Cyclic AMP Inhibits Na^+/H^+ Exchange at the Apical Membrane of *Necturus* Gallbladder Epithelium

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ABSTRACT   The effects of elevating intracellular cAMP levels on Na^+ transport across the apical membrane of *Necturus* gallbladder epithelium were studied by intracellular and extracellular microelectrode techniques. Intracellular cAMP was raised by serosal addition of the phosphodiesterase inhibitor theophylline (3 mM) or mucosal addition of either 8-Br-cAMP (1 mM) or the adenylate cyclase activator forskolin (10 μM). During elevation of intracellular cAMP, intracellular Na^+ activity ($a\mathrm{Na}_i$) and intracellular pH (pH_i) decreased significantly. In addition, acidification of the mucosal solution, which contained either 100 or 10 mM Na^+, was inhibited by ~50%. The inhibition was independent of the presence of Cl^- in the bathing media. The rates of change of $a\mathrm{Na}_i$ upon rapid alterations of mucosal $[Na^+]$ from 100 to 10 mM and from 10 to 100 mM were both decreased, and the rate of pH_i recovery upon acid loading was also reduced by elevated cAMP levels. Inhibition was ~50% for all of these processes. These results indicate that cAMP inhibits apical membrane Na^+/H^+ exchange. The results of measurements of pH_i recovery at 10 and 100 mM mucosal $[Na^+]$ and a kinetic analysis of recovery as a function of pH_i suggest that the main or sole mechanism of the inhibitory effect of cAMP is a reduction in the maximal rate of acid extrusion. In conjunction with the increase in apical membrane electrodiffusional Cl^- permeability, produced by cAMP, which causes a decrease in net Cl^- entry (Petersen, K.-U., and L. Reuss, 1983, *J. Gen. Physiol.*, 81:705), inhibition of Na^+/H^+ exchange contributes to the reduction of fluid absorption elicited by this agent. Similar mechanisms may account for the effects of cAMP in other epithelia with similar transport properties. It is also possible that inhibition of Na^+/H^+ exchange by cAMP plays a role in the regulation of pH_i in other cell types.

INTRODUCTION

Cyclic adenosine-3′,5′-monophosphate (cAMP) inhibits NaCl absorption in gallbladder and other epithelia (Field, 1979; Frizzell et al., 1975; Petersen and

Reuss, 1983). The mechanism of this effect was believed to be inhibition of apical membrane NaCl cotransport in rabbit (Frizzell et al., 1975) and *Necturus* gallbladder (Diez de los Rios et al., 1981). At least in *Necturus* gallbladder, however, this is unlikely because NaCl entry appears to be by double exchange (Weinman and Reuss, 1982, 1984; Baerentsen et al., 1983; Reuss and Costantin, 1984; Reuss, 1984), although other investigators (Ericson and Spring, 1982; Spring and Ericson, 1982) favor a NaCl cotransport mechanism. In this preparation, the mechanism of inhibition of Cl^- transport by cAMP is the induction of an apical electrodiffusional Cl^- permeability (Petersen and Reuss, 1983). This results in conductive loss (from the cell to the mucosal solution) of the Cl^- that enters by Cl^-/HCO_3^- exchange with the end result of a fall of intracellular Cl^- activity (aCl_i). Because of this fall, Cl^- transport from the cell to the serosal solution, which appears to be at least in part by KCl cotransport (Reuss, 1983), is reduced. If the increase in apical membrane Cl^- permeability were the only mechanism of the effect of cAMP, the fall of aCl_i would result in intracellular acidification (by increased Cl^-/HCO_3^- exchange) and therefore in stimulation of Na^+ entry. However, intracellular Na^+ activity has been reported to fall, not rise (Diez de los Rios et al., 1981), and fluid transport ceases (Petersen and Reuss, 1983).

The present investigation was designed to ascertain the mechanisms by which cAMP alters Na^+ transport at the apical membrane of *Necturus* gallbladder. Conventional and ion-sensitive intra- and extracellular microelectrode techniques were employed. The results were all consistent with an inhibitory effect of cAMP on apical membrane Na^+/H^+ exchange. This causes a fall of both intracellular pH and Na^+ activity and reduces the rates of H^+ secretion and Na^+ entry across the luminal membrane.

MATERIALS AND METHODS

Preparation and Solutions

Mudpuppies (*Necturus maculosus*) of both sexes were purchased from Nasco Biologicals (Ft. Atkinson, WI), kept in large aquaria at 5–10°C, and fed goldfish periodically. The animals were anesthetized by immersion in a solution of tricaine-methane-sulfonate (1 g/ liter), and the gallbladders were rapidly excised, opened longitudinally, rinsed free of bile, and mounted as previously described (Reuss and Finn, 1975a). Two control solutions, of the following compositions (mM), were employed: (*a*) HEPES-Ringer's: 109.2 NaCl, 2.5 KCl, 1.0 CaCl₂, and 1.0 K-HEPES; (*b*) HCO₃-Ringer's: 90 NaCl, 10 NaHCO₃, 2.5 KCl, 1.8 CaCl₂, 1.0 MgCl₂, and 0.5 NaH₂PO₄. HEPES-Ringer's was equilibrated with room air and HCO₃-Ringer's with 1% CO₂/99% air. Both solutions had a pH of 7.6. HEPES-Ringer's was used for all extracellular pH measurements and HCO₃-Ringer's was used for the remaining experiments. Ionic substitutions of Na^+ with tetramethylammonium (TMA^+) or NH_4^+ and of Cl^- with cyclamate were all isomolar.

Electrical Measurements

Transepithelial voltage (V_{ms}) and resistance (R_t) were measured as previously described (Reuss and Finn, 1975a, b). In all experiments, the serosal reference electrode and current-passing electrode were both Ag/AgCl pellets separated from the solution by short

agar-Ringer's bridges. In the experiments in which extracellular pH (pH_o) changes were measured upon stopping mucosal superfusion, the mucosal voltage electrode was a calomel half-cell connected to the solution with an agar-Ringer's bridge. In the intracellular microelectrode experiments, a flowing, saturated KCl bridge was used. In these experiments, the mucosal bathing medium was continuously replaced at a fast rate.

For measurements of extracellular pH, the technique described by Weinman and Reuss (1982) was employed. Briefly, an electrode consisting of a bulb of ~500 μm diam of H^+-selective glass (Clarke Electromedical Instruments, Reading, England) fused to a lead glass pipette was positioned in the immediate vicinity of the apical surface of the tissue. Since apical membrane H^+ fluxes can cause pH gradients in the mucosal solution, the pH electrode was kept in the same position throughout the experiment. Changes in pH_o were calculated from the change in the voltage output of this electrode with respect to the agar-Ringer's bridge immersed in the same solution. To calculate apparent net H^+ fluxes into or out of the mucosal solution (J_H), the buffering power of the solution was taken into account, as previously described (Weinman and Reuss, 1982). J_H underestimates the true H^+ flux between the epithelial cells and the mucosal solution, but paired comparisons are possible in the same tissues if the position of the electrode is kept unchanged under all experimental conditions (Weinman and Reuss, 1982, 1984).

Intracellular pH and Na-sensitive microelectrodes were constructed as previously described (Reuss et al., 1983). For the pH electrodes, proton cocktail (Fluka Chemical Co., Hauppauge, NY) was employed. The Na-sensitive liquid membrane consisted of 10 mg Na ligand I (Fluka Chemical Co.) and 0.5 mg Na tetraphenylborate, dissolved to 100 mg in *o-n*-octyloxynitrobenzene. All measurements of intracellular pH (pH_i) and intracellular Na^+ activity (aNa_i) were carried out by simultaneous impalements with a conventional and an ion-sensitive microelectrode. Apical and basolateral membrane voltages (V_{mc} and V_{cs}, respectively) were referenced to the adjacent external solution. Impalements were validated by the criteria previously described (Weinman and Reuss, 1982). The rates of change of aNa_i and pH_i were estimated from the initial slopes (10–20 s) of the digitized differential intracellular voltage traces (ion-sensitive minus conventional microelectrode, both with respect to the serosal bathing medium; see Figs. 6 and 9).

The effects of drugs or ionic substitutions on intracellular voltages and ion activities were monitored by keeping the microelectrodes continuously in the same cells. The reversibility of the effects was tested in most experiments by returning to the original control solution.

The voltage measurements were digitized (model 1074 signal averager, Nicolet Instrument Corp., Madison, WI) and transferred to a microcomputer for analysis.

Drugs and Chemicals

Theophylline, 8-Br-cAMP, HEPES, and cyclamic acid and its Na and Ca salts were purchased from Sigma Chemical Co., St. Louis, MO. Forskolin was obtained from Calbiochem-Behring Corp., San Diego, CA. Amiloride was a generous gift of Merck, Sharpe & Dohme, West Point, PA. All other chemicals were purchased from Fisher Scientific Co., Pittsburgh, PA.

The concentrations of 8-Br-cAMP and theophylline employed in these studies were maximal, as estimated from their effects on apical membrane Cl^- conductance, transepithelial fluid absorption, or both (Petersen and Reuss, 1983).

Statistics

All data are presented as means ± SEM. Unless stated otherwise, statistical comparisons

were performed using the t test for paired data. A P value of <0.05 was considered significant.

RESULTS

Extracellular pH Studies

In tissues incubated in solutions buffered with 1 mM HEPES, the mucosal bathing solution is acidified by a Na-dependent, amiloride-sensitive process interpreted to correspond to apical membrane Na^+/H^+ exchange (Weinman and Reuss, 1982).

If elevation of intracellular cAMP levels inhibits Na^+/H^+ exchange, one would expect a reduction of luminal acidification. As shown in Fig. 1, serosal exposure to the phosphodiesterase inhibitor theophylline in fact decreased the acidification
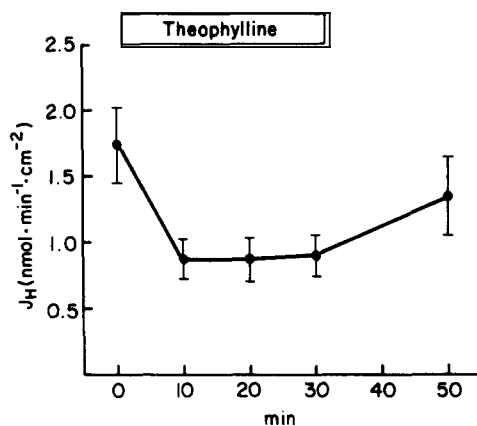

FIGURE 1.   Effect of theophylline on the apparent H^+ flux into the mucosal solution (109.2 mM Na^+) observed by stopping superfusion. Data shown are means ± SEM of six experiments. J_H was calculated as explained in Methods. Theophylline was added to the serosal solution to a final concentration of 3 mM. The values of theophylline (10, 20, and 30 min) were significantly different from control ($P <$ 0.05). The value ~20 min after removal of theophylline was not statistically different from either the control or the experimental values.

of the mucosal solution to ~50% of the control value. The effect was at least partially reversible. In Na-depleted tissues, the sudden addition of Na^+ to the mucosal side causes acidification of the mucosal solution with a half-maximal rate at ~10 mM Na^+ (Weinman and Reuss, 1982). Therefore, acidification was measured after bilateral exposure to Na-free TMA-Ringer's, upon a sudden increase in luminal $[Na^+]$ from nominally 0 to 10 mM. Theophylline reduced the degree of luminal acidification by ~50%, as summarized in Fig. 2. The acidification, both under control conditions and in theophylline, was largely inhibited by 1 mM amiloride, and the amiloride-sensitive fraction of the acidification was significantly smaller in theophylline, which indicates that the reduction in luminal acidification is at least in part due to inhibition of Na^+/H^+ exchange across the apical membrane.

Inasmuch as cAMP increases apical membrane electrodiffusional Cl$^-$ permeability and decreases intracellular Cl$^-$ activity (Petersen and Reuss, 1983), the driving force for Cl$^-$/HCO$_3^-$ exchange would be expected to increase, and hence the apical membrane HCO$_3^-$ efflux would increase as well. Therefore, the inhibition of the luminal acidification in theophylline could at least in part be caused by an increased HCO$_3^-$ net flux from the cell to the mucosal solution. This possibility was tested by two sets of experiments. First, the magnitude of the acidification was determined during exposure to HEPES-Ringer's and both 1 and 10 min after adding 1 mM amiloride to the mucosal bathing medium. We have shown (Reuss and Costantin, 1984) that shortly after exposure to amiloride,
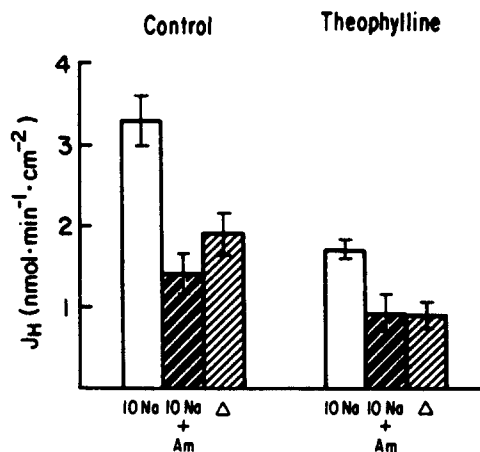

FIGURE 2. Effect of theophylline on Na$^+$ gradient-induced luminal acidification. Data shown are means ± SEM of six experiments. Before or during serosal exposure to 3 mM theophylline, the tissues were superfused on the mucosal side for 15 min with nominally Na$^+$-free Ringer's (TMA$^+$ substitution). Then the mucosal superfusate was rapidly changed to a 10 mM Na$^+$ (balance TMA$^+$) solution, without or with 1 mM amiloride. Superfusion was stopped for 3 min, and the changes in mucosal solution pH and the corresponding apparent H$^+$ fluxes were measured as described in Materials and Methods (first and second bars, respectively). The third bar of each group depicts the difference, i.e., the amiloride-sensitive component of J_H. Theophylline significantly reduced J_H in 10 mM mucosal Na$^+$ ($P < 0.005$) and in 10 mM Na$^+$ plus amiloride ($P < 0.05$). The amiloride-sensitive J_H was also significantly reduced ($P < 0.005$).

the change in luminal solution pH upon stopping superfusion reverses to alkalinization because of the persistence of Cl$^-$/HCO$_3^-$ exchange, while Na$^+$/H$^+$ exchange is inhibited. After 10 min, presumably because of the intracellular acidification produced by amiloride (Weinman and Reuss, 1982), no significant change in luminal pH was observed when superfusion was stopped. In the present experiments (Fig. 3), theophylline significantly decreased the alkalinization observed after 1 min of exposure to amiloride. This result is the opposite of that expected if Cl$^-$/HCO$_3^-$ exchange were increased. At 10 min, the pH changes under control conditions and in the presence of theophylline were not signifi-

cantly different from each other or from zero. In the second set of experiments, luminal acidification was measured in the absence of Cl^- from both bathing media upon sudden exposure to 10 mM Na^+. As shown in Fig. 4, theophylline inhibited the luminal acidification by ~46%, i.e., to a similar extent as in Cl-containing media. The results obtained in both sets of studies cannot be explained by stimulation of Cl^-/HCO_3^- exchange.

Intracellular Na^+ Activity Measurements

Inhibition of Na^+/H^+ exchange should reduce intracellular Na^+ activity (aNa_i) (Weinman and Reuss, 1984; Reuss, 1984). This was indeed observed (Fig. 5,
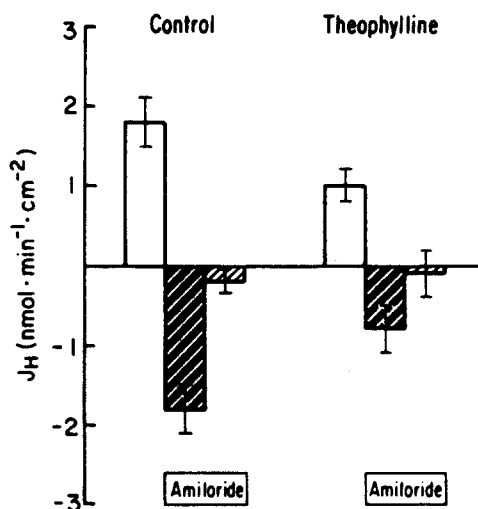

FIGURE 3. Effect of amiloride on J_H under control conditions and during treatment with theophylline. Data are means ± SEM of five experiments. J_H was measured in 109.2 mM Na^+ (first bar of each group), 1 min after mucosal addition of amiloride (second bar of each group), and after 10 min of exposure to the diuretic (third bar). Theophylline reduced significantly the acidification in 109.2 mM Na^+-Ringer's ($P < 0.01$; cf. Fig. 1). After 1 min of exposure to amiloride, alkalinization of the mucosal solution was observed, which was significantly inhibited by theophylline ($P < 0.025$). After 10 min of exposure to the diuretic, J_H was not significantly different from zero, with or without theophylline.

Table I), even though the changes, although they were statistically significant, were small. Since cell volume is likely to be reduced because aCl_i, aNa_i, and aK_i fall (Duffey et al., 1981; Petersen and Reuss, 1983; this paper; unpublished observations), the change in cell Na^+ content was probably larger than that of aNa_i. In contrast with the observation of a fall of aK_i by Duffey et al. (1981) and us (unpublished), Diez de Los Rios et al. (1981) reported an increase in aK_i (see Discussion).

To ascertain the effect of elevating intracellular cAMP levels on the rate of Na^+ entry across the apical membrane, we measured the initial rate of fall of
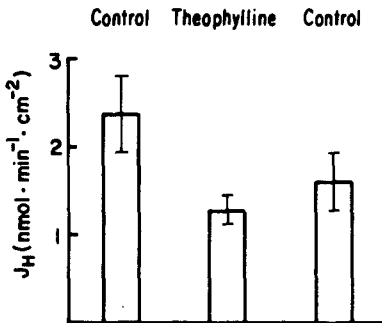
Control   Theophylline   Control

FIGURE 4. Effect of theophylline on Na^+ gradient-induced luminal acidification in the absence of Cl^-. Means ± SEM of six experiments are shown. J_H measurements were started at least 20 min after bilateral superfusion with Na-free, Cl-free media (TMA^+ and cyclamate substitution, respectively). Then the mucosal side only was superfused for 30 s with a 10 mM Na^+ (balance TMA^+, Cl-free) solution, and J_H was measured under control conditions (left), after 30 min of exposure to theophylline (center), and 30 min after theophylline removal (right). The value in theophylline was significantly different from control ($P < 0.05$).
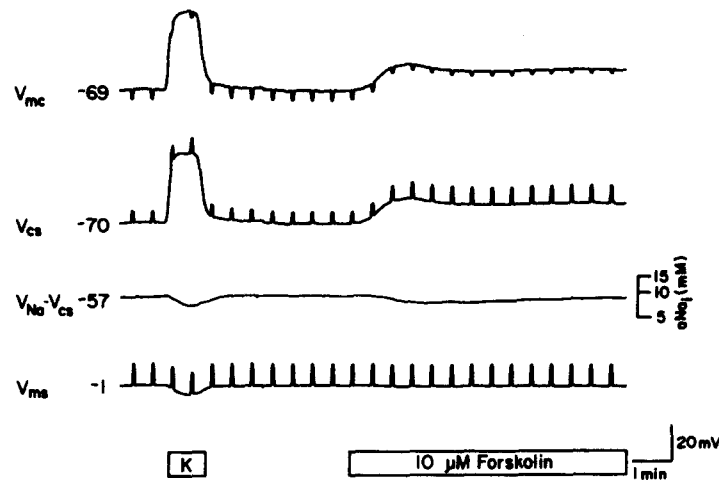

FIGURE 5. Effect of elevating intracellular cAMP levels on intracellular Na^+ activity (aNa_i). In this and other figures, the traces depict apical membrane voltage (V_{mc}), basolateral membrane voltage (V_{cs}), the difference between outputs of Na^+-sensitive microelectrode and conventional microelectrode ($V_{Na} - V_{cs}$), and transepithelial voltage (V_{ms}). Initial values are shown at the beginning of each trace (for polarity conventions, see Materials and Methods). The vertical voltage deflections are the result of transepithelial current pulses (50 μA cm^{-2}, 3 s duration). During the period indicated by the bottom bar labeled K, mucosal [K] was transiently raised from 2.5 to 92.5 mM (isomolar Na^+ substitution). Note the slow, reversible fall in aNa_i during the period of reduction of mucosal [Na^+]. Addition of the adenylate cyclase activator forskolin (second bar at the bottom) depolarized both cell membranes, reduced the apparent ratio of cell membrane resistances (apical/basolateral), and slightly decreased aNa_i. Similar results were obtained with 8-Br-cAMP (mucosal addition) and theophylline (serosal addition). See Table I.

TABLE I

Effect of Elevating Intracellular cAMP Levels on Intracellular Na^+ Activity

	Control	Experimental	Difference
	mM	mM	mM
8-Br-cAMP	10.1±1.0	8.7±1.0	1.4±0.2*
Theophylline	10.0±0.7	8.7±0.7	1.3±0.1*

Steady state values of $a Na_i$ before and during exposure to 1 mM 8-Br-cAMP (mucosal addition, $n = 4$) or to 3 mM theophylline (serosal addition, $n = 7$) are shown. Third column: paired difference between control and experimental values. In all experiments, the data were obtained from continuous impalements.
* $P < 0.01$.

$a Na_i$ upon reducing the $[Na^+]$ in the mucosal solution to a value at which Na^+/H^+ exchange should cease. For a justification of this procedure, including a discussion of errors resulting from changes in cell volume, see Reuss (1984). As illustrated in Fig. 6 and summarized in Table II, the fall in $a Na_i$ in theophylline was smaller and ~50% slower than under control conditions. Upon re-exposure to 100 mM Na^+, the rate of increase of $a Na_i$ was also 50% slower in the presence of the drug. The initial rate of fall of $a Na_i$ (if the net apical membrane Na^+ flux falls to zero) should reflect the previous steady state rate of Na^+ entry (Reuss,
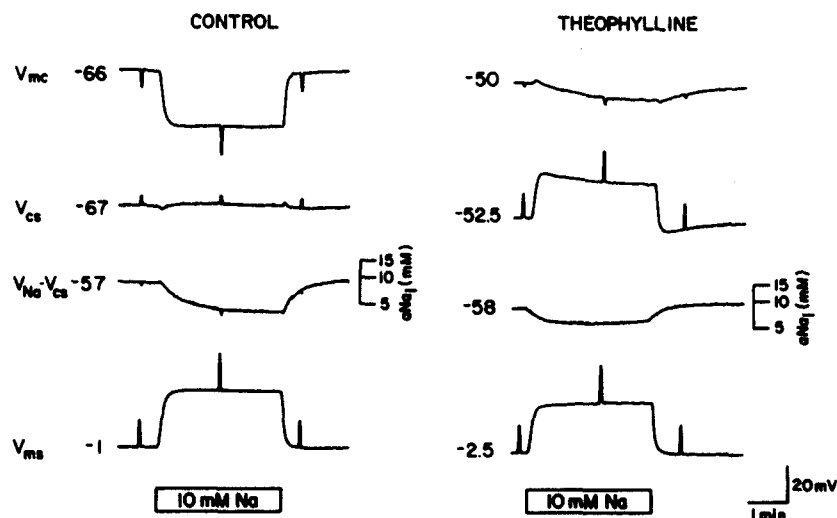

FIGURE 6. Effect of theophylline on the change in $a Na_i$ produced by lowering mucosal $[Na^+]$. Symbols are the same as in Fig. 5. Both sets of traces were obtained from the same tissue under control conditions and ~30 min after serosal addition of theophylline, respectively. During the periods indicated by the lower bars, the mucosal solution $[Na^+]$ was transiently decreased from 100 to 10 mM. Note the differences in the effects of lowering $[Na^+]$ on V_{mc}, V_{cs}, and V_{ms} (control vs. theophylline), which are caused by the high apical membrane P_{Cl} in theophylline. The change in $a Na_i$ was smaller and slower in theophylline as compared with control (see Table II).

TABLE II

Effects of Theophylline on the Reduction of Intracellular Na$^+$ Activity Produced by Lowering Mucosal [Na]

| | a Na$_i$ | | | Δa Na$_i$/Δt | |
	Ringer's	10 mM Na$^+$	Ringer's	a	b
	mM	*mM*	*mM*	*mM/min*	
Control	11.9±1.2	7.3±1.1	11.8±1.3	−5.7±1.1	9.6±1.8
Theophylline	10.5±1.0	8.8±0.9	12.4±1.2	−2.8±0.7*	4.8±1.3*

First three columns: a Na$_i$ values in HCO$_3$-Ringer's, 3 min after exposure to 10 mM Na$^+$ (mucosal side only), and 3–5 min after returning to HCO$_3$-Ringer's, in the absence (first line) and presence of 3 mM theophylline on the serosal side (second line). Δa Na$_i$/Δt: initial changes in a Na$_i$ upon lowering mucosal [Na$^+$] from 100 to 10 mM (a) and upon raising mucosal [Na$^+$] from 10 to 100 mM (b), respectively. The changes in a Na$_i$ after 3 min exposure to 10 mM Na$^+$ were 4.6 ± 0.5 and 1.8 ± 0.3 mM under control conditions and in theophylline, respectively (Δ = 2.9 ± 0.7 mM; $P < 0.001$).
* $P < 0.025$; $n = 6$ experiments.

1984). Thus, we conclude that Na$^+$ entry is reduced by ~50% when intracellular cAMP levels are elevated. The rate of Na$^+$ entry upon restoring the mucosal [Na$^+$] to 100 mM is higher than the rate of fall of a Na$_i$ upon reducing external [Na$^+$] to 10 mM, as observed before (Reuss, 1984), because during the period of exposure to low [Na$^+$], a Na$_i$ and pH$_i$ fall, which increases the net force that favors Na$^+$ entry by Na$^+$/H$^+$ exchange. In theophylline, upon restoring external [Na$^+$] to 100 mM, a Na$_i$ rose at a rate of only half of that measured in the same tissues in the absence of the drug. Although this result agrees quantitatively with the fractional inhibition of Na$^+$ entry (Table II), it is more difficult to interpret,
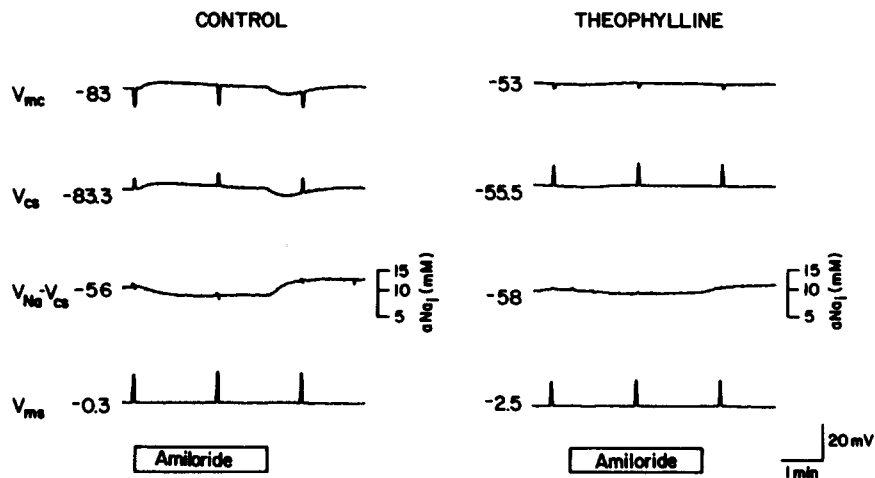

FIGURE 7.   Effect of 1 mM amiloride on a Na$_i$ under control conditions and during elevation of intracellular cAMP. Protocol and symbols as in Fig. 6. Note that the effect of amiloride on a Na$_i$ is slower and smaller in theophylline, as compared with control.

TABLE III

Effect of Theophylline on the Reduction in Intracellular Na⁺ Activity Produced by Amiloride

| | $a\text{Na}_i$ | | | $\Delta a\text{Na}_i/\Delta t$ | |
	Control	Amilor-ide	Control	a	b
	mM	*mM*	*mM*	*mM/min*	
Control	11.9±1.2	9.6±1.1	12.9±1.3	−2.4±0.4	4.0±1.1
Theophylline	10.3±1.1	9.6±0.9	10.8±1.4	−1.3±0.4*	2.1±0.5*

Protocol as in Table II. Amiloride exposure (1 mM, mucosal side only) lasted 3 min. *$P < 0.05$. The falls in $a\text{Na}_i$ produced by amiloride were 2.3 ± 0.5 and 0.7 ± 0.3 mM under control conditions and in theophylline, respectively ($\Delta = 1.6 ± 0.6$ mM; $P < 0.05$).

because the $a\text{Na}_i$ values, and perhaps also the pH_i values, were not the same when external Na^+ was raised.

Further evidence that the Na^+ entry process inhibited by cAMP is Na^+/H^+ exchange was obtained by studying the effect of amiloride (1 mM added to the mucosal solution only) on $a\text{Na}_i$. As illustrated in Fig. 7 and summarized in Table III, theophylline significantly decreased the rate of fall of $a\text{Na}_i$ upon addition of amiloride and the rate of rise of $a\text{Na}_i$ upon removal of the drug. At 1 mM amiloride in the presence of 100 mM Na^+, Na^+ entry was partially inhibited, but did not stop. This is compatible with previous observations (Weinman and Reuss, 1984; Reuss, 1984) and can be ascribed to the kinetics of the amiloride inhibition.

Intracellular pH Measurements

Since the pH of these cells is decreased by inhibition of apical membrane Na^+/H^+ exchange (Weinman and Reuss, 1982), elevated cAMP levels should have a similar effect. As shown in Table IV, both 8-Br-cAMP and theophylline did produce a slight, but significant, intracellular acidification. When the serosal

TABLE IV

Effects of Elevations of Intracellular cAMP Levels on Membrane Voltages and Intracellular pH

	V_{ms}	V_{cs}	pH_i
	mV		
Control	−0.4±0.2	−76±3	7.33±0.04
8-Br-cAMP	−2.1±0.3*	−64±3*	7.27±0.06*
Control	−0.6±0.2	−75±2	7.40±0.04
Theophylline	−1.7±0.2*	−57±3*	7.30±0.05*

The data shown are steady state values obtained from continuous impalements under control conditions and after 3–5 min of exposure to 8-Br-cAMP (1 mM, mucosal side, $n = 5$) or 10–20 min of exposure to theophylline (3 mM, serosal side, $n = 8$). * $P < 0.05$ or better.

surface was exposed to 3 mM theophylline (Fig. 8), pH$_i$ first rose (probably because of entry of the drug, pK$_a$ 8.8) and then fell. These results support the conclusion that cAMP inhibits Na$^+$/H$^+$ exchange, but they could also be explained in principle by stimulation of Cl$^-$/HCO$_3^-$ exchange and hence by an increased net HCO$_3^-$ flux from the cells to the mucosal solution. The extracellular pH measurements described above allow us to rule out the second possibility and therefore to conclude that the steady state intracellular acidification produced by 8-Br-cAMP or theophylline is due to inhibition of Na$^+$/H$^+$ exchange.

Further support for this conclusion was obtained upon examination of the effect of theophylline on the pH$_i$ recovery from an acid load. The cells were
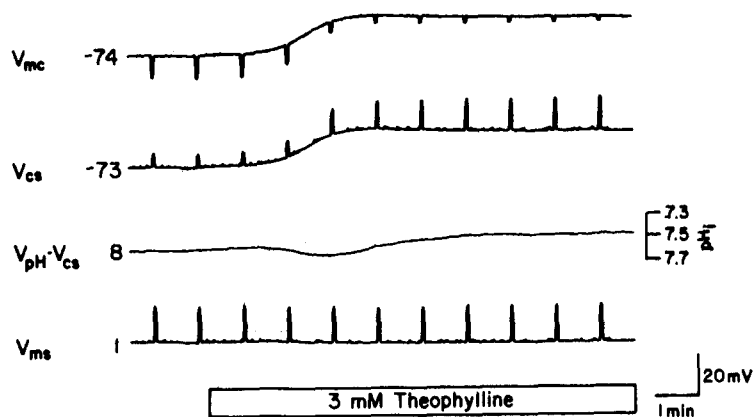

FIGURE 8.   Effect of theophylline on pH$_i$. Symbols are the same as in Figs. 5–7. $V_{pH} - V_{cs}$ denotes the difference between the voltage outputs of a liquid membrane intracellular pH microelectrode and an intracellular conventional microelectrode. The effects of theophylline on V_{mc}, V_{cs}, and the apparent ratio of cell membrane resistances are similar to those shown in Fig. 5. The delay between the change in serosal solution and the onset of the effect of theophylline is due to both the dead space in the serosal perfusion system and the subepithelial diffusion boundary layers. Note that after a brief initial alkalinization, the cell becomes slightly acidified by exposure to theophylline. See text and Table IV.

acidified by exposure to NH$_4$Cl (Boron and De Weer, 1976). As illustrated in Fig. 9, exposure of the mucosal surface of the tissue to NH$_4$Cl causes first a rapid intracellular alkalinization, attributable to influx of NH$_3$, followed by a slower change in pH$_i$ in the acid direction ("plateau acidification"), probably caused by NH$_4^+$ influx. Upon removal of external NH$_4$Cl, NH$_3$ leaves the cell more rapidly than NH$_4^+$, which causes intracellular acidification (NH$_4^+$ ⇌ NH$_3$ + H$^+$), followed by recovery of pH$_i$ to the value before exposure to NH$_4$Cl. In 100 mM Na$^+$, this rate of pH$_i$ recovery is considerably reduced by theophylline. Fig. 10 shows typical experiments in which the mucosal [Na$^+$] was reduced to 10 mM at the time of NH$_4$Cl removal. After a steady rate of pH$_i$ recovery was obtained, external [Na$^+$] was raised to 100 mM. The rate of pH$_i$ recovery in 10 mM Na$^+$
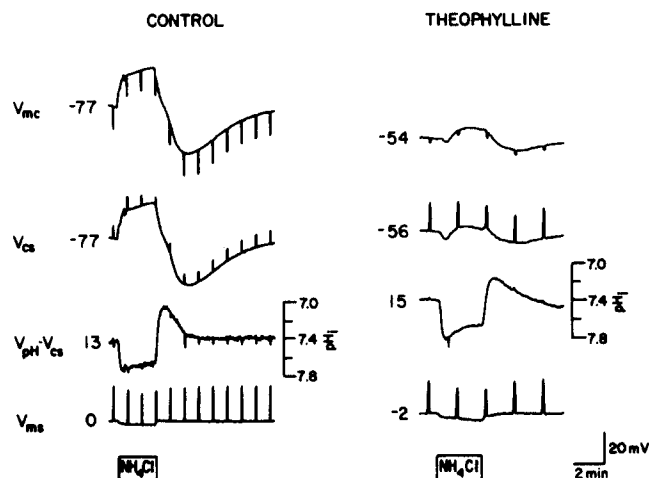
CONTROL   THEOPHYLLINE

FIGURE 9.   Recovery of pH_i from an acid load under control conditions and in theophylline. Symbols are the same as in Fig. 8. During exposure to 25 mM NH_4Cl (3 min, indicated by the bars) the cells alkalinize rapidly (NH_3 entry) and then pH_i recovers somewhat (NH_4^+ entry), while the cell membranes depolarize. Upon NH_4Cl removal, pH_i falls below its control value (exit of both intracellular NH_3 and NH_4^+ in the form of NH_3) and then recovers. Simultaneously, both cell membranes transiently hyperpolarize. Note the differences between the control traces and those in theophylline, i.e., the smaller changes in cell membrane voltages and the slower pH_i recovery after NH_4Cl removal. See Tables V and VI and text.
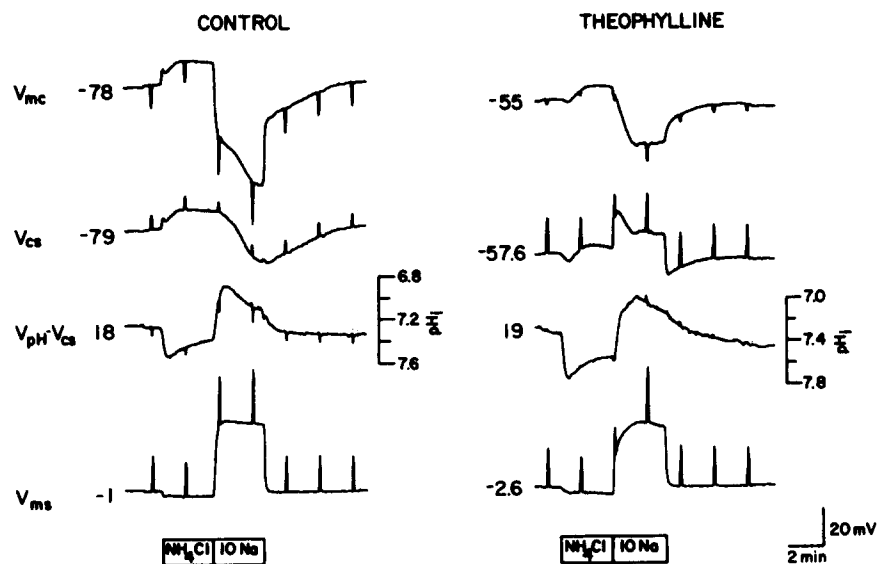

CONTROL   THEOPHYLLINE

FIGURE 10.   Recovery of pH_i from an acid load, at low mucosal [Na], under control conditions, and in theophylline. Protocol is the same as in Fig. 11, but mucosal [Na] was reduced to 10 mM for 3 min starting immediately after NH_4Cl removal and raised to 100 mM at the end of this period. Note the differences in the membrane voltage traces and in the rates of pH_i recovery. cf. Fig. 9 and see Table V.

was less than in 100 mM Na^+ and was further reduced by theophylline. Table V summarizes the results obtained in all experiments such as those depicted in Figs. 9 and 10. The average values of pH_i before exposure to NH_4Cl were not statistically different under control conditions from those measured after treatment with theophylline. The maximum acidification obtained upon removal of NH_4Cl was also unchanged by the drug. The rate of pH_i recovery in 100 mM Na^+ was halved by theophylline. In 10 mM Na^+, recovery rates in the absence and presence of theophylline were ~60% of those measured in the same tissues in 100 mM Na^+. This indicates that, with or without the drug, half-maximal recovery takes place at a mucosal $[Na^+]$ slightly lower than 10 mM.

In 10 mM mucosal Na^+, 1 mM mucosal amiloride reduced pH_i recovery from 0.10 ± 0.03 to 0.02 ± 0.01 ($n = 3$), which indicates that pH_i recovery after intracellular acidification with the NH_4Cl loading technique is largely the result of apical membrane Na^+/H^+ exchange.

TABLE V
Effect of Theophylline on pH_i Recovery from an Acid Load

	pH_i		
	Control	Peak acidification	$\Delta pH_i/\Delta t$
			min^{-1}
100 Na^+			
Control	7.37±0.03	7.03±0.05	−0.16±0.03
Theophylline	7.32±0.05	6.95±0.08	−0.08±0.01*
10 Na^+			
Control	7.38±0.06	6.96±0.10	−0.09±0.01
Theophylline	7.35±0.04	7.00±0.06	−0.05±0.01*

Protocol as in Figs. 9 and 10. All values were obtained in the same tissues ($n = 4$). $\Delta pH_i/\Delta t$ is the initial rate of recovery upon NH_4Cl removal.
* $P < 0.01$.

To ascertain the kinetic characteristics of the inhibition by cAMP of pH_i recovery, recovery rates were measured at different pH_i values in the presence of 10 mM Na^+ in the mucosal solution. Different degrees of intracellular acidification were obtained by replacing 5–90 mM of the 90 mM TMA^+ in this solution with NH_4^+ for periods ranging from 2 to 10 min. The results obtained in eight tissues are summarized in Fig. 11. The initial rate of pH_i recovery is enhanced as pH_i decreases, both with and without theophylline, as expected. Recovery tends to saturate. Least-squares fits to the Michaelis-Menten equation indicate apparent K_m values of 81.3 ± 11.4 and 33.4 ± 4.3 nM for H_i^+ and apparent V_{max} values of 0.17 ± 0.01 and 0.08 ± 0.003 min^{-1} in control and theophylline, respectively. Interpretation of these data in terms of removal of H^+ equivalents requires knowledge of the intracellular buffering power under control conditions and in theophylline at all the pH_i values depicted in Fig. 11. Such a measurement is difficult in this preparation because the thick serosal unstirred layer prevents rapid equilibration of the NH_3 molecule throughout the
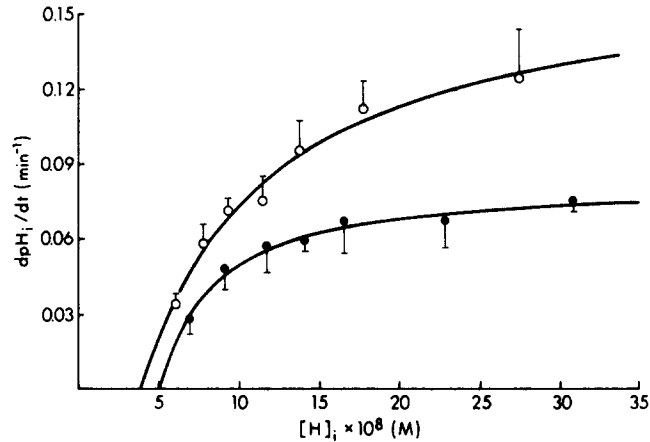
FIGURE 11. Effect of theophylline treatment on the rate of pH_i recovery from intracellular acid loads as a function of $[H]_i$. Open symbols: control data; filled symbols: theophylline data obtained in the same tissues. Each data point represents the mean of three to eight measurements. Different degrees of intracellular acidification were obtained by varying the concentration of NH_4^+ (from 5 to 90 mM) and the duration of the exposure to mucosal NH_4Cl (from 2 to 10 min). The standard errors on the [H] axis (not shown) were ≤ 0.02 pH units, with the exception of the highest control value (SEM = 0.05). The lines correspond to the least-squares fits of the data to the equation $dpH_i/dt = [dpH_i/dt(\text{max}) \times \Delta H_i]/(\Delta H_i + K'_H)$, where ΔH_i is the change in $[H^+]_i$ produced by acid loading and K'_H is the apparent K_m. The intercepts on the abscissa correspond to the mean pH_i values (control and theophylline) in the eight tissues employed in these experiments. See text.
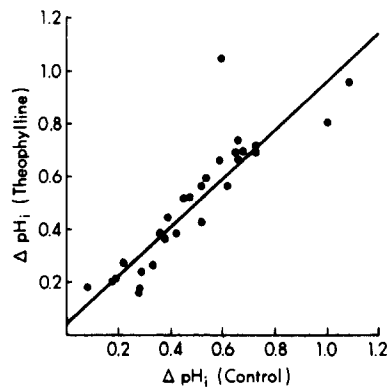

FIGURE 12. Effect of theophylline treatment on the peak alkalinization produced by exposure to NH_4Cl. Each point depicts a paired comparison with the same $[NH_4Cl]$ in a given tissue. The data were fit by the line $y = (0.05 \pm 0.04) + (0.91 \pm 0.08)x$; $r = 0.89$.

fluid phases of the preparation. However, the possibility of a difference in intracellular buffering power produced by theophylline was tested by comparing the peak intracellular alkalinization early during exposure to NH_4Cl under the two conditions. The result of this comparison is shown in Fig. 12. The slope of the line is 0.91 ± 0.08 and the y-axis intercept is 0.05 ± 0.04, which indicates an immeasurably small change in intracellular buffering power. Even if intracellular buffering power increased by ~10%, this change would be far too small to account for the differences between the curves in Fig. 11.

Theophylline treatment, exposure to NH_4Cl, and removal of this salt had striking effects on membrane voltages and the apparent ratio of membrane resistances, as illustrated in Figs. 9 and 10. The membrane voltages and the apparent ratio of cell membrane resistances (apical/basolateral) before exposure to NH_4Cl were significantly reduced by theophylline and the transepithelial voltage (lumen to bath) was significantly more negative. These results, summarized in Table VI, are in agreement with previous ones (Petersen and Reuss,

TABLE VI

Effects of Exposure to NH_4Cl on Transepithelial and Cell Membrane Voltages

	Control			Theophylline		
	V_{ms}	V_{mc}	V_{cs}	V_{ms}	V_{mc}	V_{cs}
	mV			mV		
Control	0.3±0.3	−73±2	−72±1	−1.6±0.3	−54±3	−55±2
End NH$_4$Cl	−1.6±0.2*	−61±2*	−62±2*	−4.9±0.3*	−47±4*	−52±4
Peak hyperpolarization	0.5±0.2	−93±3*	−93±3*	−1.4±0.2	−67±4*	−68±4*

Protocol as in Fig. 9; $n = 6$. * Values significantly different from control ($P < 0.05$ or better).

1983) and can be explained by the cAMP-induced increase in apical membrane P_{Cl}. During exposure to NH_4Cl, both cell membranes depolarized significantly; in theophylline, the depolarization was about half of that observed under control conditions. Upon removal of NH_4Cl, a striking transient hyperpolarization was observed in the absence of theophylline. On the average, the apical membrane voltage reached a maximum ~23 mV more negative than before NH_4Cl and returned slowly toward control. In theophylline, the hyperpolarization was smaller. In 10 mM Na$^+$, removal of NH_4Cl gave rise to similar changes in membrane voltages, but their interpretation is more difficult because of the large paracellular diffusion potential produced by the reduction of mucosal [Na$^+$]. The mechanisms of these voltage changes will be discussed below.

DISCUSSION

Results from this laboratory strongly support the notion that NaCl entry in *Necturus* gallbladder epithelial cells is due to the parallel, but independent, operation of Na$^+$/H$^+$ and Cl$^-$/HCO$_3^-$ exchangers (Weinman and Reuss, 1982, 1984; Reuss and Costantin, 1984; Reuss, 1984). This view is also held by Baerentsen et al. (1983). Spring and co-workers (Ericson and Spring, 1982; Spring and Ericson, 1982) hold that NaCl entry is the result of ternary complex

NaCl cotransport. The reasons for these differences are not clear (for a discussion, see Reuss, 1984). The inhibition of salt and fluid transport produced by cAMP in several fluid-absorbing epithelia (Field, 1979; Frizzell et al., 1979; Petersen et al., 1982) was attributed to inhibition of coupled NaCl entry (Frizzell et al., 1975; Diez de los Rios et al., 1981). In the studies of Diez de los Rios et al. on *Necturus* gallbladder, this interpretation was based on the measurement of decreases in both $a\text{Na}_i$ and $a\text{Cl}_i$ in the absence of changes in membrane voltage. However, we (Petersen and Reuss, 1983) and Duffey et al. (1981) found that cAMP causes cell depolarization and a dramatic fall in the apparent ratio of cell membrane resistances (apical/basolateral). We showed that the mechanism of these effects is an increase in apical membrane electrodiffusional Cl^- permeability (P^a_{Cl}) (Petersen and Reuss, 1983). Diez de los Rios et al. (1981) compared cell membrane voltages measured in different cells before and after 45–60 min of exposure to cAMP and did not measure the cell membrane resistance ratio. Conceivably, a 12-mV depolarization (Table IV) can be missed unless paired observations are made in the same cells. Systematic errors in the measurement of membrane voltage will of course result in errors in the calculation of intracellular ion activities. We concluded that the reduction in NaCl absorption in response to cAMP in *Necturus* gallbladder is at least in part due to the fall in intracellular Cl^- activity secondary to electrodiffusional backflux of Cl^- across the apical membrane, which causes a reduction in Cl^- transport from the cells to the serosal solution. However, the effect of cAMP on P^a_{Cl} does not explain the cessation of net transepithelial Na^+ transport. The fall in $a\text{Cl}_i$ should stimulate $\text{Cl}^-/\text{HCO}_3^-$ exchange, acidify the cell interior, and thus stimulate Na^+ entry by Na^+/H^+ exchange. Therefore, inasmuch as cAMP abolishes fluid absorption (Petersen and Reuss, 1983) and, moreover, since $a\text{Na}_i$ has been reported to fall (Diez de los Rios et al., 1981), it was reasonable to hypothesize that cAMP inhibits Na^+ entry. The experiments reported here support this hypothesis by demonstrating that cAMP inhibits Na^+/H^+ exchange.

Evidence for Inhibition of Na^+/H^+ Exchange by cAMP

(*a*) During elevation of intracellular cAMP levels, acidification of the mucosal solution, measured in 100 mM mucosal Na^+, was inhibited by ~50% (Figs. 1 and 2). Since the cation and anion exchangers operate in parallel, this might be due either to inhibition of Na^+/H^+ exchange or to stimulation of $\text{Cl}^-/\text{HCO}_3^-$ exchange and hence of the HCO_3^- flux from cells to mucosal medium. The second possibility was ruled out: (*i*) In Na^+-depleted epithelia, pH_i falls (Weinman and Reuss, 1982) and therefore $\text{Cl}^-/\text{HCO}_3^-$ exchange should decrease. However, under these conditions, theophylline again caused an ~50% reduction of luminal acidification, measured upon sudden exposure to 10 mM Na^+. In addition, the fraction of acidification sensitive to 1 mM amiloride was significantly reduced by theophylline (Fig. 2). (*ii*) We have observed that immediately upon exposure to amiloride, luminal acidification initially reverses to alkalinization (Reuss and Costantin, 1984), which indicates persistence of $\text{Cl}^-/\text{HCO}_3^-$ exchange. Hence, if theophylline were to stimulate $\text{Cl}^-/\text{HCO}_3^-$ exchange, mucosal solution alkalinization would be stimulated immediately after exposure to amiloride. Contrary

to this expectation, less alkalinization was observed (Fig. 3), which suggests that Cl^-/HCO_3^- exchange was inhibited rather than stimulated (see below). (*iii*) Finally, theophylline also inhibited mucosal acidification by ~50% after prolonged Cl^- removal from both sides of the tissue. This indicates that Cl^- is not required for the inhibition.

(*b*) Additional support for an inhibitory effect of cAMP on apical membrane Na^+/H^+ exchange was obtained from measurements of intracellular Na^+ activity. Raising intracellular cAMP produced a small, but significant, fall of $a\,Na_i$ (Table I). Inasmuch as fluid absorption is inhibited by cAMP, this fall indicates that the main effect is a reduction of entry and not inhibition of the Na^+,K^+ pump, which by itself would cause an elevation of $a\,Na_i$. It should be noted that $a\,Na_i$ fell, although the intracellular pH was lowered by theophylline (Table IV). By itself, intracellular acidification would be expected to stimulate Na^+/H^+ exchange because of the more favorable chemical gradient for H^+ efflux and because of the possibility of allosteric activation of the exchanger (Aronson et al., 1982). Intracellular Na^+ content may fall more than $a\,Na_i$ because of the likely reduction in cell volume during elevation of intracellular cAMP levels.

The experiments summarized in Table II show that the steady state rate of Na^+ entry, estimated from the initial rate of fall of $a\,Na_i$ upon reducing mucosal [Na] to 10 mM (Reuss, 1984), and also the rate of increase of $a\,Na_i$ upon restoring 100 mM Na^+ to the mucosal solution, were reduced by ~50% by theophylline treatment. Since Na^+/H^+ exchange is the main or sole mechanism of Na^+ entry (Weinman and Reuss, 1984; Reuss, 1984), the inescapable conclusion is that cAMP inhibits this process. As expected, 1 mM amiloride was less effective in changing $a\,Na_i$ during exposure to theophylline than under control conditions (Table III).

(*c*) Exposure to either 8-Br-cAMP or theophylline caused a significant steady state fall of pH_i (Table IV). As discussed above, this result cannot be ascribed to stimulation of Cl^-/HCO_3^- exchange. Theophylline also significantly reduced the rate of pH_i recovery from an acid load. As shown in Table V, the inhibition was 50% in 100 mM mucosal Na^+ and similar, i.e., 44%, in 10 mM Na^+.

During pH_i recovery from acid loading, there was a large transient hyperpolarization of both cell membranes, which was slightly delayed with respect to the recovery. As illustrated in Figs. 9 and 10 and summarized in Table VI, the hyperpolarization was smaller, but still significant, in theophylline. The apparent ratio of cell membrane resistances during the hyperpolarization was not different from that before exposure to NH_4Cl, which indicates that the ion permeability changes responsible for this hyperpolarization occurred at both membranes. A possible explanation for these voltage changes is a transient increase in $a\,Ca_i$. Berg and Ellis (1982) observed such an effect upon removal of NH_4Cl from the solution bathing sheep Purkinje fibers, and Lea and Ashley (1981) observed an increase in $a\,Ca_i$ in barnacle muscle fibers upon CO_2-induced acidification. Since in *Necturus* gallbladder there is a Ca^{2+}-dependent P_K at both membranes (Bello-Reuss et al., 1981), such an elevation of $a\,Ca_i$ would increase P_K at both barriers and thereby hyperpolarize the cells. Of course, direct measurements of $a\,Ca_i$ will be necessary to test this hypothesis.

Characteristics of the Inhibition of Na^+/H^+ Exchange

The preceding discussion demonstrates that elevating intracellular cAMP levels inhibits apical membrane Na^+/H^+ exchange. This effect is independent of the operation of the anion exchanger. The inhibition cannot be attributed to a change in the driving force for Na^+/H^+ exchange, since cAMP causes reductions in both aNa$_i$ and pH$_i$, effects that would have resulted, by themselves, in stimulation of the exchange. We conclude, therefore, that cAMP exerts its effects by altering the kinetic properties of the Na^+/H^+ exchanger itself.

The high theophylline concentration employed produced similar fractional inhibitions (~50%) of the three rate measurements made at a [Na^+] of the mucosal solution of 100 mM: the rate of mucosal acidification (Figs. 1, 3, and 4), the rate of change of aNa$_i$ upon reducing mucosal [Na^+] (Fig. 6, Table II), and the rate of pH$_i$ recovery from an acid load (Fig. 9, Table V). This agreement lends support to the view that apical membrane Na^+/H^+ exchange was inhibited, and that this process is responsible for most of the apical Na^+ influx and H^+ efflux. However, since it is possible that Cl^-/HCO_3^- exchange is inhibited by cAMP (Fig. 3), the results of the extracellular pH measurements in 109.2 mM Na^+ may well reflect a combination of two effects of cAMP. Therefore, the effect of theophylline was also examined in Na^+-depleted tissues upon exposure to 10 mM Na^+. Because Na^+ depletion acidifies the cells (Weinman and Reuss, 1982), the contribution of Cl^-/HCO_3^- exchange to the pH$_o$ change should be minimized. In addition, at this Na^+ concentration, the rate of luminal acidification is approximately half-maximal (Weinman and Reuss, 1982). Under these conditions, theophylline again reduced J_H ~50%, as it did in similar experiments in Cl-free media.

A more direct test of the mechanism of the inhibition was obtained by comparing the rates of pH$_i$ recovery upon intracellular acidification in the same tissues, with or without theophylline, at two external Na^+ concentrations. In the absence of theophylline, the rate in 10 mM Na^+ was 56% of that in 100 mM Na^+. This is in reasonable agreement with the estimate of the K_m for Na^+, ~11 mM, derived from measurements of mucosal acidification (Weinman and Reuss, 1982), and with the K_m found in proximal tubule brush border vesicles (Kinsella and Aronson, 1981). It also indicates that pH$_i$ recovery from an acid load in this epithelium is mostly or solely due to apical membrane Na^+/H^+ exchange. If other transport systems contributed to this recovery, the Na^+ dependence of pH$_i$ recovery would be less than the Na^+ dependence of luminal acidification, and thus the apparent K_m values would differ. The almost complete inhibition of pH$_i$ recovery in 10 mM Na^+ by 1 mM amiloride also supports this conclusion.

In both 100 and 10 mm Na^+, theophylline reduced the rates of pH$_i$ recovery by ~50% (Table V), which indicates that cAMP did not alter the K_m of Na^+/H^+ exchange for external Na^+.

We also observed that in 10 mM Na^+, theophylline roughly halved the maximal rate of recovery from an acid load and decreased the pH$_i$ value at which recovery occurs at a half-maximal rate (Fig. 11). Quantitative interpretation of these results is difficult because of the possibility of a cAMP-induced change in

intracellular buffering power. Such a change is likely if one considers the change of pH_i (Table VI) and the probable reduction of cell volume (see above). However, the measurements of rapid intracellular alkalinization upon exposure to NH_4Cl suggest that theophylline had no significant effect on intracellular buffering power (Fig. 12). In conclusion, these data suggest that the inhibition of Na^+/H^+ exchange by elevating intracellular cAMP involves predominantly or solely a decrease in V_{max}, rather than changes in apparent K_m for external Na^+ or internal H^+, since the concentrations of these ions that yield half-maximal recovery are unchanged in the case of the Na^+ and are decreased in the case of H^+.

Significance of the Results

The demonstration of inhibition of apical membrane Na^+/H^+ exchange by cAMP complements our study of the effect of this agent on apical membrane Cl^- transport (Petersen and Reuss, 1983). Whether both effects involve apical membrane recycling or modifications of the transport proteins involved in Cl^- electrodiffusion and Na^+/H^+ exchange remains to be determined. The explanation for the fall of intracellular activities of both Na^+ and Cl^- (Diez de los Rios et al., 1981; Petersen and Reuss, 1983; and this paper) is a dual effect of the cyclic nucleotide at the apical membrane: there is an increase in electrodiffusional Cl^- permeability, which increases the cell-to-lumen Cl^- flux, and a reduction of Na^+/H^+ exchange, which decreases the lumen-to-cell Na^+ flux. Thus, net NaCl entry is inhibited, resulting in reduced salt and water absorption. It is possible that one or both of these mechanisms of inhibition of fluid absorption operate in other salt- (and water-) absorbing epithelia. For instance, in mammalian proximal renal tubule, parathyroid hormone (PTH), which is known to increase intracellular cAMP, selectively inhibits the fraction of fluid absorption ascribable to HCO_3^- transport (Dennis, 1976), i.e., related to luminal membrane Na^+/H^+ exchange (Kinsella and Aronson, 1980; Boron and Boulpaep, 1983). In a recent preliminary communication, Kahn et al. (1984) have shown that both cAMP and PTH inhibit Na^+/H^+ exchange in apical membrane vesicles from rabbit proximal tubule. We are unaware of data supporting such a possibility in other so-called leaky epithelia.

Two observations suggest that the inhibition of fluid transport by cAMP may well involve other transport steps, either directly or indirectly. First, the fall of aNa_i observed in the experiments reported here was rather modest compared with the abolishment of fluid transport (Petersen and Reuss, 1983). Thus, cAMP may inhibit the basolateral Na,K pump as well. This could occur directly or via the intracellular acidification. Eaton et al. (1984) have recently provided evidence for a steep pH_i sensitivity of the rate of the Na,K pump in rabbit urinary bladder. Second, contrary to our expectation, based on the fall of aCl_i, Cl^-/HCO_3^- exchange appeared to be inhibited by cAMP, as suggested by the reduced mucosal solution surface alkalinization observed immediately after exposure of the mucosal surface to amiloride. A firm conclusion on this issue will require additional studies. If inhibition of Cl^-/HCO_3^- exchange does indeed occur, again the possibilities of a direct effect or one mediated by pH_i should be considered.

From the point of view of regulation of pH_i, the effect of cAMP in *Necturus* gallbladder is inhibitory, i.e., opposite to that demonstrated in barnacle muscle fibers (Boron et al., 1978). In this preparation, cAMP enhanced the recovery from an acid load by stimulating anion exchange.

In conclusion, our results indicate that elevation of cAMP levels in *Necturus* gallbladder epithelium inhibits apical membrane Na^+/H^+ exchange by a mechanism that cannot be ascribed to changes in pH_i or aNa_i. This effect is reversible and involves predominantly a reduction of V_{max}. In conjuction with the increase in apical membrane P_{Cl}, the inhibition of apical membrane cation exchange contributes to the inhibition of fluid absorption. It is possible that this mechanism accounts for effects of cAMP on fluid absorption in other epithelia and on pH_i regulation in other cell types.

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