J. Membrane Biol. 74, 123–129 (1983)

The Journal of
Membrane Biology

Bumetanide Inhibition of NaCl Transport by *Necturus* Gallbladder

Mikael Larson and Kenneth R. Spring
Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

Summary. Salt transport by the *Necturus* gallbladder epithelium is the result of the coupled entry of NaCl into the cells across the apical membrane and the active transport of Na out of the cells across the basolateral membrane. The NaCl entry step was studied by measuring the rate of cell volume increase accompanying ouabain inhibition of the Na–K-ATPase in the basolateral membrane. When bumetanide, a diuretic analog of furosemide, was added to the mucosal bathing solution it reversibly blocked the entry of NaCl into the cells and abolished fluid transport. A dose-response relationship showed half-maximal inhibition of NaCl entry at a bumetanide concentration of 10^{-9} M; complete inhibition of coupled NaCl movement occurred with as little as 10^{-7} M bumetanide. Partial substitution of Na or Cl in the mucosal solution failed to demonstrate competition between bumetanide and either of the ions. The drug was also effective in blocking NaCl entry in the absence of ouabain; addition of the diuretic to the mucosal bathing solution resulted in prompt cell shrinkage and a decrease in intracellular NaCl. Cell volume decrease followed bumetanide addition to the mucosal bath because NaCl entry was blocked but active Na transport continued for several minutes until the intracellular Na transport pool was depleted.

Key Words epithelial cell · apical cotransport · cell volume

Introduction

The mechanism of transepithelial NaCl and water transport in leaky epithelia has been the subject of many investigations [2, 3, 6, 7, 9, 10, 16, 20, 21]. In spite of the well-established fact that NaCl enters the cell across the apical membrane, the specific mechanisms of this entry step have not been defined. Evidence from a number of tissues has been reported [2, 3, 6, 7, 10] indicating NaCl entry into cells of leaky epithelia results from the coupled transport of the two ions, mediated by a carrier in the apical membrane. The driving force for NaCl entry has been proposed to be the favorable chemical gradient for the two ions [3, 7, 19]. We recently confirmed that NaCl entry during transepithelial fluid transport by *Necturus* gallbladder results from the coupled cotransport of NaCl into the cell across the apical membrane [3]. We also reported that NaCl uptake was reversibly inhibited by the diuretic, bumetanide. This drug is an analog of furosemide and is much more potent than most of its analogs and derivatives [1]. The mechanism underlying the action of bumetanide has previously been investigated in other systems, such as the avian and human erythrocyte [12, 13]. The drug appears to be a highly specific inhibitor of ionic cotransport in epithelial cells and other tissues.

In this report we investigate the characteristics of bumetanide inhibition of NaCl entry in the *Necturus* gallbladder epithelium. We utilize several independent methods for the study of fluid and electrolyte transport by this epithelium. Fluid transport was measured in sac preparations, NaCl entry was evaluated by analysis of cell volume changes and associated changes in intracellular ionic activaties. It had previously been reported that inhibition of active Na transport lead to cell swelling [3]. Swelling results from the increase in cell solute content due to the continuing entrance of NaCl across the apical membrane. Thus NaCl entry could be studied by determination of cell volume changes following the addition of ouabain to the serosal bathing solution. The present investigation demonstrates that:

1) Bumetanide completely inhibits transepithelial fluid transport when added to the lumen of sac preparations of *Necturus* gallbladder.

2) The dose-response relation between bumetanide and the rate of NaCl and fluid movement into the cell yields a half-maximal dose of 1 nM.

3) The effect of bumetanide was not potentiated by a reduction in the concentration of Na or Cl, suggesting that the drug does not compete with either of these ions for a binding site.

4) There was no dependence of NaCl entry on the presence of K in the mucosal bath.

5) The addition of bumetanide to the bathing solution, in the absence of ouabain, caused rapid cell shrinkage, as would be expected from the inhibition of NaCl entry. Bumetanide caused a decrease in intracellular Na, Cl and an increase in intracellular K as measured with ion-sensitive microelectrodes.

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 oxygenated Ringer solution.

Sac Preparations

Transepithelial fluid transport was calculated from the change in weight of the gallbladder tied as a sac to the end of a polyethylene tube. The sac was suspended in Ringer solution, blotted, and weighed every 10 min. After a control period of 40–60 min, the luminal solution was switched to Ringer to which 10^{-4} M bumetanide had been added. After 40–60 min 10^{-4} ouabain was added to the serosal bathing solution, and the weight of the sac was determined for the next 60 min. To rule out leaks due to damaged areas of the epithelium, experiments were considered acceptable only if ouabain completely abolished fluid transport. The rate of transepithelial volume flow was calculated from a least squares fit of the change in weight as a function of time. The surface area of the sac was calculated from the volume of the luminal fluid assuming a spherical shape.

Cell Volume Measurements

The epithelium was dissected free of most of its connective tissue and mounted in a miniature Ussing chamber as previously described [3, 14]. The epithelial cells were visualized and analyzed with a microscope-video system [14]. A recording of the cell was made every 60 sec. 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 [14].

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, $CaCl_2$; 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 serosal bath was perfused either by control Ringer solution or by the same solution to which 10^{-4} M ouabain (Sigma Chemical Co., St. Louis, Mo.) had been added. The diuretic, bumetanide, was added to the mucosal perfusion solution in concentrations varying from 10^{-11} to 10^{-5} M. Bumetanide was a generous gift from Hoffman-LaRoche, Inc., Nutley, N.J. A 10^{-3} M bumetanide stock solution was prepared by dissolving the drug in a small volume of dilute NaOH solutions at pH 10.

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, CT) and filled with 1 M KCl. Single-barrel, ion-sensitive electrodes were pulled from the same glass, equilibrated for 1–3 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 (Orion) and equilibrated overnight in 1 M NaCl. K and Na electrodes were filled and used immediately. The K ion exchanger was 5% K-tetrakis-(*p*-chlorophenyl)-borate in 3-O-nitroxylene. The Na ion exchanger, 10% sodium ligand (ETH 227 Fluka Chemical, Hauppauge, N.Y.) in 2-nitrophenyloctyl ether (Fluka) with 0.5% Na-tetraphenylboron, (Sigma Chemical, St. Louis, Mo.) was a generous gift of Drs. S. Weinman and L. Reuss (Washington University, St. Louis, Mo.). The electrodes were calibrated in pure solutions of NaCl or KCl and tested in Ringer solution. The chloride electrodes had an average slope of 52.4 ± 1.0 mV/decade change in Cl activity. The potassium electrodes had an average slope of 56.3 ± 0.4 mV/decade and a selectivity over Na of 50.1 ± 8.0. The Na electrodes had a slope of 52.7 ± 1.6 mV/decade and a selectivity of 20.4 ± 4.0 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.

The gallbladder was either mounted in a fast flow chamber as previously described [5] or pinned serosal side down in a dish. Two cells were punctured simultaneously, one with a voltage-sensitive electrode and one with an ion-sensitive electrode. Voltage electrode punctures were accepted only when the potential dropped quickly 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 withdrawl 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 [18, 21]. 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 [18, 21]. Agreement of the voltage divider ratio of ion-selective and voltage-sensitive electrodes was used as a criterion for acceptability in the initial measurements. Voltage divider ratio was not measured in subsequent experiments performed with the tissue pinned down in a dish.

Data Analysis

A linear regression from at least 4 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 $\pm$ SEM. The Student's t test was used to test the significance of difference.

Results

Six different series of experiments were performed to characterize the action of bumetanide on the NaCl-coupled entry step across the apical membrane. First, fluid transport was measured in sac

preparations and the inhibitory action of the drug evaluated. Second, a dose-response relation was developed for bumetanide inhibition of coupled NaCl entry. Third, we tested for competition between bumetanide and Na or Cl. Fourth, the K dependence of NaCl entry was tested by determination of the rate of ouabain-induced swelling in the presence of a K free mucosal bath. Fifth, we examined whether NaCl entry was blocked by bumetanide while the basolateral Na−K-ATPase was operating normally. Sixth, we determined changes in intracellular ionic composition caused by inhibition of the entry step by bumetanide.

Transepithelial Fluid Transport

The rate of transepithelial fluid transport measured in nine sac preparations bathed in normal Ringer solution was 6.55 ± 1.19 μl/cm^2·hr. As shown in Fig. 1, the addition of 10^{-4} M bumetanide to the luminal bathing solution reduced the fluid transport rate to 0.7 ± 0.5 μl/cm^2·hr, not significantly different from zero. The control transepithelial fluid transport rate is less than that previously reported from this laboratory, 8.86 ± 0.93 μl/cm^2·hr [14]. The stringent criterion that all acceptable preparations must be completely inhibited by ouabain eliminated several preparations whose rapid weight loss was probably due to damage to the epithelium. Elimination of these damaged preparations reduced the average rate of fluid transport compared to our previous observations. The rate observed in the present study, 6.5 μl/cm^2·hr, probably more closely represents the transepithelial fluid flux in our preparations.

Dose-Response

The ouabain-induced swelling of epithelial cells previously described [3] was used to determine the rate of NaCl and water movement into the cell across the apical cell membrane, J_v. In each experimental series the mucosal bath was perfused for 30 min with solutions containing bumetanide. Addition of 10^{-4} M ouabain to the serosal bath resulted in rates of cell swelling which depended on the concentration of bumetanide present in the mucosal bath. Bumetanide concentrations lower than 10^{-10} M resulted in values of J_v not statistically different from control (Fig. 2). The control influx agreed well with the previously measured rates of NaCl and water influx into cells of the *Necturus* gallbladder [3, 14]. Bumetanide concentrations of 10^{-9} and 10^{-8} M resulted in significant inhibition of swelling. Bumetanide concentrations greater than 10^{-7} M resulted in total inhibition of

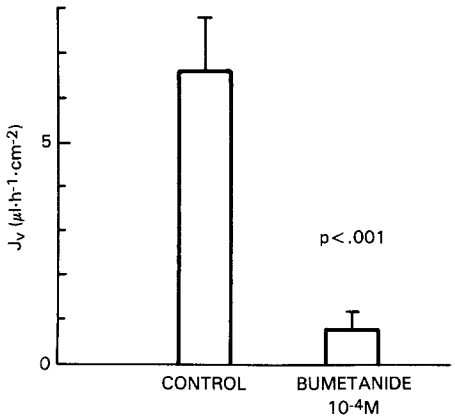

Fig. 1. Rate of transepithelial fluid transport measured in sac preparations bathed in control Ringer during the exposure of the mucosal surface of the bladder to 10^{-4} M bumetanide

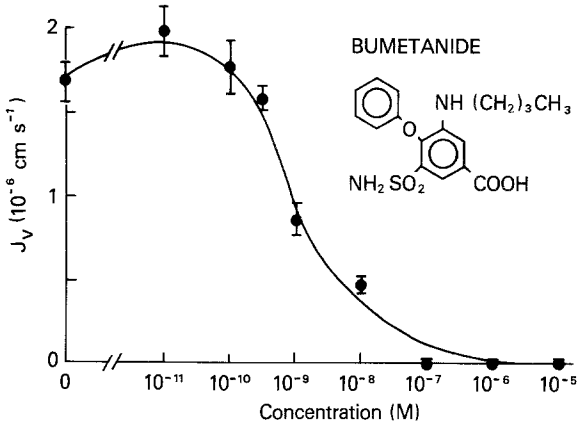

Fig. 2. Effect of bumetanide (structure shown) on the rate of volume flow into the gallbladder epithelial cell as a result of ouabain inhibition of active transport. The volume flow rate, J_v is plotted on the ordinate against the concentration of bumetanide in the mucosal perfusate (abscissa). Each point shows the mean and standard error. The line was drawn by eye

ouabain-induced cell swelling. The dose-response relationship exhibited a typical sigmoid curve, with a half-maximal effective dose IC_{50}, of 1.0×10^{-9} M (Fig. 2). Bumetanide did not cause any adverse changes in the appearance of the cells, even at doses of 10^{-4} M.

Competition

The Na or Cl concentration in the mucosal perfusion solutions was reduced to test for competition between bumetanide and either of these ions. It was not technically feasible to vary the concentration of inhibitor and Na or Cl over a wide range to evaluate any interaction between the drug and the transport ions. We chose instead to examine the effectiveness of two doses of inhibitor in the presence of a reduction in the Na or Cl concentra-

tion of the mucosal perfusate. The concentration dependence of the NaCl entry step has previously been reported [3]; J_v was reported to be reduced by about 50% when the Na or Cl concentration in the mucosal solution was lowered to about 20 mM. We tested the effectiveness of bumetanide in the presence of a lowered Na or Cl concentration in the mucosal perfusate, Na was replaced by N-methyl-D-glucamine and Cl was substituted by gluconate. The rationale of this experimental procedure was the following: If there were competition between bumetanide and Na or Cl, we would expect to find that the drug would be more effective at lowered Na or Cl concentration. A bumetanide concentration (0.5×10^{-9} M) was chosen which would not cause significant inhibition unless competition occurred with Na or Cl. As shown in Fig. 3 there was no potentiation of the action of bumetanide by reduction in Cl or Na. To verify that the bumetanide was an effective inhibitor when the mucosal perfusate contained a low concentration of Cl, the half-maximal inhibitory dose, 1 nM, was added to a mucosal perfusate containing 17 mM Cl. Ouabain-induced swelling was inhibited to the same extent as expected for the unpotentiated effect of bumetanide alone. This was again consistent with a lack of competition between bumetanide and Na or Cl in agreement with a previous report about noncompetitive inhibition of transport in the avian erythrocyte [13]. The extremely high affinity of the NaCl transporter for bumetanide made the direct demonstration of competition very difficult. We can conclude only from our results that competition could not be detected rather than that the drug inhibits noncompetitively.

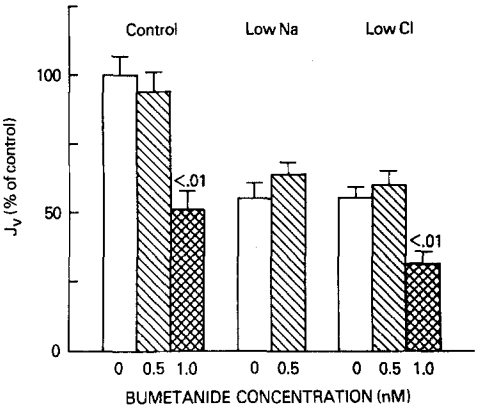

Fig. 3. Relative rate of volume flow into the gallbladder epithelial cells after ouabain inhibition. The effect of two doses of bumetanide (0.5 and 1.0 nM) on J_v are compared when the mucosal bath contained normal Ringer or Ringer in which the Cl or Na concentration was reduced to about 20 mM. Rates of volume flow in low Na or Cl solution, in the absence of bumetanide, are taken from reference 3

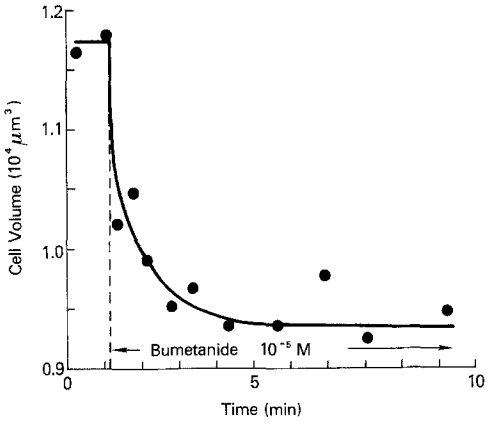

Fig. 4. An example is shown of the effect of bumetanide (10^{-5} M) on cell volume in the absence of ouabain. Cell volume is plotted on the ordinate against time; at the vertical dashed line 10^{-5} M bumetanide was added to the mucosal perfusate. The points are individual measurements of the volume of one epithelial cell. The line was drawn by eye

Effect of K-free Mucosal Fluid

The NaCl cotransport system in rabbit cortical thick ascending limb [8] and avian erythrocytes [11, 13] requires K in the bathing solution. We tested whether neutral coupled NaCl entry in the *Necturus* gallbladder was dependent on the presence of K in the mucosal bath. The rate of ouabain-induced swelling was measured when mucosal bath was perfused with nominally K-free Ringer solution. The K concentration, measured in perfusion fluid and mucosal effluent, were equal and ranged from 0.03 to 0.06 mM. If K were required either as a cotransported cation or as a cofactor for normal operation of the luminal NaCl transport, the rate of ouabain-induced swelling should be decreased in the absence of K. The volume flow rate in the absence of K was $1.50 \pm 0.09 \times 10^{-6}$ cm/sec ($n = 14$), not significantly different from the control value of $1.69 \pm 0.12 \times 10^{-6}$ cm/sec ($n = 17$). Steady-state cell volume prior to ouabain addition was not altered by the absence of K from the mucosal perfusate. Neutral, coupled NaCl entry across the apical membrane of the *Necturus* gallbladder epithelium does not require luminal K.

Inhibition in the Absence of Ouabain

The fifth experimental series was conducted to see if there was any influence of the basolateral Na–K-pump on the bumetanide inhibition of the NaCl coupled entry across the apical membrane. If bu-

Table. Effects of bumetanide on intracellular Na, K and Cl

Ion	Mucosal perfusate		
	Control	10^{-5} M Bumetanide	P
Cell volume μm^3	10267 ±232 (16)	8516 ±190 (16)	<0.001
a_{Cl} mM	22.4± 1.2 (43)	7.1± 0.5 (44)	<0.001
$Q_{Cl} \times 10^{-15}$ moles	295 ± 28	78 ± 9	<0.001
$J_{Cl} \times 10^{-12}$ moles/cm^2·sec	–	−369	
a_{Na} mM	11.6± 1.7 (15)	3.8± 1.0 (15)	<0.001
$Q_{Na} \times 10^{-15}$ moles	153 ± 23	41 ± 11.5	<0.01
$J_{Na} \times 10^{-12}$ moles/cm^2·sec	–	−255	
a_K mM	97 ± 6.7 (15)	110.9± 8 (15)	<0.001
$Q_K \times 10^{-15}$ moles	1277 ± 94	1210 ± 92	ns
$J_K \times 10^{-12}$ moles/cm^2·sec	–	−145	

a_i, steady-state activity of ion i before or 3 to 5 min after bumetanide addition, Q_i, quantity of ion in cell, J_i, flux of ion from cell for a 2-min period after bumetanide addition (negative value indicates efflux). The flux is expressed as a function of the apical surface area of the cell; flux across the basolateral membrane is approximately $^1/_3$ that across the apical membrane.

metanide had its action soley on the entry step, we would expect to see a cell volume shrinkage when perfusing the mucosal bath with a solution to which bumetanide, at an effective dose, had been added. Inhibition of NaCl entry leads to cell shrinkage because active transport continues and depletes the cell of NaCl. The rate of shrinkage is therefore a direct measure of the rate of water and solute loss from the cell and the magnitude of the shrinkage reflects the quantity of solute leaving the cell [11, 19]. After Ringer solution perfusion of the mucosal bath, the solution was switched to one containing 10^{-5} M bumetanide. The shrinkage seen in a typical experiment is shown in Fig. 4. The most striking finding here is the rapid onset of the bumetanide effect. A few seconds after the switch, the cell volume has decreased significantly. The rate of this rapid shrinkage was estimated at $3.11 \pm 0.22 \times 10^{-6}$ cm/sec ($n=16$), from linear regression of the first three to five points during the volume decrease. This rate is comparable to the previously reported rate of active NaCl transport by the *Necturus* gallbladder epithelium [3, 14]. The Table shows that 2–4 min after the addition of bumetanide the cell volume reached its minimum and the change amounted to $-17.2 \pm 0.6\%$ ($n=16$). It could be concluded that the inhibitory action of bumetanide on the apical membrane was not dependent on the presence of ouabain in the serosal bath.

Inhibition of NaCl entry by bumetanide and subsequent cell shrinkage had significant effects on the intracellular activities of Na, Cl and K. As shown in Fig. 5, the addition of bumetanide to the mucosal bathing solution caused a rapid decrease

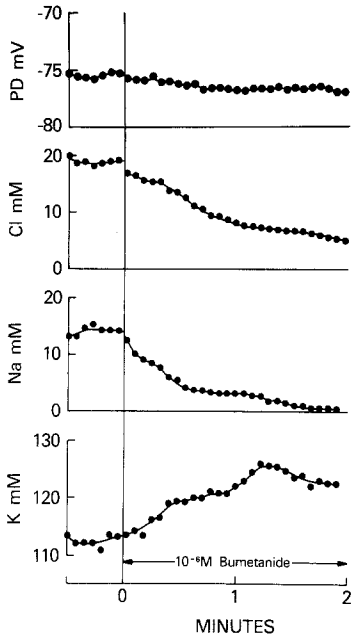

Fig. 5. Examples are shown of the effect of bumetanide (10^{-5} M) on intracellular ionic activities of Cl, Na and K as well as the apical membrane potential (PD). Each tracing shows measurements at 5-sec intervals (indicated by dots) connected by lines drawn by eye. Measurements were made by simultaneously puncturing two cells in the same region of the gallbladder and determining the PD in one cell and the intracellular ion activity in another

in intracellular Na and Cl and an increase in intracellular K. There was no significant change in the apical membrane potential from the control value of -71.9 ± 1.5 mV ($n=79$). The Table indicates that approximately 75% of the intracellular NaCl was lost during the first 90 sec after addition of

the inhibitor. Intracellular K activity increased during that time period, but the total quantity of K did not change significantly. Paired analysis showed that the increase in K activity amounted to $14.3 \pm 1.8\%$, slightly less than would be predicted from the cell volume decrease.

Discussion

Our results offer additional evidence that NaCl enters the *Necturus* gallbladder cell across the apical membrane by means of mediated cotransport. No requirement for potassium could be detected, ruling out the possibility that a Na, K, Cl carrier is responsible for salt and water absorption by the gallbladder. Previous experiments from our laboratory demonstrated that NaCl cotransport was not the result of parallel Na−H and Cl−HCO_3 exchange [3]. All of the evidence from our present and previous investigations of NaCl entry support the conclusion that salt entry occurs by means of the coupled, mediated movement of Na and Cl ions across the apical membrane. The dose of bumetanide needed to achieve a 50% inhibition of NaCl cotransport by *Necturus* gallbladder, 1×10^{-9} M, is substantially less than that required in other cells [4, 12]. It is useful to have a specific inhibitor of NaCl entry for further characterization of the NaCl cotransport process. Bumetanide is ideally suited to the analysis of apical NaCl entry. The inhibitor has an extremely high affinity for the transporter in *Necturus* gallbladder, inhibits rapidly, and could not be shown to compete with Na or Cl. Recently Weinman and Reuss [22] reported the existence of Na−H exchange in the apical membrane of *Necturus* gallbladder epithelium. They suggest that a fraction of Na entry during transepithelial salt and water transport may be due to this exchange process. The effectiveness of bumetanide as an inhibitor of transepithelial fluid transport in the sac preparation and the rapid and complete inhibition of NaCl entry in the presence or absence of ouabain all argue strongly against the possibility that Na−H exchange is involved in transepithelial Na transport. It seems more likely that this exchange process is required for the maintenance of intracellular pH as discussed by these authors [22]. It was previously shown that the inhibitory effect of bumetanide was also rapidly reversible [3]. About 80 analogs of bumetanide have been synthesized and tested for diuretic potency [4, 12]; many of these agents are readily available for binding displacement studies. It seems possible that bumetanide and its analogues could be used as a probe for the future isolation of the NaCl cotransporter in the apical membrane of *Necturus* gallbladder epithelium.

The addition of bumetanide to the mucosal perfusate causes the rapid depletion of intracellular NaCl. The resultant cell shrinkage reflects the depletion of the cell Na pool resulting from active Na transport. Two points are of interest: (i) The Na pool is rapidly and virtually emptied by the pump. (ii) The time course of Cl loss parallels that of Na. These results show that all of the intracellular NaCl is available for transport and that the cell has only enough NaCl in it to sustain transport for 1–2 min after cessation of entry. The total quantity of intracellular Cl is approximately twice that of intracellular Na (Table). The loss of three-fourths of the intracellular Na and Cl leads therefore to a Cl flux which is twice that of Na. Some of the Cl is probably lost together with K, possibly by a cotransport process at the basolateral membrane [15, 16]. Although the intracellular K concentration rose during bumetanide-induced cell shrinkage, the increase in K was less than expected from the volume change alone. If no change in the quantity of intracellular K occurred during the inhibition of NaCl entry, cell K activity should have risen to 117 mM. The K activity observed, 110.9 ± 8 mM, is less than this value, but scatter in the measurements precludes any detailed quantitative analysis of this issue. Potassium actively transported into the cell must normally be lost, because intracellular K activity is in a steady state and above equilibrium (16 and Table). Inasmuch as both the apical and basolateral cell membranes of *Necturus* gallbladder epithelial cells have substantial K conductance [15, 17], the loss of K could be largely due to diffusion. The exit of Cl is not so easily understood.

Several investigators have examined the mode of Cl movement across epithelial cells [2, 3, 6, 7, 15–18, 21]. The means by which Cl moves across the basolateral membrane of the *Necturus* gallbladder epithelium is not known. It has been shown that the Cl conductance of the basolateral membrane is insufficient to explain the observed Cl flux by diffusion [15]. Similar results have been reported for *Necturus* proximal tubule epithelial cells [18]. Although the mechanism of Cl exit is unknown, the results in Fig. 5 and the Table show that Cl leaves the cell rapidly after inhibition of entry.

In summary, the diuretic bumetanide is a valuable agent for the study of NaCl entry and exit from *Necturus* gallbladder epithelial cells. The extraordinarily high affinity of the inhibitor suggests that this compound will be of great use in the isola-

tion and characterization of the NaCl cotransporter in the apical membrane.

References

1. Cohen, M.R., Hinsch, E., Vergona, R., Ryan, J., Kolis, S.J., Schwartz, M.A. 1976. A comparative diuretic and tissue distribution study of bumetanide and furosemide in the dog. *J. Pharmacol. Exp. Ther.* **197(3):**697–702
2. Cremaschi, D., Henin, S. 1975. Na^+ and Cl^- transepithelial routes in rabbit gallbladder. *Pfluegers Arch.* **361:**33–41
3. Ericson, A.-C., Spring, K.R. 1982. Coupled NaCl entry into *Necturus* gallbladder epithelial cells. *Am. J. Physiol.* **243:**C140–*C*145
4. Feit, P.W. 1971. Amino benzoic acid diuretics, 2. 4-substituted-3-amino-5-sulfamylbenzoic acid derivatives. *J. Med. Chem.* **14(5):**432–439
5. Fisher, R.S., Persson, B.-E., Spring, K.R. 1981. Epithelial cell volume regulation-bicarbonate dependence. *Science* **214:**1357–1359
6. Frizzell, R.A., Field, M., Schultz, S.F. 1979. Sodium-coupled chloride transport by epithelial tissues. *Am. J. Physiol.* **236:***F*1–*F*8
7. Garcia-Diaz, J.F., Armstrong, W.McD. 1980. The steady-state relationship between sodium and chloride transmembrane electrochemical potential differences in *Necturus* gallbladder. *J. Membrane Biol.* **55:**213–222
8. Greger, R., Schlatter, E. 1981. Presence of luminal K^+, a prerequisite for active NaCl transport in the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pfluegers Arch.* **392:**92–94
9. Hill, A. 1980. Salt-water coupling in leaky epithelia. *J. Membrane Biol.* **55:**117–182
10. Kimura, G., Spring, K.R. 1979. Luminal Na^+ entry into *Necturus* proximal tubule cells. *Am. J. Physiol.* **236:***F*295–*F*301
11. Kregenow, F.M. 1981. Osmoregulatory salt transporting mechanisms: Control of cell volume in anisotonic media. *Annu. Rev. Physiol.* **43:**493–505
12. Nielsen, O.B.T., Feit, P.W. 1978. Structure-activity relationship of aminobenzoic acid diuretics and related compounds (1) *In:* Diuretic Agents. E.J. Cragoe, Jr., editor. ACS Symposium Series 83, pp. 12–23. American Chemical Society, Washington, D.C.
13. Palfrey, H.C., Feit, P.W., Greengard, P. 1980. cAMP-stimulated cation cotransport in avian erythrocytes: Inhibition by 'loop' diuretics. *Am. J. Physiol.* **238:***C*139–*C*148
14. Persson, B.-E., Spring, K.R. 1982. Gallbladder epithelial cell hydraulic water permeability and volume regulation. *J. Gen. Physiol.* **79:**481–505
15. Reuss, L. 1979. Electrical properties of the cellular transepithelial path way in *Necturus* gallbladder: III. Ionic permeability of the basolateral cell membrane. *J. Membrane Biol.* **47:**239–259
16. Reuss, L., Grady, T.P. 1979. Effects of external sodium and cell membrane potential on intracellular chloride activity in gallbladder epithelium. *J. Membrane Biol.* **51:**15–31
17. Reuss, L., Weinman, S.A. 1979. Intracellular ionic activities and transmembrane electrochemical potential differences in gallbladder epithelium. *J. Membrane Biol.* **49:**345–362
18. Shindo, T., Spring, K.R. 1981. Chloride movement across the basolateral membrane of proximal tubule cells. *J. Membrane Biol.* **58:**35–42
19. Spring, K.R., Ericson, A.-C. 1982. Epithelial cell volume modulation and regulation. J. Membrane Biol. **69:**167–176
20. Spring, K.R., Giebisch, G. 1977. Kinetics of Na^+ transport in *Necturus* proximal tubule. *J. Gen. Physiol.* **70:**307–328
21. Spring, K.R., Kimura, G. 1978. Chloride reabsorption by renal proximal tubules of *Necturus*. *J. Membrane Biol.* **38:**233–254
22. Weinman, S., Reuss, L. 1982. $Na^+ - H^+$ exchange at the apical membrane of *Necturus* gallbladder. *J. Gen. Physiol.* **80:**299–321

Received 27 July 1982; revised 24 November 1982; revised again 18 January 1983