Cyclic AMP Inhibits Cl⁻/HCO₃⁻ Exchange at the Apical Membrane of Necturus Gallbladder Epithelium

LUIS REUSS

From the Department of Physiology and Biophysics, The University of Texas Medical Branch, Galveston, Texas 77550

ABSTRACT Intracellular microelectrode techniques were employed to study the effect of cyclic AMP on apical membrane Cl⁻/HCO₃⁻ exchange and electrodiffusive HCO₃⁻ transport in Necturus gallbladder epithelium. Intracellular cAMP levels were raised by addition of either the phosphodiesterase inhibitor theophylline (3 × 10⁻⁵ 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 showed acidification, owing to secretion of both H⁺ and HCO₃⁻. When the same experiment is performed after addition of amiloride or removal of Na⁺ from the mucosal bathing medium, alkalinization is observed since H⁺ transport is either inhibited or reversed, whereas HCO₃⁻ 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 Cl⁻ activity (aCl⁻) upon reducing mucosal solution [Cl⁻] to either 10 or 0 mM, although membrane voltage and resistance measurements were consistent with stimulation of apical membrane electrodiffusive Cl⁻ permeability. Estimates of the conductive flux, obtained by either reducing simultaneously mucosal [Cl⁻] and [HCO₃⁻] or lowering [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 Cl⁻ uptake upon increasing mucosal Cl⁻ from nominally zero to levels ranging from 2.5 to 100 mM suggest that the mechanism of inhibition is a decrease in Vₘₕₐₓ. Consistent with these results, the rate of intracellular alkalinization upon reducing external Cl⁻ was also inhibited significantly by theophylline. Reducing mucosal solution [HCO₃⁻] from 10 to 1 mM under control conditions caused intracellular acidification and an increase in aCl⁻. Theophylline inhibited both changes, by 62 and 32%, respectively. These data indicate that elevation of intracellular cAMP inhibits apical membrane anion (Cl⁻/HCO₃⁻) exchange. Studies of the effects of rapid changes in mucosal [HCO₃⁻] on membrane voltages and the apparent ratio of membrane resistances, both in the presence and in

Address reprint requests to Dr. Luis Reuss, Dept. of Physiology and Biophysics, The University of Texas Medical Branch, Galveston, TX 77550.

J. GEN. PHYSIOL. © The Rockefeller University Press. 0022-1295/87/08/0173/24 $2.00

Volume 90  August 1987  173-196
In the absence of theophylline, with or without Cl\(^-\) in the mucosal solution, do not support the hypothesis that cAMP produces a sizable increase in apical membrane electrodiffusive 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 Na\(^+\) and 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 Na\(^+\) and Cl\(^-\) activities were observed, and there were no changes in membrane voltage (Díez de los Ríos 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 Cl\(^-\) activity, that cAMP activates an apical membrane Cl\(^-\) conductance (Petersen and Reuss, 1983).

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

Since cAMP was shown to inhibit NaCl absorption by gallbladder epithelium, the demonstration that Na\(^+\)/H\(^+\) exchange is the main or sole mechanism of Na\(^+\) entry suggested that cAMP might inhibit this process. Intracellular microelectrode studies, including measurements of intracellular 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 Na\(^+\)/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 Cl\(^-\)/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 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⁻/HCO₃⁻ exchange and on a putative HCO₃⁻ conductive pathway are addressed directly. The results indicate that cAMP inhibits Cl⁻/HCO₃⁻ exchange, but does not induce a sizable HCO₃⁻ 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, 1975a, b; Weinman and Reuss, 1982). The control bathing solution, hereafter referred to as Ringer’s, had the following composition (millimolar): 90 NaCl, 10 NaHCO₃, 2.5 KCl, 1.8 CaCl₂, 1.0 MgCl₂, and 0.5 NaH₂PO₄; the pH was ~7.65 (gassed with a mixture of 1% CO₂ and 99% air), and the osmolality was ~200 mosmol/kg. Low-Cl⁻ solutions and low-HCO₃⁻ solutions were prepared by isomolar replacement of Cl⁻ or HCO₃⁻ with cyclamate. In some experiments, the solution (nominally HCO₃⁻ 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 × 10⁻³ and 10⁻⁶ M, respectively (serosal side only). Amiloride, added to the mucosal solution at a final concentration of 10⁻³ M, was a generous gift of Merck, Sharpe & Dohme (West Point, PA).

**Measurements of Electrical Potentials**

Transepithelial (Vₑ) and cell membrane voltages (apical, Vₐ; basolateral, Vᵦ) were measured as previously described (Reuss and Finn, 1975a, 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 Vₑ, and the adjacent bathing solution for Vₐ and Vᵦ. Transepithelial current pulses of 50 μA·cm⁻² (1–5 s duration) were passed by means of Ag/AgCl electrodes to measure the transepithelial resistance (Rₑ) and the apparent ratio of cell membrane resistances (Rᵦ/Rₐ). 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 HCO₃⁻ secretion across the apical membrane, the control solution was rapidly replaced with Na⁺-free medium (TMA⁺ substitution) or with HEPES-Ringer’s plus 10⁻⁸ 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⁺ 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 Cl⁻ activity and intracellular pH (Reuss et al., 1987). The silanized barrel was injected with Corning 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 Cl-sensitive microelectrodes, and with 3 M KCl in the pH-sensitive microelectrodes. The 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 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 ± 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 Cl⁻/HCO₃⁻ 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 HCO₃⁻ efflux into the mucosal medium, the apical surface was exposed for 1 min to a nominally Na⁺-free HEPES-Ringer's solution (TMA⁺ 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 H⁺ flux (ΔH) was computed and normalized to that determined under control conditions (NaCl HEPES-Ringer's, before theophylline). In the absence of theophylline, both amiloride and Na⁺-free medium reversed the luminal acidification to luminal alkalization, as previously reported (Reuss and Costantin, 1984). The larger alkalization observed in Na⁺-free medium compared with amiloride is due to reversal of the Na⁺/H⁺ exchange in the former condition. In theophylline, the ΔH in amiloride was reduced by 50%, and that in Na⁺-free medium fell by ~26%, compared with the respective values in the absence of theophylline. Most significantly, the Cl⁻-dependent alkalization (ΔH in TMA Cl minus ΔH in TMA cyclamate) was decreased by 63%. These results are consistent with the hypothesis that the elevation of intracellular cAMP levels inhibits apical membrane Cl⁻/HCO₃⁻ 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 HCO₃⁻ efflux in these experiments is due to a reduction of the net driving force for anion exchange. To test this
Inhibition of Cl⁻/HCO₃⁻ Exchange in Necturus Gallbladder Epithelium

Figure 1. Initial apparent fluxes of H⁺ equivalents into the mucosal bath. Open bars: control conditions (see Methods). Hatched bars: pretreatment with theophylline (3 × 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 (ΔpHₐ), and the buffering power of the solution (β), the apparent net H⁺ flux (ΔH) was calculated (ΔH = −ΔpHₐ/β). The data were normalized in each tissue to the value observed in Na⁺ Ringer's in the absence of theophylline (ΔH = 1.0). Means ± SEM for six experiments are shown. Upward bars denote mucosal solution acidification. Theophylline causes significant decreases of the mucosal solution acidification in Na⁺ Ringer's, and the alkalinization in amiloride-containing or Na⁺-free medium. The Cl⁻-dependent alkalinization (TMA CI⁻ TMA cyclamate) is also significantly reduced.

Possibility, pHᵢ and aClᵢ were measured in tissues bathed in the same solutions employed in the pHᵢ studies, both under control conditions and after exposure to theophylline. The results are summarized in Table I. Theophylline reduced aClᵢ from 18.3 to 12.4 mM, and pHᵢ from 7.54 to 7.50. The ratio of driving forces for HCO₃⁻ secretion (theophylline:control) is given by

$$\frac{aClᵢ(\text{control})}{aClᵢ(\text{theo})} \cdot \exp[pHᵢ(\text{theo}) - pHᵢ(\text{control})].$$

The result of the calculation is 1.35. In other words, in the presence of theophylline, the driving force is more favorable for HCO₃⁻ secretion. Therefore, the effect of theophylline on the initial rate of luminal alkalinization cannot be attributed to a reduced driving force.

Table I

<table>
<thead>
<tr>
<th>Condition</th>
<th>Vₘₑ</th>
<th>Vₘᵢ</th>
<th>Vₘᵢ</th>
<th>Rₒ/Rₐ</th>
<th>aClᵢ</th>
<th>pHᵢ</th>
<th>Rᵢ</th>
<th>D.cm²⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1±0.5</td>
<td>-65±1</td>
<td>-65±1</td>
<td>1.22±0.56</td>
<td>18.3±1.4</td>
<td>7.54±0.03</td>
<td>138±5</td>
<td></td>
</tr>
<tr>
<td>Theophylline</td>
<td>-1.7±0.5</td>
<td>-46±4</td>
<td>-47±4</td>
<td>0.11±0.06</td>
<td>12.4±2.4</td>
<td>7.50±0.05</td>
<td>200±21</td>
<td></td>
</tr>
<tr>
<td>Difference</td>
<td>-1.8±0.2*</td>
<td>17±*</td>
<td>18±*</td>
<td>-1.11±0.32*</td>
<td>-5.9±2.2*</td>
<td>-0.04±0.005*</td>
<td>62±23*</td>
<td></td>
</tr>
</tbody>
</table>

Values in theophylline were measured at 15-20 min. aClᵢ n = 5; pHᵢ n = 5.

* The difference (theophylline - control) is significantly different from zero.
Effects of cAMP on the Changes in Intracellular Cl\(^-\) Activity Elicited by Changes in Luminal [Cl\(^-\)]

It has been previously shown (Petersen and Reuss, 1983) that cAMP causes a fall in aCl\(_i\) to values near electrochemical equilibrium in tissues incubated in a low-buffering-power solution. In a 10 mM 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 \(P_{Cl}\), a

\[
\begin{align*}
V_{mc} & : -66 \\
V_{cs} & : -65 \\
V_{cl} - V_{cs} & : 33 \\
V_{ms} & : 1.1 \\
\end{align*}
\]

\[15\text{ mV} \quad 20\text{ mV} \quad 1\text{ min}\]

FIGURE 2. Effects of forskolin (10\(^{-5}\) M, mucosal side) on membrane voltages and resistances and intracellular Cl\(^-\) activity (aCl\(_i\)) 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 aCl\(_i\) is the difference between the voltage outputs of the 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 aCl\(_i\) toward equilibrium levels. These changes are similar to those elicited by theophylline in low-HCO\(_3\) media (see Petersen and Reuss, 1983).

sudden reduction of extracellular [Cl\(^-\)] should cause conductive Cl\(^-\) exit and, depending on the magnitude of the reduction of external [Cl\(^-\)], either a decrease in Cl\(^-\) entry via anion exchange or a net Cl\(^-\) efflux by this pathway. If the rate of anion exchange is unchanged by theophylline, reducing external [Cl\(^-\)] in theophylline-treated tissues would be expected to result in a faster fall of aCl\(_i\) than in untreated preparations. As shown in Fig. 3, under control conditions, reducing [Cl\(^-\)] to 10 mM causes slow cell membrane hyperpolarization and a rapid fall in aCl\(_i\). In theophylline, the membrane voltage changes are strikingly
different: a rapid depolarization of both cell membranes is observed upon lowering [Cl⁻], which is followed by relaxation toward the preceding voltage; upon restoration of mucosal [Cl⁻] to control levels, a fast hyperpolarization is seen, followed by a slow depolarization to the control values. The initial rate of fall of aClᵢ, however, does not differ significantly from that seen under control conditions.

Figure 3. Effects of reducing mucosal solution [Cl⁻] to 10 mM on membrane voltages, resistances, and aClᵢ, 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⁻ medium causes hyperpolarization of both cell membranes, little change in Vᵢₘ, Rᵢ, or Rₒ/Rᵢₒ, and a rapid fall of aClᵢ. In theophylline, the same maneuver causes rapid depolarization of both cell membranes, followed by repolarization during the period of exposure to the low-Cl⁻ medium, and a spiking, transient hyperpolarization upon returning to control solution. In contrast with the control traces, Rₒ/Rᵢₒ is very low in theophylline and rises when luminal [Cl⁻] is lowered. The initial rates of fall of aClᵢ 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 aClᵢ changes is much greater in 10 mM HCO₃⁻ 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⁻ solution. In theophylline, the membrane resistance ratio is very low in high-Cl⁻ medium and increases in the low-Cl⁻ solution. Summaries of voltage, resistance, and aClᵢ data are presented...
in Table II. These results are consistent with the induction of an apical membrane Cl⁻-conductive pathway. Although the Cl⁻ conductance is evident only in theophylline-treated tissues, the initial rate of fall of aClᵢ upon lowering luminal solution [Cl⁻] is not different from control. Consistent results were obtained when the luminal [Cl⁻] was reduced to nominally zero. In six such experiments, the initial rates of fall of aClᵢ were 27.7 ± 5.7 and 33.2 ± 9.6 mM/min, under control conditions and in theophylline, respectively.

These results suggest the possibility that in theophylline-treated tissues the additional 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 aClᵢ values immediately before lowering external [Cl⁻] are different under control conditions and in theophylline (see Table II). To obtain a more direct indication of the rates of Cl⁻ via the exchanger and via

### Table II

<table>
<thead>
<tr>
<th>Condition</th>
<th>Vmem</th>
<th>Vnc</th>
<th>Vm</th>
<th>Rₑ/Rₑ</th>
<th>R₁</th>
<th>aClᵢ</th>
<th>ΔaClᵢ/Δt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mV</td>
<td>mV</td>
<td>mV</td>
<td>Ω cm²</td>
<td>mM</td>
<td>mM/min</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>98 Cl</td>
<td>0.5±0.2</td>
<td>65±1</td>
<td>65±1</td>
<td>5.20±0.78</td>
<td>178±21</td>
<td>18.7±1.4</td>
<td></td>
</tr>
<tr>
<td>10 Cl</td>
<td>0.4±0.3</td>
<td>72±2*</td>
<td>72±2*</td>
<td>6.48±1.15</td>
<td>200±23*</td>
<td>11.5±1.3*</td>
<td>-11.9±1.7</td>
</tr>
<tr>
<td>Difference</td>
<td>0.1±0.2</td>
<td>-7±2</td>
<td>-7±3</td>
<td>1.27±0.76</td>
<td>22±3</td>
<td>-7.3±1.1</td>
<td></td>
</tr>
<tr>
<td>Theophylline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>98 Cl</td>
<td>-1.5±0.2</td>
<td>-50±3</td>
<td>-51±3</td>
<td>0.09±0.06</td>
<td>205±23</td>
<td>11.1±1.1</td>
<td></td>
</tr>
<tr>
<td>10 Cl</td>
<td>-1.5±0.3</td>
<td>-45±3</td>
<td>-47±3</td>
<td>0.31±0.11</td>
<td>229±25</td>
<td>8.0±1.5</td>
<td>-11.6±1.7</td>
</tr>
<tr>
<td>Difference</td>
<td>0.1±0.4</td>
<td>5±3*</td>
<td>5±2*</td>
<td>0.22±0.07</td>
<td>24±5</td>
<td>-3.1±0.5</td>
<td></td>
</tr>
</tbody>
</table>

Values in 10 mM Cl⁻ were measured at 2 or 3 min. *Difference* is values in 10 mM Cl⁻ minus values in 98 mM Cl⁻. ΔaClᵢ/Δt represents the initial rate of the fall of aClᵢ upon reduction in mucosal [Cl⁻] to 10 mM. n = 6.

* Significantly different from values in 98 mM Cl⁻.

The conducative pathway, experiments were carried out in which mucosal solution [Cl⁻] and [HCO₃⁻] 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 Cl⁻ efflux should increase by 58 mV (Δμ/ F). As shown in Fig. 4 and summarized in Table III, under control conditions, this maneuver causes no significant fall of aClᵢ (ΔaClᵢ/Δt = -0.3 ± 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 Vₘₑ were the same when reducing mucosal [Cl⁻] alone or mucosal [Cl⁻] and [HCO₃⁻] (28 ± 2 and 27 ± 1 mV, respectively), after treatment with theophylline or forskolin, the initial rate of change of aClᵢ was -6.5 ± 1.3 mM/min, in contrast with the rate measured, also in theophylline, upon lowering [Cl⁻] to 10 mM at constant [HCO₃⁻], which was -11.6 ± 1.7 mM/min. The difference, which
estimates the nonconductive initial rate of Cl⁻ loss upon lowering luminal [Cl⁻] to 10 mM, is -5.1 mM/min. Therefore, in theophylline, ~56% of the fall in aClᵢ 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>
<thead>
<tr>
<th>Condition</th>
<th>Vᵣᵣ</th>
<th>Vᵢᵢ</th>
<th>Vᵢₐ</th>
<th>Rᵢ/Rₒ</th>
<th>Rᵢ</th>
<th>aClᵢ</th>
<th>ΔaClᵢ/Δt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-66 ± 1</td>
<td>-67 ± 3</td>
<td>-67 ± 3</td>
<td>6.51 ± 1.87</td>
<td>157 ± 8</td>
<td>18.5 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>10 CI/1 HCO₃</td>
<td>-64 ± 2*</td>
<td>-66 ± 2*</td>
<td>-66 ± 3</td>
<td>8.19 ± 1.81*</td>
<td>174 ± 10*</td>
<td>18.7 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Difference</td>
<td>-2 ± 0.8</td>
<td>2 ± 1</td>
<td>1 ± 1</td>
<td>1.69 ± 0.50</td>
<td>17 ± 4</td>
<td>0.1 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

| Theophylline    | -45 ± 2* | -47 ± 2* | 0.07 ± 0.01* | 214 ± 24 | 12.6 ± 1.5 |
| 10 CI/1 HCO₃   | -54 ± 2* | -54 ± 2* | 0.11 ± 0.04* | 238 ± 28 | 8.7 ± 1.5* |
| Difference     | -4 ± 1.1* | 17 ± 4 | 15 ± 3* | 0.05 ± 0.04* | 24 ± 5 | -4 ± 0.6* |

*Values in 10 Cl⁻, 1 HCO₃ solution were measured at 1 min. ΔaClᵢ/Δt represents the initial rate of fall of aClᵢ upon lowering luminal [Cl⁻] and [HCO₃]; n = 4. For other symbols, see Table II.
these conditions is immeasurably small). Since the average intracellular Cl− activities before reducing mucosal Cl− were 18.7 and 11.1 mM, the rate coefficients for the aCl\(_i\) changes mediated by nonconductive pathways are 0.64 and 0.46 min\(^{-1}\) in control and theophylline, respectively.

Further support for an inhibition of Cl\(^-\)/HCO\(_3\) exchange was obtained from estimates of the initial rate of Cl− entry as a function of luminal [Cl\(^-\)]. In these experiments, both under control conditions and after pretreatment with either theophylline or forskolin, aCl\(_i\) was first reduced by exposure to a Cl\(^-\)-free luminal bathing solution. As shown in Fig. 5, elevating luminal [Cl\(^-\)] to 10 or 50 mM resulted in rapid increases in aCl\(_i\). The rate of change of aCl\(_i\) was greater after theophylline, particularly at the higher Cl\(^-\) concentration. The kinetics of this process, for four experiments, are shown in Fig. 6. The apparent Cl\(^-\) influx, measured as ΔaCl\(_i\)/Δt during the first 2 s, was normalized in each tissue to the value observed when [Cl\(^-\)] was raised to 100 mM under control conditions. In control tissues, there was a hyperbolic saturating relationship between influx and luminal [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 Cl⁻/HCO₃⁻ exchange, i.e., the saturating component observed after forskolin treatment, and that the mechanism of this effect is a decrease in V_max.

**Effects of cAMP on the Changes in Intracellular pH Elicited by Lowering Mucosal \([Cl^-]\)**

Experiments were carried out as described in the preceding section, but pH_i was measured instead of aCl_i. As shown in Fig. 7 and summarized in Table IV, the change in pH_i elicited by decreasing mucosal solution \([Cl^-]\) to 10 mM was reduced in theophylline, compared with control conditions. From these data, the rate of change of intracellular HCO₃⁻ activity can be estimated, using the initial rate of change of 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 CO₂ from 1 to 5% (27 mM; Reuss, L., unpublished results) and the CO₂/HCO₃⁻ buffering power, equal to 2.3 [HCO₃⁻] (Roos and Boron, 1981). It is also assumed that the intracellular P_CO₂ 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 HCO₃⁻ activity upon lowering external \([Cl^-]\) to 10 mM was 14.3 ± 3.4 mM/min, which is not statistically different from the initial rate of change of aCl_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 ± 1.9 mM/min (Table IV). The fact that theophylline alters the HCO\(_3\) influx while the Cl\(^-\) TABLE IV

<table>
<thead>
<tr>
<th>Condition</th>
<th>(V_{mc})</th>
<th>(V_{mc})</th>
<th>(V_{mc})</th>
<th>(R_a/R_b)</th>
<th>(R_b)</th>
<th>(pH)</th>
<th>(\Delta pH/\Delta t)</th>
<th>(\Delta HCO_3/\Delta t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1±0.1</td>
<td>-68±1</td>
<td>-68±1</td>
<td>6.18±1.5</td>
<td>251±14</td>
<td>7.39±0.06</td>
<td>7.34±0.06</td>
<td>0.32±0.07</td>
</tr>
<tr>
<td>98 Cl</td>
<td>-1.1±0.0</td>
<td>-79±2*</td>
<td>-80±1*</td>
<td>5.72±1.59</td>
<td>202±11</td>
<td>7.60±0.06</td>
<td>0.51±0.12</td>
<td>14.5±3.4</td>
</tr>
<tr>
<td>Difference</td>
<td>1.2±1.0</td>
<td>-11±1</td>
<td>-12±1</td>
<td>-0.46±0.98</td>
<td>4±4</td>
<td>0.21±0.03</td>
<td>11±1</td>
<td></td>
</tr>
<tr>
<td>Theophylline</td>
<td>-1.8±0.3</td>
<td>-49±3*</td>
<td>-51±3*</td>
<td>0.14±0.05</td>
<td>251±29*</td>
<td>7.34±0.06</td>
<td>0.32±0.07</td>
<td>8.7±1.9</td>
</tr>
<tr>
<td>10 Cl</td>
<td>-3.6±0.8*</td>
<td>-49±4*</td>
<td>-53±3*</td>
<td>0.57±0.25</td>
<td>240±24*</td>
<td>7.45±0.07</td>
<td>0.32±0.07</td>
<td>8.7±1.9</td>
</tr>
<tr>
<td>Difference</td>
<td>-1.8±1.0</td>
<td>0±2*</td>
<td>-2±2*</td>
<td>0.45±0.20</td>
<td>9±5</td>
<td>0.12±0.04</td>
<td>11±1</td>
<td></td>
</tr>
</tbody>
</table>

Values in 10 mM Cl\(^-\) were measured at 2 or 5 min. \(\Delta pH/\Delta t\) represents the initial rate of rise of pH upon reduction in mucosal [Cl\(^-\)] to 10 mM. \(\Delta HCO_3/\Delta t\) was calculated as explained in the text, and \(\Delta pH/\Delta t\) has been defined above. n = 6. For other symbols, see Table II.
efflux is unchanged suggests that an HCO₃⁻-independent Cl⁻ efflux mechanism is operative when intracellular cAMP levels are raised.

In summary, theophylline reduces the initial rate of change in aHCO₃⁻ elicited by reducing luminal [Cl⁻]. This result strongly supports the conclusion that cAMP inhibits apical membrane Cl⁻/HCO₃⁻ exchange.

**Effects of Reduction of Mucosal [HCO₃⁻] on aClᵢ and pHᵢ under Control Conditions and in Theophylline**

If cAMP inhibits Cl⁻/HCO₃⁻ exchange, then the changes in pHᵢ and aClᵢ produced by lowering external [HCO₃⁻] (at constant Pco₂) 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.

Consistent with the preceding data, which suggested inhibition of Cl⁻/HCO₃⁻ exchange in theophylline, the initial rates of change of aClᵢ and pHᵢ in response to lowering mucosal [HCO₃⁻] were significantly reduced. A calculation of the initial rates of fall of aHCO₃ᵢ 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⁻/HCO₃⁻ exchange, they are subject to alternative interpretations. First, it is conceivable that the changes in pHᵢ upon lowering external [HCO₃⁻] are totally or partly the result of a change in the rate and/or direction of Na⁺/H⁺ exchange (see Discussion). Second, the elevation of aClᵢ could be underestimated by conductive efflux of the Cl⁻ that enters via anion exchange.
CONTROL

\( V_{\text{mc}} \) -72

\( V_{\text{cs}} \) -72

\( V_{\text{h}} - V_{\text{cs}} \) 16

\( V_{\text{ms}} \) 0.3

THEOPHYLLINE

\( V_{\text{mc}} \) -50

\( V_{\text{cs}} \) -52

\( V_{\text{h}} - V_{\text{cs}} \) 16

\( V_{\text{ms}} \) -2.0

\( \text{I HCO}_3 \) 20 mV

\( \text{I HCO}_3 \) 20 mV

\( \text{I min} \)

**TABLE V**

| Condition | \( V_{\text{mc}} \) | \( V_{\text{mc}} \) | \( V_{\text{h}} - V_{\text{cs}} \) | \( R_c / R_b \) | \( R_c \) | \( c_{\text{Cl}} \) | \( \Delta c_{\text{Cl}} / \Delta \text{t} \)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>mV</td>
<td>mV</td>
<td>mV</td>
<td>( \Omega \cdot \text{cm}^2 )</td>
<td>mM</td>
<td>mM/min</td>
<td></td>
</tr>
<tr>
<td>10 HCO(_3)</td>
<td>0.2±0.1</td>
<td>-69±6</td>
<td>-69±6</td>
<td>5.16±1.66</td>
<td>166±10</td>
<td>22.1±2.0</td>
<td></td>
</tr>
<tr>
<td>1 HCO(_3)</td>
<td>0.2±0.1</td>
<td>-67±6*</td>
<td>-67±6*</td>
<td>4.91±1.51</td>
<td>174±10*</td>
<td>25.1±1.5*</td>
<td></td>
</tr>
<tr>
<td>Difference</td>
<td>0.0±0.1</td>
<td>5±1</td>
<td>3±1</td>
<td>0.25±0.27</td>
<td>9±1</td>
<td>3.1±0.8</td>
<td></td>
</tr>
<tr>
<td>Theophylline</td>
<td>-1.8±0.4</td>
<td>-49±4</td>
<td>-51±4</td>
<td>0.21±0.13</td>
<td>204±16</td>
<td>12.6±1.0</td>
<td></td>
</tr>
<tr>
<td>1 HCO(_3)</td>
<td>-1.2±0.4</td>
<td>-47±2*</td>
<td>-49±2*</td>
<td>0.23±0.14</td>
<td>221±32*</td>
<td>14.2±1.3</td>
<td></td>
</tr>
<tr>
<td>Difference</td>
<td>-0.6±0.4</td>
<td>2±1</td>
<td>3±1</td>
<td>0.02±0.01</td>
<td>17±22</td>
<td>1.6±0.4</td>
<td></td>
</tr>
</tbody>
</table>

Values in 1 mM HCO\(_3\) were measured at 2 or 3 min. \( \Delta \text{pH}/\Delta \text{t} \) is the initial rate of change of pH, upon lowering mucosal [HCO\(_3\)] to 1 mM. \( n = 4 \). For other symbols, see Table II.
minal \([ \text{HCO}_3^- ]\) 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 \([ \text{HCO}_3^- ]\) to 1 mM was not altered by theophylline treatment, and the apparent ratio of membrane resistances in 1 mM \(\text{HCO}_3^-\) was not significantly different from that measured in 10 mM \(\text{HCO}_3^-\), either in the absence or in the presence of theophylline. These observations contradict the proposal that CAMP increases apical membrane electrodiffusive \(\text{HCO}_3^-\) permeability.

To maximize the possible effect of luminal solution \([ \text{HCO}_3^- ]\) on apical membrane voltage and resistance, additional experiments were carried out in which \([ \text{HCO}_3^- ]\) was changed from 10 to 1 mM in tissues in which the \(\text{Cl}^-\) in the luminal solution had been previously replaced with cyclamate. As illustrated in Fig. 10

<table>
<thead>
<tr>
<th>Condition</th>
<th>(V_m)</th>
<th>(V_m)</th>
<th>(V_a)</th>
<th>(R_a/R_b)</th>
<th>(R_b)</th>
<th>(pH)</th>
<th>(\Delta pH/\Delta t)</th>
<th>(\Delta [\text{HCO}_3^-]/\Delta t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 (\text{HCO}_3^-)</td>
<td>0.1±0.2</td>
<td>-68±2</td>
<td>-68±2</td>
<td>9.62±1.88</td>
<td>195±13</td>
<td>7.38±0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (\text{HCO}_3^-)</td>
<td>-0.2±0.4</td>
<td>-65±2*</td>
<td>-65±2*</td>
<td>9.70±1.22</td>
<td>198±14</td>
<td>7.27±0.07</td>
<td>-0.22±0.03</td>
<td>-6.2±0.8</td>
</tr>
<tr>
<td>Difference</td>
<td>-0.4±0.3</td>
<td>5±0.4</td>
<td>3±0.3</td>
<td>0.08±0.95</td>
<td>3±2</td>
<td>-0.11±0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theophylline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 (\text{HCO}_3^-)</td>
<td>-1.6±0.4</td>
<td>-47±4</td>
<td>-49±4*</td>
<td>0.04±0.02*</td>
<td>241±23*</td>
<td>7.28±0.06*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (\text{HCO}_3^-)</td>
<td>-1.9±0.5*</td>
<td>-45±4</td>
<td>-45±4*</td>
<td>0.06±0.02*</td>
<td>248±24*</td>
<td>7.17±0.06*</td>
<td>-0.15±0.03</td>
<td>-4.0±0.8*</td>
</tr>
<tr>
<td>Difference</td>
<td>-0.4±0.1</td>
<td>4±1</td>
<td>4±1</td>
<td>0.02±0.01</td>
<td>7±2*</td>
<td>-0.11±0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values in 1 mM \(\text{HCO}_3^-\) were measured at 2 or 3 min. \(n = 6\). For other symbols, see Tables II and IV.

**FIGURE 10.** Effects of reducing mucosal \([ \text{HCO}_3^- ]\) to 1 mM on membrane voltages and resistances in a nominally \(\text{Cl}^-\)-free mucosal medium. As in the presence of \(\text{Cl}^-\), exposure to 1 mM \(\text{HCO}_3^-\) causes depolarization of both cell membranes, with no change in \(R_a/R_b\). The depolarization is somewhat greater in the theophylline. See text and Table VII.
TABLE VII

Effects of Lowering Mucosal [HCO₃⁻] to 1 mM, in a Cl⁻-free Medium, on Membrane Voltages and Resistances under Control Conditions and during Exposure to Theophylline

<table>
<thead>
<tr>
<th>Condition</th>
<th>V_{mc}</th>
<th>V_{nc}</th>
<th>V_{ca}</th>
<th>R_{a}/R_{b}</th>
<th>R_a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Cl</td>
<td>-0.9±0.7</td>
<td>-95±3</td>
<td>-96±3</td>
<td>7.71±0.44</td>
<td>205±11</td>
</tr>
<tr>
<td>0 Cl, 1 HCO₃</td>
<td>-1.1±0.7</td>
<td>-88±3*</td>
<td>-89±3*</td>
<td>7.77±0.73</td>
<td>204±10</td>
</tr>
<tr>
<td>Difference</td>
<td>-0.2±0.1</td>
<td>7±2</td>
<td>6±2</td>
<td>0.06±0.51</td>
<td>-1±1</td>
</tr>
</tbody>
</table>

Theophylline

<table>
<thead>
<tr>
<th>Condition</th>
<th>V_{mc}</th>
<th>V_{nc}</th>
<th>V_{ca}</th>
<th>R_{a}/R_{b}</th>
<th>R_a</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Cl</td>
<td>-3.0±0.9$^z$</td>
<td>-58±5$^z$</td>
<td>-61±5$^z$</td>
<td>1.09±0.39$^z$</td>
<td>256±26$^z$</td>
</tr>
<tr>
<td>0 Cl, 1 HCO₃</td>
<td>-3.5±1.0$^z$</td>
<td>-49±5$^*z$</td>
<td>-53±5$^*z$</td>
<td>0.89±0.25$^z$</td>
<td>266±29$^{*z}$</td>
</tr>
<tr>
<td>Difference</td>
<td>-0.5±0.2</td>
<td>9±2</td>
<td>8±2</td>
<td>0.20±0.14</td>
<td>10±3</td>
</tr>
</tbody>
</table>

Values in 1 HCO₃ 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-HCO₃ solution was greater than in the presence of 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 HCO₃ solution was, on
average, larger than in the absence of the drug (9 ± 2 vs. 7 ± 2 mV). As discussed below, this result is expected from the cAMP-induced drop in $R_{\text{b}}/R_{\text{b}}$ without a need to postulate the induction of an $\text{HCO}_3^-$ electrodiffusive permeability. Furthermore, the apparent ratio of cell membrane resistances was not changed by the reduction in external $[\text{HCO}_3^-]$.

Finally, the previous conclusion of a large cAMP-induced electrodiffusive $P_{\text{Cl}}$ 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 $\text{Cl}^-$-free mucosal solution. The latter was replaced, for 1-min periods, with luminal bathing media containing $\text{Cl}^-$ at concentrations ranging from 2.5 to 100 mM. Throughout, extracellular $[\text{HCO}_3^-]$ 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 $[\text{HCO}_3^-]$, changing luminal $[\text{Cl}^-]$ elicits rapid changes in membrane voltage, which are apparent even at 2.5 mM. The conclusion of a high electrodiffusive apical membrane $P_{\text{Cl}}$ is strongly supported by these results.

**DISCUSSION**

The results presented above indicate that, in *Necturus* gallbladder epithelium, elevation of intracellular cAMP inhibits apical membrane $\text{Cl}^-/\text{HCO}_3^-$ exchange, but does not induce a sizable apical membrane electrodiffusive $\text{HCO}_3^-$ permeability, a possibility previously suggested by others (Zeldin et al., 1985). Additional evidence for stimulation of an apical membrane $\text{Cl}^-$-conductive pathway by cAMP has been presented.

*Inhibition of Apical Membrane Anion Exchange*

The evidence for inhibition of $\text{Cl}^-/\text{HCO}_3^-$ exchange was obtained from measurements of both extracellular pH and intracellular pH and $\text{Cl}^-$ activity. In most experiments, the main parameter determined was an initial rate of change (of $\text{pH}_o$, $\text{pH}_i$, or $a\text{Cl}_i$) 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 $a\text{Cl}_i$ measurements were performed with ion-sensitive microelectrodes, which are not perfectly selective for $\text{Cl}^-$ (Baumgarten, 1981). At low $a\text{Cl}_i$ values, the contribution of intracellular $\text{HCO}_3^-$, and perhaps of other intracellular anions, can be significant and hence result in overestimates of the true $a\text{Cl}_i$. However, both under control conditions and in theophylline-treated tissues, the initial value of $a\text{Cl}_i$ was of the order of 10 mM or higher, and in this region the response of the $\text{Cl}^-$-sensitive microelectrode to $\text{Cl}^-$ is near-Nernstian; i.e., the $\text{HCO}_3^-$ interference is minimal. Therefore, it is believed that the estimates of initial rates of change of $a\text{Cl}_i$ are correct. The problem of insufficient selectivity of the $\text{Cl}^-$-sensitive microelectrode at low $a\text{Cl}_i$ also complicates the quantitative interpretation of the $\text{Cl}^-$ uptake measurements shown in Figs. 5 and 6. At the low apparent $a\text{Cl}_i$ values measured in a $\text{Cl}^-$-free luminal solution, the slope of the $\text{Cl}^-$-sensitive microelectrode is probably lower than the value predicted from
the Nernst equation, and therefore the rate of change in \( aCl \) 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 \( aCl \). Hence, I feel that the comparison shown in Fig. 6 is valid.

Second, the changes in \( aCl \) 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\(^+\)-loading technique (Reuss, 1985) revealed immeasurably small cell volume changes upon reducing mucosal solution \([Cl^-]\) 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 \( Cl^-\)-conductive pathway that is not present under control conditions, these results suggest that cell shrinkage involves conductive loss of \( Cl^-\) (and probably of \( K^+\)); since cell volume is not reduced by lowering \([Cl^-]\) under control conditions, the \( Cl^-\) efflux responsible for the shrinkage must occur via the conductive pathway. In other words, these preliminary results strengthen the conclusion of a large conductive \( Cl^-\) 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 \( Na^+\)-free medium on that side have been implicitly assumed to result from effluxes of \( HCO_3^-\) into that solution. In principle, the change in \( pH_o\) could be due to a net \( H^+\) flux from the lumen to the cells, to a net flux of base (not necessarily \( HCO_3^-\)) 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 \( H^+\) influx (lumen to cell), because in tissues exposed to \( 10^{-3}\) M amiloride in the presence of 100 mM \( Na^+\), the net \( H^+\) flux, although reduced, is from cell to lumen (Weinman and Reuss, 1984; Reuss, 1984). (c) Under conditions comparable to those in the \( pH_o\) experiments, the changes in \( pH_i\) and \( aCl\) are consistent with electroneutral anion exchange (Reuss and Costantin, 1984; this article).

Fourth, the \( pH_i\) changes elicited by a reduction of luminal \([HCO_3^-]\) were attributed to a change in the direction of \( Cl^-/HCO_3^-\) exchange. Alternatively, inasmuch as luminal \( [H^+]\) is raised in this experiment, the cell acidification could result from a decrease in the net \( H^+\) efflux via \( Na^+/H^+\) exchange, or from a reversal of this flux. The reduction in theophylline could simply be due to the inhibitory effect of cAMP on \( Na^+/H^+\) exchange (Reuss and Peterson, 1985). This possibility was tested by comparing the initial rates of change of \( pH_i\) upon exposure to 1 mM \( HCO_3^-\) or to 1 mM \( HCO_3^-\) plus 1 mM amiloride. If cell acidification is due to a change in \( H^+\) flux via \( Na^+/H^+\) exchange, it should be less in amiloride. However, amiloride caused an increase in \( \Delta pH/\Delta t\), from -0.17
± 0.02 to −0.23 ± 0.02 min⁻¹ (p < 0.05; n = 4). This result could mean that, in
the absence of amiloride, the drop in pH, upon reduction of mucosal [HCO₃⁻]
activates the Na⁺/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 ΔpHₒ 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 CO₂ 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 H⁺ fluxes under several
experimental conditions. These studies support the hypothesis of inhibition of
Cl⁻/HCO₃⁻ exchange by theophylline treatment because the rate of luminal
solution alkalinization observed shortly after adding amiloride or removing Na⁺
from the lumen was reduced significantly by theophylline. As stated above,
measurements of αClᵢ and pHᵢ under the same conditions indicate that the
driving force for Cl⁻/HCO₃⁻ exchange may be slightly elevated in theophylline-
treated tissues. In other words, the fall in the HCO₃⁻ 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 αClᵢ in response to reductions in luminal
[Cl⁻] also support the hypothesis that Cl⁻/HCO₃⁻ exchange is cAMP inhabitable.
In theophylline, the initial rate of fall of αClᵢ when mucosal [Cl⁻] was reduced
remained unchanged, compared with control, although cAMP clearly induces a
Cl⁻-conductive pathway. Estimation of the 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 αClᵢ upon reducing
mucosal [Cl⁻] to 10 mM and upon reducing mucosal [Cl⁻] to 10 mM and
[HCO₃⁻] to 1 mM were compared. As shown in the Results, the initial rates of
fall of αClᵢ were the same in the absence and presence of theophylline. Since in
theophylline the conductive 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 αClᵢ in theophylline. Second, the conductive Cl⁻ loss was estimated
by comparing the values of ΔαClᵢ/Δt upon reducing mucosal [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 ± 1.7 to
−7.1 ± 0.8 mM/min. Since at this concentration the effect of DPC is not maximal,
there is probably a residual Cl⁻ flux via the exchanger, which is probably similar
to that observed when control tissues are exposed to DPC at the same concen-
tration (~1.4 ± 0.4 mM/min). Hence, the conductive flux can be estimated to
be the difference between 7.1 and 1.4, i.e., about −5.7 mM/min, which again is
~50% of the total net Cl⁻ flux. Again, the precise degree of inhibition is difficult
to assess because of the different values of αClᵢ in control and theophylline-
treated tissues. In sum, both estimates of conductive Cl⁻ fluxes suggest that Cl⁻/HCO₃⁻ exchange is inhibited when intracellular cAMP is elevated. The preliminary measurements of cell volume changes elicited by lowering mucosal solution [Cl⁻] to 10 mM suggest that the conductive 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 [Cl⁻] is reduced in theophylline-treated tissues, part of the 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 [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 Vₘₐₓ in forskolin-treated tissues. The apparent Kₘ of the process decreased dramatically. By itself, this effect would stimulate Cl⁻ transport by anion exchange, so that it cannot be responsible for the overall inhibition. The determination of the Kₘ 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 ΔaClᵢ/Δt at low external [Cl⁻]. Hence, the value of the apparent Kₘ is quite uncertain. In any event, it is interesting to observe that a similar result, namely decreases in both Vₘₐₓ and Kₘ, was obtained in our investigation of the effect of cAMP on Na⁺/H⁺ exchange (Reuss and Petersen, 1985).

The measurements of changes in pH in response to reductions of luminal [Cl⁻] to 10 mM, as well as in response to a lowering of luminal [HCO₃⁻] to 1 mM, also support the conclusion of inhibition of apical membrane anion exchange. When external [Cl⁻] was lowered, the cells alkalinized (Cl⁻ efflux coupled to HCO₃⁻ influx). The rate of alkalinization was significantly lowered by theophylline, as illustrated in Fig. 7 and summarized in Table IV. Reducing luminal [HCO₃⁻] from 10 to 1 mM under control conditions caused intracellular acidification and a rise in aClᵢ. 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 Na⁺/H⁺ exchange in the effects of lowering mucosal [HCO₃⁻] was ruled out by the amiloride experiments reported above.

**Arguments against Induction of an HCO₃⁻-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 HCO₃⁻ conductance. They showed that in a Cl⁻-free, HCO₃⁻-free medium, IBMX produced monotonic cell membrane depolarization; however, in the presence of 25 mM HCO₃⁻ 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 HCO₃⁻-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 HCO₃⁻ permeability, then the HCO₃⁻ 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 [HCO3\textsuperscript{-}] of 25 mM, the intracellular HCO3\textsuperscript{-} concentration would be ≤1.8 mM, and pH\textsubscript{t} (assuming equal values of P\textsubscript{CO2} in extracellular and intracellular phases) would be, at most, 6.26. This possibility is unlikely on the basis of pH\textsubscript{t} 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 pH\textsubscript{t} reach values as low as 6.5 (Reuss and Petersen, 1985). Under more physiologic conditions, pH\textsubscript{t} 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 HCO3\textsuperscript{-} conductance at the apical membrane (Petersen, K.-U., personal communication).

The hypothesis of a cAMP-induced apical membrane HCO3\textsuperscript{-} conductance was tested directly by assessing the effect of a reduction in mucosal solution [HCO3\textsuperscript{-}] on membrane voltages, the apparent ratio of cell membrane resistances, and pH\textsubscript{t} and aCl\textsubscript{t} both under control conditions and during exposure to theophylline. The mucosal bathing solution was control Ringer's, NaHCO3 was partially replaced with Na cyclamate, and the P\textsubscript{CO2} was maintained constant. As shown above (Figs. 8 and 9, and Tables V and VI), the depolarization elicited by reducing mucosal [HCO3\textsuperscript{-}] from 10 to 1 mM, in the presence of Cl\textsuperscript{-}, was not significantly changed by theophylline. In addition, the apparent ratio of membrane resistances was not altered by the reduction in external [HCO3\textsuperscript{-}], and the initial rate of intracellular acidification was not increased, but was reduced in theophylline. Although all three observations argue against an HCO3\textsuperscript{-}-conductive pathway, a relatively small electrodiffusive HCO3\textsuperscript{-} permeability could exist and be masked by the native K\textsuperscript{+} permeability and the cAMP-induced Cl\textsuperscript{-} permeability. Since the latter is the dominant apical membrane conductive pathway during elevation of cAMP levels, the effect of lowering [HCO3\textsuperscript{-}] to 1 mM on membrane voltages and on the ratio of resistances was tested after Cl\textsuperscript{-} removal from the mucosal solution. As shown in the Results (Fig. 10 and Table VII), the apical membrane depolarization elicited by lowering external [HCO3\textsuperscript{-}] was slightly increased in theophylline, but it is doubtful that this result is an indication of a conductive pathway for HCO3\textsuperscript{-} across the apical membrane. First, the voltage change is slow, compared with that induced by changes in external [Cl\textsuperscript{-}] (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 [HCO3\textsuperscript{-}] could cause a larger change in V\textsubscript{mc} simply because of the lower value of R\textsubscript{a}/R\textsubscript{b} in theophylline. If the primary effect of cAMP were to reduce basolateral P\textsubscript{K} and hence to increase R\textsubscript{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 pH 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 \(K^+\) 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 \( \text{Na}^+ \), \( \text{K}^+ \) pump activity and \( P_K \) may be inhibited \textit{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).

I am grateful for the comments of Calvin U. Cotton and Yoav Segal on preliminary versions of this manuscript, the technical help of Joianne E. Bazile and James L. Costantin, and the secretarial help of Ann L. Pearce.

This research was supported by National Institutes of Health grant DK-38734.

Original version received 22 January 1987 and accepted version received 27 April 1987.

REFERENCES


Reuss, L. 1987. Salt and water transport by the gallbladder epithelium. In Handbook of 

Reuss, L., and J. L. Costantin. 1984. Cl⁻/HCO₃⁻ exchange at the apical membrane of Necturus 

In press.

Reuss, L., and A. L. Finn. 1975a. Electrical properties of the cellular transepithelial pathway 
in Necturus gallbladder. I. Circuit analysis and steady-state effects of mucosal solution ionic 

Reuss, L., and A. L. Finn. 1975b. Electrical properties of the cellular transepithelial pathway 
in Necturus gallbladder. II. Ionic permeability of the apical cell membrane. Journal of 


244:C336–C347.


Saito, Y., and E. M. Wright. 1983. Bicarbonate transport across the frog choroid plexus and 

Saito, Y., and E. M. Wright. 1984. Regulation of bicarbonate transport across the brush border 

321.


Zeldin, D. C., A. Corcia, and W. McD. Armstrong. 1985. Cyclic AMP-induced changes in 