (Reprinted from *Science* with permission.) Initial cell volumes are representative of the range of values observed. No bicarbonate-dependent difference in average cell volume was observed

molality; cell volume increased at a rate similar to the initial rate of shrinkage which represented osmotic gain of water by the cell. After the osmotically induced increase of cell volume, regulation to the control volume was again observed (Persson & Spring, 1982).

Figure 2 (bottom) is a representative plot of the volume response to hypertonicity for cells bathed in a bicarbonate-free Ringers' solution. The rate of osmotically induced shrinkage was similar to that observed when HCO_3 was present, but no volume regulation was observed (Fisher et al., 1981). Cell volume decreased for about 40 sec and then remained unchanged for the duration of the hypertonic exposure. This volume decrease averaged 14.4% of the control value, similar to the average decrease (13.3%) observed in the presence of HCO_3. When tissues bathed in bicarbonate-free solutions were returned to isotonic conditions, cell volume increased for 40 sec back to the original volume without further time-dependent changes.

Electrical Measurements

Intracellular potential and activity measurements were obtained under identical experimental conditions to those of the volume measurements. The mucosal osmolality was increased 18% for tissues bathed in either HCO_3 or HCO_3-free Ringers's solutions. Since ion-selective electrodes are sensitive to the intracellular PD (V_c) as well as intracellular ion activities, it was necessary to correct for any changes of V_c in response to mucosal hypertonicity. Examples of changes in V_c are shown in Fig. 3 (top). In the presence of HCO_3, V_c hyperpolarized slightly and then stabilized; in contrast, no change of V_c was observed for tissues bathed in HCO_3-free solutions. These data are summarized in Table 1. V_c averaged -67 mV in HCO_3 Ringer's solution; 40 sec after exposure to mucosal hypertonicity when the cell had reached its minimum volume, V_c had hyperpolarized to an average value of -68 mV. During the subsequent period of cell volume regulation (40 to 130 sec), V_c did not change significantly. Cells bathed in HCO_3-free Ringer's solutions did not hyperpolarize during mucosal hypertonicity.

The PD traces in Fig. 3 also show the changes of V_c after returning to the control, isotonic Ringer's solution; V_c underwent a transient hyperpolarization which reached a peak in about 40 sec and averaged 4.1 ± 0.6 (27) mV and 4.6 ± 0.7 (30) mV in HCO_3 and HCO_3-free solutions, respectively. The cause of this hyperpolarization is unknown. These changes of V_c are clearly not related to the volume regulatory increase since they were observed in bicarbonate-free solutions.

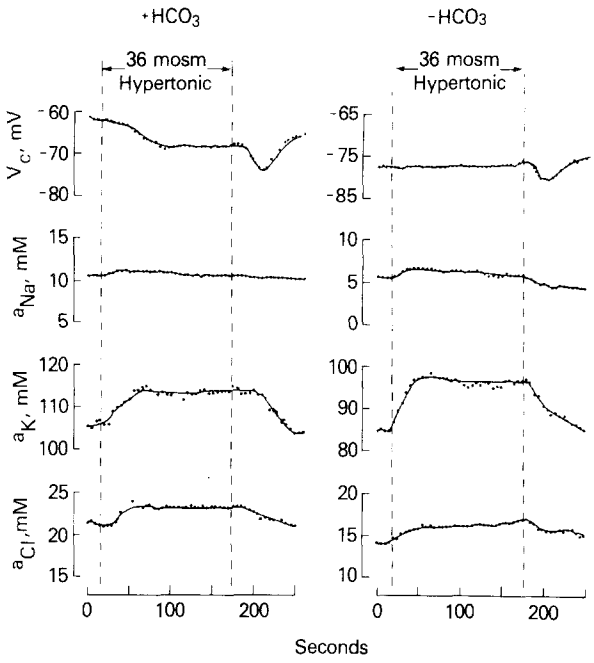

Fig. 3. Typical tracings of intracellular PD (V_c) and activities of Na (a_{Na}), K (a_K) and Cl (a_{Cl}) plotted as a function of time in the presence and absence of bicarbonate. Each tracing was obtained in a separate study. The time course of PD and activity changes upon first exposing tissues to hypertonic mannitol solutions varied depending on flow rate and electrode position in the chamber

Because gallbladder epithelial cells are electrically coupled (Frömter, 1972; Reuss & Finn, 1975*a*) and the changes of V_c in response to hypertonicity were small, we considered it feasible to determine intracellular activity with PD-sensitive and ion-sensitive electrodes recording from two nearby cells. However, during the transient hyperpolarization upon return to isotonic Ringer's solution, slight differences in the time courses recorded from the two electrodes yielded incorrectly calculated values of activity. Therefore we were not able to characterize, with confidence, the intracellular activity changes during these voltage transients.

Also given in Table 1 are the values of voltage divider ratio ($VDR = \Delta V_c / \Delta V_t$), transepithelial resistance (R_t), and transepithelial voltage (V_t). VDR averaged about 0.5 for both control solutions and was unchanged during hypertonicity. R_t, in bicarbonate Ringer's solution, averaged 200 ± 12 Ω-cm^2 and also did not change. In bicarbonate-free solutions, however, R_t increased reversibly during hypertonicity from an average of 131 to 183 Ω cm^2. The cause of the differences in R_t in the absence of bicarbonate is unknown. V_t hyperpolarized slightly in both HCO_3-containing and HCO_3-free

Table 1. Electrical parameters during hypertonic exposure[a, b]

Seconds in hypertonicity	V_c (mV)	VDR	V_t (mV)	R_t (Ω cm^2)
HCO_3 Ringer's solution				
Control	-67.0 ± 0.9	0.55 ± 0.06	$+0.3 \pm 0.5$	200 ± 12
40	$-68.7^c \pm 0.9$	0.55 ± 0.06	-0.6 ± 0.5	204 ± 11
130	$-70.2^c \pm 0.9$	0.54 ± 0.06	-1.1 ± 0.6	212 ± 12
(No. tissues, obs.)	(22, 53)	(5, 13)	(5, 13)	(5, 13)
HCO_3-Free Ringer's Solution				
Control	-68.1 ± 1.6	0.46 ± 0.02	-0.2 ± 0.1	131 ± 8
40	-67.5 ± 1.7	0.47 ± 0.01	$-1.9^c \pm 0.2$	$152^c \pm 9$
130	-66.6 ± 1.7	0.46 ± 0.01	$-1.9^c \pm 0.1$	$183^c \pm 8$
(No. tissues, obs.)	(16, 44)	(5, 16)	(5, 16)	(5, 16)

[a] V_c, intracellular voltage; VDR, voltage divider ratio; V_t, transepithelial voltage; R_t, transepithelial resistance.
[b] Values are mean $\pm$ SEM.
[c] Different from control value ($P < 0.01$)

Table 2. Cell volume and intracellular activities[a]

Condition	Cell volume $\times 10^3$ μm^3	a_{Na}	a_K (mmol/liter)	a_{Cl}
$+HCO_3$	10.9 ± 0.5 (19)	13.4 ± 1.5 (5, 15)	100.9 ± 5.3 (6, 15)	24.9 ± 1.8 (12, 23)
$-HCO_3$	11.3 ± 1.0 (13)	7.8 ± 0.7 (5, 17)	98.5 ± 4.2 (5, 16)	19.1 ± 1.8 (6, 11)
HCO_3 + ouabain[b]	10.6 ± 0.7 (17)	22.8 ± 2.3 (8, 12)	71.3 ± 4.1 (8, 11)	[c]

[a] Values are mean $\pm$ SEM (No. tissues, No. observations).
[b] Ouabain (10^{-4} M) was added to the serosal bathing solution and the NaCl or Cl concentration of the mucosal bathing solution was reduced to prevent cell swelling.
[c] Not measured.

hypertonic Ringer's solution. The change was consistent with a Na-dependent streaming potential through a cation-selective tight junction caused by transepithelial osmotic water flow (Reuss & Finn, 1975*a*).

All of the electrical changes during exposure to hypertonic solutions were relatively small, suggesting that large changes in cell membrane conductance were not occurring. These observations are therefore supportive of our previous contention that the volume regulatory increase occurs via the operation of electrically silent transporters which increase salt and water flow into these cells in response to an osmotic challenge (Fisher et al., 1981; Ericson & Spring 1982b; Spring & Ericson 1982).

Intracellular Activities and Quantities

It was previously shown that volume regulatory increase required Na and Cl in the mucosal bath and that volume regulation probably involved the uptake of NaCl across the apical membrane (Ericson & Spring 1982*b*). We wished to determine whether an increase of intracellular Na and Cl occurred during volume regulation. The intracellular activites of the primary osmotic constituents, Na, K and Cl, were measured during the exposure of the tissue to a hypertonic mucosal bath. Control values of intracellular activities in the presence and absence of HCO_3 are given in Table 2. The measured activities of all three ions were similar to those reported previously by other investigators (Graf & Giebisch 1979; Reuss & Weinman 1979; Garcia-Diaz & Armstrong, 1980; Zeuthen, 1982).

Representative examples of changes in a_{Na}, a_K and a_{Cl} are shown in Fig. 3. On the left side of the Figure are records of tissues bathed in bicarbonate Ringer's solution. When cells were exposed to 36 mOsm hypertonicity, a_K and a_{Cl} increased concurrently with the period of cell shrinkage. During the subsequent period of volume regulation, no further changes in a_K or a_{Cl} were observed. On the other hand, a_{Na} increased only slightly during cell shrinkage from 10.4 to 11.0 mM and then, during volume regulation, decreased slowly back to control. Upon return to isotonic conditions, a_K and a_{Cl} decreased to control levels; no changes of a_{Na} were observed during this period.

Similar changes were observed when tissues were bathed in bicarbonate-free solutions (Fig. 3, right). The rate of increase in activities was independent of the presence of HCO_3 and is consistent with the idea that the initial increase of ionic activities was due simply to osmotic water loss. In the absence of bicarbonate, and therefore volume regulation, there were no further changes in a_{Na}, a_K, or a_{Cl}.

Changes of intracellular ionic activity are summarized in Fig. 4; 40 and 130 sec were chosen as representative times which correspond to completion of the shrinkage and regulatory periods, respectively. For reference, the mean volume changes (as a percent of control) are plotted at the top of this Figure. Ion activities increased during cell shrinkage; larger changes were measured for all three ions in the absence of HCO_3. Indeed, the percentage increase in activity (Na = 13%, K =

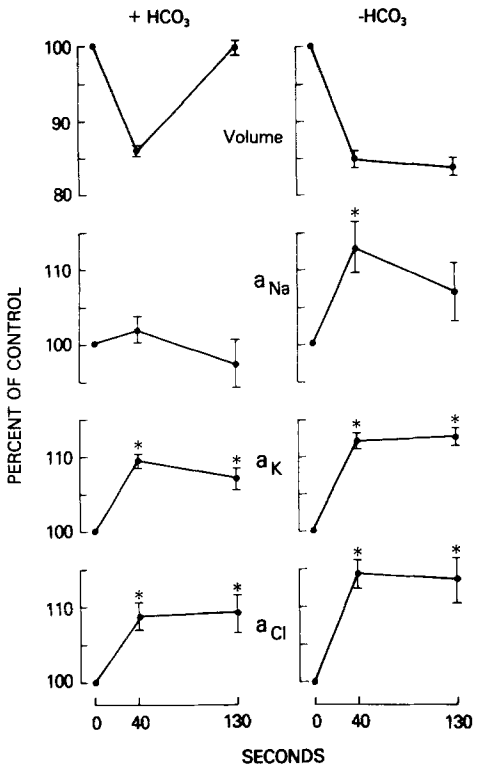

Fig. 4. Average changes of cell volume and intracellular activities during mucosal hypertonic exposure are plotted versus time. Values are expressed as a percent of control. (*) indicates significantly different from 0 sec value ($P<0.01$)

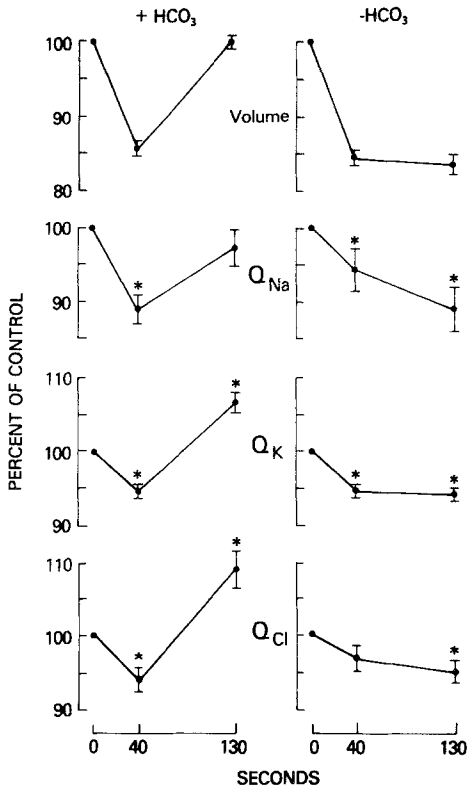

Fig. 5. Average changes of intracellular quantities of Na, K and Cl during mucosal hypertonic exposure are plotted versus time. Average values of cell volume are replotted for comparison. Values are expressed as a percent of control. (*) indicates significantly different from 0 sec value ($P<0.01$)

12% and Cl = 14%) approached that of cell shrinkage (14.4%) in the absence of HCO_3. This indicates that cell shrinkage, in the absence of HCO_3, resulted in relatively small losses of Na, K and Cl from the cell. In Fig. 5 are given plots of the intracellular quantities at the same times as the activity plots shown in Fig. 4. Quantities were calculated for each experiment from the measured activity and the mean volume as stated in Materials and Methods. During cell shrinkage, intracellular Na content (Q_{Na}) decreased 5.7% in the absence of HCO_3 compared to 11.7% in the presence of HCO_3. Similarly, the cells did not lose a significant quantity of Cl in the absence of HCO_3 as opposed to a decrease of 5.8% with HCO_3 present. Loss of K (Q_K) was comparable under both conditions averaging about 5%. Thus, during the period of cell shrinkage, the quantities of Na, K and Cl decreased showing that these osmotically active solutes were lost from the cell or sequestered as a result of cell shrinkage. The loss of these ions may be simply due to an acceleration of their normal exit processes induced by the increase in their intracellular activities during cell shrinkage.

The subsequent period of cell volume recovery was not accompanied by additional significant changes of ion activities (*cf.* Fig. 4, left). This was surprising given the Na and Cl requirement for regulatory cell volume increase; but activity is not representative of ion movement when volume is changing. Indeed volume regulatory increase resulted in significant increases of Q_{Na}, Q_K and Q_{Cl} (Fig. 5, left) averaging about 8, 12 and 15%, respectively, above the minimum levels attained during osmotic cell shrinkage. However, the gain of these ions was far less than expected if volume regulation were solely due to an increase in intracellular NaCl as it is in the *Amphiuma* red blood cell. These results suggest that Na, K and Cl are not the only substances responsible for the rise in cell osmolality after volume regulation is completed.

In the absence of HCO_3 no volume increase was observed after shrinkage (Fisher et al., 1981) and in parallel, no significant changes of a_K, a_{Cl}, Q_K, or Q_{Cl} were detected (Figs. 4 and 5, right side). However, a_{Na} and Q_{Na} decreased slightly over this time interval. It is possible that under these circum-

stances, the initial rise of a_{Na} stimulated the basolateral Na pump which in turn decreased Q_{Na}.

Effect of Ouabain

Since it was surprising that tissues bathed in HCO_3 Ringer's solution exhibited no measurable increase in a_{Na} during shrinkage (*cf.* Fig. 5), it seemed possible that increases of a_{Na} during the hyperosmotic volume response were prevented by stimulation of the pump. Stimulation of the Na/K pump also was suggested by the fact that Q_K increased during volume regulation (*cf.* Fig. 5). Thus, it was possible that most of the incoming Na immediately was pumped out of the cell in exchange for K. The net effect would be an increase of osmotically active intracellular solutes with relatively little change of a_{Na}.

The role of the basolateral Na/K pump in the hypertonic response was examined by pretreating tissues with the specific inhibitor ouabain (Glynn & Karlish, 1975). Ouabain causes an increase of *Necturus* gallbladder cell volume because solute exit ceases at the basolateral membrane while entry at the apical membrane continues. Thus, ouabain increases a_{Na} in gallbladder and other tissues because initially the electrochemical gradients still favor NaCl entry (Ericson & Spring, 1982a). To reduce these changes of volume and activity, tissues were bathed in a bicarbonate Ringer's solution in which the Na and/or Cl concentration of the mucosal bath was reduced to 20 mM. Similar studies of volume under these conditions demonstrated that ouabain does not alter the time course or magnitude of the volume regulatory response to hypertonicity (Ericson & Spring, 1982b). As shown in Table 2, cell volume in ouabain-treated tissue was not changed even though a_{Na} had almost doubled (13.4 to 22.8 mM) and a_K was depressed (100.9 to 71.3 mM). A representative trace of a_{Na} in response to mucosal hypertonicity in the presence of ouabain is shown in Fig. 6 (bottom). For comparison, the response of an untreated tissue is shown in the top of this figure. Clearly, in the presence of ouabain, hypertonicity caused a large increase of a_{Na} which was reversed upon return to isotonic conditions. The average changes of Na and K activities and quantities after ouabain are shown in Fig. 7. A striking increase of a_{Na} was observed during cell shrinkage averaging 17.8% (compare with the 2% increase of a_{Na} in the absence of ouabain; Fig. 4, left side). No change of Q_{Na} was calculated

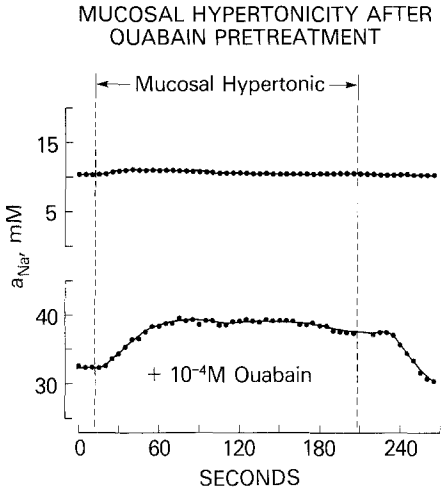

Fig. 6. Intracellular Na activity plotted as a function of time in response to mucosal hypertonicity. Tissues were incubated in HCO_3 Ringer's solution in the absence (upper trace) or presence (lower trace) of ouabain in the serosal bathing solution. Mannitol (36 mM) was added to the mucosal perfusate between dotted lines

for this period indicating that Na exit during shrinkage in the absence of ouabain was due to stimulation of the Na/K pump. In contrast, changes of a_K and Q_K during shrinkage were similar to those observed for untreated tissues which suggests that the loss of K during cell shrinkage is independent of the pump and may in fact be diffusional.

Changes of intracellular Na and K content during volume regulatory increase for tissues pretreated with ouabain are also shown in Fig. 7. Under these conditions, Q_{Na} increased 12.1% above the control value, compared to no change observed during volume regulation in the absence of ouabain. Thus, inhibition of the Na/K pump unmasked substantial increases of Q_{Na} during the hypertonic exposure period. It should be noted that the increase in Q_{Na} in the presence of ouabain was still substantially less than that expected if cell volume regulation were due solely to the influx of NaCl. Surprisingly, ouabain inhibition did not prevent the increase of Q_K during volume recovery (Fig. 7). This indicated that the increase of Q_K during this period is not related to the Na-dependent stimulation of the pump (*see* Discussion).

Discussion

Osmotic Balance Sheet

This work was designed to determine the role of Na, K and Cl in volume regulation by gallbladder

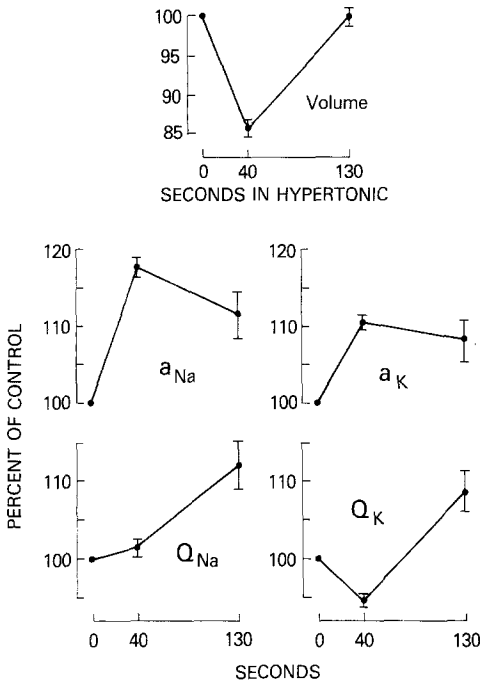

Fig. 7. Effect of ouabain on average changes of cell volume, Na and K activities and quantities in response to mucosal hypertonicity. Values are expressed as percent of control. (*) indicates significantly different from 0 sec value ($P < 0.01$)

epithelial cells during a mucosal hyperosmotic challenge. In general, there was a loss of these ions during the initial osmotic shrinkage followed by a gain during the subsequent volume regulatory period. When the volume regulatory period was completed, the cell had returned to its original volume and intracellular K and Cl were increased above their control concentrations. An understanding of the mechanism of cell volume regulation in response to mucosal hypertonicity requires a quantitative assessment of the measured changes of the intracellular ion content and a determination of whether the changes in these quantities were sufficient to account for the increased fluid entry during volume recovery. Such as quantitative analysis is shown diagrammatically in Fig. 8 where the intracellular quantities of Na, K and Cl for the control (0 sec) and hypertonic conditions (40 and 130 sec) are given. It was assumed that under steady-state, control conditions, the intracellular osmolality was identical to that of the bathing solutions and that the intracellular activity coefficients of the ions were unchanged throughout the experiment. Knowledge of the cell volume permitted us to determine the fraction of intracellular osmotically active solutes which could be attributed to Na, K and Cl. In this Figure we have plotted the average absolute quantities of Na, K and Cl. The difference between the measured quantities and the calculated total quantity of osmotically active solutes is given in the upper panels ("X"). Under control conditions, in tissues bathed in HCO_3 Ringer's, Na, K and Cl ions account for 84% of the intracellular osmotically active constituents. In the absence of HCO_3, 76% of the osmotically active solutes may be attributed to Na, K and Cl. We do not know why intracellular Na and Cl are reduced in the absence of HCO_3 compared to their levels in the presence of HCO_3. The Cl electrode readings could be artefactually increased by interference from HCO_3 ions. Although the composition of *"X"* is unknown, it probably consists of other inorganic salts and organic solutes, primarily the intracellular free amino acids. Indeed, in other vertebrate nonmarine cell systems where the intracellular contents have been examined, free amino acids generally constitute 10 to 20% of the intracellular osmotically active solutes (Gilles, 1979).

Also shown in Fig. 8 are the calculated changes of the intracellular quantities of Na, K, Cl and *"X"* during the hypertonic exposure period. After 40 sec of hypertonicity, intracellular osmolality again was assumed to be identical to that of the mucosal bathing solution. This seemed reasonable because the initial period of cell shrinkage was complete within 40 sec (*cf.* Fig. 2), and the cell behaves as an osmometer with reference to the mucosal bathing solution (Persson & Spring, 1982). For tissues bathed in HCO_3 solutions, *"X"* increased 45% (from 35 to 51×10^{-14} osmoles), during cell shrinkage. In the absence of HCO_3, the fraction of intracellular osmolality attributed to Na, K and Cl was reduced and therefore the quantity of unaccounted solutes (*"X"*) was larger in isotonic HCO_3-free Ringer's. A small (16%) additional increase in "*X*" occurred during cell shrinkage when HCO_3 was not present. The increases in *"X"* which accompany cell shrinkage suggest that shrinkage *per se* results in an increase in these unknown osmotically active intracellular solutes. It is possible that because cell shrinkage concentrates intracellular proteins it leads to an increase in the osmotic coefficient of these proteins. The osmotic coefficient of hemoglobin has been shown to increase as a function of concentration and to alter significantly the osmolality of red blood cells (Freedman & Hoffman, 1979; Siebens, 1983). During volume regulation the gain of Na, K and Cl

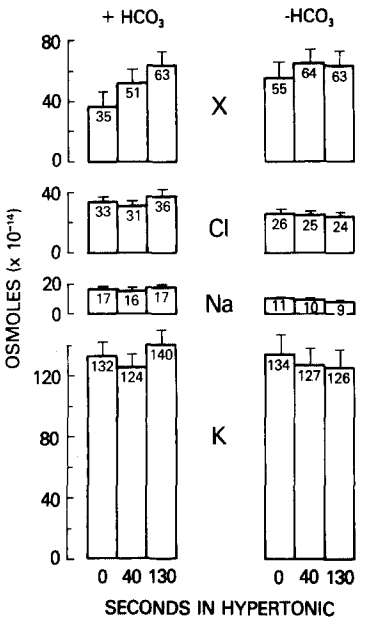

Fig. 8. Calculated (X) and measured (Na, K and Cl) intracellular quantities of osmotically active solutes are plotted versus time in hypertonicity. Values for Na, K and Cl were obtained using mean values for activity and volume. Errors shown reflect standard errors of the product of two means. Values of "X" represent the difference between the expected intracellular quantity of osmotically active solutes and the combined total measured quantities of Na+K+Cl. It was assumed that the intracellular osmolarity was identical to the mucosal bath osmolarity for each period (*see* text)

was accompanied by a further calculated increase of "X" (to 63×10^{-14} osmoles). Thus unknown solutes contribute significantly to the increase in cellular osmotically active substances during volume regulatory increase.

Loss of Ions During Cell Shrinkage

The mechanisms of the loss of ions during the shrinkage period is of interest. Previous studies indicated that the apical cell membrane hydraulic conductivity in gallbladder epithelial cells is 0.055 cm/sec (Persson & Spring, 1982). This value is about 4 orders of magnitude higher than the permeability of these cells to Na, K or Cl (Reuss & Finn, 1975*b*; Reuss, 1979), and it seems unlikely that the predominant mode of salt exit during shrinkage could be attributed to solvent drag. The loss of these substances during shrinkage may be the result of an increase in the rate of their normal, steady-state exit mechanism. For example, Na exit from gallbladder epithelial cells proceeds primarily across the basolateral membrane and is an active process mediated by the ouabain-sensitive Na pump (Reuss, Bello-Reuss & Grady, 1979). Inhibition of the pump by ouabain eliminated Na loss during cell shrinkage suggesting that small increases of a_{Na} during cell shrinkage in the absence of ouabain stimulated Na exit via the pump. Similarly, it is possible that loss of K and Cl during osmotic cell shrinkage also resulted from enhancement of their normal steady-state exit processes. The specific mechanisms are unknown but may involve KCl cotransport (van Os & Slegers, 1975; Reuss, 1979). Thus, cell shrinkage, which increases the intracellular concentration of KCl, could in turn augment the rate of KCl exit.

The precise kinetic characteristics of the Na pump in epithelial cells is a subject of current investigation and it is interesting that our results indicate that the pump rate must be very sensitive to small changes in the intracellular Na concentration. In other systems, the intracellular Na activity which leads to half-maximal rates of Na transport (K_m) ranges from about 5 to 30 mM (Gary & Garrahan, 1973; De Weer, 1975; Eaton, 1981; Trachtenberg, Packey & Sweeney, 1981; Nielsen, 1982; Saito & Wright, 1982). Although similar measurements have not been made in *Necturus* gallbladder, our results show that the pump serves to hold the intracellular Na activity virtually constant at a value which is probably close to its K_m. Constancy of intracellular Na activity may be important as part of a feedback control mechanism for regulation of membrane permeability (Taylor & Windhager, 1979) or modulation of intracellular enzyme activity (Suelter, 1970).

Zeuthen (1982) measured the intracellular activities of Na, K and Cl in *Necturus* gallbladder epithelium during the perfusion of anisotonic solutions. The osmolality of the mucosal or serosal bathing solution was altered by the addition or removal of mannitol and the resultant rate of change of intracellular ionic activity was determined. The perfusion solutions were equilibrated with 100% O_2 and were virtually bicarbonate free. Volume regulatory increase probably did not occur in these experiments because of the lack of bicarbonate. Zeuthen measured the initial rate of change of the intracellular activities during the anisotonic period and observed significant loss of K during cell shrinkage. He did not detect losses of intracellular Na or Cl during osmotically induced volume changes. Our results do not agree with those of Zeuthen because we find significant loss of all three ions during cell shrinkage, but it is important to note that realization of the loss of

these ions was only possible when the volume and activity measurements were combined. Zeuthen concluded that osmotic water flow resulted in solvent drag of K; we have attributed the loss of K to an acceleration of the normal K exit mechanism as described above.

Ionic Changes During Cell Volume Regulation

The increase of cell volume after osmotically induced shrinkage was accompanied by a significant gain of intracellular K with substantially smaller accumulation of Na and Cl. Together these three ions contributed about 65% of the requisite increase of intracellular osmolality with K alone representing over 70% of the observed gain of these ions. The gain of NaCl accounted for about 20% of the increase of intracellular osmotically active solutes during volume regulatory increase. This was unexpected because volume regulation has been shown to require Na and Cl in the mucosal bathing solution (Ericson & Spring, 1982b). If the fluid entering cells during volume regulation consisted only of isotonic NaCl, the intracellular activities of Na and Cl should have approximately doubled from their control values of 13 and 25 mM to 31 and 43 mM, respectively[1]. Because the quantities of intracellular NaCl did not increase as expected we must reject our hypothesis that volume regulatory increase is simply due to the intracellular accumulation of NaCl (Fisher et al. 1981; Ericson & Spring, 1982b). The results from the ouabain-treated tissues show that although an increase in cell Na during volume regulation was prevented by the action of the Na, K pump, inhibition of the pump did not result in the predicted doubling of cell Na. We conclude that, although volume regulatory increase involves the stimulation of NaCl entry via Na/H and Cl/HCO_3 exchange, NaCl plays only a minor role in the increased cellular solute content which results.

[1] The expectation that cell NaCl activities should have doubled during volume regulatory increase is based on the assumption that the cell volume changes in response to mucosal hypertonicity in the present studies were identical to the volume changes measured optically in our previous studies. This is supported by the measured changes of intracellular Na activity in response to hypertonicity after ouabain pretreatment. Under these conditions, it was expected that no substantial Na loss should occur during cell shrinkage. The intracellular Na activity increased 18% during the period of osmotic cell shrinkage indicating that an 18% decrease of cell volume must have occurred during this period. It was concluded that cell shrinkage occurred within the same time period and to the same extent as our previous optical studies of these cells.

Table 3. Electrochemical potential differences[a]

Seconds in hypertonicity	$\Delta\tilde{\mu}_{Na}/F$	$\Delta\tilde{\mu}_{K}/F$	$\Delta\tilde{\mu}_{Cl}/F$
HCO_3 Ringer's Solution			
Control	-109.4 ± 3.8	33.3 ± 1.7	42.0 ± 2.2
40	-110.2 ± 3.7	34.6 ± 2.0	46.5 ± 2.4[b]
130	-113.8 ± 3.7[b]	33.1 ± 2.2	49.9 ± 2.5[b]
(*N*)	(15)	(15)	(15)
HCO_3-Free Ringer's Solution			
Control	-121.2 ± 5.4	30.3 ± 1.0	37.0 ± 2.4
40	-117.2 ± 5.6[b]	32.9 ± 1.0[b]	39.9 ± 2.7[b]
130	-117.7 ± 5.7[b]	34.1 ± 0.9[b]	38.7 ± 2.7[b]
(N)	(17)	(16)	(11)

[a] Values are in millivolts. Negative values signify a gradient which favors entry into cells.
[b] Values significantly different from control ($P<0.01$) on a paired basis.

The gain of K during volume regulatory increase was originally attributed to the action of the Na, K-ATPase which exchanged Na for K. However, the gain of cell K was unaffected by ouabain suggesting that the K came from intracellular stores or that the increased reading was artefactual. No direct evidence has been obtained indicating a substantial amount of intracellular binding of Na and K, but the issue remains unresolved (Civan, 1978). Artefacts because of interfering ions may be a significant problem with regard to the changes of K content during volume regulation. We found that K-sensitive liquid ion exchange microelectrodes are 2 to 3 orders of magnitude more sensitive to quarternary amines than to K (*unpublished observations*). Thus, if any metabolic processes during volume regulatory increase generate small (1 to 2 mM) changes in the intracellular concentrations of these substances, an overestimate of intracellular K activity would result.

Na, K and Cl Electrochemical Gradients and Volume Regulation

It is worthwhile to ascertain whether significant changes in the transmembrane electrochemical gradients for Na, K and Cl are involved in volume regulation. In Table 3 are shown the electrochemical potential differences ($\Delta\tilde{\mu}/F$) for Na, K and Cl during the hypertonic exposure in the presence and absence of HCO_3. Under all conditions, the gradients favor Na entry as well as K and Cl loss. Osmotic cell shrinkage caused no major changes in the magnitude of these gradients suggesting that volume regulatory ion flows cannot be attributed

simply to a proportional increase of the electrochemical driving forces for Na, K and Cl. While it is clear from previous studies that inhibition of Na/H or Cl/HCO_3 exchange by a number of approaches blocks volume regulatory increase, it is not apparent why these transport processes play such a pivotal role in volume regulation yet contribute only a small fraction of the increase in intracellular osmotically active solutes. In summary, volume regulatory increase by *Necturus* gallbladder cells involves the activation of both solute entry and exit processes as well as the release of osmotically active substances which increase the cellular osmolarity. While it seems clear that Na, K and Cl are all involved in the volume regulatory process it is not apparent what other solutes participate.

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