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# Feedback Inhibition of NaCl Entry in *Necturus* Gallbladder Epithelial Cells

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**Summary.** When *Necturus* gallbladder epithelium is treated with ouabain the cells swell rapidly for 20-30 minutes then stabilize at a cell volume 30% greater than control. The cells then begin to shrink slowly to below control size. During the initial rapid swelling phase cell Na activity, measured with microelectrodes, rises rapidly. Calculations of the quantity of intracellular Na suggest that the volume increase is due to NaCl entry. Once the peak cell volume is achieved, the quantity of Na in the cell does not increase, suggesting that NaCl entry has been inhibited. We tested for inhibition of apical NaCl entry during ouabain treatment either by suddenly reducing the NaCl concentration in the mucosal bath or by adding bumetanide to the perfusate. Both maneuvers caused rapid cell shrinkage during the initial phase of the ouabain experiment, but had no effect on cell volume if performed during the slow shrinkage period. The lack of sensitivity to the composition of the mucosal bath during the shrinkage period occurred because of apparent feedback inhibition of NaCl entry. Another maneuver, reduction of the Na in the serosal bath to 10 mM, also resulted in inhibition of apical NaCl uptake. The slow shrinkage which occurred after one or more hours of ouabain treatment was sensitive to the transmembrane gradients for K and Cl across the basolateral membrane and could be inhibited by bumetanide. Thus during pump inhibition in *Necturus* gallbladder epithelium cell Na and volume first increase due to continuing NaCl entry and then cell volume slowly decreases due to inhibition of the apical NaCl entry and activation of basolateral KCl exit.

**Key Words** NaCl cotransport · Na/Ca exchange · cell volume · intracellular Na activity · ouabain · KCl transport

## Introduction

Most epithelial cells are confronted by substantial inflows of solutes and water across the apical cell membrane; the exit of these solutes and water must exactly balance the influx for cellular homeostasis. Evidence in tight epithelia has been available for some time which indicates that Na entry into the cells across the apical membrane is subject to calcium-dependent inhibition [21]. More recently it has been shown that the rate of fluid absorption by rabbit renal proximal tubule also appears to be subject to feedback regulation [7]. The proposed mechanism of regulation of Na entry in epithelial cells involves alterations in intracellular calcium level [13, 21]. The general scheme of feedback control of Na entry proposed by Blaustein [2] is as follows: Increases in cell Na (due for example to an increased rate of Na entry or a decreased rate of Na extrusion) lead to a decrease in Ca/Na exchange at the basolateral membrane of the epithelial cells. Ca/Na exchange normally is thought to operate so as to extrude Ca in exchange for Na. Any increase in cell Na reduces the transmembrane Na gradient, slowing Ca/Na exchange, and increasing cell calcium [13]. The increase in cell calcium is thought to reduce the rate of Na entry either directly [3] or through intermediates [21]. This negative feedback scheme stabilizes cellular Na by altering the rate of Na entry and hence transepithelial Na transport.

Two classes of experiments have been employed in epithelia to study negative feedback control of Na entry. The first involves alterations of the Na gradient across the basolateral membrane by either raising cell Na or reducing the Na in the serosal bath [21]. The second experimental approach utilizes inhibitors of calcium-mediated events or calcium ionophores to verify the involvement of calcium ions [21]. Previous evidence for feedback regulation of fluid absorption by rabbit renal proximal tubule was obtained by reducing the Na concentration of the peritubular bathing solution; the rate of fluid absorption was found to decrease to 40% of control [7]. We had previously observed that *Necturus* gallbladder epithelial cells exhibited behavior consistent with feedback inhibition of NaCl entry [4]. In these experiments, the tissue was treated with ouabain and the rate and magnitude of cell swelling were determined. Ouabain-treated cells swelled rapidly (4.3%/min) for about 10 min but did not increase beyond 145% of their control volume. We calculated that favorable Na and Cl

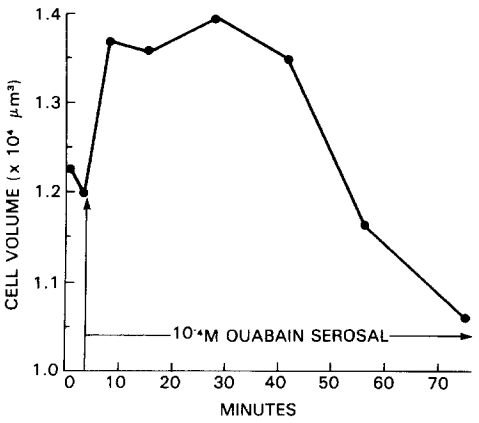


**Fig. 1.** Representative experiment showing the effect of ouabain on gallbladder cell volume. The initial fast swelling is followed by a plateau phase and then slow shrinkage to below control volume

gradients still existed after cell swelling had ceased and hypothesized that feedback inhibition of NaCl entry may have occurred [4].

NaCl entry into *Necturus* gallbladder epithelial cells is electroneutral and carrier mediated [4, 17]. The exact nature of the entry step is in dispute. Two modes of NaCl entry have been proposed: NaCl cotransport [4] and parallel Na-H, Cl-OH exchange [19]. Strong evidence has been presented to support both modes of NaCl entry [15, 19] and it seems possible that the two entry mechanisms coexist or are interconvertible. It is not clear at present what factors could be involved in the activation or inactivation of either entry mechanism. The present results show that NaCl entry by cotransport is subject to feedback inhibition which results in the cessation of entry when intracellular Na exceeds 30 mM. Once NaCl entry has been inhibited the increases in intracellular NaCl and cell volume lead to the activation of KCl exit from the cell across the basolateral membrane and subsequent cell shrinkage.

## 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 methane-sulfonate (Finquel, Ayerst, N.Y.). The gallbladder was removed, drained of bile, and kept in oxygenated Ringer solution.

### Solutions

The composition of the control Ringer solution was the following (in mM): 90, NaCl; 2.5, KCl; 10, $NaHCO_3$; 0.5 $NaH_2PO_4$; 1.8, CaCl; 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. High K Ringer contained 25 mM K instead of the 2.5 mM normally present; Na was reduced accordingly. Low Na Ringer contained 90 mM N-methyl-D-glucamine Cl in place of NaCl; the Na concentration of the Ringer was reduced to 10 mM. Low NaCl Ringer contained mannitol in place of 90 mM NaCl, the NaCl concentration of the solution was approximately 10 mM.

Two bicarbonate-free Ringer solutions were also used. Phosphate Ringer contained (in mM): 100 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 1 $K_2HPO_4$, 0.3 K $H_2PO_4$. Phosphate Ringer was bubbled with air, had a pH of 7.6 and an osmolality of 196 mOsm/liter. High K-low Cl Ringer had the following composition (in mM): 67.5 mM Na gluconate, 2.5 KCl, 22.5 K gluconate, 1.8 $CaCl_2$, 6.2 Ca lactate, 10.5 $NaH_2PO_4$ and sufficient mannitol to give a final osmolality of 196 mOsmol/liter. High K-low Cl Ringer was equilibrated with air and had a pH of 7.6. The calcium concentration of the low Cl Ringer was increased from the control value of 1.8 to 8.0 mM. This was necessary to maintain the free calcium activity near control levels. Calcium activity was measured with an ion-sensitive electrode (Orion, Cambridge, MA).

Bumetanide was added to the perfusates at a concentration of $10^{-5}$ M. A $10^{-3}$ M bumetanide stock solution was prepared by dissolving the drug in a small volume of dilute NaOH solution at pH 9. Quinidine (Sigma) was added to the serosal perfusate at a concentration of $10^{-4}$ M. Ouabain (Sigma) was added to the serosal perfusate at $10^{-4}$ M.

### Cell Volume Measurements

The epithelium was mounted in a miniature Ussing chamber as previously described [4, 17]. The epithelial cells were visualized and analyzed with a microscope-video system [17]. Cell volume was determined by planimetry of stored video images of "optical sections" of the epithelial cell. 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 [17].

### Electrophysiological Methods

Microelectrode measurements were performed in separate experiments from those for volume determination. Voltage-sensitive microelectrodes were fashioned from glass capillaries (Kwik-Fil, WPI Instruments, New Haven, CT) and filled with 1 M KCl. Single-barrel, Na-sensitive electrodes were pulled from the same glass, equilibrated for 1-3 hr at 25% relative humidity, and siliconized by immersing the tip for 5 sec in a 50/50 mixture of xylene and trimethylchlorosilane (Pierce Chemical Co., Rockford, Ill). Na-sensitive electrodes were filled and used immediately. The Na ion exchanger 10% solution ligand (ETH 227 Fluka Chemical, Hauppauge, N.Y.) in 2-nitrophenyloctylether (Fluka) with 0.5% Na tetraphenylboron (Sigma). The electrodes were calibrated in pure solutions of NaCl or KCl and tested in Ringer solution. The Na electrodes had a slope of 55.6 ± 0.3 mV/decade and a selectivity of 40.7 ± 3.2 over K. The Na activities were calculated from the electrode voltage after correction for membrane potential and K interference caused by an assumed cell K activity of 97 mM [5].

The gallbladder was mounted in a fast flow chamber as previously described [3, 10]. Two cells were punctured simultaneously, one with a voltage-sensitive electrode and one with a

Na-sensitive electrode. Voltage electrode punctures were accepted only when the potential dropped promptly to a stable value greater than −50 mV 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 [5]. Ion-sensitive punctures were accepted only if the initial voltage deflection upon puncture was followed by the rapid attainment of a steady reading ascertained by the agreement of two successive readings taken 8 sec apart which did not differ by more than 0.1 mV [5]. Agreement of the voltage divider ratio of ion-selective and voltage-sensitive electrodes was used as a criterion for acceptability in some measurements.

### Data Analysis

A linear regression from at least 3 points during the linear phase of cell volume change was used to calculate the rate of swelling of the cells. The rate of the water movement, $J_v$, was calculated from the rate of swelling and the apical surface area of the epithelial cell. All data are presented as mean ± SEM. The Student's $t$ test was used to test the significance of difference.

## Results

As shown in Fig. 1, addition of $10^{-4}$ M ouabain to the serosal perfusate causes rapid cell swelling, as previously described [4, 10]. The cell stops swelling after about 20 min of ouabain treatment and then begins a slower shrinkage. The first part of the results deals with the cessation of cell swelling and the second part with the cause of the cell shrinkage.

### Ouabain-Induced Cell Swelling

The average cell volume changes observed in 59 *Necturus* gallbladders treated with ouabain are given in Table 1. The maximum swelling occurred 28 ± 3 min after ouabain addition and the maximum volume achieved was 127 ± 5% of control. The rate of cell volume increase in Table 1, 0.41 ± $10^{-6}$ cm/sec, is about 25% of that previously reported in *Necturus* gallbladder [4, 10]. The rates of cell swelling measured in the present experiments were an underestimate of the initial rate previously determined for two reasons: (i) The sample interval was long (5-10 min) in the present experiments because we did not attempt to determine initial rates of cell swelling. The rate of swelling was not linear, the initial rate was more rapid than that measured after 10 min or more in ouabain. In a few experiments ($n$ = 8) we measured the initial rate by sampling frequently and compared the results to those obtained by sampling at the 5-min interval utilized in the present experiments. The initial slope was 2.3 ± 0.2 times greater than the slope determined from the less frequent samples. (ii) The subepithelial connective tissue layers were not removed in the present experiments. We could not strip off the connective tissue in the present experiments because tissue folding and instability developed during long periods of ouabain treatment of stripped preparations. We were concerned that the presence of the subepithelial connective tissue could have prevented or delayed complete inhibition of the Na,K-ATPase by ouabain. We therefore repeated the experiments utilizing $10^{-3}$ M ouabain in the serosal perfusate. As shown in Table 1, although the rate of cell swelling was not significantly increased by the higher dose of ouabain, the time to peak volume was reduced from 28 ± 3 min to 15 ± 2 min. Thus it appears that pump inhibition may not be complete in the present experiments until several minutes after the start of ouabain treatment.

**Table 1.** Ouabain-induced cell swelling

| Ouabain concentration (M) | Time to $V_{max}$ (min) | $V_{max}/V_o$ | $J_v$ ($10^{-6}$ cm/sec) |
|---|---|---|---|
| $10^{-4}$ | 28 ± 3 (18) | 1.27 ± 0.05 | 0.41 ± 0.06 |
| $10^{-3}$ | 15 ± 2 (10)[a] | 1.23 ± 0.02 | 0.51 ± 0.09 |

[a] $P < 0.01$, $V_{max}$ = maximum cell volume, $V_o$ = initial cell volume. Ouabain added to the serosal perfusate at time zero. $J_v$ determined from least squares fit of 3-5 points during the cell swelling phase.

### Intracellular Na Activity During Ouabain Treatment

Figure 2 shows the time course of changes in intracellular Na activity and apical membrane potential difference (PD) from 119 measurements in eight gallbladders. Each determination of cell Na and PD was the average of five or more punctures obtained within a 2- to 3-min period. The line drawn through the Na activity points is a polynomial fitted to the data by the method of least squares. The PD data could be adequately fitted by a linear regression line. A similar time course for the depolarization of *Necturus* gallbladder caused by ouabain has been previously described by Reuss and coworkers [15, 16]. The control Na activity ($a^{\circ}_{Na}$) was 12.1 ± 1.0 mM ($n$ = 8) and the rate of increase of Na activity $\left(\frac{da_{Na}}{dt}\right)$ was 0.62 ± 0.08 mM/min ($n$ = 45) during the first 30 min of ouabain treatment. The relative rate of change of cell volume, $\frac{1}{V}\left(\frac{dV}{dt}\right)$ calculated from the data in Table 1, was equal to 9.2 ± 1.6 × $10^{-3}$ $min^{-1}$

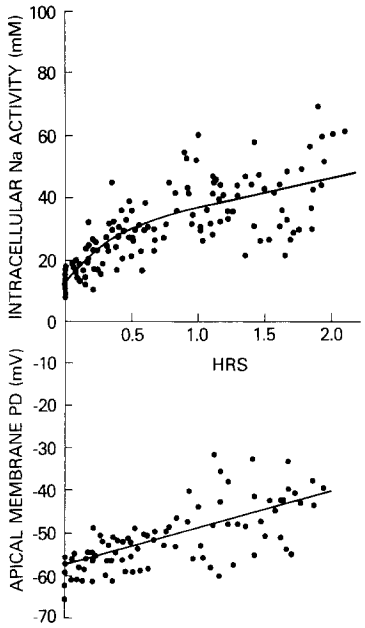


**Fig. 2.** Measurements of intracellular Na activity (top) and apical membrane potential (bottom) for eight ouabain treated gallbladders. 119 points are shown, each is the average of at least five punctures of individual cells within a 2-3 min period. The top curve is drawn by the method of polynomial regression and has the equation:

$$y = 11.84 + 57.2x - 49.65x^2 + 16.33x^3$$

where $x$ is time in hours. The correlation coefficient is 0.8. The lower curve is drawn by the method of linear least squares and has the equation:

$$y = -59.7 + 9.8x$$

where $x$ is time in hours. The correlation coefficient is 0.74

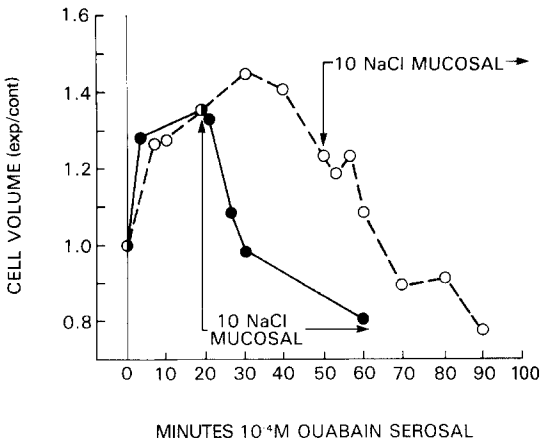


**Fig. 3.** Time course of cell volume changes for two experiments in which the NaCl concentration of the mucosal perfusate was suddenly reduced from 100 to 10 mM. Shown in the solid circles and line in an experiment in which the NaCl reduction was performed while the cell was still in the swelling phase caused by ouabain treatment. The open circles and dashed line show an experiment in which the concentration change was made after the ouabain-induced swelling had stopped and the cell began to shrink

($n$ = 18). $C_{Na}$, the concentration of Na in the fluid entering the cell during the swelling phase, is given by

$$C_{Na} = \frac{1}{\gamma}\left[a^{\circ}_{Na} + \frac{da_{Na}}{dt}\Big/\frac{dV}{Vdt}\right] \quad (1)$$

where $\gamma$ is the activity coefficient for Na, assumed to be equal to the free solution value of 0.78. $C_{Na}$ is calculated from Eq. (1) to be 101 ± 19 mM, identical to the Na concentration in Ringer solution. Thus, during the swelling phase, the quantity of cell Na increases together with cell volume as if the incoming fluid contained the same Na concentration as the mucosal bath. Once the cell swelling has stopped (at approximately 30 min after ouabain treatment) the intracellular Na activity has risen to only 30 mM. Therefore cell swelling did not stop because of dissipation of the Na gradient across the apical membrane.

The measured intracellular Na activity continued to increase slowly after the initial rapid rise in the first 30 min. The rate of cell Na increase during the next 90-min period was calculated from the polynomial regression line to be 0.23 ± 0.04 mM/min ($n$ = 59). The rate of cell volume change was $-5.1 \pm 0.07$ min$^{-3}$, the negative sign indicates that cell shrinkage was occurring. The concentration of Na in the transported fluid during this period was calculated from Eq. (1) using an initial Na concentration ($a^{\circ}_{Na}$) of 30 mM, which was the value determined 30 min after ouabain treatment. The calculated Na concentration of the fluid leaving the cell was −19 ± 13 mM, not significantly different from zero. These results show that the quantity of intracellular Na did not change significantly during the cell shrinkage phase and that the increase in cell Na activity during this period was accounted for by the cell volume decrease. We concluded from these results that Na entry into the cell probably ceased after 30 min of ouabain treatment, and we performed experiments to test this conclusion.

## Evidence for Feedback Inhibition of Na Entry

Two experiments were performed to test whether inhibition of Na entry was the cause of the cessation of cell swelling. In the first approach, illustrated in Fig. 3, the NaCl concentration in the mucosal per-

**Table 2.** Cellular volume flows during ouabain treatment

| Condition | $J_v$ at $t < 30$ min ($10^{-6}$ cm/sec) | $J_v$ at $t > 30$ min ($10^{-6}$ cm/sec) |
|---|---|---|
| Ouabain (*S*) Ringer (*M*) | 0.41 ± 0.06 (18) | −0.25 ± 0.04 (58) |
| Ouabain (*S*) 10 mM NaCl (*M*) | −0.85 ± 0.07 (11)[a] | −0.19 ± 0.03 (17) |
| Ouabain (*S*) Bumetanide (*M*) | −1.15 ± 0.11 (19)[a] | −0.35 ± 0.06 (18) |

(*S*) = serosal perfusate; (*M*) = mucosal perfusate.
[a] $P < 0.001$ *vs.* Ringer; negative sign indicates cell shrinkage.

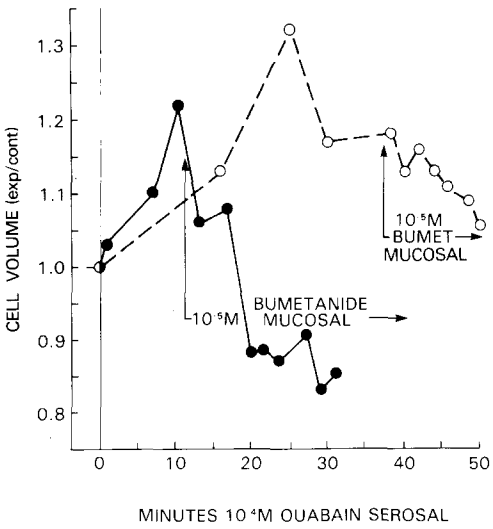


**Fig. 4.** Time course of cell volume changes for two experiments in which $10^{-5}$ M bumetanide was added to the mucosal perfusate at the time indicated by the vertical arrow. Bumetanide addition during the cell swelling phase (solid line and filled circles) causes immediate shrinkage. Addition of the inhibitor at later times is shown by the open circles and dashed line

fusate was suddenly reduced from 100 to 10 mM. If a reduction in the NaCl concentration of the mucosal bath is performed in control conditions, cell shrinkage occurs [18]. In ouabain-treated tissues the response depends on the time elapsed after the ouabain has been applied. As shown in Table 2 reduction of mucosal perfusate NaCl during the ouabain-induced cell swelling phase causes a reversal of swelling and rapid cell shrinkage. A similar reduction in the NaCl concentration of the mucosal perfusate does not significantly alter the rate of cell volume change if performed during the shrinking phase. Reduction of the NaCl of the mucosal perfusate could cause shrinkage for two reasons: (i) cell NaCl exceeds mucosal NaCl, and salt may leave the cell across the apical membrane by reversal of the NaCl entry step; (ii) additional exit steps could be activated which lead to the rapid efflux of solute and shrinkage.

The second test for feedback inhibition of Na entry during ouabain-induced swelling was the exposure of the tissue to $10^{-5}$ M bumetanide in the mucosal perfusate. The results obtained are illustrated in Fig. 4 and summarized in Table 2. Bumetanide is a potent inhibitor of NaCl cotransport by *Necturus* gallbladder [4, 10] and immediately blocks the ouabain-induced swelling if applied within the first 30-min period. Surprisingly, bumetanide leads to rapid cell shrinkage, with a similar time course to that observed with a low NaCl concentration in the mucosal bath. The rapid shrinkage which resulted from bumetanide addition was unexpected; the cause of this shrinkage is not apparent at this time. We speculate that the sudden inhibition of NaCl entry activated a rapid solute exit process, such as the efflux of KCl across the basolateral cell membrane. We did not further investigate the changes in cell composition associated with the cell shrinkage caused by bumetanide or low NaCl. The objective of these experiments was the determination of whether feedback inhibition had occurred as a response to the increased cell Na and cell volume resulting from ouabain treatment. Addition of bumetanide during the shrinkage phase after ouabain treatment did not significantly alter the rate of the cell volume changes (Table 2). This insensitivity of cell volume to the addition of a potent inhibitor of the NaCl entry also indicates that apical NaCl cotransport has been inhibited prior to the bumetanide treatment.

### Inhibition of Apical NaCl Entry by a Reduction in Serosal Bath NaCl

It has previously been demonstrated that reduction of the NaCl concentration of the mucosal perfusate to 10 mM from the control value of 100 mM causes cell shrinkage [18]. The cause of the shrinkage has been attributed to continued Na pump activity resulting in NaCl exit across the basolateral membrane [10, 17, 18]. As long as the mucosal perfusate NaCl concentration remains at 10 mM, cell volume is reduced to about 85% of control [18]. When normal NaCl Ringer is reintroduced into the mucosal bath, the cell quickly swells back to its original volume. An example of such as experiment is shown in Fig. 5 (filled circles and solid line). If the same experiment is performed when the NaCl concentration in both bathing solutions are simultaneously reduced to 10 mM, the cell fails to return to its original volume (Fig. 5, open circles and dashed line). In

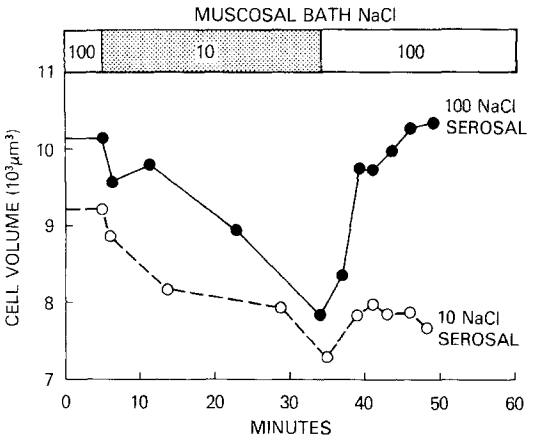


**Fig. 5.** Effects on cell volume of reduction of the NaCl concentration in the mucosal perfusate from 100 to 10 mM followed by an increase to 100 mM. Two experiments are shown. When the serosal perfusate is normal Ringer (100 mM NaCl) the cell shrinks when the NaCl in the mucosal bath is reduced and then swells rapidly when the NaCl concentration is increased (solid line and filled circles). When the mucosal and serosal perfusates are switched to 10 mM NaCl Ringer (open circles and dashed line), the initial shrinkage is not significantly different from control, but the cell volume fails to increase when the mucosal NaCl concentration is returned to control

12 experiments in which the tissue was exposed to 10 mM NaCl Ringer in both the mucosal and serosal bath, cells shrank to 82.4 ± 1.7% of control volume. These results are in excellent agreement with previous results in which the serosal perfusate was 100 mM NaCl Ringer [18]. However, when only the mucosal bath NaCl was returned to 100 mM (i.e., the serosal bath contained 10 mM NaCl Ringer) there was no significant volume flow into cells ($J_v = -1.1 \pm 0.5 \times 10^{-7}$ cm/sec, NS different from zero). Thus, reduction of serosal bath NaCl lead to inhibition of NaCl entry into the cell across the apical membrane, a result predicted by the feedback inhibition hypothesis [2, 21].

In summary, two classes of experiments, inhibition of NaCl entry and reduction of serosal bath NaCl, indicate that apical NaCl entry is subject to regulatory inhibition.

## The Characteristics of Ouabain-Induced Shrinkage

As shown in Fig. 1, long exposures of gallbladder cells to ouabain lead to cell shrinkage below control volume. For 16 experiments in which cells were monitored for 100 min or more after ouabain treatment, cell volume at that time averaged 91 ± 4% of control (significantly less than 100%, $P < 0.05$). We

**Table 3.** Estimated changes in cell K and PD after ouabain addition

| Time (hr) | K* (mM) | PD* (mV) | Measured PD (mV) | K** (mM) |
|---|---|---|---|---|
| 0 | 103.8 | −59.7 | −59.7 | 103.8 |
| 0.5 | 85.6 | −56.8 | −54.8 | 74.9 |
| 1.0 | 79.9 | −55.8 | −49.9 | 54.0 |
| 1.5 | 74.5 | −54.7 | −45.0 | 39.0 |
| 2.0 | 57.0 | −50.7 | −40.1 | 28.1 |

Where: time is the elapsed time in hours after adding ouabain, K* is the cell K activity estimated from the assumption of a constant total cation content and utilizing the control value of cell K activity from reference 11, PD* is the membrane potential calculated from K* and the Nernst equation using a slope of 35 mV/decade, measured PD is taken from the equation fitted to the data in Fig. 2, and K** is the K activity calculated from the measured PD assuming a slope of 35 mV/decade.

wished to determine the cause of the cell shrinkage and first tested whether it was simply a long term effect of ouabain treatment. It was previously shown that ouabain did not cause cell swelling when the mucosal perfusate contained 10 mM NaCl [4]. Presumably, swelling did not occur because of the absence or reduction of the NaCl gradient across the apical membrane [4, 17]. We treated nine preparations with ouabain in the serosal perfusate, while the mucosal bath contained 10 mM NaCl. All experiments were conducted for 100 min or longer. There was no significant cell volume change at any time during this period ($J_v = 0.38 \pm 0.22 \times 10^{-7}$ cm/sec, NS different from zero). Ouabain did not cause cell swelling or shrinkage when the mucosal bath contained 10 mM NaCl Ringer. Thus it seemed that ouabain-induced cell volume and Na increase were required to activate the shrinkage seen after 30 min ouabain treatment.

## Estimated K Activity during Ouabain Treatment

Intracellular K activity has been measured in *Necturus* gallbladder epithelium by several investigators [5, 10, 11, 16]. Measurements of K activity in our laboratory under control conditions similar to those used in the present experiments yielded a value of 103.8 ± 4.5 mM [11]. Reuss et al. [16] studied the changes in cell K activity that followed ouabain addition. They found that intracellular K activity fell from the control value of 86 ± 4.9 mM to 49.9 ± 5.3 mM 30 min after adding ouabain. Basolateral membrane PD also decreased from a control level of −61.5 ± 4.7 mV to −57.1 ± 3.2 mV during this period [16]. We estimated the intracellular K activ-

**Table 4.** Effect of K and Cl on ouabain-induced shrinkage

| Serosal bath[a] composition | $J_v$ ($10^{-6}$ cm/sec) | Paired $t$ *vs.* first period |
|---|---|---|
| *A.* | | |
| Ouabain 2.5 mM K | −0.18 ± 0.03 (11) | — |
| Ouabain 25 mM K | +0.74 ± 0.13 (11) | $P < 0.001$ |
| Ouabain 2.5 mM K | −0.21 ± 0.03 (9) | NS |
| *B.* | | |
| Ouabain 2.5 mM K, 100 mM Cl | −0.27 ± 0.03 (15) | — |
| Ouabain 25 mM K, 8 mM Cl | −0.51 ± 0.09 (15) | $P < 0.001$ |
| Ouabain 2.5 mM K, 100 mM Cl | −0.26 ± 0.05 (13) | NS |

Negative $J_v$ indicates volume flow out of the cell.
[a] Serosal bath contained Ringer solution plus $10^{-4}$ M ouabain. K was increased at the expense of Na; Cl was replaced by gluconate, and calcium was increased to 8 mM.

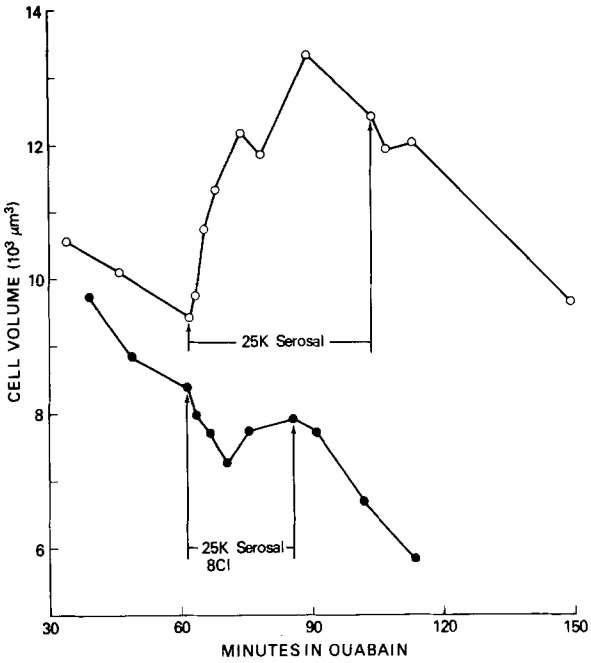


**Fig. 6.** Time course of ouabain-induced shrinkage when the K or K and Cl concentrations of the serosal bathing solution are changed. Ouabain-induced shrinkage is shown for the period from 30 to 120 min after addition of the inhibitor to the serosal bath. At the point indicated by the vertical arrow, the K concentration of the serosal bath was increased to 25 mM (open circles). The shrinkage reversed and the cell began to swell rapidly. Reduction of the serosal perfusate K to control levels (2.5 mM) restored the shrinkage to control rates. When the serosal bath K is raised to 25 mM and the Cl is reduced to 8 mM, the shrinkage rate is nearly the same as under control conditions

ity in our ouabain experiments by two methods: (i) from the assumption that total cation content of the cell is constant and that cell K falls as cell Na rises, or (ii) from the measured membrane PD assuming no change in K selectivity after ouabain. Table 3 lists the K activities calculated by both methods as well as the membrane PD predicted from the K activities assuming constant cation content. In the first 30-min period following ouabain addition cell K activity calculated by either method fell by 20-30%. During this period the data in Table 1 shows that cell volume increased by 27%. Thus dilution of the cell K consequent to the ouabain-induced cell swelling can account for all of the estimated change in cell K during this time period. Reuss et al. [16] measured a 40% decrease in cell K activity in the first 30 min after ouabain addition, most of this decrease was probably due to the cell volume increase. The data in Table 3 also suggest that K activity begins to fall more rapidly than predicted from the constant cation assumption at times longer than 30 min after adding ouabain. These results suggest that K loss is accelerated after the first half-hour and that K exit may be the cause of the cell shrinkage observed in our experiments.

### Ouabain-Induced Shrinkage is Due to KCl Exit

Cell volume decrease in most cells is due to the loss of KCl [8, 9]. We tested the K dependence of ouabain-induced shrinkage by elevating K concentration of the serosal bath. The K concentration was increased tenfold to 25 mM during the shrinkage phase. As shown in Fig. 6 (open circles), increase of the K in the serosal bath reversed the ouabain-induced shrinkage and caused cell swelling. It has been previously shown that increasing the K concentration in the serosal perfusate to 25 mM under control conditions has no significant effect on steady-state cell volume [11]. Table 4*A* lists the rate of ouabain-induced shrinkage in the periods before, during, and after elevation of the serosal bath K concentration. The rate of K-induced swelling was three times the rate of ouabain-induced shrinkage.

The K-dependent reversal of cell shrinkage could be prevented by simultaneous changes in both the K and Cl concentrations of the serosal perfusate. A tenfold reduction in bath Cl concentration was combined with the tenfold increase in K concentration utilized above. As shown in Fig. 6 (solid line and filled circles), the combination of offsetting

changes in the K and Cl gradients across the basolateral membrane eliminated the K-induced swelling. Table 4*B* lists the rates of cell shrinkage before, during, and after the KCl concentration changes. The rate of cell shrinkage in the presence of high K and low Cl was somewhat faster than control rates. The high K, low Cl Ringer was bicarbonate-free, utilizing a phosphate buffer. This was necessary because of the high calcium concentrations required to maintain the calcium activity in the gluconate Ringer (*see* Materials and Methods). The mucosal perfusate was also a phosphate buffer Ringer solution. As a control for these experiments, seven tissues were bathed in bicarbonate-free Ringer with normal ionic composition and treated with $10^{-4}$ M ouabain. The time course and rates of cell volume change were not significantly different from those obtained in bicarbonate Ringer. The interaction of the transmembrane gradients for K and Cl suggested that the exit of the two ions may be directly linked or interdependent.

We tested the possibility that the ouabain-dependent shrinkage was due to KCl cotransport across the basolateral cell membrane. The diuretic bumetanide has been shown to inhibit cotransport of KCl and NaCl by *Necturus* gallbladder [10, 11] and in other tissues [1, 12]. We added $10^{-5}$ M bumetanide to the serosal perfusate during the ouabain-induced shrinkage period. Table 5 shows that bumetanide inhibited the shrinkage and presumably the KCl transport responsible for the volume decrease. Surprisingly, bumetanide caused a slow cell swelling, the mechanism of which is not apparent at this time. The combination of bumetanide sensitivity and interdependent effects of the K and Cl gradients across the basolateral cell membrane supports the conclusion that the efflux of K and Cl, induced by ouabain treatment are mediated.

**Table 5.** Effect of bumetanide on ouabain-induced shrinkage

| Serosal bath[a] composition | $J_v$ ($10^{-6}$ cm/sec) | Paired *t vs.* first period |
|---|---|---|
| Ouabain | −0.18 ± 0.05 (11) | — |
| Ouabain and bumetanide | +0.25 ± 0.03 (10) | $P < 0.001$ |
| Ouabain | −0.19 ± 0.04 (3) | NS |

Negative $J_v$ indicates volume flow out of the cell.
[a] Serosal bath contained Ringer solution plus drugs indicated.

### Effect of Quinidine on the Ouabain-Induced Volume Changes

Quinidine has been widely used as an inhibitor of calcium-mediated processes as well as an agent to increase cell calcium [21]. Although the drug has a number of actions, it has been shown previously to inhibit Na entry in epithelia and to increase cell calcium in *Necturus* proximal tubule [21]. We therefore added $10^{-4}$ M quinidine to both perfusates in *Necturus* gallbladder at the time of ouabain treatment of the preparation. We wished to determine whether quinidine would reduce the cell swelling rate because of direct or indirect effects on the rate of NaCl entry. Table 6 shows that quinidine had no effect on the rate or magnitude of the ouabain-induced cell swelling, but the drug significantly slowed the cell shrinkage which followed the swelling phase. The lack of effect of quinidine on the swelling phase of the ouabain-induced volume changes is not consistent with the calcium-dependent feedback regulation hypothesis [21]. The inhibitory action of quinidine in the shrinkage phase is consistent with other investigations on *Necturus* gallbladder which show that quinidine blocks the KCl-dependent volume regulatory decrease which follows a reduction in the osmolality of the mucosal bath [6].

## Discussion

Prolonged inhibition of the Na,K-ATPase by ouabain caused *Necturus* gallbladder cells first to swell,

**Table 6.** Effect of quinidine on ouabain-induced volume changes

| Serosal bath[a] composition | $J_v$ at $t < 30$ min ($10^{-6}$ cm/sec) | Time to $V_{max}$ (min) | $\frac{V_{max}}{V_o}$ | $J_v$ at $t > 30$ min ($10^{-6}$ cm/sec) |
|---|---|---|---|---|
| Ouabain | 0.41 ± 0.06 (18) | 28 ± 3 | 1.27 ± 0.05 | −0.25 ± 0.04 |
| Ouabain and quinidine | 0.45 ± 0.04 (10) | 23 ± 4 | 1.27 ± 0.05 | −0.10 ± 0.01 (8)[b] |

[a] Serosal perfusate was *Necturus* Ringer plus the drugs indicated.
[b] Significantly different from $10^{-4}$ M ouabain alone at $P < 0.05$. Negative sign of $J_v$ indicates volume flow out of the cell.

as a result of NaCl entry across the apical cell membrane, and then to shrink, as a result of KCl exit across the basolateral membrane. In the classic picture of changes in cellular composition due to pump inhibition, intracellular Na concentration rises and K concentration falls as if one cation replaces the other [14, 15]. The mechanism and driving forces for these compositional changes are usually attributed to passive ionic movements as the ionic gradients created by the pump are dissipated [14, 15]. In *Necturus* gallbladder, and possibly in many other tissues, this simple picture is incorrect on several grounds. First, the ionic movements are not diffusional, but are mediated by specific moieties in the relevant cell membrane; both NaCl entry and KCl exit appear to involve membrane-bound transporters. Second, the stability of cell volume and composition is not due to inherently low rates of transmembrane solute movements but instead to specific regulatory feedback mechanisms. The combination of inhibition of NaCl entry and activation of KCl exit serves to stabilize the cell volume and to oppose cell swelling due to the Donnan effects of the intracellular macromolecules. Third, the driving forces which determine the rate and magnitude of the volume flows resulting from pump inhibition are not those of Na and K alone. The combined gradients for both cations and anions are involved. In *Necturus* gallbladder epithelial cells these salt flows are polarized, being directed inwardly across the apical cell membrane and outwardly across the basolateral cell membrane.

## Feedback Inhibition of NaCl Entry

Most of the results obtained from *Necturus* gallbladder are consistent with the feedback inhibition hypothesis [2, 21]. NaCl entry is inhibited after 30 min of ouabain treatment, and this inhibition is not due to dissipation of the ionic gradients for NaCl entry. It has been proposed that feedback regulation of Na entry in other epithelia is responsible for the maintenance of a constant cell Na despite fluctuations in the rate of transepithelial Na transport [21]. Recent observations in *Necturus* gallbladder epithelial cells undergoing volume regulatory increase indicated that cell Na activity did not change during fluctuations of cell volume [5]. The constancy of cell Na activity was abolished in the presence of ouabain suggesting that Na pump rate variation was responsible for the regulation of intracellular Na activity [5]. Feedback inhibition of NaCl entry in *Necturus* gallbladder, on the other hand, appears to be activated only after a threefold increase in Na activity to 30 mM has been achieved. A similar observation has been made in rabbit urinary bladder where cell Na activity had to rise to greater than 25 mM before feedback inhibition of Na entry was detectable [20]. These results call into question the significance of feedback inhibition of Na or NaCl entry as a meaningful controlling factor in the maintenance of a constant cell Na.

A reduction of the NaCl concentration of the serosal perfusate resulted in inhibition of apical NaCl entry in good agreement with the predictions of the feedback hypothesis [21]. However, quinidine, an agent thought to both increase cellular calcium concentrations and block calcium-activated membrane events [21] had no effect on the time course or magnitude of NaCl entry inhibition in *Necturus* gallbladder. Thus, it is not clear whether NaCl entry is inhibited in this preparation by a calcium-mediated sequence of events.

## Activation of KCl Exit

The KCl exit and associated cell shrinkage that are detectable after 30 min or more of ouabain treatment are remarkable. The exit appears to be due to a transport process across the basolateral cell membrane. The cell preserves its volume by loosing KCl. Typical *Necturus* gallbladder cell K content is $1.2 \times 10^{-12}$ moles [5, 10]. The rate of K loss during cell shrinkage, calculated from the shrinkage rate ($J_v = -0.18 \times 10^{-6}$ cm/sec) and the cell K activity (~100 mM), is $9 \times 10^{-17}$ moles/sec; total depletion of cell K would take about 3.7 hours if the efflux rate remained constant. Thus the cell looses K, does not gain Na, and therefore must gradually become depleted of Cl. The availability of anions may limit the magnitude and duration of the volume change. The Na concentration rises during this time period only because of cellular shrinkage. The intracellular K concentration and membrane potential both tend to remain high hours after ouabain treatment [5, 15, 16], because the cell shrinkage parallels the K loss. Our results do not permit us to determine when KCl exit begins after the addition of ouabain to the preparation. The Na activity measurements (Fig. 2) suggest that virtually all of the cell volume change was due to NaCl entry and that K activity was reduced by dilution. The calculations in Table 3 support this conclusion and suggest a small magnitude for the K or KCl efflux in the first 30 min after ouabain. As described in results, when the NaCl concentration in the mucosal perfusate was reduced to 10 mM prior to the addition of ouabain, no significant change in cell volume occurred for nearly two hours after ouabain treatment. This observation suggests that large amounts of K are not lost unless cell swelling occurs to activate the KCl exit process. The unexpected actions of bume-

tanide (Tables 2 and 5) may also involve KCl movement which is activated in response to inhibition of the cotransport pathway by bumetanide.

The KCl exit which occurs after 30 min of ouabain treatment, resembles the efflux seen during volume regulatory decrease [11]. This KCl exit process appears to occur across the basolateral cell membrane of the *Necturus* gallbladder epithelium. Volume regulatory decrease can be blocked by raising the K concentration in the serosal bath, or it can be restored by simultaneously lowering Cl and raising K [11]. Microelectrode and volume measurements indicated that volume regulatory decrease was the result of the activation of KCl exit with a stoichiometry of 3K and 2Cl [11]. This process could be blocked with quinidine [6] and other inhibitors of calcium or calmodulin-mediated processes [6]. We do not know the stoichiometry of KCl exit during ouabain treatment, but it is tempting to speculate that KCl efflux is mediated by the same system in both circumstances. It has been difficult to rationalize the need for powerful volume regulatory transport systems which respond to osmotic stress in a tissue which rarely, if ever, sees anisotonic solutions [17]. It is possible that the osmotically activated transporters are present to deal with the sequelae of anoxia-induced pump inhibition. The tissue could be protected against substantive cell volume changes for several hours and therefore might be able to withstand the anoxic or ischemic period.

In summary, our results show that *Necturus* gallbladder epithelial cells respond to pump inhibition by the gain of NaCl across the apical membrane and the subsequent loss of KCl across the basolateral cell membrane. Both of these salt flows are mediated by transporters in the cell membrane and are activated under the appropriate circumstances. There is strong evidence to support the feedback regulation hypothesis, but several inconsistencies between our results and the hypothesis still exist. The main point is that feedback inhibition in *Necturus* gallbladder epithelium is activated only after cell Na has nearly tripled and cell volume increased almost 30%. It is hard to understand how such a system could serve a meaningful function in the homeostasis of cell Na under normal conditions.

## References


1. Aull, F. 1981. Potassium chloride cotransport in steady-state ascites tumor cells. *Biochim. Biophys. Acta* **643**:339–345
2. Blaustein, M.P. 1974. The interrelationship between sodium and calcium fluxes across cell membranes. *Rev. Physiol. Biochem. Pharmacol.* **70**:33–82
3. Chase, H.S., Al-Awqati, Q. 1981. Regulation of the sodium permeability of the luminal border of toad bladder by intracellular sodium and calcium. *J. Gen. Physiol.* **77**:693–712
4. Ericson, A.-C., Spring, K.R. 1982. Coupled NaCl entry into *Necturus* gallbladder epithelial cells. *Am. J. Physiol.* **243**:*C*140–*C*145
5. Fisher, R.S., Spring, K.R. 1984. Intracellular activities during volume regulation by *Necturus* gallbladder. *J. Membrane Biol.* **78**:187–199
6. Foskett, J.K., Spring, K.R. 1984. Possible roles for calcium and cytoskeleton in epithelial volume regulation. *Am. J. Physiol. (in press)*
7. Friedman, P.A., Figueiredo, J.F., Maack, T., Windhager, E.E. 1981. Sodium-calcium interactions in the renal proximal convoluted tubule of the rabbit. *Am. J. Physiol.* **240**:*F*558–*F*568
8. Grinstein, S., Dupre, A., Rothstein, A. 1982. Volume regulation by human lymphocytes. *J. Gen. Physiol.* **79**:849–868
9. Hoffman, E. 1977. Control of cell volume. *In:* Transport of Ions and Water in Animals. B.L. Gupta, R.B. Moreton, J.L. Oschman, and B.J. Wall, editors. pp. 285–332. Academic Press, London
10. Larson, M., Spring, K.R. 1983. Bumetanide inhibition of NaCl transport by *Necturus* gallbladder. *J. Membrane Biol.* **74**:123–129
11. Larson, M., Spring, K.R. 1984. Volume regulation by *Necturus* gallbladder: Basolateral KCl exit. *J. Membrane Biol. (in press)*
12. Lauf, P.K. 1984. Thiol-dependent passive K/Cl transport in sheep red cells: IV. Furosemide inhibition as a function of external $Rb^+$, $Na^+$, and $Cl^-$. *J. Membrane Biol.* **77**:57–62
13. Lee, C.O., Taylor, A., Windhager, E.E. 1980. Cytosolic calcium ion activity in epithelial cells of *Necturus* kidney. *Nature (London)* **287**:859–861
14. Macknight, A.D.C., Leaf, A. 1977. Regulation of cellular volume. *Physiol. Rev.* **57**:510–573
15. Reuss, L., Bello-Reuss, E., Grady, T.P. 1979. Effects of ouabain on fluid transport and electrical properties of *Necturus* gallbladder. *J. Gen. Physiol.* **73**:385–402
16. Reuss, L., Weinman, S.A., Grady, T.P. 1980. Intracellular $K^+$ activity and its relation to basolateral membrane ion transport in *Necturus* gallbladder epithelium. *J. Gen. Physiol.* **76**:33–52
17. Spring, K.R., Ericson, A.-C. 1982. Epithelial cell volume modulation and regulation. *J. Membrane Biol.* **69**:167–176
18. Spring, K.R., Hope, A. 1979. Fluid transport and the dimensions of cells and interspaces of living *Necturus* gallbladder. *J. Gen. Physiol.* **73**:287–305
19. Weinman, E., Reuss, L. 1984. $Na^+$-$H^+$ exchange and $Na^+$ entry across the apical membrane of *Necturus* gallbladder. *J. Gen. Physiol.* **83**:57–74
20. Wills, N.K., Lewis, S.A. 1980. Intracellular $Na^+$ activity as a function of $Na^+$ transport rate across a tight epithelium. *Biophys. J.* **30**:181–186
21. Windhager, E.E., Taylor, A. 1983. Regulatory role of intracellular calcium ions in epithelial Na transport. *Annu. Rev. Physiol.* **45**:511–532


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