

# Cyclic AMP Inhibits $\text{Cl}^-/\text{HCO}_3^-$ Exchange at the Apical Membrane of *Necturus* Gallbladder Epithelium

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**ABSTRACT** Intracellular microelectrode techniques were employed to study the effect of cyclic AMP on apical membrane  $\text{Cl}^-/\text{HCO}_3^-$  exchange and electrodiffusive  $\text{HCO}_3^-$  transport in *Necturus* gallbladder epithelium. Intracellular cAMP levels were raised by addition of either the phosphodiesterase inhibitor theophylline ( $3 \times 10^{-3}$  M) or the adenylate cyclase activator forskolin ( $10^{-5}$  M) to the serosal bathing solution. Measurements of pH in a poorly buffered control mucosal solution upon stopping superfusion show acidification, owing to secretion of both  $\text{H}^+$  and  $\text{HCO}_3^-$ . When the same experiment is performed after addition of amiloride or removal of  $\text{Na}^+$  from the mucosal bathing medium, alkalization is observed since  $\text{H}^+$  transport is either inhibited or reversed, whereas  $\text{HCO}_3^-$  secretion persists. The changes in pH in both amiloride or Na-free medium were significantly decreased in theophylline-treated tissues. Theophylline had no effect on the initial rates of fall of intracellular  $\text{Cl}^-$  activity ( $a\text{Cl}_i$ ) upon reducing mucosal solution  $[\text{Cl}^-]$  to either 10 or 0 mM, although membrane voltage and resistance measurements were consistent with stimulation of apical membrane electrodiffusive  $\text{Cl}^-$  permeability. Estimates of the conductive flux, obtained by either reducing simultaneously mucosal  $[\text{Cl}^-]$  and  $[\text{HCO}_3^-]$  or lowering  $[\text{Cl}^-]$  alone in the presence of a blocker of anion exchange (diphenylamine-2-carboxylate), indicate that elevation of intracellular cAMP inhibited the anion exchanger by ~50%. Measurements of net  $\text{Cl}^-$  uptake upon increasing mucosal  $\text{Cl}^-$  from nominally zero to levels ranging from 2.5 to 100 mM suggest that the mechanism of inhibition is a decrease in  $V_{\text{max}}$ . Consistent with these results, the rate of intracellular alkalization upon reducing external  $\text{Cl}^-$  was also inhibited significantly by theophylline. Reducing mucosal solution  $[\text{HCO}_3^-]$  from 10 to 1 mM under control conditions caused intracellular acidification and an increase in  $a\text{Cl}_i$ . Theophylline inhibited both changes, by 62 and 32%, respectively. These data indicate that elevation of intracellular cAMP inhibits apical membrane anion ( $\text{Cl}^-/\text{HCO}_3^-$ ) exchange. Studies of the effects of rapid changes in mucosal  $[\text{HCO}_3^-]$  on membrane voltages and the apparent ratio of membrane resistances, both in the presence and in

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the absence of theophylline, with or without  $\text{Cl}^-$  in the mucosal solution, do not support the hypothesis that cAMP produces a sizable increase in apical membrane electrodiffusive  $\text{HCO}_3^-$  permeability.

#### INTRODUCTION

Elevation of intracellular cyclic AMP inhibits net NaCl uptake across the apical membrane of gallbladder epithelial cells. This effect was first attributed to inhibition of apical membrane NaCl cotransport, on the basis of measurements of transepithelial tracer fluxes and apical membrane  $\text{Na}^+$  and  $\text{Cl}^-$  unidirectional uptakes (Frizzell et al., 1975). Support for this view was later obtained in studies in which membrane voltages and intracellular ion activities were determined before and after exposure to cAMP. The expected falls of intracellular  $\text{Na}^+$  and  $\text{Cl}^-$  activities were observed, and there were no changes in membrane voltage (Diez de los Rios et al., 1981). In contrast with these observations, Duffey et al. (1981), in a study primarily designed to assess the effect of cAMP on the junctional complexes of *Necturus* gallbladder epithelium, found a moderate depolarization of the cell membranes and a large fall in the ratio of cell membrane resistances (apical:basolateral). We subsequently confirmed these results and showed, from measurements of membrane voltages, membrane conductances, and intracellular  $\text{Cl}^-$  activity, that cAMP activates an apical membrane  $\text{Cl}^-$  conductance (Petersen and Reuss, 1983).

Although this cAMP-induced pathway can explain the reduction in  $\text{Cl}^-$  transport, the mechanism of inhibition of  $\text{Na}^+$  absorption was not obvious. In parallel with these studies, we demonstrated the existence of an  $\text{Na}^+/\text{H}^+$  exchanger at the apical membrane of *Necturus* gallbladder epithelium, whose rate under control conditions accounts for at least 75% of  $\text{Na}^+$  entry (Weinman and Reuss, 1982, 1984; Reuss, 1984). This cation exchanger operates in parallel with a  $\text{Cl}^-/\text{HCO}_3^-$  exchanger (Reuss and Costantin, 1984), which in turn accounts for the rate of transepithelial  $\text{Cl}^-$  transport (Reuss, 1984).

Since cAMP was shown to inhibit NaCl absorption by gallbladder epithelium, the demonstration that  $\text{Na}^+/\text{H}^+$  exchange is the main or sole mechanism of  $\text{Na}^+$  entry suggested that cAMP might inhibit this process. Intracellular microelectrode studies, including measurements of intracellular  $\text{Na}^+$  activity and pH, and determination of acid/base apical membrane transport from extracellular pH measurements, confirmed this hypothesis. Maximal doses of 8-bromo-cAMP, of the phosphodiesterase inhibitor theophylline, or of the adenylate cyclase activator forskolin inhibited  $\text{Na}^+/\text{H}^+$  exchange by ~50%. The effect involved a decrease in the  $V_{\text{max}}$  of the exchanger (Reuss and Petersen, 1985).

At that time, we also suggested the possibility of inhibition of  $\text{Cl}^-/\text{HCO}_3^-$  exchange by cAMP (Reuss and Petersen, 1985). This suggestion was based on the observation of a reduction, in theophylline-treated preparations, of the secretion of base equivalents into the mucosal solution observed shortly after exposure to 1 mM amiloride. Zeldin et al. (1985) recently suggested that cAMP might also elicit an  $\text{HCO}_3^-$ -conductive pathway at the apical membrane of *Necturus* gallbladder. We have argued that these conclusions are incorrect (Reuss and Stoddard, 1987; Reuss, 1987). In this article, the questions of the effects of

cAMP on apical membrane Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange and on a putative HCO<sub>3</sub><sup>-</sup>-conductive pathway are addressed directly. The results indicate that cAMP inhibits Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange, but does not induce a sizable HCO<sub>3</sub><sup>-</sup> electrodiffusive permeability.

#### METHODS

Mudpuppies (*Necturus maculosus*), purchased from Nasco Biologicals (Ft. Atkinson, WI), were kept in a large aquarium at ~5°C. The animals were anesthetized with tricaine methanesulfonate and the gallbladders were excised and mounted in a chamber as previously described (Reuss and Finn, 1975*a, b*; Weinman and Reuss, 1982). The control bathing solution, hereafter referred to as Ringer's, had the following composition (millimolar): 90 NaCl, 10 NaHCO<sub>3</sub>, 2.5 KCl, 1.8 CaCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, and 0.5 NaH<sub>2</sub>PO<sub>4</sub>; the pH was ~7.65 (gassed with a mixture of 1% CO<sub>2</sub> and 99% air), and the osmolality was ~200 mosmol/kg. Low-Cl<sup>-</sup> solutions and low-HCO<sub>3</sub><sup>-</sup> solutions were prepared by isomolar replacement of Cl<sup>-</sup> or HCO<sub>3</sub><sup>-</sup> with cyclamate. In some experiments, the solution (nominally HCO<sub>3</sub><sup>-</sup>-free) was buffered with HEPES (1 mM) and equilibrated with room air (see below). Theophylline (Sigma Chemical Co., St. Louis, MO) and forskolin (Calbiochem-Behring Corp., San Diego, CA) were employed at final concentrations of 3 × 10<sup>-3</sup> and 10<sup>-5</sup> M, respectively (serosal side only). Amiloride, added to the mucosal solution at a final concentration of 10<sup>-3</sup> M, was a generous gift of Merck, Sharpe & Dohme (West Point, PA).

#### *Measurements of Electrical Potentials*

Transepithelial ( $V_{ms}$ ) and cell membrane voltages (apical,  $V_{mc}$ ; basolateral,  $V_{cs}$ ) were measured as previously described (Reuss and Finn, 1975*a, b*; Weinman and Reuss, 1982). The reference electrode, positioned in the serosal bathing solution, was an Ag/AgCl pellet in series with a short Ringer's-agar bridge. The mucosal electrode was a flowing, saturated-KCl bridge connected to a calomel half-cell. This technique was used in order to minimize liquid junction potentials arising from changes in the mucosal bathing medium. References were the serosal side for  $V_{ms}$ , and the adjacent bathing solution for  $V_{mc}$  and  $V_{cs}$ . Transepithelial current pulses of 50 μA · cm<sup>-2</sup> (1–3 s duration) were passed by means of Ag/AgCl electrodes to measure the transepithelial resistance ( $R_t$ ) and the apparent ratio of cell membrane resistances ( $R_a/R_b$ ). Appropriate corrections were made for bathing solution series resistances. Intracellular microelectrodes were prepared from inner-fiber borosilicate glass, filled with 3 M KCl, and used within a few hours of filling. Their tip resistances ranged from 40 to 80 MΩ.

#### *Measurements of Extracellular pH*

In order to assess acid-base transport across the apical membrane, the pH of the mucosal solution was measured as previously described (Weinman and Reuss, 1982). For these experiments, gallbladders were incubated in a 1 mM HEPES-Ringer's solution pre-equilibrated with room air (pH ~7.65). The pH of the mucosal bathing solution (volume, 100–200 μl) was measured with a small glass electrode upon stopping superfusion. To estimate HCO<sub>3</sub><sup>-</sup> secretion across the apical membrane, the control solution was rapidly replaced with Na<sup>+</sup>-free medium (TMA<sup>+</sup> substitution) or with HEPES-Ringer's plus 10<sup>-3</sup> M amiloride, and superfusion was stopped (see Reuss and Costantin, 1984). The buffering powers of the solutions were determined by titration. The results, expressed as the initial rate of secretion of H<sup>+</sup> equivalents, were normalized to the acidification observed in each tissue upon stopping superfusion with the control HEPES-Ringer's solution.

### *Measurements of Intracellular Ionic Activities*

Double-barreled microelectrodes were employed to measure intracellular  $\text{Cl}^-$  activity and intracellular pH (Reuss et al., 1987). The silanized barrel was injected with Corning  $\text{Cl}^-$  exchanger 477913 (Corning Medical, Medfield, MA) or Fluka proton cocktail (Tridom/Fluka, Hauppauge, NY). The reference barrel was injected with a solution consisting of 1 M Na formate and 10 mM KCl in the  $\text{Cl}^-$ -sensitive microelectrodes, and with 3 M KCl in the pH-sensitive microelectrodes. The  $\text{Cl}^-$ -sensitive barrel and the pH-sensitive barrel were backfilled with Ringer's and with the solution recommended by Amman et al. (1981), respectively. Electrical connections were made with Ag/AgCl wires. Microelectrode calibration, impalement validation, calculations of the activity coefficients of the solutions, and calculations of intracellular  $\text{Cl}^-$  activity and intracellular pH were made as described before (Reuss et al., 1983; Reuss, 1984; Weinman and Reuss, 1984).

### *Statistics*

Results are presented as means  $\pm$  SEM. Unless stated otherwise, statistical comparisons were made by conventional *t* tests on paired data. A value of  $p < 0.05$  was considered significant.

## RESULTS

### *Extracellular pH Measurements*

A modification of the technique described by Weinman and Reuss (1982) was used to assess the rate of  $\text{Cl}^-/\text{HCO}_3^-$  exchange under control conditions and after elevating intracellular cAMP levels by exposure to theophylline. The results are summarized in Fig. 1. As observed previously, stopping superfusion with a control NaCl-HEPES medium elicited mucosal solution acidification, which was inhibited by theophylline (Reuss and Petersen, 1985). In order to unmask the  $\text{HCO}_3^-$  efflux into the mucosal medium, the apical surface was exposed for 1 min to a nominally  $\text{Na}^+$ -free HEPES-Ringer's solution (TMA<sup>+</sup> substitution) or to control HEPES-Ringer's plus  $10^{-3}$  M amiloride. The superfusion was stopped and the change in solution pH was measured for 1–3 min. From the initial rate of change of the extracellular pH and the buffering power of the solution, an apparent  $\text{H}^+$  flux ( $\Delta\text{H}$ ) was computed and normalized to that determined under control conditions (NaCl HEPES-Ringer's, before theophylline). In the absence of theophylline, both amiloride and  $\text{Na}^+$ -free medium reversed the luminal acidification to luminal alkalization, as previously reported (Reuss and Costantin, 1984). The larger alkalization observed in  $\text{Na}^+$ -free medium compared with amiloride is due to reversal of the  $\text{Na}^+/\text{H}^+$  exchange in the former condition. In theophylline, the  $\Delta\text{H}$  in amiloride was reduced by 50%, and that in  $\text{Na}^+$ -free medium fell by ~26%, compared with the respective values in the absence of theophylline. Most significantly, the  $\text{Cl}^-$ -dependent alkalization ( $\Delta\text{H}$  in TMA Cl minus  $\Delta\text{H}$  in TMA cyclamate) was decreased by 63%. These results are consistent with the hypothesis that the elevation of intracellular cAMP levels inhibits apical membrane  $\text{Cl}^-/\text{HCO}_3^-$  exchange. It must be noted, however, that the ionic composition of the intracellular compartment is different in control and in theophylline-treated preparations (Petersen and Reuss, 1983; Reuss and Petersen, 1985). Therefore, it is possible that the reduction in  $\text{HCO}_3^-$  efflux in these experiments is due to a reduction of the net driving force for anion exchange. To test this

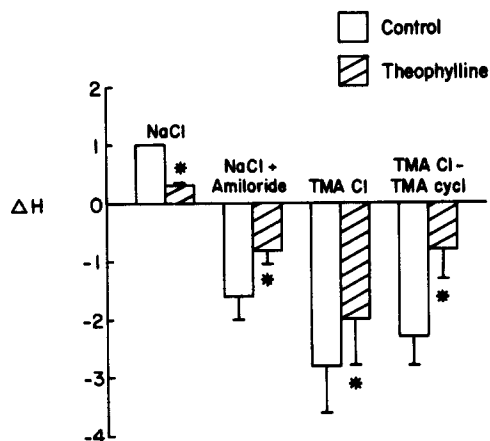


FIGURE 1. Initial apparent fluxes of H<sup>+</sup> equivalents into the mucosal bath. Open bars: control conditions (see Methods). Hatched bars: pretreatment with theophylline ( $3 \times 10^{-3}$  M serosal side) for at least 30 min. From the initial rate of change of the pH of the mucosal solution upon stopping superfusion ( $\Delta\text{pH}_o$ ) and the buffering power of the solution ( $\beta$ ), the apparent net H<sup>+</sup> flux ( $\Delta\text{H}$ ) was calculated ( $\Delta\text{H} = -\Delta\text{pH}_o/\beta$ ). The data were normalized in each tissue to the value observed in Na<sup>+</sup> Ringer's in the absence of theophylline ( $\Delta\text{H} = 1.0$ ). Means  $\pm$  SEM for six experiments are shown. Upward bars denote mucosal solution acidification. Theophylline causes significant decreases of the mucosal solution acidification in Na<sup>+</sup> Ringer's, and the alkalization in amiloride-containing or Na<sup>+</sup>-free medium. The Cl<sup>-</sup>-dependent alkalization (TMA Cl - TMA cyclamate) is also significantly reduced.

possibility,  $\text{pH}_i$  and  $a\text{Cl}_i$  were measured in tissues bathed in the same solutions employed in the  $\text{pH}_o$  studies, both under control conditions and after exposure to theophylline. The results are summarized in Table I. Theophylline reduced  $a\text{Cl}_i$  from 18.3 to 12.4 mM, and  $\text{pH}_i$  from 7.54 to 7.50. The ratio of driving forces for HCO<sub>3</sub><sup>-</sup> secretion (theophylline:control) is given by

$$\frac{a\text{Cl}_i(\text{control})}{a\text{Cl}_i(\text{theo})} \exp[\text{pH}_i(\text{theo}) - \text{pH}_i(\text{control})].$$

The result of the calculation is 1.35. In other words, in the presence of theophylline, the driving force is more favorable for HCO<sub>3</sub><sup>-</sup> secretion. Therefore, the effect of theophylline on the initial rate of luminal alkalization cannot be attributed to a reduced driving force.

TABLE I  
*Effects of Theophylline on Membrane Voltages and Resistances,  
 $a\text{Cl}_o$  and  $\text{pH}_i$  in Tissues Bathed in 1 mM HEPES-Ringer's*

Condition	$V_m$ mV	$V_{mc}$ mV	$V_o$ mV	$R_o/R_b$	$a\text{Cl}_i$ mM	$\text{pH}_i$	$R_i$ $\Omega\text{-cm}^2$
Control	$0.1 \pm 0.3$	$-63 \pm 1$	$-63 \pm 1$	$1.22 \pm 0.36$	$18.3 \pm 1.4$	$7.54 \pm 0.03$	$198 \pm 5$
Theophylline	$-1.7 \pm 0.5$	$-46 \pm 4$	$-47 \pm 4$	$0.11 \pm 0.06$	$12.4 \pm 2.4$	$7.50 \pm 0.03$	$200 \pm 21$
Difference	$-1.8 \pm 0.2^*$	$17 \pm 4^*$	$15 \pm 4^*$	$-1.11 \pm 0.32^*$	$-5.9 \pm 2.2^*$	$-0.04 \pm 0.003^*$	$62 \pm 23^*$

Values in theophylline were measured at 15–20 min.  $a\text{Cl}_i$ :  $n = 5$ ;  $\text{pH}_i$ :  $n = 3$ .  
\* The difference (theophylline - control) is significantly different from zero.

*Effects of cAMP on the Changes in Intracellular  $\text{Cl}^-$  Activity Elicited by Changes in Luminal  $[\text{Cl}^-]$*

It has been previously shown (Petersen and Reuss, 1983) that cAMP causes a fall in  $a\text{Cl}_i$  to values near electrochemical equilibrium in tissues incubated in a low-buffering-power solution. In a 10 mM  $\text{HCO}_3^-$  Ringer's, a similar result was observed, as illustrated by the experiment depicted in Fig. 2, in which forskolin was added to the luminal bathing solution to a final concentration of  $10^{-5}$  M. Once cAMP has caused an increase in apical membrane electrodiffusive  $P_{\text{Cl}}$ , a

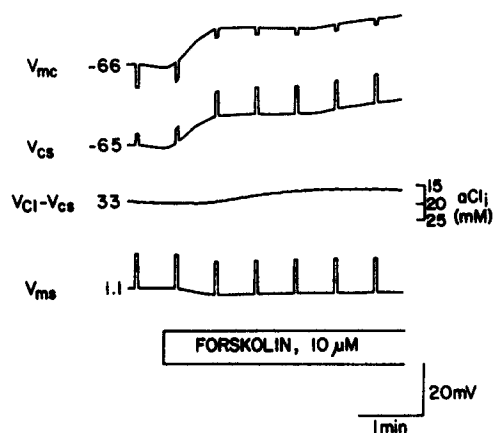


FIGURE 2. Effects of forskolin ( $10^{-5}$  M, mucosal side) on membrane voltages and resistances and intracellular  $\text{Cl}^-$  activity ( $a\text{Cl}_i$ ) in a tissue incubated in control Ringer's solution. In this and in the following figures, the cell membrane voltages (apical,  $V_{mc}$ ; basolateral,  $V_{cs}$ ) and the transepithelial voltage ( $V_{ms}$ ) are shown. Their starting values are indicated at the beginning of each trace (references:  $V_{ms}$ , serosal solution;  $V_{mc}$  and  $V_{cs}$ , external solution adjacent to the membrane). Current pulses of  $50 \mu\text{A} \cdot \text{cm}^{-2}$  and 3 s duration were applied at a frequency of 2/min. The transepithelial resistance ( $R_t$ ) and the apparent ratio of cell membrane resistances ( $R_a/R_b$ ) (see Boulpaep and Sackin, 1980) can be calculated after correction for series resistance. The trace denoting  $a\text{Cl}_i$  is the difference between the voltage outputs of the  $\text{Cl}^-$ -sensitive and reference barrels of a double-barreled microelectrode. Theophylline causes a slow depolarization of both cell membranes, a mucosal side-negative change in transepithelial voltage, an increase in  $R_t$ , a decrease in  $R_a/R_b$ , and a fall in  $a\text{Cl}_i$  toward equilibrium levels. These changes are similar to those elicited by theophylline in low- $\text{HCO}_3^-$  media (see Petersen and Reuss, 1983).

sudden reduction of extracellular  $[\text{Cl}^-]$  should cause conductive  $\text{Cl}^-$  exit and, depending on the magnitude of the reduction of external  $[\text{Cl}^-]$ , either a decrease in  $\text{Cl}^-$  entry via anion exchange or a net  $\text{Cl}^-$  efflux by this pathway. If the rate of anion exchange is unchanged by theophylline, reducing external  $[\text{Cl}^-]$  in theophylline-treated tissues would be expected to result in a faster fall of  $a\text{Cl}_i$  than in untreated preparations. As shown in Fig. 3, under control conditions, reducing  $[\text{Cl}^-]$  to 10 mM causes slow cell membrane hyperpolarization and a rapid fall in  $a\text{Cl}_i$ . In theophylline, the membrane voltage changes are strikingly

different: a rapid depolarization of both cell membranes is observed upon lowering [Cl<sup>-</sup>], which is followed by relaxation toward the preceding voltage; upon restoration of mucosal [Cl<sup>-</sup>] to control levels, a fast hyperpolarization is seen, followed by a slow depolarization to the control values. The initial rate of fall of  $a\text{Cl}_i$ , however, does not differ significantly from that seen under control conditions.

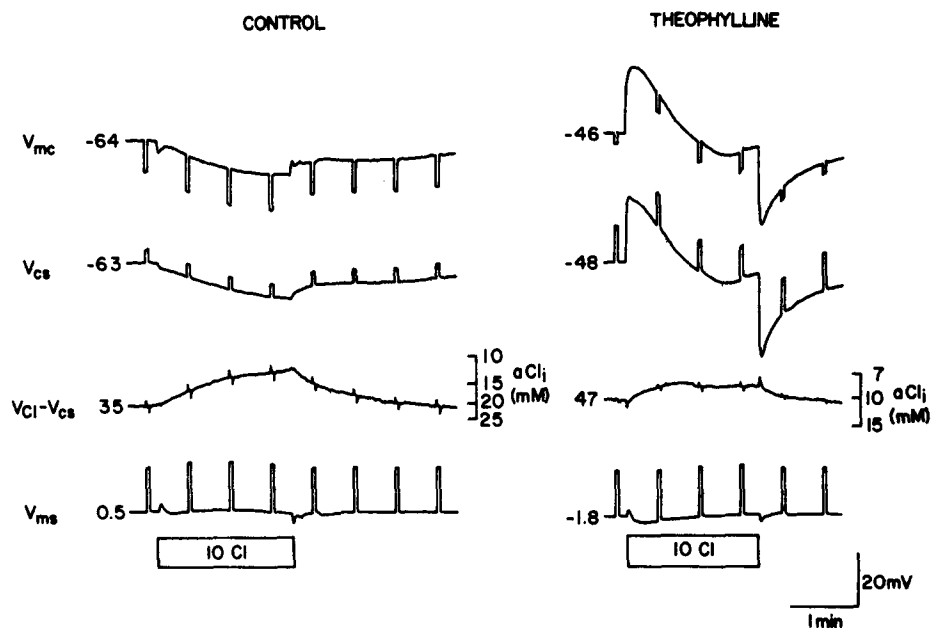


FIGURE 3. Effects of reducing mucosal solution [Cl<sup>-</sup>] to 10 mM on membrane voltages, resistances, and  $a\text{Cl}_i$ , under control conditions and in theophylline. Both sets of traces were obtained in the same preparation, before and ~20 min after exposure to theophylline. Under control conditions, the low-Cl<sup>-</sup> medium causes hyperpolarization of both cell membranes, little change in  $V_{ms}$ ,  $R_i$ , or  $R_a/R_b$ , and a rapid fall of  $a\text{Cl}_i$ . In theophylline, the same maneuver causes rapid depolarization of both cell membranes, followed by repolarization during the period of exposure to the low-Cl<sup>-</sup> medium, and a spiking, transient hyperpolarization upon returning to control solution. In contrast with the control traces,  $R_a/R_b$  is very low in theophylline and rises when luminal [Cl<sup>-</sup>] is lowered. The initial rates of fall of  $a\text{Cl}_i$  are similar in the two conditions. See Table II.

These voltage and activity changes are qualitatively similar to those previously observed in tissues bathed in low-buffering-power solutions, but the magnitude of the voltage and  $a\text{Cl}_i$  changes is much greater in 10 mM HCO<sub>3</sub><sup>-</sup> medium (compare Fig. 9, Petersen and Reuss, 1983). As seen also in Fig. 3, the apparent ratio of membrane resistances is high in control conditions, and does not change appreciably during exposure to the 10 mM Cl<sup>-</sup> solution. In theophylline, the membrane resistance ratio is very low in high-Cl<sup>-</sup> medium and increases in the low-Cl<sup>-</sup> solution. Summaries of voltage, resistance, and  $a\text{Cl}_i$  data are presented

in Table II. These results are consistent with the induction of an apical membrane  $\text{Cl}^-$ -conductive pathway. Although the  $\text{Cl}^-$  conductance is evident only in theophylline-treated tissues, the initial rate of fall of  $a\text{Cl}_i$  upon lowering luminal solution  $[\text{Cl}^-]$  is not different from control. Consistent results were obtained when the luminal  $[\text{Cl}^-]$  was reduced to nominally zero. In six such experiments, the initial rates of fall of  $a\text{Cl}_i$  were  $27.7 \pm 5.7$  and  $33.2 \pm 9.6$  mM/min, under control conditions and in theophylline, respectively.

These results suggest the possibility that in theophylline-treated tissues the additional  $\text{Cl}^-$  efflux via the conductive pathway is compensated by a reduced electroneutral efflux via the exchanger. However, interpretation of these observations is difficult because the  $a\text{Cl}_i$  values immediately before lowering external  $[\text{Cl}^-]$  are different under control conditions and in theophylline (see Table II). To obtain a more direct indication of the rates of  $\text{Cl}^-$  via the exchanger and via

TABLE II  
*Effects of Reducing Luminal  $[\text{Cl}^-]$  to 10 mM on Membrane Voltages and Resistances and  $a\text{Cl}_i$  under Control Conditions and during Exposure to Theophylline*

Condition	$V_{ms}$ mV	$V_{mc}$ mV	$V_{cs}$ mV	$R_a/R_b$	$R_t$ $\Omega \cdot \text{cm}^2$	$a\text{Cl}_i$ mM	$\Delta a\text{Cl}_i/\Delta t$ mM/min
Control							
98 Cl	$0.3 \pm 0.2$	$-65 \pm 1$	$-65 \pm 1$	$5.20 \pm 0.78$	$178 \pm 21$	$18.7 \pm 1.4$	
10 Cl	$0.4 \pm 0.3$	$-72 \pm 2^*$	$-72 \pm 2^*$	$6.48 \pm 1.15$	$200 \pm 23^*$	$11.5 \pm 1.3^*$	$-11.9 \pm 1.7$
Difference	$0.1 \pm 0.2$	$-7 \pm 2$	$-7 \pm 3$	$1.27 \pm 0.76$	$22 \pm 3$	$-7.3 \pm 1.1$	
Theophylline							
98 Cl	$-1.5 \pm 0.2^\ddagger$	$-50 \pm 3^\ddagger$	$-51 \pm 3^\ddagger$	$0.09 \pm 0.06^\ddagger$	$205 \pm 23^\ddagger$	$11.1 \pm 1.1^\ddagger$	
10 Cl	$-1.5 \pm 0.3^\ddagger$	$-45 \pm 3^\ddagger$	$-47 \pm 3^\ddagger$	$0.31 \pm 0.11^{*\ddagger}$	$229 \pm 25^{*\ddagger}$	$8.0 \pm 1.3^*$	$-11.6 \pm 1.7$
Difference	$0.1 \pm 0.4$	$5 \pm 3^\ddagger$	$5 \pm 2^\ddagger$	$0.22 \pm 0.07$	$24 \pm 5$	$-3.1 \pm 0.5^\ddagger$	

Values in 10 mM  $\text{Cl}^-$  were measured at 2 or 3 min. "Difference" is values in 10 mM  $\text{Cl}^-$  minus values in 98 mM  $\text{Cl}^-$ .  $\Delta a\text{Cl}_i/\Delta t$  represents the initial rate of the fall of  $a\text{Cl}_i$  upon reduction in mucosal  $[\text{Cl}^-]$  to 10 mM.  $n = 6$ .

\* Significantly different from values in 98 mM  $\text{Cl}^-$ .

‡ Significantly different from the corresponding value in the absence of theophylline.

the conductive pathway, experiments were carried out in which mucosal solution  $[\text{Cl}^-]$  and  $[\text{HCO}_3^-]$  were reduced simultaneously to 10% of the control values. In theory, in this experiment, the net driving force for anion exchange should not change, whereas that for conductive  $\text{Cl}^-$  efflux should increase by 58 mV ( $\Delta\bar{\mu}/F$ ). As shown in Fig. 4 and summarized in Table III, under control conditions, this maneuver causes no significant fall of  $a\text{Cl}_i$  ( $\Delta a\text{Cl}_i/\Delta t = -0.3 \pm 0.5$  mM/min), an observation consistent with the assumption that the net fluxes via the exchanger are unchanged under these conditions. Although the peak changes in  $V_{mc}$  were the same when reducing mucosal  $[\text{Cl}^-]$  alone or mucosal  $[\text{Cl}^-]$  and  $[\text{HCO}_3^-]$  ( $28 \pm 2$  and  $27 \pm 1$  mV, respectively), after treatment with theophylline or forskolin, the initial rate of change of  $a\text{Cl}_i$  was  $-6.5 \pm 1.3$  mM/min, in contrast with the rate measured, also in theophylline, upon lowering  $[\text{Cl}^-]$  to 10 mM at constant  $[\text{HCO}_3^-]$ , which was  $-11.6 \pm 1.7$  mM/min. The difference, which



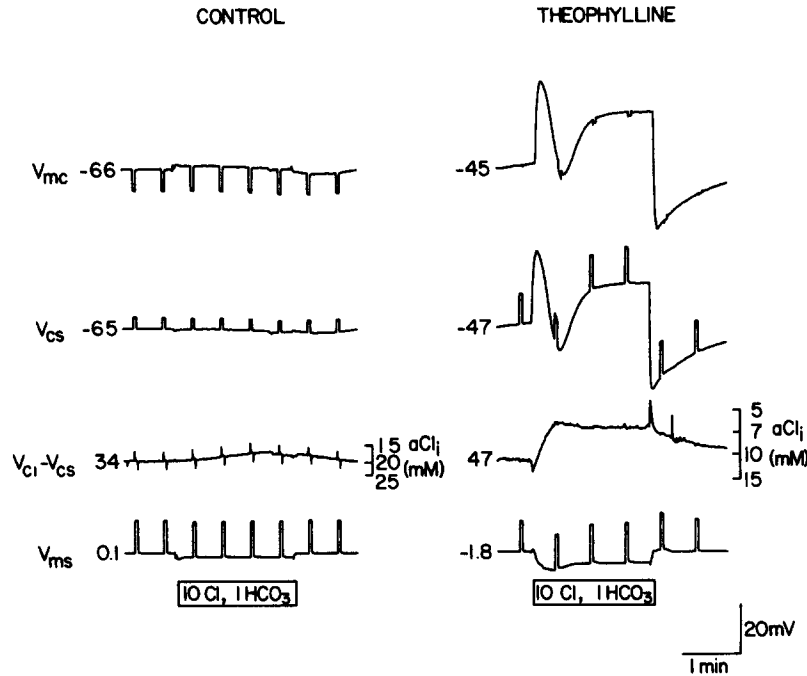


FIGURE 4. Effects of simultaneous reductions of mucosal solution  $[Cl^-]$  and  $[HCO_3^-]$  on membrane voltages and resistances and  $aCl_i$ , under control conditions and after pretreatment with forskolin ( $10^{-5}$  M, serosal side). Symbols and protocol as in Fig. 2. See Table III.

estimates the nonconductive initial rate of  $Cl^-$  loss upon lowering luminal  $[Cl^-]$  to 10 mM, is  $-5.1$  mM/min. Therefore, in theophylline,  $\sim 56\%$  of the fall in  $aCl_i$  upon reducing luminal solution  $[Cl^-]$  appears to be via the  $Cl^-$ -conductive pathway. Analogously, the initial rate of nonconductive  $Cl^-$  loss in the absence of theophylline is estimated to be  $-11.9$  mM/min (the conductive  $Cl^-$  loss under

TABLE III

*Effects of Simultaneous Reductions in Luminal  $[Cl^-]$  and  $[HCO_3^-]$  on Membrane Voltages and Resistances and  $aCl_i$  under Control Conditions and during Exposure to Theophylline*

Condition	$V_m$ mV	$V_{mc}$ mV	$V_{cs}$ mV	$R_a/R_o$	$R_i$ $\Omega \cdot cm^2$	$aCl_i$ mM	$\Delta aCl_i/\Delta t$ mM/min
Control							
98 Cl/10 HCO <sub>3</sub>	$0.4 \pm 0.1$	$-67 \pm 3$	$-67 \pm 3$	$6.51 \pm 1.87$	$157 \pm 8$	$18.5 \pm 2.3$	
10 Cl/1 HCO <sub>3</sub>	$-2.4 \pm 0.7^*$	$-64 \pm 2^*$	$-66 \pm 3$	$8.19 \pm 1.81^*$	$174 \pm 10^*$	$18.7 \pm 2.5$	$-0.3 \pm 0.5$
Difference	$-2.8 \pm 0.8$	$4 \pm 1$	$1 \pm 1$	$1.69 \pm 0.30$	$17 \pm 4$	$0.1 \pm 0.4$	
Theophylline							
98 Cl/10 HCO <sub>3</sub>	$-1.2 \pm 0.4^\ddagger$	$-45 \pm 2^\ddagger$	$-47 \pm 2^\ddagger$	$0.07 \pm 0.01^\ddagger$	$214 \pm 24$	$12.9 \pm 1.5$	
10 Cl/1 HCO <sub>3</sub>	$-5.9 \pm 0.7^{*\ddagger}$	$-28 \pm 2^{*\ddagger}$	$-34 \pm 2^{*\ddagger}$	$0.11 \pm 0.04^\ddagger$	$238 \pm 28$	$8.7 \pm 1.5^{*\ddagger}$	$-6.5 \pm 1.3^\ddagger$
Difference	$-4.7 \pm 1.1^\ddagger$	$17 \pm 4^\ddagger$	$13 \pm 3^\ddagger$	$0.05 \pm 0.04^\ddagger$	$24 \pm 8$	$-4.2 \pm 0.6^\ddagger$	

Values in 10  $Cl^-$ , 1  $HCO_3^-$  solution were measured at 1 min.  $\Delta aCl_i/\Delta t$  represents the initial rate of fall of  $aCl_i$  upon lowering luminal  $[Cl^-]$  and  $[HCO_3^-]$ .  $n = 4$ . For other symbols, see Table II.

these conditions is immeasurably small). Since the average intracellular  $\text{Cl}^-$  activities before reducing mucosal  $\text{Cl}^-$  were 18.7 and 11.1 mM, the rate coefficients for the  $a\text{Cl}_i$  changes mediated by nonconductive pathways are 0.64 and  $0.46 \text{ min}^{-1}$  in control and theophylline, respectively.

Further support for an inhibition of  $\text{Cl}^-/\text{HCO}_3^-$  exchange was obtained from estimates of the initial rate of  $\text{Cl}^-$  entry as a function of luminal  $[\text{Cl}^-]$ . In these experiments, both under control conditions and after pretreatment with either theophylline or forskolin,  $a\text{Cl}_i$  was first reduced by exposure to a  $\text{Cl}^-$ -free luminal bathing solution. As shown in Fig. 5, elevating luminal  $[\text{Cl}^-]$  to 10 or 50 mM resulted in rapid increases in  $a\text{Cl}_i$ . The rate of change of  $a\text{Cl}_i$  was greater after

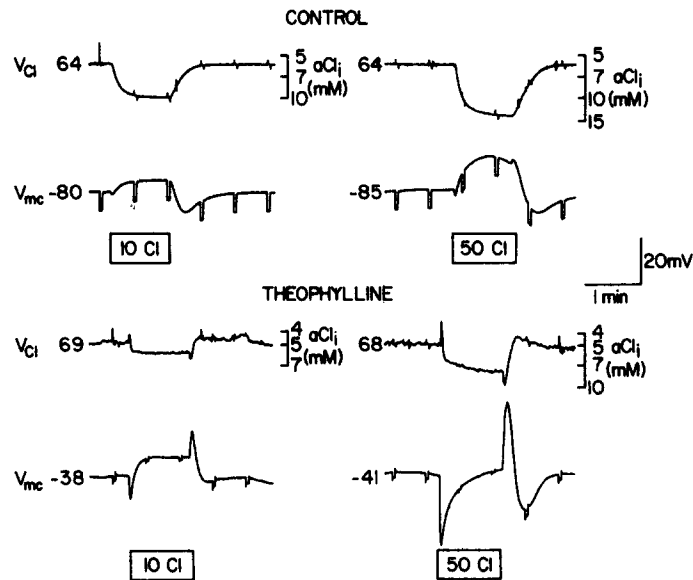


FIGURE 5. Effects of elevating mucosal solution  $[\text{Cl}^-]$  from nominally 0 to 10 and 50 mM on  $V_{\text{mc}}$  and  $a\text{Cl}_i$ , under control conditions and in theophylline. In both conditions, the preparation was exposed to a  $\text{Cl}^-$ -free mucosal bathing solution until a steady state reduction of  $a\text{Cl}_i$  was obtained.

theophylline, particularly at the higher  $\text{Cl}^-$  concentration. The kinetics of this process, for four experiments, are shown in Fig. 6. The apparent  $\text{Cl}^-$  influx, measured as  $\Delta a\text{Cl}_i/\Delta t$  during the first 2 s, was normalized in each tissue to the value observed when  $[\text{Cl}^-]$  was raised to 100 mM under control conditions. In control tissues, there was a hyperbolic saturating relationship between influx and luminal  $[\text{Cl}^-]$ . Fitting the data to the Michaelis-Menten equation yielded a normalized  $V_{\text{max}}$  of 1.07 and an apparent  $K_m$  of 11.3 mM. After elevation of cAMP levels, the results could be fitted by an equation containing a Michaelis-Menten term and a linear term.  $V_{\text{max}}$  and  $K_m$  were reduced to 0.42 and 0.7 mM, respectively. The quantitative aspects of these measurements will be discussed in more detail below; taken at face value, they support the conclusion that cAMP

inhibits  $\text{Cl}^-/\text{HCO}_3^-$  exchange, i.e., the saturating component observed after forskolin treatment, and that the mechanism of this effect is a decrease in  $V_{\text{max}}$ .

*Effects of cAMP on the Changes in Intracellular pH Elicited by Lowering Mucosal  $[\text{Cl}^-]$*

Experiments were carried out as described in the preceding section, but  $\text{pH}_i$  was measured instead of  $a\text{Cl}_i$ . As shown in Fig. 7 and summarized in Table IV, the change in  $\text{pH}_i$  elicited by decreasing mucosal solution  $[\text{Cl}^-]$  to 10 mM was reduced in theophylline, compared with control conditions. From these data, the rate of change of intracellular  $\text{HCO}_3^-$  activity can be estimated, using the initial

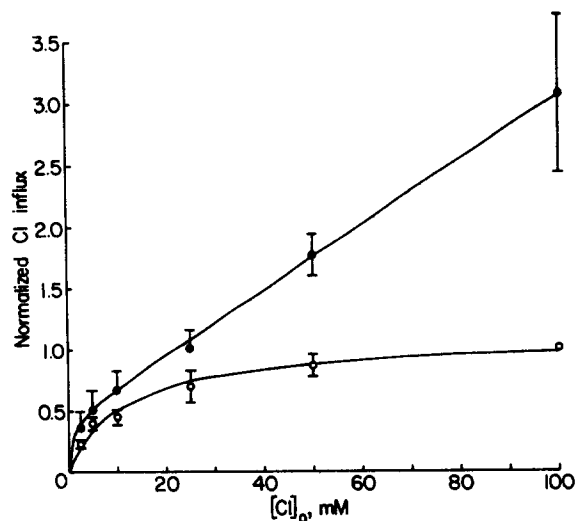


FIGURE 6. Initial apparent rates of rise of  $a\text{Cl}_i$  upon elevating mucosal solution  $[\text{Cl}^-]$  from nominally zero to 2.5, 5, 10, 25, 50, or 100 mM, under control conditions (O) and in theophylline- or forskolin-treated tissues (●). Values are means  $\pm$  SEM of four experiments, normalized to the control value of  $\Delta a\text{Cl}_i/\Delta t$  with 100 mM  $\text{Cl}^-$ . The curves are the least-squares fits to the Michaelis-Menten equation (control data) and the Michaelis-Menten equation plus a linear component (theophylline data). See text.

rate of change of  $\text{pH}_i$ , the total buffering power ( $\beta_T$ ), and an intracellular activity coefficient of 0.75.  $\beta_T$  was calculated as the sum of the nonbicarbonate buffering power measured by elevating external  $\text{CO}_2$  from 1 to 5% (27 mM; Reuss, L., unpublished results) and the  $\text{CO}_2/\text{HCO}_3^-$  buffering power, equal to 2.3  $[\text{HCO}_3^-]_i$  (Roos and Boron, 1981). It is also assumed that the intracellular  $P_{\text{CO}_2}$  is equal to that in the extracellular fluid and that the nonbicarbonate intracellular buffering power is unchanged by treatment with theophylline (Reuss and Petersen, 1985). Under control conditions, the initial rate of rise of intracellular  $\text{HCO}_3^-$  activity upon lowering external  $[\text{Cl}^-]$  to 10 mM was  $14.3 \pm 3.4$  mM/min, which is not statistically different from the initial rate of change of  $a\text{Cl}_i$  in the

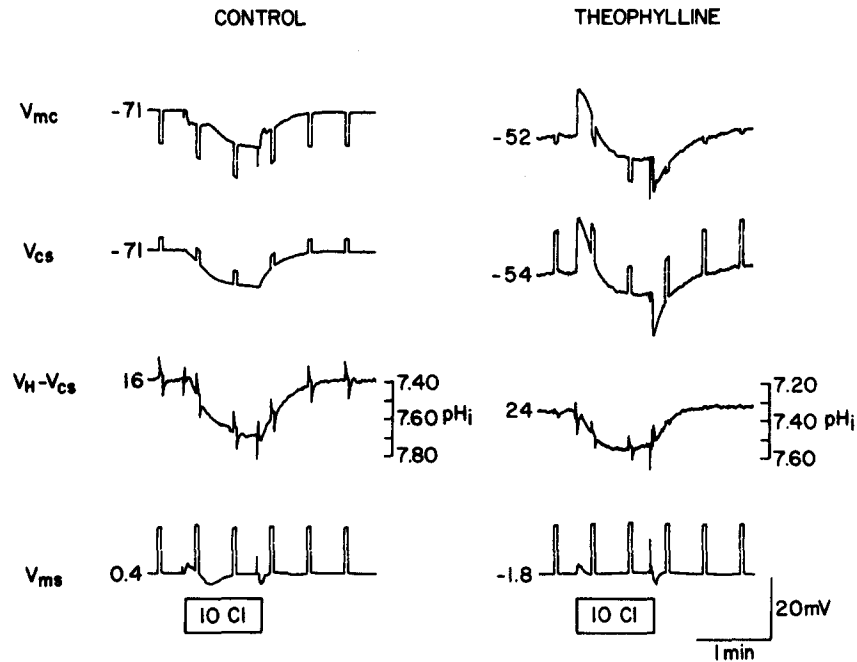


FIGURE 7. Effects of reducing mucosal  $[Cl^-]$  to 10 mM on membrane voltages and resistances and  $pH_i$  (measured with a double-barreled, liquid-membrane, pH-sensitive microelectrode). Voltage and resistance changes are similar to those in Fig. 3. Note that the initial rate of cell alkalinization is reduced by theophylline. See Table IV.

analogous experiment (Table II). These results support the idea that the major mechanism of apical membrane  $Cl^-$  and  $HCO_3^-$  transport in these conditions is electroneutral (1:1)  $Cl^-/HCO_3^-$  exchange. In theophylline, the initial rate of rise of intracellular  $HCO_3^-$  activity decreased significantly to  $8.7 \pm 1.9$  mM/min (Table IV). The fact that theophylline alters the  $HCO_3^-$  influx while the  $Cl^-$

TABLE IV  
Effects of Reducing Luminal  $[Cl^-]$  to 10 mM on Membrane Voltages and Resistances and  $pH_i$  under Control Conditions and during Exposure to Theophylline

Condition	$V_m$ mV	$V_{mc}$ mV	$V_{cs}$ mV	$R_o/R_b$	$R_i$ $\Omega \cdot cm^2$	$pH_i$	$\Delta pH_i/\Delta t$ $min^{-1}$	$\Delta \Delta HCO_3^-/\Delta t$ mM/min
Control								
98 Cl	$0.1 \pm 0.1$	$-68 \pm 1$	$-68 \pm 1$	$6.18 \pm 1.31$	$198 \pm 14$	$7.39 \pm 0.06$		
10 Cl	$-1.1 \pm 1.0$	$-79 \pm 2^*$	$-80 \pm 1^*$	$5.72 \pm 1.39$	$202 \pm 11$	$7.60 \pm 0.06^*$	$0.51 \pm 0.12$	$14.3 \pm 3.4$
Difference	$1.2 \pm 1.0$	$-11 \pm 1$	$-12 \pm 1$	$-0.46 \pm 0.98$	$4 \pm 4$	$0.21 \pm 0.03$		
Theophylline								
98 Cl	$-1.8 \pm 0.3^*$	$-49 \pm 3^*$	$-51 \pm 3^*$	$0.14 \pm 0.05$	$231 \pm 29^*$	$7.34 \pm 0.06$		
10 Cl	$-3.6 \pm 0.8^*$	$-49 \pm 4^*$	$-53 \pm 3^*$	$0.57 \pm 0.25^*$	$240 \pm 24^*$	$7.43 \pm 0.07^*$	$0.32 \pm 0.07^*$	$8.7 \pm 1.9^*$
Difference	$-1.8 \pm 1.0$	$0 \pm 2^*$	$-2 \pm 2^*$	$0.43 \pm 0.20$	$9 \pm 5$	$0.12 \pm 0.04^*$		

Values in 10 mM  $Cl^-$  were measured at 2 or 3 min.  $\Delta pH_i/\Delta t$  represents the initial rate of rise of  $pH_i$  upon reduction in mucosal  $[Cl^-]$  to 10 mM.  $\Delta \Delta HCO_3^-/\Delta t$  was calculated as explained in the text, and  $\Delta pH_i/\Delta t$  has been defined above.  $n = 6$ . For other symbols, see Table II.

efflux is unchanged suggests that an HCO<sub>3</sub><sup>-</sup>-independent Cl<sup>-</sup> efflux mechanism is operative when intracellular cAMP levels are raised.

In summary, theophylline reduces the initial rate of change in *a*HCO<sub>3i</sub> elicited by reducing luminal [Cl<sup>-</sup>]. This result strongly supports the conclusion that cAMP inhibits apical membrane Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange.

*Effects of Reduction of Mucosal [HCO<sub>3</sub><sup>-</sup>] on *a*Cl<sub>i</sub> and *p*H<sub>i</sub> under Control Conditions and in Theophylline*

If cAMP inhibits Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange, then the changes in *p*H<sub>i</sub> and *a*Cl<sub>i</sub> produced by lowering external [HCO<sub>3</sub><sup>-</sup>] (at constant *P*<sub>CO<sub>2</sub></sub>) should be less in theophylline, compared with control. Typical experimental records are shown in Figs. 8 and 9, and the data from all of the experiments are summarized in Tables V and VI.

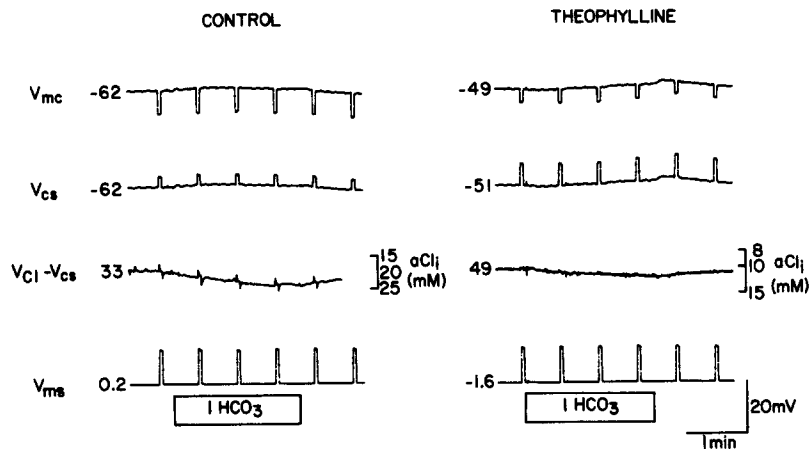


FIGURE 8. Effects of reducing mucosal [HCO<sub>3</sub><sup>-</sup>] to 1 mM on membrane voltages and resistances and *a*Cl<sub>i</sub>. Under control conditions and in theophylline, lowering mucosal [HCO<sub>3</sub><sup>-</sup>] causes a small depolarization of both cell membranes, with no appreciable change in *R<sub>a</sub>*/*R<sub>b</sub>* or *R<sub>i</sub>*. The initial rate of rise of *a*Cl<sub>i</sub> is reduced by theophylline. See Table V.

Consistent with the preceding data, which suggested inhibition of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange in theophylline, the initial rates of change of *a*Cl<sub>i</sub> and *p*H<sub>i</sub> in response to lowering mucosal [HCO<sub>3</sub><sup>-</sup>] were significantly reduced. A calculation of the initial rates of fall of *a*HCO<sub>3i</sub> in these experiments yields values of 6.2 ± 0.8 and 4.0 ± 0.8 mM/min, in the absence and presence of theophylline, respectively. Although these results are consistent with an effect of cAMP in Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange, they are subject to alternative interpretations. First, it is conceivable that the changes in *p*H<sub>i</sub> upon lowering external [HCO<sub>3</sub><sup>-</sup>] are totally or partly the result of a change in the rate and/or direction of Na<sup>+</sup>/H<sup>+</sup> exchange (see Discussion). Second, the elevation of *a*Cl<sub>i</sub> could be underestimated by conductive efflux of the Cl<sup>-</sup> that enters via anion exchange.

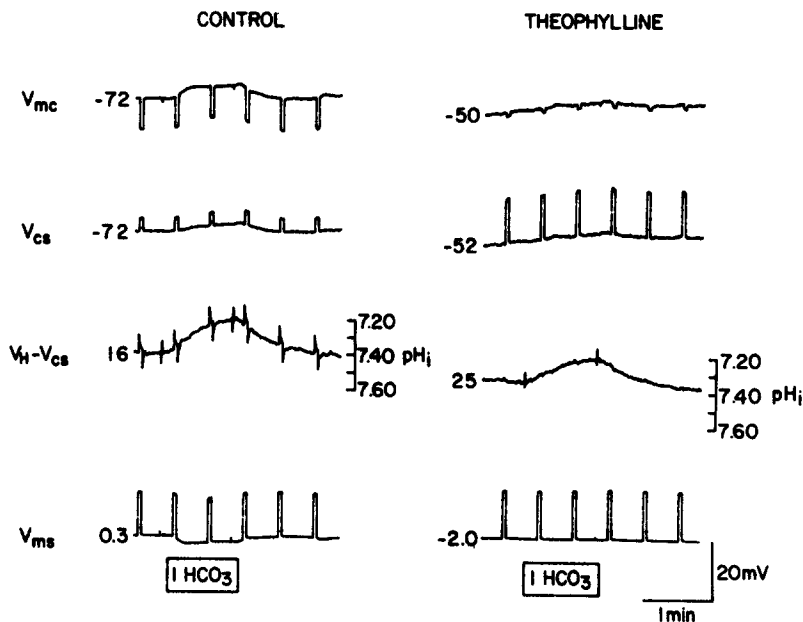


FIGURE 9. Effects of reducing mucosal  $[\text{HCO}_3^-]$  to 1 mM on membrane voltages and resistances and  $\text{pH}_i$ . The initial rate of cell acidification is less in theophylline. See Table VI.

*Effects of cAMP on Apical Membrane Electrodifusive Anion Permeability*

Because of the recent conclusions of Zeldin et al. (1985), the possibility of a cAMP-dependent apical membrane electrodiffusive  $\text{HCO}_3^-$  permeability was further examined. If cAMP induces or enhances an apical membrane  $\text{HCO}_3^-$  conductance, then reducing luminal  $[\text{HCO}_3^-]$  should cause a greater cell membrane depolarization than under control conditions. In addition, lowering lu-

TABLE V  
*Effects of Reducing Luminal  $[\text{HCO}_3^-]$  on Membrane Voltages and Resistances and  $a\text{Cl}_i$  under Control Conditions and during Exposure to Theophylline*

Condition	$V_{ms}$ mV	$V_{mc}$ mV	$V_{cs}$ mV	$R_a/R_b$	$R_i$ $\Omega \cdot \text{cm}^2$	$a\text{Cl}_i$ mM	$\Delta a\text{Cl}_i/\Delta t$ mM/min
Control							
10 $\text{HCO}_3^-$	$0.2 \pm 0.1$	$-69 \pm 6$	$-69 \pm 6$	$5.16 \pm 1.66$	$166 \pm 10$	$22.1 \pm 2.0$	
1 $\text{HCO}_3^-$	$0.2 \pm 0.1$	$-67 \pm 6^*$	$-67 \pm 6^*$	$4.91 \pm 1.51$	$174 \pm 10^*$	$25.1 \pm 1.5^*$	$4.5 \pm 1.2$
Difference	$0.0 \pm 0.1$	$3 \pm 1$	$3 \pm 1$	$0.25 \pm 0.27$	$9 \pm 1$	$3.1 \pm 0.8$	
Theophylline							
10 $\text{HCO}_3^-$	$-1.8 \pm 0.4^\ddagger$	$-49 \pm 4^\ddagger$	$-51 \pm 4^\ddagger$	$0.21 \pm 0.13^\ddagger$	$204 \pm 16^\ddagger$	$12.6 \pm 1.0^\ddagger$	
1 $\text{HCO}_3^-$	$-1.2 \pm 0.4^\ddagger$	$-47 \pm 2^{*\ddagger}$	$-49 \pm 3^{*\ddagger}$	$0.23 \pm 0.14^\ddagger$	$221 \pm 32^*$	$14.2 \pm 1.3^{*\ddagger}$	$1.7 \pm 0.2^\ddagger$
Difference	$-0.6 \pm 0.4$	$2 \pm 1$	$3 \pm 1$	$0.02 \pm 0.01$	$17 \pm 22$	$1.6 \pm 0.4^\ddagger$	

Values in 1 mM  $\text{HCO}_3^-$  were measured at 2 or 3 min.  $\Delta\text{pH}_i/\Delta t$  is the initial rate of change of  $\text{pH}_i$  upon lowering mucosal  $[\text{HCO}_3^-]$  to 1 mM.  $n = 4$ . For other symbols, see Table II.

TABLE VI  
*Effects of Reducing Luminal [HCO<sub>3</sub><sup>-</sup>] on Membrane Voltages and Resistances and pH<sub>i</sub> under Control Conditions and during Exposure to Theophylline*

Condition	V <sub>m</sub> mV	V <sub>mc</sub> mV	V <sub>ca</sub> mV	R <sub>a</sub> /R <sub>b</sub>	R <sub>i</sub> Ω·cm <sup>2</sup>	pH <sub>i</sub>	ΔpH <sub>i</sub> /Δt min <sup>-1</sup>	Δ[HCO <sub>3</sub> <sup>-</sup> ]/Δt mM/min
Control								
10 HCO <sub>3</sub> <sup>-</sup>	0.1±0.2	-68±2	-68±2	9.62±1.88	195±13	7.38±0.05		
1 HCO <sub>3</sub> <sup>-</sup>	-0.2±0.4	-65±2*	-65±2*	9.70±1.22	198±14	7.27±0.07	-0.22±0.03	-6.2±0.8
Difference	-0.4±0.3	3±0.4	3±0.3	0.08±0.95	3±2	-0.11±0.03		
Theophylline								
10 HCO <sub>3</sub> <sup>-</sup>	-1.6±0.4 <sup>‡</sup>	-47±4 <sup>‡</sup>	-49±4 <sup>‡</sup>	0.04±0.02 <sup>‡</sup>	241±25 <sup>‡</sup>	7.28±0.06 <sup>‡</sup>		
1 HCO <sub>3</sub> <sup>-</sup>	-1.9±0.3**	-43±4**	-45±4**	0.06±0.02 <sup>‡</sup>	248±24**	7.17±0.06**	-0.15±0.03 <sup>‡</sup>	-4.0±0.8 <sup>‡</sup>
Difference	-0.4±0.1	4±1	4±1	0.02±0.01	7±2 <sup>‡</sup>	-0.11±0.03		

Values in 1 mM HCO<sub>3</sub><sup>-</sup> were measured at 2 or 3 min. n = 6. For other symbols, see Tables II and IV.

minal [HCO<sub>3</sub><sup>-</sup>] should result in an increase in the electrical resistance of the apical membrane, and hence in an elevation in the apparent ratio of cell membrane resistances. As shown in Figs. 8 and 9 and Tables V and VI, the change in apical membrane voltage produced by lowering external [HCO<sub>3</sub><sup>-</sup>] to 1 mM was not altered by theophylline treatment, and the apparent ratio of membrane resistances in 1 mM HCO<sub>3</sub><sup>-</sup> was not significantly different from that measured in 10 mM HCO<sub>3</sub><sup>-</sup>, either in the absence or in the presence of theophylline. These observations contradict the proposal that cAMP increases apical membrane electrodiffusive HCO<sub>3</sub><sup>-</sup> permeability.

To maximize the possible effect of luminal solution [HCO<sub>3</sub><sup>-</sup>] on apical membrane voltage and resistance, additional experiments were carried out in which [HCO<sub>3</sub><sup>-</sup>] was changed from 10 to 1 mM in tissues in which the Cl<sup>-</sup> in the luminal solution had been previously replaced with cyclamate. As illustrated in Fig. 10

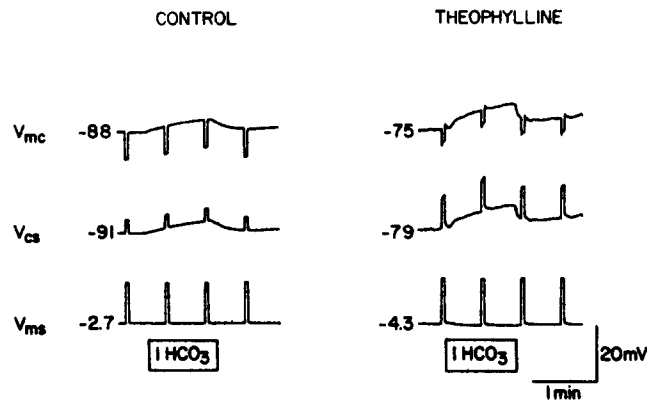


FIGURE 10. Effects of reducing mucosal [HCO<sub>3</sub><sup>-</sup>] to 1 mM on membrane voltages and resistances in a nominally Cl<sup>-</sup>-free mucosal medium. As in the presence of Cl<sup>-</sup>, exposure to 1 mM HCO<sub>3</sub><sup>-</sup> causes depolarization of both cell membranes, with no change in R<sub>a</sub>/R<sub>b</sub>. The depolarization is somewhat greater in theophylline. See text and Table VII.

TABLE VII

*Effects of Lowering Mucosal  $[\text{HCO}_3^-]$  to 1 mM, in a  $\text{Cl}^-$ -free Medium, on Membrane Voltages and Resistances under Control Conditions and during Exposure to Theophylline*

Condition	$V_{ms}$ mV	$V_{mc}$ mV	$V_{cs}$ mV	$R_a/R_b$	$R_t$ $\Omega \cdot \text{cm}^2$
Control					
0 Cl	$-0.9 \pm 0.7$	$-95 \pm 3$	$-96 \pm 3$	$7.71 \pm 0.44$	$205 \pm 11$
0 Cl, 1 $\text{HCO}_3^-$	$-1.1 \pm 0.7$	$-88 \pm 3^*$	$-89 \pm 3^*$	$7.77 \pm 0.73$	$204 \pm 10$
Difference	$-0.2 \pm 0.1$	$7 \pm 2$	$6 \pm 2$	$0.06 \pm 0.51$	$-1 \pm 1$
Theophylline					
0 Cl	$-3.0 \pm 0.9^\ddagger$	$-58 \pm 5^\ddagger$	$-61 \pm 5^\ddagger$	$1.09 \pm 0.39^\ddagger$	$256 \pm 26^\ddagger$
0 Cl, 1 $\text{HCO}_3^-$	$-3.5 \pm 1.0^\ddagger$	$-49 \pm 5^{*\ddagger}$	$-53 \pm 5^{*\ddagger}$	$0.89 \pm 0.25^\ddagger$	$266 \pm 29^{*\ddagger}$
Difference	$-0.5 \pm 0.2$	$9 \pm 2$	$8 \pm 2$	$0.20 \pm 0.14$	$10 \pm 3$

Values in 1  $\text{HCO}_3^-$  were measured at 1 min.  $n = 6$ . For other symbols, see Table II.

and summarized in Table VII, the depolarization elicited by exposure to the low- $\text{HCO}_3^-$  solution was greater than in the presence of  $\text{Cl}^-$ , both under control conditions and in theophylline. In the presence of the drug, the steady state apical membrane voltage change induced by the 1 mM  $\text{HCO}_3^-$  solution was, on

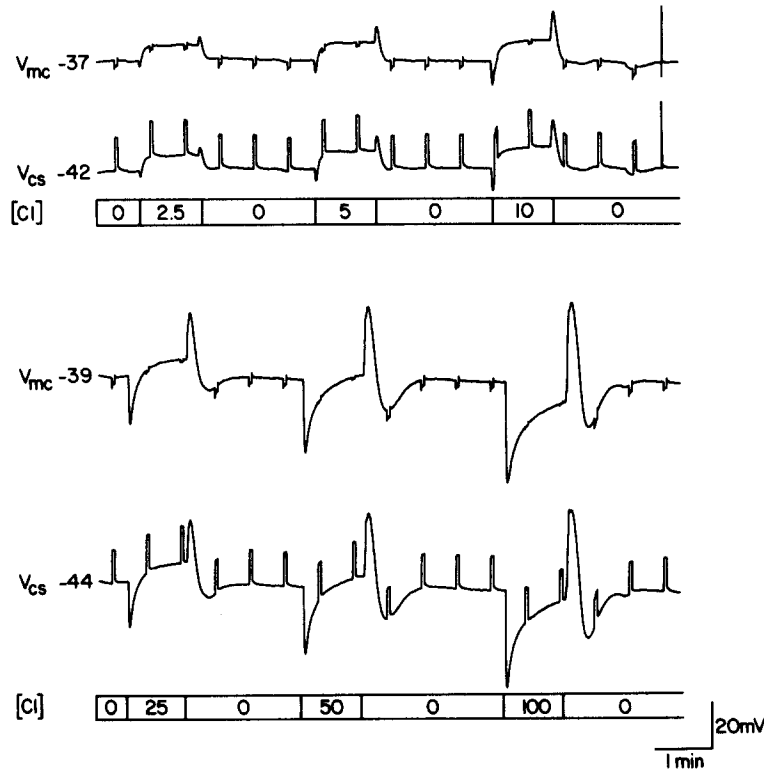


FIGURE 11. Effects of elevating mucosal solution  $[\text{Cl}^-]$  from nominally zero to the values indicated on membrane voltages and the apparent ratio of cell membrane resistances in a forskolin-treated preparation.



average, larger than in the absence of the drug ( $9 \pm 2$  vs.  $7 \pm 2$  mV). As discussed below, this result is expected from the cAMP-induced drop in  $R_a/R_b$ , without a need to postulate the induction of an HCO<sub>3</sub><sup>-</sup> electrodiffusive permeability. Furthermore, the apparent ratio of cell membrane resistances was not changed by the reduction in external [HCO<sub>3</sub><sup>-</sup>].

Finally, the previous conclusion of a large cAMP-induced electrodiffusive  $P_{Cl}$  of the apical membrane (Petersen and Reuss, 1983) was examined again. For these experiments, tissues under control conditions or exposed to theophylline or forskolin were incubated in a Cl<sup>-</sup>-free mucosal solution. The latter was replaced, for 1-min periods, with luminal bathing media containing Cl<sup>-</sup> at concentrations ranging from 2.5 to 100 mM. Throughout, extracellular [HCO<sub>3</sub><sup>-</sup>] was 10 mM. The results of such an experiment are shown in Fig. 11. Note that, in contrast to the slow and small effects of changing luminal [HCO<sub>3</sub><sup>-</sup>], changing luminal [Cl<sup>-</sup>] elicits rapid changes in membrane voltage, which are apparent even at 2.5 mM. The conclusion of a high electrodiffusive apical membrane  $P_{Cl}$  is strongly supported by these results.

#### DISCUSSION

The results presented above indicate that, in *Necturus* gallbladder epithelium, elevation of intracellular cAMP inhibits apical membrane Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange, but does not induce a sizable apical membrane electrodiffusive HCO<sub>3</sub><sup>-</sup> permeability, a possibility previously suggested by others (Zeldin et al., 1985). Additional evidence for stimulation of an apical membrane Cl<sup>-</sup>-conductive pathway by cAMP has been presented.

##### *Inhibition of Apical Membrane Anion Exchange*

The evidence for inhibition of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange was obtained from measurements of both extracellular pH and intracellular pH and Cl<sup>-</sup> activity. In most experiments, the main parameter determined was an initial rate of change (of pH<sub>o</sub>, pH<sub>i</sub>, or  $aCl_i$ ) in response to a rapid alteration in the ionic composition of the mucosal bathing solution. This experimental design allows for quantitative analysis of the net fluxes across the apical membrane before large changes in intracellular composition can take place. Several technical limitations must be considered before interpretation of the results.

First, the  $aCl_i$  measurements were performed with ion-sensitive microelectrodes, which are not perfectly selective for Cl<sup>-</sup> (Baumgarten, 1981). At low  $aCl_i$  values, the contribution of intracellular HCO<sub>3</sub><sup>-</sup>, and perhaps of other intracellular anions, can be significant and hence result in overestimates of the true  $aCl_i$ . However, both under control conditions and in theophylline-treated tissues, the initial value of  $aCl_i$  was of the order of 10 mM or higher, and in this region the response of the Cl<sup>-</sup>-sensitive microelectrode to Cl<sup>-</sup> is near-Nernstian; i.e., the HCO<sub>3</sub><sup>-</sup> interference is minimal. Therefore, it is believed that the estimates of initial rates of change of  $aCl_i$  are correct. The problem of insufficient selectivity of the Cl<sup>-</sup>-sensitive microelectrode at low  $aCl_i$  also complicates the quantitative interpretation of the Cl<sup>-</sup> uptake measurements shown in Figs. 5 and 6. At the low apparent  $aCl_i$  values measured in a Cl<sup>-</sup>-free luminal solution, the slope of the Cl<sup>-</sup>-sensitive microelectrode is probably lower than the value predicted from

the Nernst equation, and therefore the rate of change in  $a\text{Cl}_i$  may be greater than the apparent value. However, such an error should be similar in control and experimental conditions. In addition, making the measurements in the 2 s after the ionic substitution minimizes changes in microelectrode slope owing to the change in  $a\text{Cl}_i$ . Hence, I feel that the comparison shown in Fig. 6 is valid.

Second, the changes in  $a\text{Cl}_i$  in response to external ionic substitutions are useful for these purposes if they denote net ion fluxes. One possible source of error is that, in addition to the ionic activity changes, there are significant changes in cell volume. Preliminary experiments carried with the TMA<sup>+</sup>-loading technique (Reuss, 1985) revealed immeasurably small cell volume changes upon reducing mucosal solution  $[\text{Cl}^-]$  to 10 mM in the absence of theophylline (Cotton, C. U., and L. Reuss, unpublished observations). In the presence of theophylline, the same maneuver caused cell shrinkage at an average initial rate of 2%/s. Inasmuch as theophylline induces an apical  $\text{Cl}^-$ -conductive pathway that is not present under control conditions, these results suggest that cell shrinkage involves conductive loss of  $\text{Cl}^-$  (and probably of  $\text{K}^+$ ); since cell volume is not reduced by lowering  $[\text{Cl}^-]$  under control conditions, the  $\text{Cl}^-$  efflux responsible for the shrinkage must occur via the conductive pathway. In other words, these preliminary results strengthen the conclusion of a large conductive  $\text{Cl}^-$  pathway and support the notion of inhibition of anion exchange by cAMP (see below).

Third, the changes in mucosal solution pH in tissues treated with amiloride or exposed to an  $\text{Na}^+$ -free medium on that side have been implicitly assumed to result from effluxes of  $\text{HCO}_3^-$  into that solution. In principle, the change in  $\text{pH}_o$  could be due to a net  $\text{H}^+$  flux from the lumen to the cells, to a net flux of base (not necessarily  $\text{HCO}_3^-$ ) from the cells to the lumen, or to a combination of two or more fluxes, with the net result of removal of acid equivalents from the mucosal bathing solution. Several arguments support the notion that the luminal alkalization observed under these conditions is principally or exclusively due to apical membrane anion exchange: (a) It is inhibited by disulfonic stilbene derivatives and other anion exchange inhibitors (Reuss and Costantin, 1984, and unpublished observations). (b) It cannot be ascribed to an  $\text{H}^+$  influx (lumen to cell), because in tissues exposed to  $10^{-5}$  M amiloride in the presence of 100 mM  $\text{Na}^+$ , the net  $\text{H}^+$  flux, although reduced, is from cell to lumen (Weinman and Reuss, 1984; Reuss, 1984). (c) Under conditions comparable to those in the  $\text{pH}_o$  experiments, the changes in  $\text{pH}_i$  and  $a\text{Cl}_i$  are consistent with electroneutral anion exchange (Reuss and Costantin, 1984; this article).

Fourth, the  $\text{pH}_i$  changes elicited by a reduction of luminal  $[\text{HCO}_3^-]$  were attributed to a change in the direction of  $\text{Cl}^-/\text{HCO}_3^-$  exchange. Alternatively, inasmuch as luminal  $[\text{H}^+]$  is raised in this experiment, the cell acidification could result from a decrease in the net  $\text{H}^+$  efflux via  $\text{Na}^+/\text{H}^+$  exchange, or from a reversal of this flux. The reduction in theophylline could simply be due to the inhibitory effect of cAMP on  $\text{Na}^+/\text{H}^+$  exchange (Reuss and Petersen, 1985). This possibility was tested by comparing the initial rates of change of  $\text{pH}_i$  upon exposure to 1 mM  $\text{HCO}_3^-$  or to 1 mM  $\text{HCO}_3^-$  plus 1 mM amiloride. If cell acidification is due to a change in  $\text{H}^+$  flux via  $\text{Na}^+/\text{H}^+$  exchange, it should be less in amiloride. However, amiloride caused an increase in  $\Delta\text{pH}_i/\Delta t$ , from  $-0.17$

$\pm 0.02$  to  $-0.23 \pm 0.02 \text{ min}^{-1}$  ( $p < 0.05$ ;  $n = 4$ ). This result could mean that, in the absence of amiloride, the drop in  $\text{pH}_i$  upon reduction of mucosal  $[\text{HCO}_3^-]$  activates the  $\text{Na}^+/\text{H}^+$  exchanger, and that this effect is inhibited by amiloride. Activation of the exchanger by titration of an internal site has been demonstrated in renal brush border vesicles (Aronson et al., 1982).

In sum, although there are some potential limitations in the techniques employed, they have allowed assessment of the rate of apical membrane anion exchange under control conditions and after elevation of intracellular cAMP levels.

The  $\Delta\text{pH}_o$  measurements do not provide quantitative estimates of the acid/base fluxes across the apical membrane because of the unavoidable presence of permeant buffers and the loss of  $\text{CO}_2$  to the gas phase (Weinman and Reuss, 1982). However, paired measurements in the same tissues allow for comparisons of both the magnitude and direction of the apparent  $\text{H}^+$  fluxes under several experimental conditions. These studies support the hypothesis of inhibition of  $\text{Cl}^-/\text{HCO}_3^-$  exchange by theophylline treatment because the rate of luminal solution alkalization observed shortly after adding amiloride or removing  $\text{Na}^+$  from the lumen was reduced significantly by theophylline. As stated above, measurements of  $a\text{Cl}_i$  and  $\text{pH}_i$  under the same conditions indicate that the driving force for  $\text{Cl}^-/\text{HCO}_3^-$  exchange may be slightly elevated in theophylline-treated tissues. In other words, the fall in the  $\text{HCO}_3^-$  flux is not caused by a reduced driving force, but must depend on a change in the functional properties of the carrier itself.

The measurements of changes in  $a\text{Cl}_i$  in response to reductions in luminal  $[\text{Cl}^-]$  also support the hypothesis that  $\text{Cl}^-/\text{HCO}_3^-$  exchange is cAMP inhibitable. In theophylline, the initial rate of fall of  $a\text{Cl}_i$  when mucosal  $[\text{Cl}^-]$  was reduced remained unchanged, compared with control, although cAMP clearly induces a  $\text{Cl}^-$ -conductive pathway. Estimation of the  $\text{Cl}^-$  fluxes via the exchanger and the channel was necessary to assess the effect of cAMP on anion exchange. This was done in two different ways. First, the rates of change of  $a\text{Cl}_i$  upon reducing mucosal  $[\text{Cl}^-]$  to 10 mM and upon reducing mucosal  $[\text{Cl}^-]$  to 10 mM and  $[\text{HCO}_3^-]$  to 1 mM were compared. As shown in the Results, the initial rates of fall of  $a\text{Cl}_i$  were the same in the absence and presence of theophylline. Since in theophylline the conductive  $\text{Cl}^-$  was ~56% of the total flux, the rate of anion exchange is reduced by cAMP to ~44% of control, but part of this effect is due to the lower  $a\text{Cl}_i$  in theophylline. Second, the conductive  $\text{Cl}^-$  loss was estimated by comparing the values of  $\Delta a\text{Cl}_i/\Delta t$  upon reducing mucosal  $[\text{Cl}^-]$  to 10 mM with and without diphenylamine-2-carboxylate (DPC) (Reuss et al., 1987). In the presence of theophylline, DPC ( $10^{-4}$  M) reduced this rate from  $-11.5 \pm 1.7$  to  $-7.1 \pm 0.8 \text{ mM/min}$ . Since at this concentration the effect of DPC is not maximal, there is probably a residual  $\text{Cl}^-$  flux via the exchanger, which is probably similar to that observed when control tissues are exposed to DPC at the same concentration ( $-1.4 \pm 0.4 \text{ mM/min}$ ). Hence, the conductive flux can be estimated to be the difference between 7.1 and 1.4, i.e., about  $-5.7 \text{ mM/min}$ , which again is ~50% of the total net  $\text{Cl}^-$  flux. Again, the precise degree of inhibition is difficult to assess because of the different values of  $a\text{Cl}_i$  in control and theophylline-

treated tissues. In sum, both estimates of conductive  $\text{Cl}^-$  fluxes suggest that  $\text{Cl}^-/\text{HCO}_3^-$  exchange is inhibited when intracellular cAMP is elevated. The preliminary measurements of cell volume changes elicited by lowering mucosal solution  $[\text{Cl}^-]$  to 10 mM suggest that the conductive  $\text{Cl}^-$  loss in theophylline could be even larger than these estimates, and therefore the exchange flux would be even smaller (see above). It is possible that when external  $[\text{Cl}^-]$  is reduced in theophylline-treated tissues, part of the  $\text{Cl}^-$  efflux via the channel is recycled by the exchanger.

Further information on the mechanism of the inhibition of anion exchange by cAMP was obtained in the experiments in which luminal  $[\text{Cl}^-]$  was raised from nominally zero to values ranging from 2.5 to 100 mM (Fig. 6). The results are consistent with a decrease in  $V_{\max}$  in forskolin-treated tissues. The apparent  $K_m$  of the process decreased dramatically. By itself, this effect would stimulate  $\text{Cl}^-$  transport by anion exchange, so that it cannot be responsible for the overall inhibition. The determination of the  $K_m$  during elevation of cAMP is difficult because of the errors in determining the slope of the conductive flux, which cause large fractional errors in estimating  $\Delta a\text{Cl}_i/\Delta t$  at low external  $[\text{Cl}^-]$ . Hence, the value of the apparent  $K_m$  is quite uncertain. In any event, it is interesting to observe that a similar result, namely decreases in both  $V_{\max}$  and  $K_m$ , was obtained in our investigation of the effect of cAMP on  $\text{Na}^+/\text{H}^+$  exchange (Reuss and Petersen, 1985).

The measurements of changes in  $\text{pH}_i$  in response to reductions of luminal  $[\text{Cl}^-]$  to 10 mM, as well as in response to a lowering of luminal  $[\text{HCO}_3^-]$  to 1 mM, also support the conclusion of inhibition of apical membrane anion exchange. When external  $[\text{Cl}^-]$  was lowered, the cells alkalinized ( $\text{Cl}^-$  efflux coupled to  $\text{HCO}_3^-$  influx). The rate of alkalinization was significantly lowered by theophylline, as illustrated in Fig. 7 and summarized in Table IV. Reducing luminal  $[\text{HCO}_3^-]$  from 10 to 1 mM under control conditions caused intracellular acidification and a rise in  $a\text{Cl}_i$ . Both effects were significantly reduced by theophylline (see Figs. 8 and 9, and Tables V and VI). These observations are also consistent with inhibition of anion exchange by cAMP. The possibility of a role of  $\text{Na}^+/\text{H}^+$  exchange in the effects of lowering mucosal  $[\text{HCO}_3^-]$  was ruled out by the amiloride experiments reported above.

#### *Arguments against Induction of an $\text{HCO}_3^-$ -conductive Pathway*

Zeldin et al. (1985) argued that elevation of intracellular cAMP, induced in *Necturus* gallbladder epithelium by exposure to isobutylmethylxanthine (IBMX), produced an increase in apical membrane  $\text{HCO}_3^-$  conductance. They showed that in a  $\text{Cl}^-$ -free,  $\text{HCO}_3^-$ -free medium, IBMX produced monotonic cell membrane depolarization; however, in the presence of 25 mM  $\text{HCO}_3^-$  in the bathing solutions (at constant pH), IBMX caused a transient hyperpolarization, followed by depolarization. These authors interpreted the hyperpolarization as being due to an apical membrane  $\text{HCO}_3^-$ -conductive pathway. This argument is incorrect. In their experiments, the cells hyperpolarized, on average, from  $-67$  to  $-70$  mV; if this hyperpolarization were due to an increase in electrodiffusive  $\text{HCO}_3^-$  permeability, then the  $\text{HCO}_3^-$  equilibrium potential would have to be greater

than the baseline zero-current voltage of the apical membrane. Inasmuch as the transepithelial voltage was mucosa-positive before IBMX, the zero-current voltage of the apical membrane must be greater than the measured voltage, i.e.,  $>67$  mV. At an external  $[\text{HCO}_3^-]$  of 25 mM, the intracellular  $\text{HCO}_3^-$  concentration would be  $\leq 1.8$  mM, and  $\text{pH}_i$  (assuming equal values of  $P_{\text{CO}_2}$  in extra- and intracellular phases) would be, at most, 6.26. This possibility is unlikely on the basis of  $\text{pH}_i$  measurements in *Necturus* gallbladder under a variety of experimental conditions (Weinman and Reuss, 1982; Reuss and Costantin, 1984; Reuss and Petersen, 1985). Only during acid-loading experiments by the ammonium chloride prepulse technique (see Boron and De Weer, 1976) did the  $\text{pH}_i$  reach values as low as 6.5 (Reuss and Petersen, 1985). Under more physiologic conditions,  $\text{pH}_i$  ranges from 7.33 to 7.55. Hence, the mechanism of the hyperpolarization observed by Zeldin et al. cannot be the one they proposed (see Reuss, 1987; Reuss and Stoddard, 1987). Petersen and his associates have reached a similar conclusion concerning the results of Zeldin and co-workers, although their studies of the effect of cAMP on the electrical properties of guinea pig gallbladder are consistent with the possibility of induction of an  $\text{HCO}_3^-$  conductance at the apical membrane (Petersen, K.-U., personal communication).

The hypothesis of a cAMP-induced apical membrane  $\text{HCO}_3^-$  conductance was tested directly by assessing the effect of a reduction in mucosal solution  $[\text{HCO}_3^-]$  on membrane voltages, the apparent ratio of cell membrane resistances, and  $\text{pH}_i$  and  $\alpha\text{Cl}_i$  both under control conditions and during exposure to theophylline. The mucosal bathing solution was control Ringer's,  $\text{NaHCO}_3$  was partially replaced with Na cyclamate, and the  $P_{\text{CO}_2}$  was maintained constant. As shown above (Figs. 8 and 9, and Tables V and VI), the depolarization elicited by reducing mucosal  $[\text{HCO}_3^-]$  from 10 to 1 mM, in the presence of  $\text{Cl}^-$ , was not significantly changed by theophylline. In addition, the apparent ratio of membrane resistances was not altered by the reduction in external  $[\text{HCO}_3^-]$ , and the initial rate of intracellular acidification was not increased, but was reduced in theophylline. Although all three observations argue against an  $\text{HCO}_3^-$ -conductive pathway, a relatively small electrodiffusive  $\text{HCO}_3^-$  permeability could exist and be masked by the native  $\text{K}^+$  permeability and the cAMP-induced  $\text{Cl}^-$  permeability. Since the latter is the dominant apical membrane conductive pathway during elevation of cAMP levels, the effect of lowering  $[\text{HCO}_3^-]$  to 1 mM on membrane voltages and on the ratio of resistances was tested after  $\text{Cl}^-$  removal from the mucosal solution. As shown in the Results (Fig. 10 and Table VII), the apical membrane depolarization elicited by lowering external  $[\text{HCO}_3^-]$  was slightly increased in theophylline, but it is doubtful that this result is an indication of a conductive pathway for  $\text{HCO}_3^-$  across the apical membrane. First, the voltage change is slow, compared with that induced by changes in external  $[\text{Cl}^-]$  (see Fig. 11). Second, the change in apical membrane voltage in response to luminal ionic substitutions becomes closer to the change in zero-current voltage when the apical membrane conductance is elevated; i.e., an indirect effect of  $[\text{HCO}_3^-]$  could cause a larger change in  $V_{\text{mc}}$  simply because of the lower value of  $R_a/R_b$  in theophylline. If the primary effect of cAMP were to reduce basolateral  $P_{\text{K}}$  and hence to increase  $R_b$  (Zeldin et al., 1985), the same argument could be made.

Third, there was no significant change in the ratio of cell membrane resistances upon reducing mucosal  $[\text{HCO}_3^-]$  in theophylline-treated preparations incubated in a  $\text{Cl}^-$ -free mucosal medium. In sum, these arguments suggest that the cell membrane depolarization produced by lowering mucosal  $[\text{HCO}_3^-]$  is not conductive in nature, but results from effects of mucosal  $[\text{HCO}_3^-]$  on other transport pathways, perhaps a pH-mediated effect on  $P_K$  at both cell membranes. The possibility of an apical membrane  $\text{HCO}_3^-$ -conductive pathway cannot be completely ruled out. However, its magnitude would be too small to be measurable with the present techniques, and hence such a pathway cannot be invoked to explain the results of Zeldin et al. (1985).

#### *Significance of the Results*

The demonstration of an effect of intracellular cAMP levels on apical membrane  $\text{Cl}^-/\text{HCO}_3^-$  exchange in *Necturus* gallbladder is, as far as I am aware, the first demonstration of inhibition of anion exchange by this nucleotide. Prior to this finding, Boron et al. (1978) found that, in barnacle muscle fibers, cAMP stimulates  $\text{pH}_i$  recovery from an acid load by stimulating anion exchange. Previously, it was also demonstrated that, in *Necturus* gallbladder, cAMP stimulates an apical membrane  $\text{Cl}^-$ -conductive pathway (Petersen and Reuss, 1983) and inhibits apical membrane  $\text{Na}^+/\text{H}^+$  exchange (Reuss and Petersen, 1985). A similar conclusion was reached in studies carried out on renal cortex microvillus membrane vesicles (Kahn et al., 1985). In addition to these already complicated results, Zeldin et al. (1985) have suggested the additional possibilities of stimulation of an apical membrane  $\text{HCO}_3^-$  conductance, like that described in choroid plexus by Saito and Wright (1983, 1984), and inhibition of basolateral membrane  $\text{K}^+$  permeability. It has been shown above that their main argument in favor of an  $\text{HCO}_3^-$ -conductive pathway is in error. The proposal of a decrease in basolateral membrane  $P_K$  rests on more solid grounds, particularly because addition of  $\text{Ba}^{2+}$  to the serosal solution abolished the depolarizing effect of IBMX, although by itself  $\text{Ba}^{2+}$  caused only a 7-mV depolarization. Interpretation of this result is complicated by the finding that IBMX did decrease the ratio of cell membrane resistances after  $\text{Ba}^{2+}$ . The possibility of an effect of cAMP on basolateral membrane  $P_K$  is also suggested by our observation of a relatively low apparent ratio of cell membrane resistances in theophylline- or forskolin-treated tissues, even when exposed to a  $\text{Cl}^-$ -free mucosal bathing medium (Figs. 5, 10, and 11, and data not shown).

On the basis of the results in this article and in previous studies (Petersen and Reuss, 1983; Reuss and Petersen, 1985), I am convinced of at least three effects of cAMP at the apical membrane of *Necturus* gallbladder epithelial cells, namely stimulation of an electrodiffusive  $P_{\text{Cl}}$ , inhibition of  $\text{Na}^+/\text{H}^+$  exchange, and inhibition of  $\text{Cl}^-/\text{HCO}_3^-$  exchange. It has also been ruled out that cAMP induces a sizable  $\text{HCO}_3^-$ -conductive path at the apical membrane. The possibility of an additional effect at the basolateral membrane, which would include inhibition of the  $\text{Na}^+$  pump, was suggested by us (Reuss and Petersen, 1985) on the basis of the rather small effect of cAMP on intracellular  $\text{Na}^+$  activity, compared with the abolishment of transepithelial salt and fluid transport. Such a possibility will

require further investigation. It is tempting to speculate that basolateral membrane Na<sup>+</sup>,K<sup>+</sup> pump activity and P<sub>K</sub> may be inhibited *pari passu* by cAMP. Such coordinated changes in the activity of the two major ion transport pathways in basolateral membranes of epithelial cells have been suggested in other preparations (Lau et al., 1984; Hudson and Schultz, 1984).

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