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Volume Regulation by *Necturus* Gallbladder: Basolateral KCl Exit

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Summary. Swelling of the epithelial cells of *Necturus* gallbladder caused by an 18% reduction in the osmolality of the mucosal bath is followed by rapid volume readjustment. This volume regulatory decrease requires Cl and is sensitive to the K and Cl gradients across the basolateral cell membrane. Volume regulatory decrease is not inhibited by amiloride, SITS, ouabain or bicarbonate removal. The process is blocked by bumetanide in the serosal bath. Measurement of the intracellular activities of K and Cl and the rate of volume regulation under five different experimental conditions showed that KCl exited from the cell across the basolateral membrane with a stoichiometry of 3 K to 2 Cl. This KCl exit process appears to be transiently activated following the reduction in osmolality of the mucosal perfusate.

Key Words volume regulatory decrease · epithelial cell swelling · ion-sensitive microelectrodes · quantitative microscopy · bumetanide

Introduction

When *Necturus maculosus* gallbladder epithelial cells are exposed to anisotonic media they undergo an initial osmotically induced volume change followed by a recovery to their original volume [14–16, 35]. The recovery occurs despite the fact that the bathing solution osmolality remains different from that of control Ringer's. The return of the cell to its original volume has been termed "volume regulation"; similar behavior has been described in red blood cells [8–10, 26, 30, 34], ascites tumor cells [4, 5, 19, 27], isolated rabbit proximal tubules [20, 21, 33], lymphocytes [7, 11, 22–25] and frog skin epithelium [41]. The volume regulatory increase which occurs during exposure of the apical surface of the cells to hypertonic perfusates has been previously studied by light microscopic [14, 35] and electrophysiologic approaches [15, 16]. Volume regulatory increase requires Na and Cl in the mucosal bath as well as bicarbonate in the bathing solutions [14–16, 35]. Recent work indicates that volume regulatory increase involves increases in intracellular quantities of Na, Cl and K as well as in other, unidentified, solutes [16]. The mechanism of the volume regulatory decrease which occurs after cell swelling in hyposmotic bathing solutions, has not previously been investigated in *Necturus* gallbladder.

Volume regulatory decrease occurs in virtually all cells in response to a reduction in the osmolality of the bathing solutions. In most cells the decrease in cell volume is accomplished by the loss of KCl and water [6–12, 19–26, 28–30, 34]. Although *Necturus* gallbladder epithelial cells exhibit rapid volume regulatory decrease, the ionic basis for the decrease of cell volume has not been evaluated.

In the present work, we determined the ionic dependence, inhibitor sensitivity and sidedness of volume regulatory decrease. We show that *Necturus* gallbladder epithelial cells reduce their volume after osmotic swelling by the loss of KCl across the basolateral cell membrane and that this KCl exit has an apparent stoichiometry of 3 K to 2 Cl ions.

Materials and Methods

The experiments were performed on adult *Necturus maculosus* that had been kept in an aquarium at 15°C for at least one month prior to the experiment. They were anesthetized by immersion in a 0.1% solution of tricaine methanesulfonate (Finquel, Ayerst, N.Y.). The gallbladder was removed, drained of bile and kept in Ringer's solution.

Solutions

The composition of the control Ringer's in the serosal perfusate was the following (in mM): 90 NaCl; 2.5 KCl; 10 $NaHCO_3$; 0.5 NaH_2PO_4; 1.8 $CaCl_2$; and 1 $MgCl_2$. The solution was gassed with 99% air and 1% CO_2, pH adjusted to 7.6, osmolality about 200 mOsm/kg. The control mucosal perfusate was identical to the serosal perfusate except that the NaCl concentration was reduced to 72 mM and replaced by 36 mM mannitol. Chloride-free

Ringer's was made by replacement of all Cl by gluconate. High K solutions were made by replacing some of the NaCl in the control Ringer's by KCl. Bicarbonate-free Ringer's contained the following (in mM): 100 NaCl; 2.3 KCl; 1 HPO_4^{-2}; 0.3 $H_2PO_4^-$; 1 $CaCl_2$; 1 $MgCl_2$. The solution was gassed with air, pH adjusted to 7.6, osmolality about 200 mOsm/kg. The following drugs were added to one or both perfusates in different experiments: ouabain (Sigma Chemical Co., St. Louis, Mo.) was added at 10^{-4} M to the serosal perfusate; bumetanide (Hoffman-LaRoche, Nutley, N.J.) was added to either perfusate at concentrations of 10^{-5} or 10^{-4} M; amiloride (Merck Sharp and Dohme, West Point, Pa.) was added to both perfusates at 10^{-3} M; SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid, US Biochemicals, Cleveland, Ohio) was added to both perfusates at 10^{-4} M; perhydrohistionicatoxin (a gift from Dr. Arnold Brossi, NIH) was added to both perfusates at concentrations ranging from 10^{-8} to 10^{-6} M.

Cell Volume Measurements

The epithelium was dissected free of most of its connective tissue and mounted in a miniature Ussing chamber as previously described [13, 32, 35]. The epithelial cells were visualized and analyzed with a microscope-video system [35, 39]. Cell volume was determined by planimetry of stored video images of "optical sections" of the epithelial cells. The area and perimeter of each optical section were determined from tracings of the cell outline. Cell volume was computed from the areas and displacements of focus as previously described [35, 39].

Osmotic experiments were conducted as previously described [14, 35]. In brief, the osmolality of the mucosal bath was suddenly reduced by 36 mOsm and a record of cell volume was made every 8 to 10 sec. In most studies utilizing inhibitors or changes in the ionic composition of the perfusate, the tissue was exposed to the change for 30 min prior to the osmotic perturbation. When the tissue was treated with ouabain, the drug was added to the serosal bath 2 to 3 min prior to the change in osmolality. Each preparation was exposed only once to a reduction in mucosal perfusate osmolality because multiple exposures to hypotonic challenges altered the rate of volume regulatory decrease (*see* Results).

Electrophysiological Methods

Microelectrode measurements were performed in separate experiments from those used for volume determination. Voltage-sensitive microelectrodes were fashioned from glass capillaries (Kwik-Fil, WPI Instruments, New Haven, Conn.) and filled with 1 M KCl. Single-barrel, ion-sensitive electrodes were pulled from the same glass, equilibrated for 1 to 3 hr at 25% relative humidity, and siliconized by immersing the tips for 5 sec in a 50/50 mixture of xylene and trimethylchlorosilane (Pierce Chemical Co., Rockford, Ill.). Cl electrodes were filled with ion-exchange resin (Corning) and equilibrated overnight in 1 M NaCl. K electrodes were filled and used immediately. The K ion exchanger was 5% K-tetrakis-(*p*-chlorophenyl)-borate in 3-O-nitroxylene. Electrodes were calibrated in pure solutions of NaCl or KCl and tested in Ringer's solution. The chloride electrodes had an average slope of 52.5 ± 2.3 mV/decade change in Cl activity. The potassium electrodes had an average slope of 56.4 ± 0.2 mV/decade and a selectivity over Na of 79 ± 2. 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, New Haven, Conn.). The outputs of the electrometers were connected to separate channels of an analog-to-digital converter of a laboratory computer (Model 4051, Tektronix, Beaverton, Ore.). The mucosal bath was connected to ground through an agar bridge filled with Ringer's solution identical to the mucosal perfusate.

The gallbladder was mounted in a fast-flow chamber as previously described [15, 16]. Two groups of cells were punctured successively: first 10 to 15 cells were punctured with a voltage-sensitive electrode, and then 4 to 5 neighboring cells were impaled with an ion-sensitive electrode. Voltage electrode punctures were accepted only when the potential dropped quickly to a stable value without measurable change in input resistance, and the electrode reading returned to within 3 mV of zero upon withdrawal to the mucosal bath. Ion-sensitive electrodes were calibrated, and calculated ion activity was displayed continuously by an on-line computer system, as previously described [15, 16]. Ion-sensitive punctures were accepted only if the initial voltage deflection upon puncture was followed by a rapid attainment of a steady reading ascertained by the agreement to within 0.1 mV of two successive readings taken 8 sec apart. Intracellular ionic activities were calculated from the Nicolsky equation as previously described [16].

Data Analysis

A linear regression from at least 3 points during the linear phase of cell volume change was used together with measurement of the apical surface area of the epithelial cell to calculate the rate of the water movement J_v. All data are presented as mean ± SEM. The Student's *t*-test was used to test the significance of differences.

Results

Anion Dependence of Volume Regulatory Decrease

In these experiments the serosal perfusate was control Ringer's and the mucosal bath contained Ringer's in which 18 mM of NaCl had been substituted by mannitol. After a control period during which two or three determinations of cell volume were made, the osmolality of the mucosal perfusate was reduced 18% by removal of the mannitol. A typical record of cell volume during this experiment is shown in Fig. 1, upper left panel. When the mucosal perfusate osmolality was reduced (arrow), the cell rapidly swelled due to the osmotically induced influx of water. After reaching a peak volume, the cell spontaneously reduced its volume to control levels. The rate of swelling induced by the osmotic water flow was designated J_v^i, and the rate of cell shrinkage during volume regulatory decrease was desig-

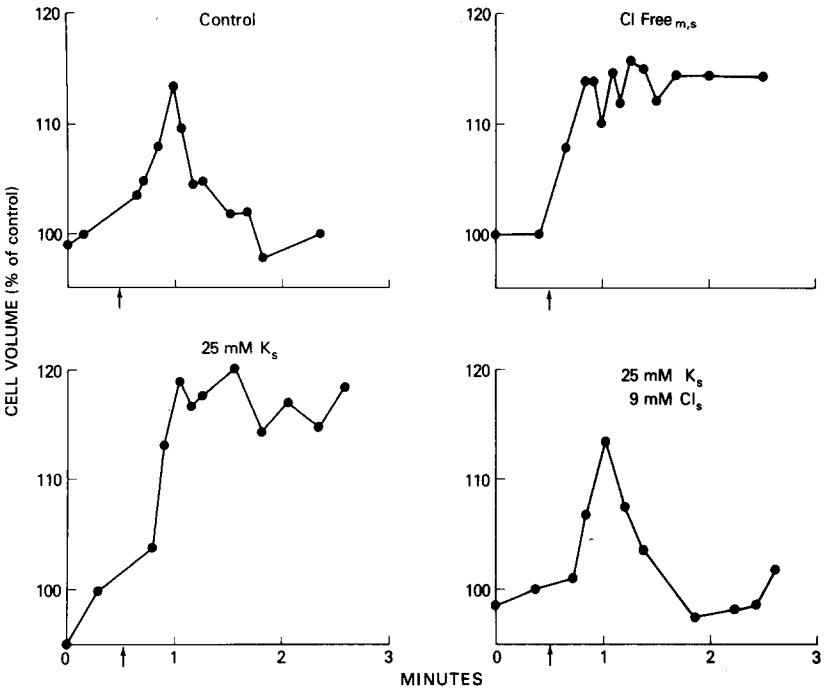

Fig. 1. Cell volume, as a percent of control, is plotted against time for four experiments. In each experiment the osmolality of the mucosal bath was decreased by 18% (36 mOsm) at 30 sec (arrow). Under control conditions (upper left), the cell quickly swelled and volume regulated back to control size. The control concentration of K was 2.5 mM and that of Cl 82 mM. The upper right panel gives an example of the response to hypotonicity when both bathing solutions were Cl-free. The lower left panel shows the response to hypotonicity when the K concentration in the serosal bath was increased to 25 mM. The lower right panel shows the response to hypotonicity when the serosal bath contained 25 mM K and 9 mM Cl instead of the control concentrations of 2.5 mM K and 100 mM Cl

Table 1. Anion dependence of volume regulatory decrease[a]

Condition	$J_v^i \times 10^{-6}$ cm/sec	$J_v^d \times 10^{-6}$ cm/sec	ΔV (%)	No. of obs.
Control	14.5 ± 1.0	−10.1 ± 1.1	15.8 ± 1.2	34
Bicarbonate-free	14.0 ± 1.2	− 8.9 ± 0.9	13.2 ± 0.8	17
Chloride-free	12.2 ± 1.3	− 0.1 ± 0.2[b]	16.7 ± 0.8	15

[a] J_v^i rate of osmotically induced cell volume increase, J_v^d rate of regulatory cell volume decrease, ΔV the magnitude of the maximum volume increase induced by the osmolality change. All values are mean ± SE. Negative values of J_v indicate volume flow out of the cell.
[b] $P < 0.05$ *vs.* control.

nated J_v^d. Table 1 shows that the mean value of J_v^i in 34 experiments was 14.5×10^{-6} cm/sec and that J_v^d averaged -10.1×10^{-6} cm/sec. The average cell volume increased 15.8%. Perfusion of both baths with a bicarbonate-free Ringer's (buffered with phosphate) did not affect the rate or magnitude of the cell volume changes induced by a reduction in the osmolality of the mucosal bath (Table 1). Removal of chloride from both bathing solutions did not significantly affect the osmotically induced swelling but completely blocked volume regulatory decrease (Table 1 and Fig. 1, upper right panel).

K Dependence of Volume Regulatory Decrease

Elevation of the K concentration in the serosal bathing solution from 2.5 mM to 25 mM also blocked volume regulatory decrease (Fig. 1, lower left panel). K was added to serosal perfusate in place of an equal quantity of Na; the Cl concentration was unchanged at 100 mM. Table 2 shows that increasing the serosal perfusate K to 25 mM did not affect the rate or magnitude of osmotically induced cell swelling but completely inhibited volume regulatory

Table 2. Effect of changes in serosal perfusate K or Cl concentration on volume regulatory decrease[a]

Condition	$J_v^i \times 10^{-6}$ cm/sec	$J_v^d \times 10^{-6}$ cm/sec	ΔV (%)	No. of obs.
25 K serosal	11.5 ± 1.2	−0.13 ± 0.15[b]	18.5 ± 0.7	17
25 K, 9 Cl serosal	9.7 ± 0.8[b]	−6.7 ± 0.8[b]	13.3 ± 1.4	15

[a] All values $\bar{x}$ ± SE. Symbols as in Table 1.
[b] $P < 0.05$ *vs.* control in Table 1.

decrease. Steady-state cell volume was not significantly affected by the increased K concentration of the serosal perfusate (i.e. cell swelling did not occur in this high K solution). Thus the block of volume regulatory decrease by increased K in the serosal bath was not simply due to the summation of two oppositely directed cell volume changes but represented instead a direct effect of the magnitude of the K electrochemical gradient on the volume regulatory process.

If the exit of K and Cl across the basolateral membrane were linked, the gradient for the anion could be adjusted to restore volume regulatory decrease. As shown in Fig. 1 (lower right panel) a 10-fold reduction in the chloride concentration of the basolateral solution to 9 mM combined with the previously described 10-fold increase in basolateral K concentration resulted in volume regulatory decrease rates only slightly below control (Table 2). Chloride was replaced by gluconate and K was increased at the expense of Na. These results suggested that the exit of KCl during volume regulatory decrease might be mediated by a cotransport or parallel ion exchange system in which the ionic gradients of the two species were interdependent.

To further evaluate the nature of the KCl exit process we altered the gradients for K or Cl across the basolateral membrane. To reduce the intracellular Cl activity, the mucosal perfusate NaCl was lowered from the control value of 82 mM to 30 mM and the osmolality difference made up with mannitol. As shown in Fig. 2 (upper left panel) and Table 3, reduction of the NaCl in the mucosal perfusate reduced the rate of volume regulatory decrease to about 40% of control without affecting the magnitude of the osmotically induced changes in cell volume. Cell Cl activity decreased by 40 to 50% when the mucosal perfusate contained 30 mM NaCl (*see* Table 6). The observed reduction in the rate of volume regulatory decrease was probably due to the lower cell chloride activity resulting from the reduced NaCl concentration in the mucosal perfusate.

Increasing the K concentration in the serosal perfusate to 15 mM slowed but did not completely block volume regulatory decrease. As shown in Fig. 2 (upper right panel) and Table 3, the rate of volume regulatory decrease was reduced to only 13% of control. Although the magnitude of the osmotically induced increase in cell volume was not significantly affected, increasing serosal bath K to 15 mM or reducing mucosal bath NaCl to 30 mM also slowed the rate of osmotically induced cell swelling J_v^i. The cause of this decrease in J_v^i is not apparent at this time.

A combination of the two perfusate composition changes, which individually slow volume regulatory decrease, lead to complete inhibition of the process. Figure 2 (lower left panel) and Table 3 show that reducing mucosal bath NaCl to 30 mM and increasing serosal bath K to 15 mM combined to block cell volume regulatory decrease. Further increases of serosal perfusate K to 25 mM in the presence of 30 mM NaCl in the mucosal bath did not produce a different result from exposing the tissue to the combination of 30 mM NaCl in the mucosal bath and 15 mM K in the serosal bath (Table 3).

Effects of Inhibitors

In previous studies of the mechanism of volume regulatory increase by *Necturus* gallbladder epithelium, it was shown that transient activation of Na/H and Cl/HCO_3 exchange played an important role in the regulatory process [14, 15]. We wished to determine whether such ion-exchange processes were responsible for the KCl movements apparently involved in volume regulatory decrease. As shown in Table 4 exposure of the tissue to 10^{-3} M amiloride in both bathing solutions did not significantly alter the osmotically induced or regulatory cell volume changes. Thus it seemed unlikely that Na/H exchange or some other amiloride-sensitive process was involved in volume regulatory decrease. Exposure of the tissue to 10^{-4} M SITS in both perfusates was also without effect on the time course of the cell volume changes (Table 4). These results indicated that Cl/HCO_3 exchange was not involved in volume regulatory decrease.

Inhibition of the Na, K-ATPase by 10^{-4} M oua-

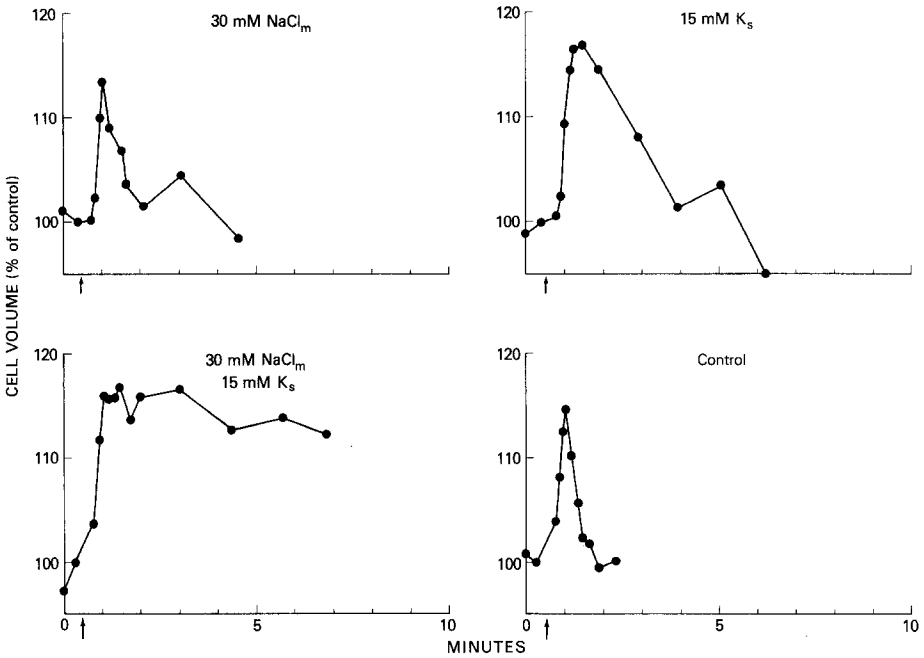

Fig. 2. Examples of cell responses to hypotonicity of the mucosal perfusate as described in Fig. 1. A control response is plotted for comparison in the lower right panel. The other panels show the effect of lowering the NaCl concentration of the mucosal perfusate from control levels of 100 mM to 30 mM (upper left), raising the K of the serosal bath from control levels of 2.5 mM to 15 mM (upper right), or combining the two maneuvers (lower left)

Table 3. Effect of alterations of the K and Cl gradients across the basolateral membrane on the rate of volume regulatory decrease[a]

Condition	$J_v^i \times 10^{-6}$ cm/sec	$J_v^d \times 10^{-6}$ cm/sec	ΔV (%)	No. of obs.
30 NaCl mucosal	11.4 ± 1.1[b]	−4.2 ± 0.4[b]	13.8 ± 0.8	13
15 K serosal	8.8 ± 1.0[b]	−1.3 ± 0.3[b]	14.3 ± 1.1	10
30 NaCl mucosal + 15 K serosal	10.9 ± 0.9[b]	−0.1 ± 0.0[b]	16.8 ± 0.9	14
30 NaCl mucosal + 25 K serosal	8.8 ± 1.0[b]	−0.1 ± 0.0[b]	17.1 ± 1.6	11

[a] All values are mean ± SE. Symbols as in Table 1.
[b] $P < 0.05$ *vs.* control in Table 1.

Table 4. Inhibitor sensitivity of volume regulatory decrease[a]

Condition	$J_v^i \times 10^{-6}$ cm/sec	$J_v^d \times 10^{-6}$ cm/sec	ΔV (%)	No. of obs.
10^{-3} M amiloride (bilateral)	12.7 ± 1.1	−8.0 ± 0.7	16.7 ± 1.3	13
10^{-4} M SITS (bilateral)	14.5 ± 1.4	−7.8 ± 0.6	16.0 ± 1.1	10
10^{-4} M ouabain (serosal)	10.2 ± 1.0[b]	−6.6 ± 0.5[b]	15.6 ± 1.3	13
10^{-5} M bumetanide (serosal)	10.4 ± 1.2[b]	−1.0 ± 0.1[b]	14.9 ± 1.0	8
10^{-4} M bumetanide (serosal)	10.8 ± 1.1[b]	−1.3 ± 0.1[b]	17.6 ± 1.9	12

[a] All values are mean ± SE. Symbols as in Table 1.
[b] $P < 0.05$ vs. control in Table 1.

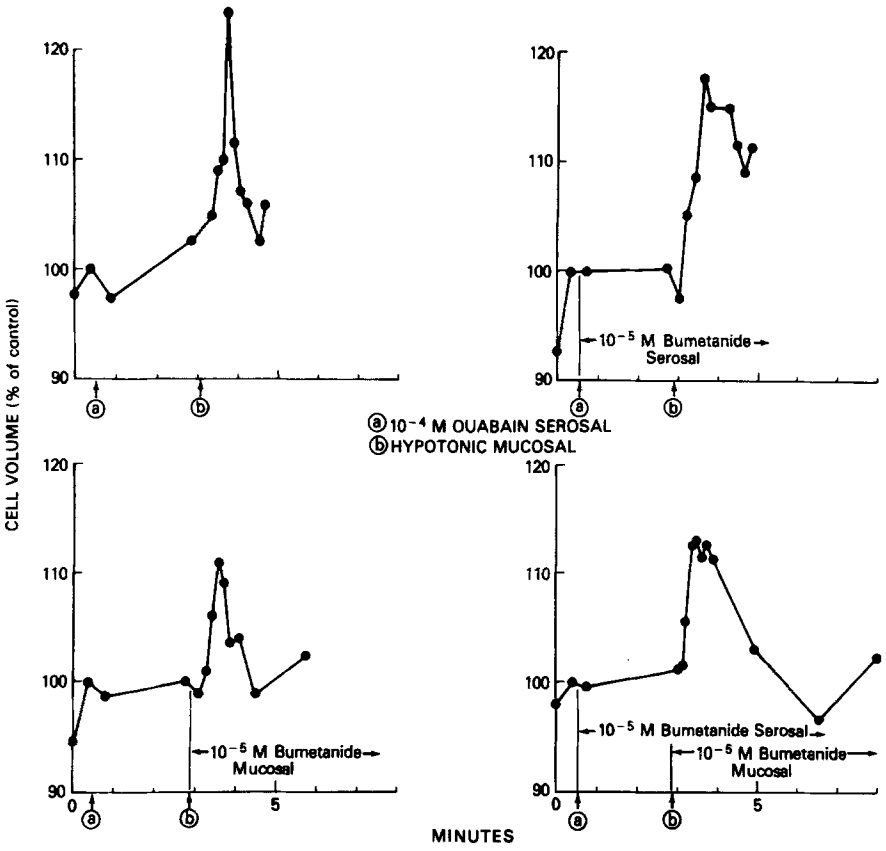

Fig. 3. Cell volume, as a percent of control, is plotted against time in minutes. In all panels 10^{-4} M ouabain was added to the serosal perfusate at arrow *a*, and the osmolality of the mucosal perfusate was reduced by 18% at arrow *b*. In the upper right and two lower panels, bumetanide (10^{-5} M) was added to one or both perfusates as indicated

bain reduced both the rate of the osmotically induced swelling as well as that of volume regulatory decrease to about $\frac{2}{3}$ of their control value (Table 4). The magnitude of the osmotically induced swelling ΔV was not significantly altered by ouabain. Since long-term exposure to ouabain alone causes cell swelling [13], these experiments were carried out by exposing the tissue to ouabain only for 2 to 3 min prior to the osmotic challenge. As shown in Fig. 3 (upper left), brief exposure to ouabain prior to and during the osmotic experiment resulted in only small cell volume increases and thereby permitted an accurate assessment of the rate of change of cell volume induced by the osmotic gradient. We conclude that ouabain does not inhibit volume regulatory decrease although it might have a small effect on cell membrane water permeability.

Bumetanide, the potent inhibitor of NaCl cotransport by *Necturus* gallbladder [13, 31], was also an effective inhibitor of volume regulatory decrease. As shown in Table 4, addition of 10^{-5} or 10^{-4} M bumetanide to the serosal bath reduced J_v^d to 10 to 13% of the control rates. Since bumetanide did not completely block volume regulatory decrease when applied from the serosal bath, we tested its effectiveness when it was present in both bathing solutions. As shown in Table 5-A, bilateral bumetanide (10^{-4} M) completely inhibited volume regulatory decrease. These results initially suggested to us that about 90% of the bumetanide-sensitive transport sites were present on the basolateral membrane and 10% on the apical surface. Further experiments presented below show that this interpretation was incorrect. Table 5-A shows that the application of bumetanide from the mucosal side only also inhibited volume regulatory decrease by 90%. Thus, these sidedness experiments with bumetanide were internally inconsistent and led us to a different approach.

Our previous results showed that intracellular Cl was essential for volume regulatory decrease. We have previously shown that exposure of the gallbladder to bumetanide in the mucosal bath reduces cell Cl activity by 75% [31]. It was possible that bumetanide was effective from the mucosal side only indirectly via a reduction in intracellular Cl activity. Thus we devised an experimental approach which allowed the exposure of the tissue to bumetanide from either bathing solution but did not permit changes in intracellular chloride activity. We showed previously that the bumetanide-induced reduction in intracellular Cl activity was due to active solute transport out of the cell mediated by the Na, K-ATPase [13, 31]. If the Na pump were inhibited

Table 5. Sidedness of bumetanide inhibition of volume regulatory decrease[a]

Condition	$J_v^i \times 10^{-6}$ cm/sec	$J_v^d \times 10^{-6}$ cm/sec	ΔV (%)	No. of obs.
A.				
10^{-4} M bumetanide (bilateral)	10.1 ± 0.8[b]	0.1 ± 0.2[b]	17.1 ± 1.1	10
10^{-4} M bumetanide (mucosal)	9.1 ± 1.0[b]	−1.0 ± 0.1[b]	14.9 ± 0.8	14
B.				
10^{-4} M ouabain + 10^{-5} M bumetanide (serosal)	7.8 ± 0.9[b]	−1.2 ± 0.2[b,c]	18.4 ± 1.2	12
10^{-4} M ouabain + 10^{-5} M bumetanide (mucosal)	7.7 ± 0.8[b]	−5.8 ± 0.7[b]	13.2 ± 1.2	12
10^{-4} M ouabain + 10^{-5} M bumetanide (bilateral)	8.7 ± 0.9[b]	−2.3 ± 0.3[b,c]	14.6 ± 1.3	14

[a] All values mean ± SE. Ouabain was added for 2 to 3 min prior to the osmotic experiment. Symbols as in Table 1.
[b] $P < 0.05$ *vs.* control in Table 1.
[c] $P < 0.05$ *vs.* ouabain in Table 4.

prior to the bumetanide exposure, changes in cell volume and presumably cell solute content could be prevented [13, 31]. As shown in Fig. 3 and Table 5-B, treatment of the tissue with ouabain for 2 to 3 min before the addition of bumetanide prevented the cell volume changes due to bumetanide-induced inhibition of NaCl entry. In Fig. 3, upper right panel, 10^{-4} M ouabain and 10^{-5} M bumetanide were added to the serosal perfusate at point *a*. Approximately 2 min later the tissue was exposed to a hypotonic mucosal perfusate (*b*) and responded by osmotic swelling and extremely slow volume regulation. The rate and magnitude of the osmotic swelling were not significantly different in the presence of both inhibitors from the results in the presence of ouabain alone. However, the rate of volume regulatory decrease was reduced to 12% of control or 18% of the rate in the presence of ouabain alone. When bumetanide was applied from the mucosal side as shown in Fig. 3, lower left panel, it was added to the hypotonic perfusate. Thus the inhibitor and the hypotonic solution arrived simultaneously at the cell membrane. There was no effect of bumetanide on the osmotic or volume regulatory flows when the drug was added to the mucosal bath (Table 5-B). Since the inhibitor action of bumetanide is instantaneous in the gallbladder [31], we felt justified in exposing the tissue to the drug together with the perfusate inasmuch as volume regulation begins after the osmotic volume flow is completed. When bumetanide was added to both perfusates the drug was slightly less effective than when it was added only to the serosal bath. This is shown in Fig. 3, lower right panel, as well as in Table 5-B. The bumetanide studies show that the volume regulatory transport sites are located on the basolateral cell membrane and that bumetanide is not a completely effective inhibitor of this transport process.[1]

Effects of Multiple Challenges on Volume Regulatory Decrease

We had previously observed that the rate of volume regulatory decrease was reduced if the tissue were challenged more than once by an osmolality decrease (Persson & Spring, *unpublished observations*). The nature and magnitude of this reduction were not previously investigated. Figure 4 shows the results of three experimental protocols in which the same cell was subjected to three osmotic challenges. The clear bars show experiments in which the osmolality of the mucosal bath was reduced

[1] We wished to obtain an effective inhibitor of volume regulatory decrease which acted at low concentrations. Because we thought there might be similarities between the KCl exit during volume regulatory decrease and the permeability changes occurring at the neuromuscular junction, we utilized an inhibitor of synaptic transmitter release, histrionicatoxin (HTX). This agent blocks synaptic transmission by interfering with the membrane permeability changes required at the synapse; at present the exact mechanism of its action is not understood [1]. This drug was an effective inhibitor of volume regulatory decrease when added to both bathing solutions and caused complete inhibition at 10^{-6} M. At 10^{-8} M the drug caused 50% inhibition of the rate of volume regulatory decrease. A small reduction in the rate of osmotically induced cell swelling was also observed in the presence of the inhibitor.

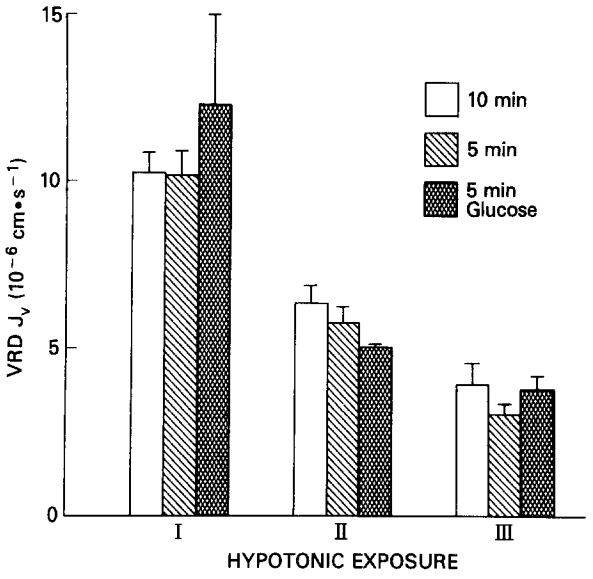

Fig. 4. The rate of volume flow out of the cell during volume regulatory decrease (VRD J_v) is shown for three successive osmotic challenges to the same cell. The time interval between the challenges was either 10 min (open bars) or 5 min (shaded bars). Glucose was added to the bathing solution for 30 min prior to the experiments indicated by the crosshatched bars

three times at 10-min intervals. The osmotically induced cell volume changes were identical in magnitude and rate for all three osmolality reductions, but the rate of volume regulatory decrease fell to 63% of control on the second challenge and to 39% of control on the third exposure. Reduction of the time between osmotic challenges to 5 min (middle bars in Fig. 4) did not significantly alter the response of the cells. Glucose is not normally present in our perfusates, and we wondered whether the cells were reducing their volume regulatory rates because of lack of a readily available energy source. The addition of 5 mM glucose to the perfusion solution had no significant effect on the osmotic or regulatory behavior of the cells. We concluded from these results that the cells were being depleted of some factor which was essential for normal rates of volume regulatory decrease.

Intracellular Ionic Activities

The intracellular activities of K and Cl were measured in gallbladder epithelial cells under conditions which were comparable to those which existed in the volume experiments prior to the osmotic challenge. Determinations of the steady-state activities of intracellular K and Cl were made in gallbladders mounted in a different chamber from that used for the cell volume measurements (*see* Materials and Methods). Five different conditions were studied in which the K and/or Cl gradients across the basolateral membrane were varied. First control measurements of apical membrane PD and intracellular activity were made; then the tissue was exposed to the test solution for 30 min and the PD and activity were determined again. At least four cells were punctured for activity measurements under each condition. PD determinations were made in at least ten cells in the control and experimental periods. Table 6 shows the PD and activity values for all five experimental conditions. The control values of PD, Cl and K activity were pooled because they did not differ significantly within experimental groups. The control PD, a_{Cl} and a_K are in good agreement with previous observations when differences in the Cl ion-exchange resins are taken into account [15, 16, 31].[2]

Table 6 lists the apical membrane potential and intracellular activities of K and Cl measured in the steady state which existed under control conditions or 30 min after switching to a test solution. As shown in Table 6 increasing the K concentration to 15 mM in the serosal bath caused the cell to depolarize and intracellular Cl activity to nearly double, but did not alter intracellular K activity. Perfusion of 25 mM K in the serosal bath caused depolarization and a further increase in cell Cl to 2.1 times its control value. Cell K activity also rose slightly during the 25-mM K perfusion. When the serosal perfusate contained 25 mM K and 9 mM Cl the membrane potential depolarized by 18 mV and intracellular Cl rose but not as high as in the previous experiments. Cell K did not change significantly under these conditions. Perfusion of 30 mM NaCl in the mucosal bath had remarkable effects on membrane potential and intracellular ion activities. The membrane potential hyperpolarized by 12 mV and cell Cl fell to 50 to 65% of control. Cell K increased by 50 to 60% when the mucosal NaCl was reduced. When the mucosal bath contained 30 mM NaCl, exposure of the tissue to 15 mM K in the serosal bath caused the membrane potential and ion activities to return to control levels. A similar result was obtained when the K concentration of the serosal bath was increased to 25 mM. The membrane potential was depolarized compared to control and cell K was slightly increased above control levels.

[2] Some previous measurements of the chloride activity in gallbladder were made with the Orion Cl resin [16, 31]. In paired comparisons with the Corning exchanger it was found that the Orion exchanger gave 9 mM higher Cl activity readings than the Corning resin presumably due to the lack of selectivity of the Orion exchanger.

Table 6. Intracellular K and Cl activities and membrane potential in cells prior to osmotic challenge

Condition	PD[b] (mV)	a_{Cl}[c] (mM)	a_K[c] (mM)
Control	−71.0 ± 1.2 (73)	14.3 ± 1.3 (38)	103.8 ± 4.5 (35)
15 K serosal	−56.2 ± 1.8[d] (16)	25.9 ± 1.6[d] (9)	104.5 ± 5.6 (7)
25 K serosal	−55.2 ± 2.8[d] (1)	30.1 ± 3.6[d] (8)	110.5 ± 5.3[d] (7)
25 K, 9 Cl serosal	−53.4 ± 2.3[d] (15)	24.1 ± 1.0[d] (8)	105.3 ± 4.1 (7)
30 NaCl mucosal	−82.6 ± 1.8[d] (14)	9.4 ± 0.7[d] (7)	155.2 ± 6.2[d] (7)
30 NaCl mucosal + 15 K serosal	−69.9 ± 1.9 (14)	14.5 ± 1.5 (7)	116.1 ± 6.0 (7)
30 NaCl mucosal	−86.7 ± 2.0[d] (13)	7.5 ± 0.5[d] (6)	159.5 ± 7.0[d] (7)
30 NaCl mucosal + 25 K serosal	−60.5 ± 0.9[d] (13)	16.1 ± 0.9 (6)	118.8 ± 5.0[d] (70)

[a] All values are mean ± SE (number of tissues).
[b] PD is the apical membrane potential difference.
[c] a_i is the activity of ion i.
[d] $P < 0.05$, for t-test against paired controls.

Table 7. Membrane PD and electrochemical gradients for K and Cl in cells prior to osmotic challenge[a]

Condition	PD[b] (mV)	$\Delta\tilde{\mu}_{Cl}/F$[c] (mV)	$\Delta\tilde{\mu}_K/F$[d] (mV)
Control	−71.0 ± 1.2 (73)	33.3 ± 2.5 (38)	−23.2 ± 1.5 (35)
15 K serosal	−56.2 ± 1.8[e] (16)	34.9 ± 2.7 (9)	4.7 ± 1.7[e] (7)
25 K serosal	−55.2 ± 2.8[e] (15)	36.4 ± 3.5 (8)	13.1 ± 2.2[e] (7)
25 K, 9 Cl serosal	−53.4 ± 2.3[e] (15)	78.9 ± 1.0[e] (8)	13.4 ± 2.5[e] (7)
30 NaCl mucosal	−82.6 ± 1.8[e] (14)	31.9 ± 3.2 (7)	−20.1 ± 1.0[e] (7)
30 NaCl mucosal + 15 K serosal	−69.9 ± 1.9 (14)	30.4 ± 3.6 (7)	17.0 ± 0.6[e] (7)
30 NaCl musocal	−86.7 ± 2.0[e] (13)	30.7 ± 2.5 (6)	−17.3 ± 1.0[e] (7)
30 NaCl mucosal + 25 K serosal	−60.5 ± 0.9[e] (13)	19.5 ± 1.3[e] (6)	23.4 ± 1.7[e] (7)

[a] All values are mean ± SE (number of tissues).
[b] PD, apical membrane potential.
[c] $\Delta\tilde{\mu}_{Cl}/F$, the electrochemical gradient for Cl across the basolateral membrane.
[d] $\Delta\tilde{\mu}_K/F$, the electrochemical gradient for K across the basolateral membrane. The electrochemical gradient for Cl always favors Cl exit as indicated by positive values; negative values for $\Delta\tilde{\mu}_K/F$ indicate a gradient which favors K exit; positive for $\Delta\tilde{\mu}_K/F$ indicate a gradient which favors K entry into the cell.
[e] $P < 0.05$ for t-test against paired controls.

The results of our microscopy experiments suggested that the rate of volume regulatory decrease was a function of the electrochemical gradients for K and Cl across the basolateral membrane. Table 7 presents the electrochemical gradients for K and Cl existing across the basolateral membrane prior to the osmotic challenge for the five experimental conditions involving alterations in K or Cl gradients. Under control conditions the electrochemical gradients for both ions favor exit from the cell. This is indicated by positive values for $\Delta\tilde{\mu}_{Cl}/F$ and negative values for $\Delta\tilde{\mu}_K/F$. It can be seen that increasing the K concentration of the serosal perfusate to 15 mM reversed the driving force for K exit but did not affect the gradient for Cl exit. A further increase of the serosal perfusate K to 25 mM did not change the Cl gradient but increased the opposing K gradient even further. Increasing the serosal perfusate K to 25 mM was one of the procedures which blocked volume regulatory decrease. When the serosal per-

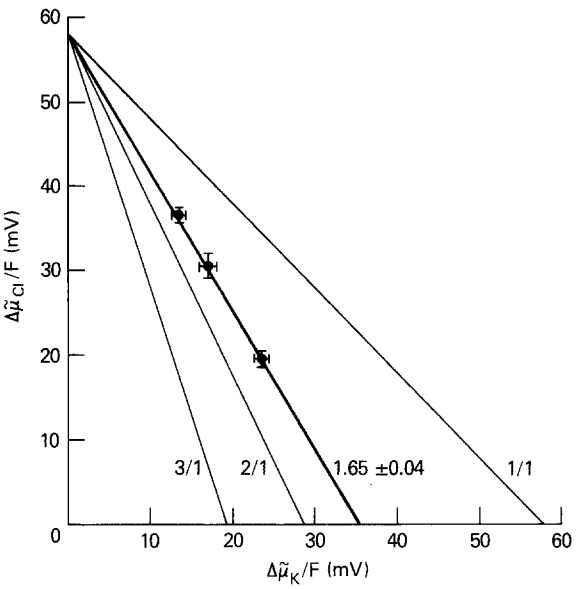

Fig. 5. The electrochemical gradient for Cl across the basolateral cell membrane ($\Delta\tilde{\mu}_{Cl}/F$) is plotted against the electrochemical gradient for K across that membrane ($\Delta\tilde{\mu}_K/F$). All activity measurements were made prior to the osmotic challenge. The electrochemical gradients were calculated from the membrane potential and activity measurements. Each average is shown with its standard error. The least-squares line, fitted to the mean values, was 1.65 ± 0.04 (SE). The data could not be adequately fitted by the lines shown for 3/1, 2/1, or 1/1 stoichiometry

fusate Cl was 9 mM and the K was 25 mM, the driving force for K opposed its exit. At the same time, the electrochemical gradient favoring Cl exit increased by a factor of 2.3 over control (78.9 *vs.* 33.3 mV). Volume regulatory decrease proceeded at $\frac{2}{3}$ of the control rate when the serosal perfusate contained 9 mM Cl and 25 mM K. Perfusion of the mucosal bath with 30 mM NaCl did not cause significant changes in $\Delta\tilde{\mu}_{Cl}/F$ and caused only small reductions in $\Delta\tilde{\mu}_K/F$. However, the addition of 15 mM K to the serosal perfusate during the exposure of the tissue to 30 mM NaCl in the mucosal bath reversed the K gradient without altering the Cl gradient. This combination of perfusion solutions also blocked volume regulatory decrease. Finally, increasing the K concentration of the serosal bath to 25 mM reduced the driving force for Cl exit and further increased the gradient opposing K exit. This solution combination also blocked volume regulatory decrease. Three of the five conditions in which the electrochemical gradients were changed lead to complete cessation of volume regulatory increase, and the other two conditions lead to reductions in the rate of volume regulation. The electrochemical gradients pertinent to volume regulatory decrease are those existing at the completion of the osmotically induced swelling. The activities of both Cl and K should be reduced approximately 15% by the influx of water. Recalculation of the electrochemical gradients shown in Table 7 for those existing at the completion of the osmotic swelling results in a 3 to 4 mV reduction in all $\Delta\tilde{\mu}/F$.

Volume regulatory decrease was not a function of the electrochemical gradient for K or Cl across the apical cell membrane. There was no correlation between these gradients and the rate of volume regulatory decrease. The gradients across the apical membrane were always of similar magnitude and were always directed so that KCl exit across that membrane was favored.

Stoichiometry of KCl Movement

The ion-sensitive electrode data showed that the electrochemical gradients for both K and Cl played a part in determining the rate of volume regulatory decrease. If the movement of the two ions are coupled the following relationship applies at equilibrium [3]:

$$-\Delta\tilde{\mu}_{Cl} = \Delta\tilde{\mu}_K \left[\frac{n_K}{n_{Cl}}\right] \quad (1)$$

where $\Delta\tilde{\mu}_i$ is the electrochemical gradient for the ion i, and n_i is the stoichiometric coefficient for each ion. We observed three conditions in which volume regulatory decrease did not occur presumably because of the change in the K and Cl electrochemical gradients. According to Eq. (1) a plot of $\Delta\tilde{\mu}_{Cl}$ *vs.* $\Delta\tilde{\mu}_K$ should yield a straight line for these three experiments; the slope of the fitted line is the ratio of the stoichiometric coefficients. Figure 5 shows such a plot. The points fall on a line with a slope of 1.65 ± 0.04 which is consistent with a ratio of K/Cl of 3/2. If the ratio of transported ions is 3 K to 2 Cl, a plot of the rate of volume regulatory decrease against the combined electrochemical gradients for two ions should be a monotonic function only for a stoichiometry of 3 K to 2 Cl. Any other stoichiometric ratio should not result in a direct proportion between driving force and flux. Table 8 lists the calculated values for the combined electrochemical gradients for K and Cl for the stoichiometry of 3 K and 2 Cl. Two sets of values are given: those calculated directly from the data in Table 7 for the combined electrochemical gradient existing prior to the osmotic challenge, and those calculated from the activities presumed to exist at the peak of the osmotic swelling. For each condition the calculated driving force for KCl movement at the peak of the osmotically induced swelling is less than that measured before the osmotic challenge. Figure 6 (top)

Table 8. Combined electrochemical gradient for K and Cl across the basolateral membrane assuming a stoichiometry of 3 K + 2 Cl

Condition	Steady state $\Delta\tilde{\mu}/F$[a]	Peak $\Delta\tilde{\mu}^*/F$[b]
Control	137.0 ± 6.9 (73)	110.0
15 K serosal	55.7 ± 8.0 (16)	35.3
25 K serosal	33.5 ± 10.0 (15)	6.6
25 K, 9 Cl serosal	117.6 ± 7.8 (15)	100.0
30 NaCl mucosal	119.0 ± 7.1 (14)	104.3
30 NaCl mucosal + 15 K serosal	9.8 ± 7.5 (14)	−3.5
30 NaCl mucosal + 25 K serosal	−31.2 ± 4.1 (13)	−12.4

[a] $\Delta\tilde{\mu}/F$ is the electrochemical gradient calculated from $3\,\Delta\tilde{\mu}_K/F + 2\,\Delta\tilde{\mu}_{Cl}/F$ using the values obtained in the steady state prior to the osmotic challenge.

[b] $\Delta\tilde{\mu}^*/F$ values are calculated from the activities estimated at the peak of the osmotic swelling. $\Delta\tilde{\mu}/F$ values are mean ± SE (number of tissues). Positive values of $\Delta\tilde{\mu}/F$ indicate a favorable driving force for KCl exit; negative values indicate a force for KCl entry into the cell.

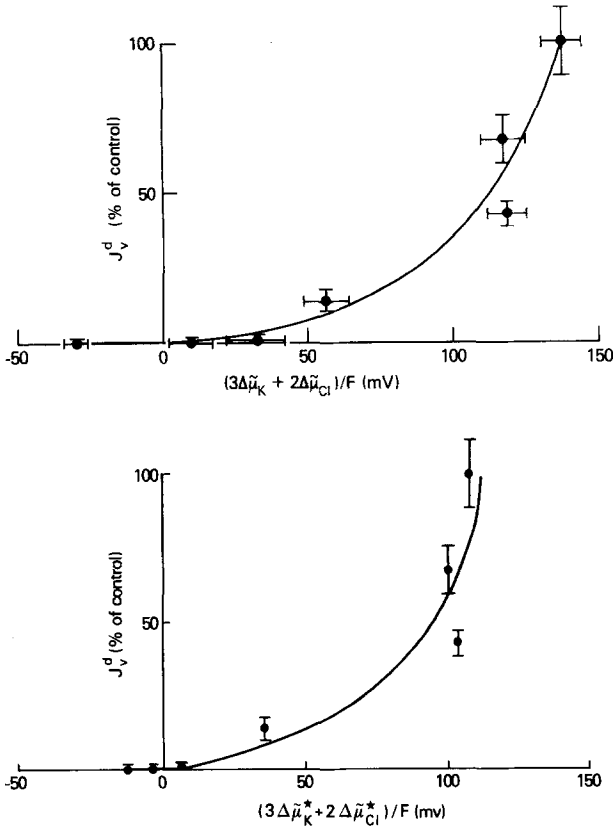

Fig. 6. (Top) The rate of volume regulatory decrease J_v^d is plotted against the combined electrochemical gradient for K and Cl across the basolateral cell membrane assuming a stoichiometry of 3 K + 2 Cl. Each point is the mean with its associated standard errors. (Bottom) J_v^d is plotted against the electrochemical gradient for K and Cl calculated to exist at the peak of the osmotically induced cell swelling

shows the relationship between the rate of volume regulatory decrease and the total electrochemical gradient provided by 3 K + 2 Cl across the basolateral membrane. The gradient was calculated for the conditions existing prior to the osmotic swelling. Figure 6 (bottom) shows a similar plot where the abscissa is the total electrochemical gradient presumed to exist at the peak of the osmotically induced cell swelling. The curves in each plot were drawn by eye and are virtually identical. Other stoichiometries (i.e. 1 K + 1 Cl, 2 K + 1 Cl, 3 K + 1 Cl) did not yield monotonic functions in which the rate of volume regulatory decrease was proportional to the combined driving force for the two ions.

Thus the ion-sensitive electrode and cell volume data are consistent with the activation of the bumetanide-sensitive transport of K and Cl out of the cell across the basolateral cell membrane in response to osmotically induced cell swelling. The stoichiometry experiments indicate that this KCl exit is not neutral and should result in hyperpolarization of the cell because of the transport of more cations than anions. It is possible to estimate the membrane potential change that would be produced by the coupled exit of 3 K + 2 Cl during volume regulatory decrease. The total K exit during volume regulation may be calculated from the rate of volume regulatory decrease, with the assumption that the concentration of K in the transported fluid is 100 mM. The rate of volume regulatory decrease J_v^d is -1.0×10^{-5} cm/sec (Table 1) yielding a K efflux of 1×10^{-9} M/cm^2-sec. If one-third of this efflux is not balanced by the efflux of anions (i.e. as would be expected for a 3 K + 2 Cl stoichiometry), the K efflux would be 0.33×10^{-9} M/cm^2-sec. Such an efflux of cations is equivalent to a current of 31.8 μA/cm^2 flowing across the basolateral cell membrane. The measured resistance of the basolateral cell membrane has recently been reported as 201 Ω cm^2 [40]. A current of 31.8 μA/cm^2 flowing across a resistance of 201 Ω cm^2 would produce a 6.4 mV hyperpolarization. Volume regulatory decrease is accompanied by a transient hyperpolarization of approximately 5 mV [15, 16] in agreement with the prediction of the stoichiometry experiments.

Discussion

Volume regulatory decrease by *Necturus* gallbladder epithelial cells occurs rapidly in response to os-

motically induced cell swelling. We characterized the rate, ionic dependence and drug sensitivity of volume regulatory decrease. On the basis of our results, we conclude that the most likely mechanism for volume regulatory decrease is the transient activation of electrogenic KCl transport out of the cell across the basolateral membrane. We will first review the evidence for our conclusions and then discuss the relevance of this KCl efflux to epithelial cell volume maintenance.

Ionic Dependence of Cell Volume Regulation

Volume regulatory decrease requires Cl and is sensitive to the electrochemical gradients of Cl and K. The insensitivity of the process to HCO_3, amiloride or SITS suggests that Cl/HCO_3 or Na/H ion exchangers are not involved. The effectiveness of bumetanide as an inhibitor of volume regulatory decrease indicates that Cl-dependent cotransport may be involved. Na,K-ATPase does not appear to be necessary for volume regulatory decrease as indicated by the ouabain experiments. In other systems, volume regulatory decrease also involves the loss of KCl from the cells. In isolated, nonperfused renal proximal tubule segments, it has been shown that osmotic cell swelling leads to a KCl efflux the exact mechanism of which has yet to be elucidated [20, 21, 33]. In the *Amphiuma* red blood cell, it has been proposed that volume regulatory decrease results from the activation of K/H exchange as well as Cl/HCO_3 exchange [8–10]. In lymphocytes, volume regulatory decrease appears to be due to changes in the passive permeability of the cell membranes to K [7, 11, 22–25]. In Ehrlich ascites tumor cells swelling leads to cotransport of Na, K and Cl out of the cell as well as to the loss of free amino acids, particularly taurine [19, 27, 28]. Thus while KCl loss is a common feature of volume regulatory decrease, the mechanism of transport of solute out of the cell differs remarkably from one preparation to another. Volume regulatory decrease in *Amphiuma* red blood cell [10] and in lymphocytes [24, 25] is a calcium-dependent phenomenon. The calcium-binding protein, calmodulin, also may be involved in the activation of KCl exit [10, 24]. Similar evidence for a role of calcium and calmodulin in the activation of volume regulatory decrease by *Necturus* gallbladder epithelium has been recently obtained [17]. Epithelial cell volume regulation is further complicated by the polarity of the cells which introduces questions of the sidedness of the KCl exit process.

Sidedness of KCl Transport During Volume Regulatory Decrease

The bumetanide experiments were designed to determine the side of the cell which participated in the KCl exit process. The results suggested that volume regulatory KCl loss occurred only across the basolateral cell membrane. Manipulation of the K and Cl concentrations in the basolateral bathing solution supported this conclusion. The rate of volume regulatory decrease was proportional to the KCl electrochemical gradient across the basolateral cell membrane and not to that across the apical membrane. These results must still be interpreted with some caution because the present experiments did not include extensive alterations of the KCl gradients across the apical membrane.

Mechanism of KCl Exit During Volume Regulatory Decrease

The electrophysiologic data are consistent with the electrogenic movement of KCl out of the cell across the basolateral membrane. Estimates of the stoichiometry of this process indicate a transport ratio of 3 K to 2 Cl. This ratio was calculated assuming that the blockage of volume regulation associated with alterations in the electrochemical gradients for K or Cl across the basolateral membrane was due only to the effects on KCl movement. Changes in the concentration or flux of free amino acids or other solutes would alter cell volume and the rate of volume regulatory decrease. We do not know whether free amino acids or other solutes play a significant role in volume regulatory decrease in *Necturus* gallbladder and cannot rule out KCl-dependent changes in amino acid flux. In previous studies of the volume regulatory increase which follows osmotically induced gallbladder cell shrinkage, it was shown that much of the cell solute change was not due to Na, K or Cl [16]. This was surprising because volume regulatory increase depended on the presence of Na and Cl in the mucosal bath and was initially thought to be the consequence of NaCl uptake [13, 35]. The electrical events associated with volume regulatory decrease and volume regulatory increase are distinctly different. Cell membrane potential was virtually unaffected during volume regulatory increase while a transient hyperpolarization was observed during volume regulatory decrease [15, 16]. Such a hyperpolarization is consistent both with an increase in cell membrane K permeability and with the net loss of cation from the cell. Our present evidence suggests that cation loss due to KCl trans-

port may be the mechanism of the hyperpolarization although this question is in need of further investigation.

Activation of KCl Cotransport During Volume Regulatory Decrease

Several investigators have suggested that KCl cotransport normally occurs across the basolateral membrane of epithelial cells [2, 18, 36–38], as well as across the membranes of other cells [4–6, 32]. The possibility of KCl cotransport in *Necturus* gallbladder was first suggested by Reuss [36] and has been recently supported by investigations involving alterations of the KCl concentrations in the serosal bath [2, 37]. Our experiments show that the bumetanide-sensitive, KCl exit process associated with volume regulatory decrease is probably quiescent in the absence of cell swelling. Bumetanide had no effect on cell volume when added to the serosal bath (Fig. 3, and *unpublished observations*) prior to the osmotic challenge. Alterations in the K concentration of the serosal bath did not cause cell swelling as might be expected from the cessation of KCl exit because of the reversal of the electrochemical gradient for K across the basolateral membrane. Although neutral cotransport of KCl across the basolateral membrane may be present under control conditions, this system must be different from that involved in volume regulatory decrease. An electrogenic KCl cotransport system with a stoichiometry of 3 K to 2 Cl could not alone explain the steady-state maintenance of intracellular K and Cl. The relevance of KCl cotransport to the transepithelial transport of Cl as well as to the maintenance of cell K concentration is not established at this time. However, it is clear from our studies that KCl cotransport plays a significant role in the readjustment of cell volume following osmotic cell swelling.

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