

Cl⁻/HCO₃⁻ Exchange at the Apical Membrane of *Necturus* Gallbladder

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ABSTRACT The hypothesis of Cl⁻/HCO₃⁻ exchange across the apical membrane of the epithelial cells of *Necturus* gallbladder was tested by means of measurements of extracellular pH (pH_o), intracellular pH (pH_i), and Cl⁻ activity (*a*Cl_i) with ion-sensitive microelectrodes. Luminal pH changes were measured after stopping mucosal superfusion with a solution of low buffering power. Under control conditions, the luminal solution acidifies when superfusion is stopped. Shortly after addition of the Na⁺/H⁺ exchange inhibitor amiloride (10⁻³ M) to the superfusate, alkalization was observed. During prolonged (10 min) exposure to amiloride, no significant pH_o change occurred. Shortly after amiloride removal, luminal acidification increased, returning to control rates in 10 min. The absence of Na⁺ in the superfusate (TMA⁺ substitution) caused changes in the same direction, but they were larger than those observed with amiloride. Removal of Cl⁻ (cyclamate or sulfate substitution) caused a short-lived increase in the rate of luminal acidification, followed by a return to control values (10–30 min). Upon re-exposure to Cl⁻, there was a transient reduction of luminal acidification. The initial increase in acidification produced by Cl⁻ removal was partially inhibited by SITS (0.5 mM). The pH_i increased rapidly and reversibly when the Cl⁻ concentration of the mucosal bathing solution was reduced to nominally 0 mM. The pH_i changes were larger in 10 mM HCO₃⁻-Ringer's than in 1 mM HEPES-Ringer's, which suggests that HCO₃⁻ is transported in exchange for Cl⁻. In both HEPES- and HCO₃⁻-Ringer's, SITS inhibited the pH_i changes. Finally, intracellular acidification or alkalization (partial replacement of NaCl with sodium propionate or ammonium chloride, respectively) caused a reversible decrease or increase of *a*Cl_i. These results support the hypothesis of apical membrane Cl⁻/HCO₃⁻ exchange, which can be dissociated from Na⁺/H⁺ exchange and operates under control conditions. The coexistence at the apical membrane of Na⁺/H⁺ and Cl⁻/HCO₃⁻ antiports suggests that NaCl entry can occur through these transporters.

INTRODUCTION

In epithelia that absorb NaCl by an electroneutral process, NaCl entry across the luminal membrane has been attributed to either direct coupling of Na⁺ and Cl⁻

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to a carrier (symport mechanism; for reviews see Frizzell et al., 1979; Warnock and Eveloff, 1982; Spring and Ericson 1982), or to the simultaneous operation of two ion exchangers, e.g., Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ (double-antiport mechanism; see Turnberg et al., 1970; Liedtke and Hopfer, 1977; Petersen et al., 1981; Warnock and Eveloff, 1982; Weinman and Reuss, 1984).

In intestinal brush border vesicles, the elegant experiments of Liedtke and Hopfer (1982*a, b*) have not confirmed the existence of an NaCl symport and have demonstrated the presence of a $\text{Cl}^-/\text{HCO}_3^-$ (or Cl^-/OH^-) antiport in parallel with a Cl^- -conductive pathway. Studies on apical membrane vesicles of *Necturus* proximal tubule have also failed to demonstrate an NaCl symport (Seifter et al., 1980), although such a transport mechanism was proposed for this segment on the basis of electrophysiologic data obtained in situ (Spring and Kimura, 1978). However, isolated-membrane studies do not provide an unequivocal answer to the question of the physiologic significance of these transport systems because of the possibilities of insertion, activation, or inactivation of such systems by the membrane isolation procedure.

Evidence consistent with electroneutral NaCl uptake has been provided in gallbladder epithelia of different species by electrophysiologic techniques, isotopic flux measurements, and optical determinations of cell volume (Cremaschi and Hénin, 1975; Frizzell et al., 1975; Duffey et al., 1978; Reuss and Grady, 1979; Garcia-Diaz and Armstrong, 1980; Ericson and Spring, 1982*a*). The results are all consistent with the symport hypothesis, but could also be explained by the double-antiport mechanism.

We have demonstrated (Weinman and Reuss, 1982) that in *Necturus* gallbladder there is an Na^+/H^+ exchanger at the apical membrane. As indicated by the observation of luminal acidification under steady state conditions and the rapid effects of high concentrations of amiloride on tracer Na^+ uptake (from lumen into epithelium) and on intracellular Na^+ activity, this mechanism operates under control conditions and appears to account for a large fraction of Na^+ entry (Weinman and Reuss, 1984). In this paper, we report experiments designed to test, in the same preparation, the hypothesis of anion exchange at the apical membrane. To this end, the following kinds of experiments were performed: (*a*) determinations of net apparent equivalent H^+ fluxes into or from the luminal bathing solution before, during, and after ionic substitutions, (*b*) measurements of intracellular pH under control conditions and upon changes in luminal solution Cl^- concentration, (*c*) measurements of intracellular Cl^- activity under control conditions and during experimentally produced changes in intracellular pH, and (*d*) examination of the sensitivity of some of these effects to a disulfonic stilbene. The results obtained support the hypothesis of apical membrane anion exchange, and indicate that this transport process operates under control conditions and can be dissociated from the parallel Na^+/H^+ antiport. A preliminary account of some of these results has been presented (Reuss et al., 1984).

MATERIALS AND METHODS

Mud puppies (*Necturus maculosus*) were purchased from Nasco Biologicals (Ft. Atkinson, WI), kept in aquaria at $\sim 10^\circ\text{C}$, and fed live goldfish. The animals were anesthetized with

Tricaine methanesulfonate and the gallbladders were excised and mounted mucosal side up in a modified Ussing chamber as previously described (Reuss and Finn, 1975, 1977). Both mucosal and serosal bathing solutions were exchanged continuously. Two control Na-Ringer's solutions were employed: (a) HEPES-Ringer's, containing (in mM): 109.2 NaCl, 2.5 KCl, 1.0 CaCl₂, and 1.0 K-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (K-HEPES); (b) HCO₃-Ringer's, containing (in mM): 90 NaCl, 10 NaHCO₃, 2.5 KCl, 1.8 CaCl₂, 1.0 MgCl₂, and 0.5 NaH₂PO₄. HEPES-Ringer's was gassed with room air and had a pH of 7.7; HCO₃-Ringer's was gassed with a 1% CO₂/99% air mixture and had a pH of 7.6. HEPES-Ringer's and HCO₃-Ringer's differ, in addition, in Ca, Mg, and phosphate concentrations and in osmolality. These differences were accepted, however, to permit direct comparison of these studies with our previous ones (Weinman and Reuss, 1982, 1984) and those of Spring and co-workers (Ericson and Spring, 1982a; Larson and Spring, 1983). Ionic substitutions of Na⁺ with tetramethylammonium (TMA⁺) and Cl⁻ with cyclamate were isomolar. When sulfate was employed to substitute chloride, the osmolality of the solution was adjusted with sucrose. Amiloride was a generous gift of Merck, Sharp & Dohme, West Point, PA; 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) was purchased from Pierce Chemical Co., Rockford, IL.

Measurements of Electrical Potentials

Transepithelial (V_{ms}), apical membrane (V_{mc}), and basolateral membrane (V_{cs}) potentials were measured as described previously (Reuss and Finn, 1975, 1977). The serosal reference electrode was an Ag-AgCl pellet separated from the bathing solution by a short Ringer's-agar bridge. The mucosal electrode in the experiments in which extracellular pH was measured was a calomel half-cell connected to the solution with an agar bridge of the same salt composition. In the experiments in which intracellular pH and/or intracellular Cl⁻ activity was measured, to minimize liquid junction potential changes upon ionic substitutions, the calomel half-cell was connected to the bathing solution by a flowing, saturated KCl bridge made of a polyethylene tube sealed at one end with a short piece of porous glass (Ultrawick; W-P Instruments, Inc., New Haven, CT). To prevent significant K⁺ concentration changes in the bathing medium, the electrode was positioned near the outflow mucosal pipette and the rate of exchange of the mucosal solution was kept high. V_{ms} was referred to the serosal side; V_{mc} and V_{cs} were referred to the respective bathing solutions. Transepithelial current pulses were passed via two Ag-AgCl electrodes to measure the transepithelial resistance and the apparent ratio of cell membrane resistances of the impaled cells.

Micropipettes were pulled from 1-mm-OD inner fiberglass capillaries (Hilgenberg, Malsfeld, Federal Republic of Germany [FRG]) on a horizontal, two-stage microelectrode puller (model PD-5; Narishige, Tokyo, Japan; or Brown-Flaming P-77; Sutter Instrument Co., San Francisco, CA) and filled with 3 or 0.5 M KCl. The cells were observed with an inverted microscope (Diavert; E. Leitz, Inc., Rockleigh, NJ) and impaled by means of remote-control micromanipulators (STM-3; Gebr. Marzhauser Wetzlar, FRG; or MO-103; Narishige). Impalements were validated as previously described (Weinman and Reuss, 1982, 1984).

Measurement of Mucosal Solution pH

Changes in pH_o upon stopping mucosal superfusion were measured with glass pH electrodes constructed as previously described (Weinman and Reuss, 1982). The transepithelial potential (V_{ms}), the voltage output of the pH electrode (V^*), both referred to the serosal bathing solution, and the difference ($V_H = V^* - V_{ms}$) were recorded continuously. The change of pH_o was calculated according to the equation $\Delta pH_o = \Delta V_H/S$, where ΔV_H

is the change in V_H measured after 3 min of stopping mucosal superfusion (in millivolts) and S is the pH electrode slope (in millivolts per pH unit). For comparisons between H^+ fluxes in the presence of different mucosal bathing media, the buffering power of the mucosal solution was taken into account as previously described (Weinman and Reuss, 1982). All comparisons involved measurements in the same tissue, with the pH electrode positioned <1 mm above the apical surface. An apparent net H^+ flux was calculated from (see Weinman and Reuss, 1982):

$$J_H = \Delta pH_o \beta v / (At),$$

where J_H is the net apparent H^+ flux ($\text{mol min}^{-1} \text{cm}^{-2}$), β is the buffering power ($\text{mol liter}^{-1} \text{pH unit}^{-1}$), ΔpH_o is the pH change, v is the volume of the mucosal solution (liters), A is the exposed tissue area (0.5 cm^2), and t is the duration of the measurement (3 min). The experimental data were normalized to the J_H value measured in HEPES-Ringer's.

Measurements of Intracellular pH and Cl Activity

To measure intracellular pH (pH_i) or intracellular Cl^- activity (aCl_i), simultaneous impalements with conventional and pH- or Cl^- -sensitive microelectrodes were employed. In most pH_i measurements, liquid membrane pH-sensitive microelectrodes were employed (Ammann et al., 1981). In a few experiments, recessed-tip glass pH microelectrodes (Thomas, 1978) were used. The results were the same with both techniques. Liquid membrane microelectrodes were preferred because they are easier to construct, have a shorter response time, and there is no risk of major changes of intracellular ionic composition immediately after impalement (dead space of the recessed-tip electrode). The electrodes were constructed from pipettes pulled as described above. After drying, the glass surface was rendered hydrophobic by exposure to hexamethyldisilazane (Sigma Chemical Co., St. Louis, MO), a small volume of pH-sensitive cocktail was injected, and an inner reference pipette was positioned near the tip, as previously described for other ion-sensitive microelectrodes (Reuss et al., 1983). The slopes of the liquid membrane electrodes ranged from 53 to 62 mV/pH unit. No significant cation interferences were found. Occasionally, responses to Cl^- and HCO_3^- were encountered. These electrodes were discarded. The slopes of the recessed-tip glass pH electrodes ranged from 57 to 59 mV/pH unit. Impalements with pH-sensitive microelectrodes were validated as described before (Weinman and Reuss, 1982, 1984).

Cl^- -sensitive microelectrodes were prepared using the exchanger 477913 (Corning Medical and Scientific, Medfield, MA), as described before (Reuss et al., 1983). The slopes ranged from 54 to 61 mV/10-fold change in Cl activity.

In both pH_i and aCl_i measurements, two cells in the same field were impaled with a conventional and an ion-sensitive microelectrode, respectively. After the voltages were stable and the impalements were validated, the mucosal bathing solution was rapidly changed, usually for ~3 min, while continuously recording both potentials. At the end of this period, the tissue was exposed again to the control bathing solution. Since the mucosal solution electrode was a flowing, saturated KCl bridge, liquid junction potentials changes were small and were neglected. Activity coefficients of the solutions and intracellular activities were calculated as described before (Reuss et al., 1983).

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

We have previously demonstrated (Weinman and Reuss, 1982, 1984) an Na^+ / H^+ antiport on the apical membrane of *Necturus* gallbladder. In the present studies, we tested the hypothesis that an anion exchange (Cl^-/HCO_3^- , Cl^-/OH^- , or an equivalent process) occurs across the apical membrane, which is independent of and parallel to the Na^+/H^+ exchange. If this is the case, several predictions concerning the magnitude and/or direction of the net acid or base flux across the apical membrane can be made for specific experimental conditions.

(a) Blocking Na^+/H^+ exchange at the apical membrane with amiloride should unmask the effect of the anion antiport and hence convert the external acidification into alkalization: the anion exchanger would move the HCO_3^- (or OH^-) from the cell into superfusate because the inward-directed Cl^- gradient (Petersen and Reuss, 1983) is larger than the opposing transmembrane pH gradient (Weinman and Reuss, 1982). As exposure to amiloride continues, the cells acidify (Weinman and Reuss, 1982), thus reducing the outward HCO_3^- (OH^-) flux. Hence, extracellular alkalization should slow with time. Removal of amiloride should restore the operation of the Na^+/H^+ antiport to a level higher than the one before amiloride, for at least two reasons: first, because of the amiloride-induced intracellular acidification (Weinman and Reuss, 1982), which makes the H^+ gradient more favorable for H^+ efflux and possibly activates the exchanger (Aronson et al., 1982); second, because amiloride causes a decrease in intracellular Na^+ activity (Weinman and Reuss, 1984) and hence the Na^+ gradient is more favorable for Na^+ entry. As both pH_i and aNa_i return to control values after amiloride removal, the rate of luminal acidification should also return to control values several minutes after removal of the drug.

Fig. 1 illustrates this sequence. When superfusion was stopped for 3 min, starting 1 min after exposure to amiloride, the luminal solution alkalized. After 10 min in amiloride, the pH_o change was practically zero (see also Weinman and Reuss, 1982). Shortly after removal of the diuretic, luminal acidification was enhanced as compared with control. Finally, 10 min after removal of amiloride, acidification had returned to the control value. In sum, all the predictions listed above were confirmed by the experimental results.

(b) Replacement of luminal Na^+ with a cation not transported by the antiport, such as TMA^+ (Weinman and Reuss, 1982), should result in a sequence of luminal pH changes similar to those produced by amiloride. However, the magnitude of the alkalization shortly after Na^+ removal should be larger than that observed after the same interval in the amiloride-treated tissue, because in this experiment that Na^+/H^+ exchange will be reversed rather than blocked. Also, luminal acidification immediately after replacing luminal Na^+ should be greater than after amiloride removal, because the changes in aNa_i and pH_i produced by Na^+ removal are larger than those obtained with amiloride (Weinman and Reuss, 1982, 1984). A comparison between TMA^+ and amiloride experiments, which is consistent with these predictions, is shown in Table I.

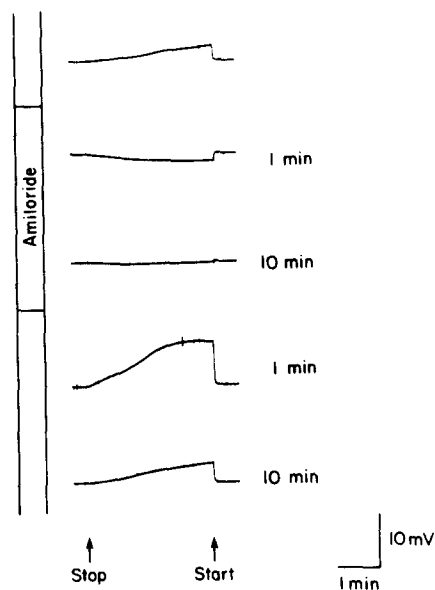


FIGURE 1. Effect of amiloride (1 mM, mucosal bathing solution) on the changes of extracellular pH produced by stopping mucosal superfusion. In this experiment, as in that illustrated in Fig. 2, the control solution was HEPES-Ringer's, and the experimental solution is indicated on the left. The traces shown correspond to the difference in voltage outputs of the mucosal pH electrode and transepithelial potential electrode (see Materials and Methods) and were obtained successively in one tissue. Upward changes in potential correspond to luminal solution acidification. Superfusion was stopped and re-started at the times denoted by the arrows. The times on the right-hand side denote the duration of exposure to HEPES-Ringer's plus amiloride or HEPES-Ringer's at the moment that superfusion was stopped. See text.

TABLE I

Effects of Addition of Amiloride or Na⁺ Removal from the Mucosal Bathing Medium on Apparent Net H⁺ Flux into the Lumen

| | Control | Normalized H ⁺ flux | | | |
|-----------------------|---------|--------------------------------|----------|---------|---------|
| | | Experimental | | Control | |
| | | 1 min | 10 min | 1 min | 10 min |
| Amiloride | 1.0 | -0.8±0.1 | -0.1±0.1 | 1.8±0.1 | 1.1±0.1 |
| Na ⁺ -free | 1.0 | -1.3±0.4 | 0.2±0.3 | 3.0±0.3 | 1.3±0.1 |

Extracellular pH changes and apparent net H⁺ flux into the lumen were measured and calculated as described in Materials and Methods. The data presented are means ± SEM normalized to the value observed in HEPES-Ringer's (1.0) (column 1). During the experimental period, amiloride was added to the mucosal bathing solution (1 mM, *n* = 14 experiments) or Na⁺ was replaced with TMA⁺ (*n* = 6 experiments) and the H⁺ flux was measured during two 3-min periods, starting 1 and 10 min after the solution change (columns 2 and 3, respectively). After this, the tissues were again exposed to HEPES-Ringer's and two more fluxes were determined, at the same intervals and for the same duration (columns 4 and 5). In both series, the two experimental values and the first value after returning to HEPES-Ringer's differed significantly from the values measured before the mucosal solution substitution. Also in both series, the experimental value at 10 min was not significantly different from zero.

(c) Removal of luminal Cl^- will reverse the Cl^- gradient and thus the direction of the apical Cl^-/HCO_3^- exchange. This should result immediately in enhanced luminal acidification upon stopping superfusion. During prolonged Cl^- removal, pH_i rises (see below) and aCl_i falls (Petersen and Reuss, 1983). Both the fall of aCl_i and the rise of pH_i reduce the magnitude of the luminal acidification. Immediately after returning to Cl^- -containing mucosal bathing medium, the rate of the cation antiport will be reduced (pH_i is elevated), whereas that of the anion antiport will be enhanced, since both Cl^- and HCO_3^- (OH^-) gradients are now more favorable for entry and exit, respectively, across the apical membrane. Later on, as the gradients are restored to control values, luminal acidification should approach the value observed before luminal Cl^- removal.

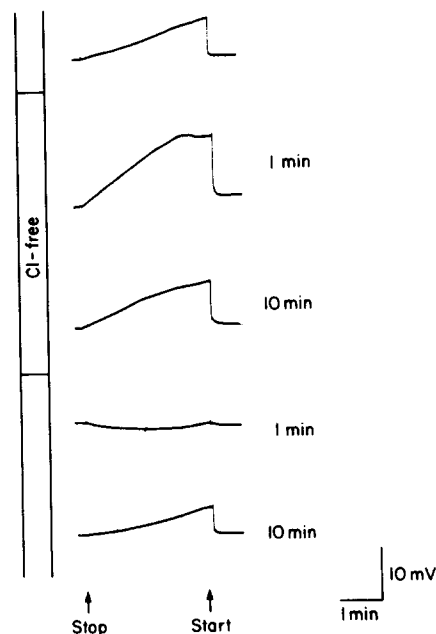


FIGURE 2. Effect of Cl^- removal from the mucosal side (isomolar substitution with cyclamate) on the changes of extracellular pH produced by stopping mucosal superfusion. See legend to Fig. 1.

That all of these predictions are borne out is shown in Fig. 2. These results are not related to the nature of the substituting anion, because similar results were obtained with sulfate (Table II).

(d) Because disulfonic stilbenes can inhibit anion exchange (Cabantchik and Rothstein, 1972), the effect of one of them, SITS, was tested. Addition of 0.5 mM SITS to the luminal solution did not significantly alter luminal acidification in HEPES-Ringer's but reduced the luminal acidification observed shortly after Cl^- removal to $64 \pm 16\%$ of the control value ($P < 0.05$, $n = 5$).

(e) The results described above, although consistent with the double-antiport hypothesis, do not rule out the possibility that each exchanger operates only

TABLE II

Effects of Cl⁻ Removal from the Mucosal Bathing Medium on Apparent Net H⁺ Flux into the Lumen

| | Normalized H ⁺ flux | | | | | |
|-----------|--------------------------------|--------------|---------|---------|---------|---------|
| | Control | Experimental | | | Control | |
| | | 1 min | 10 min | 20 min | 1 min | 10 min |
| Cyclamate | 1.0 | 2.4±0.4 | 1.3±0.2 | 1.1±0.2 | 0.4±0.3 | 0.7±0.1 |
| Sulfate | 1.0 | 1.7±0.2 | 1.0±0.1 | — | — | 0.9±0.1 |

Protocol was the same as indicated for Table I. Cl⁻ was replaced with cyclamate ($n = 6$) or sulfate ($n = 6$). In both series, the first set of values in Cl⁻-free medium was significantly different from control. In the cyclamate series, the value obtained 1 min after returning to HEPES-Ringer's was also significantly different from control.

when the other one is active. To test this possibility, tissues were bathed on both sides for up to 120 min with an Na-free, Cl-free solution, to reduce extra- and intracellular Na⁺ and Cl⁻ concentrations to essentially zero. During this period, either Na⁺ or Cl⁻ was periodically replaced in the mucosal superfusate for 1 min and the change in luminal solution pH was measured over a 3-min period, as above. As expected from the low values of both $a\text{Na}_i$ and pH_i at the time of Na⁺ addition, a high rate of luminal acidification was observed (Fig. 3), which did not decrease over the 120 min of incubation in Cl-free medium, and was not different from that observed in Cl-containing medium upon replacing Na⁺ after a 15-min period of removal. We conclude from these experiments that Na⁺/H⁺ exchange is Cl⁻ independent.

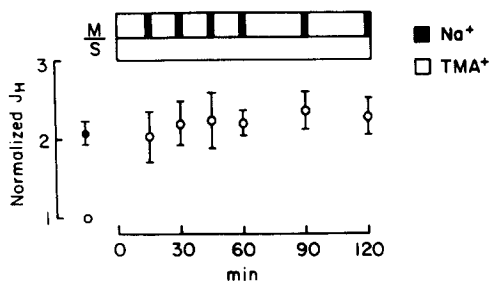


FIGURE 3. Persistence of Na⁺ gradient-induced luminal acidification during prolonged bilateral exposure to Cl⁻-free bathing media. Data shown are means \pm SEM of the net H⁺ fluxes into the luminal solution normalized to the values observed in the same tissues with HEPES-Ringer's on both sides (open circles: before $t = 0$). Starting at $t = 0$, the tissue was exposed to a TMA⁺-cyclamate solution (Na-free, Cl-free) on both sides. Luminal acidification was measured intermittently for 3-min periods after 1 min of rapid mucosal superfusion with Na-cyclamate solution (dark bars). Between the measurements, both surfaces were bathed with the TMA-cyclamate solution. Note that luminal acidification persisted at levels approximately twofold the control one for the 120-min duration of the experiments. For comparison, the magnitude of Na⁺ gradient-induced acidification in the same tissues after 15 min of pre-exposure to TMA⁺-Ringer's is shown at the beginning (closed circle). $N =$ five experiments.

In the four experiments in which Cl^- (and not Na^+) was replaced in the mucosal superfusate, luminal alkalinization (at a rate of ~70% of the control acidification) was obtained after 60 min of Na^+ removal. Thereafter, a decrease in the rate of alkalinization was observed, a result expected from the intracellular acidification produced by Na^+ removal, because of both the initial reversal of the apical membrane Na^+ gradient and the inability of the cells to extrude H^+ of metabolic origin in the absence of external Na^+ (Weinman and Reuss, 1982).

Intracellular pH Measurements

If a Cl^-/HCO_3^- exchanger operates at the luminal membrane, and if under control conditions Cl^- enters and HCO_3^- leaves the cell through this pathway, reducing or removing Cl^- from the lumen should decrease or reverse HCO_3^- transport across the apical membrane, and therefore should cause intracellular

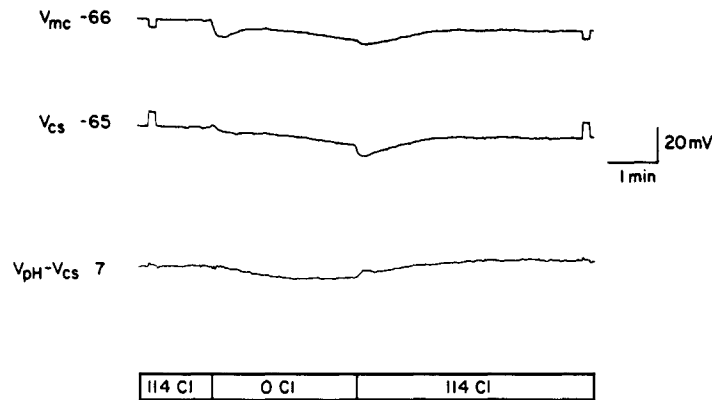


FIGURE 4. Effects of mucosal solution Cl^- removal (in HEPES-Ringer's) on intracellular pH and cell membrane potentials. The traces depict apical membrane potential (V_{mc}), basolateral membrane potential (V_{cs}), and the difference between voltage outputs of the pH and conventional intracellular microelectrodes ($V_{pH} - V_{cs}$). Traces start with both microelectrodes in cells. Initial values are indicated at the beginning of the traces. Cl^- removal (for the period indicated at the bottom) produced hyperpolarization of both membranes (downward changes in voltage) and an increase in pH_i (downward change in differential trace). All changes were reversible. Transepithelial current pulses were applied at the beginning and end of the record to compare the apparent ratio of cell membrane resistance of the two impaled cells. Note that the steady state voltage deflection in the differential trace is negligible.

alkalinization. Figs. 4 and 5 show examples of the effects of luminal Cl^- removal (cyclamate substitution) on cell membrane potentials and pH_i in HEPES-Ringer's and HCO_3^- -Ringer's, respectively. In both experiments, the cell interior became more alkaline during superfusion of the apical side with a Cl^- -free bathing solution. A steady state new pH_i value was attained ~2 min after the substitution. In both sets of experiments, re-exposure to Cl^- -containing medium caused a transient acidification of the cell, which was more prominent in HCO_3^- -Ringer's.

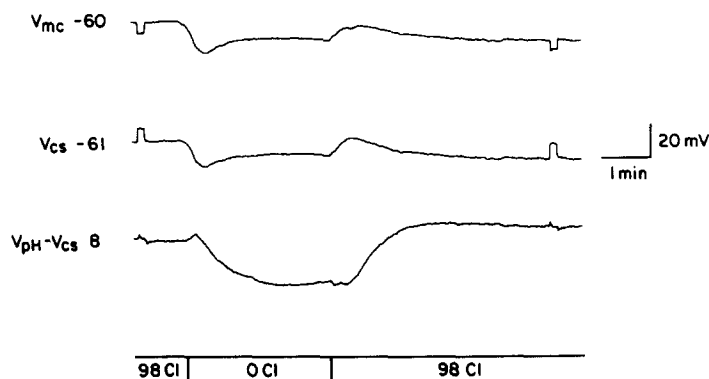


FIGURE 5. Effects of mucosal solution Cl^- removal (in HCO_3^- -Ringer's) on intracellular pH and cell membrane potentials. Symbols and protocol are as in Fig. 4. Note that the cell membrane potential and pH_i changes are larger than in HEPES-Ringer's, and that pH_i undergoes a notable transient fall after re-exposure to Cl^- -containing medium.

As summarized in Table III, the intracellular alkalinization produced by Cl^- removal was larger in HCO_3^- -Ringer's than in HEPES-Ringer's, although the intracellular buffering power must be higher in the former condition. This indicates that the equivalent base flux into the cells during exposure to the low Cl^- solutions was much greater in HCO_3^- -Ringer's, and therefore that HCO_3^- itself participates in the exchange (see Discussion).

Addition of SITS to the mucosal bathing medium reduced significantly the steady state change in pH_i caused by removal of Cl^- . In HEPES-Ringer's, addition of 0.5 mM SITS at the time of Cl^- removal abolished the pH_i change ($\Delta\text{pH}_i = 0.01 \pm 0.01$, $n = 4$); in HCO_3^- -Ringer's, the same experimental maneuver reduced the intracellular alkalinization to 0.14 ± 0.05 ($n = 3$), i.e., to 40% of that observed in the absence of SITS (Table III).

Intracellular Cl^- Activity Measurements

Acidifying the cell should decrease intracellular HCO_3^- activity and therefore, by making the gradient less favorable for Cl^- entry, should cause a fall in $a\text{Cl}_i$. Intracellular alkalinization, by increasing intracellular HCO_3^- , should enhance the operation of the anion antiport and cause an increase in $a\text{Cl}_i$. Both predictions were tested in tissues exposed initially to HEPES-Ringer's or HCO_3^- -Ringer's.

TABLE III
Effect of Cl^- Removal on Intracellular pH

| | Control | Cl-free | Difference |
|--|-----------------|-----------------|------------------|
| HEPES-Ringer's ($n = 7$) | 7.55 ± 0.04 | 7.78 ± 0.06 | -0.23 ± 0.05 |
| HCO_3^- -Ringer's ($n = 4$) | 7.53 ± 0.02 | 7.89 ± 0.04 | -0.35 ± 0.03 |

Shown are means \pm SEM of pH_i values under steady state conditions (control) and 3 min after nominally complete removal of Cl^- from the mucosal side (Cl-free, cyclamate substitution), while maintaining continuous impalements.

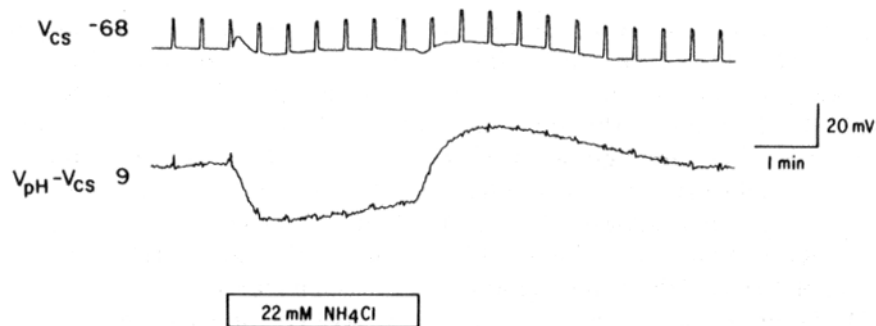


FIGURE 6. Effects of NH_4Cl on intracellular pH and basolateral membrane potential. Symbols and polarity are as in Figs. 4 and 5. Tissue was exposed to 22 mM NH_4Cl for the period indicated at the bottom. Note the rapid alkalization immediately after the solution change, the slow acidification during NH_4Cl , and the "overshoot" acidification after removal of NH_4Cl . Compare the time course of aCl_i in Fig. 7.

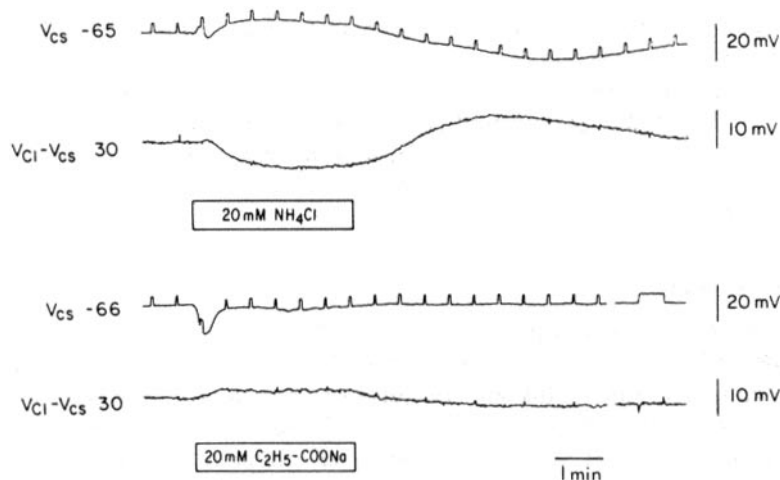


FIGURE 7. Effects of intracellular pH changes on aCl_i in HCO_3 -Ringer's. The top and bottom traces of each pair depict basolateral membrane potential (V_{cs}) and the difference between voltage outputs of Cl -sensitive and conventional microelectrodes ($V_{Cl} - V_{cs}$), respectively. Ammonium chloride (top) causes an increase of aCl_i (negative deflection in differential trace), whereas sodium propionate (bottom) causes a decrease of aCl_i (steady state positive deflection in differential trace). The aCl_i changes were reversible. Note the transient fall of aCl_i upon removal of ammonium chloride. Membrane potential changes are discussed in the text. As in Figs. 4-6, transepithelial current pulses were applied for measurement of the apparent ratio of cell membrane resistances. At the end of the bottom records, the effects of these pulses are shown with a faster time base. Note the negligible steady state voltage deflection in the differential trace. See text and Table IV.

Intracellular acidification was produced by substituting on the mucosal side, at constant pH_o , 20% of the NaCl with sodium propionate. Intracellular alkalization was produced by substituting, also at constant pH_o , 20% of the NaCl with NH_4Cl . By themselves, the small decreases of bathing medium Na^+ or Cl^- concentration have no significant effects on intracellular activities. Both salts have been used to change pH_i in other cells (Boron and De Weer, 1976; Keifer, 1981; Boron and Boulpaep, 1983*a, b*). Ammonium chloride initially causes alkalization (NH_3 entry), followed by slight acidification (NH_4^+ entry). Upon removal of NH_4Cl , NH_3 leaves rapidly, and the cell is transiently acidified (Boron and De Weer, 1976). In two experiments in HEPES-Ringer's (one is shown in Fig. 6), the mean peak alkalization produced by NH_4Cl was 0.32 pH unit, and the mean acidification observed after removal was also 0.32 pH unit. Sodium propionate, at the concentrations employed here, acidifies *Necturus* gallbladder epithelial cells by 0.1–0.2 pH unit (K.-U. Petersen and L. Reuss, unpublished observations).

The effects of these maneuvers on aCl_i are illustrated in Fig. 7. Intracellular alkalization (top panel) caused, as expected, an increase in aCl_i . Upon NH_4Cl removal, aCl_i fell to a value lower than the initial one and then returned to control. This transient decrease of aCl_i , predictable from the transient intracellular acidification upon NH_4Cl removal (see above), was more marked in HCO_3^- -Ringer's than in HEPES-Ringer's.

The bottom panel of Fig. 7 depicts the effects of Na propionate. In both HEPES- and HCO_3^- -Ringer's, small reversible decreases of aCl_i were observed. Note that since liquid membrane Cl^- -sensitive microelectrodes are also sensitive to propionate (Saunders and Brown, 1977), propionate influx causes an underestimate of the aCl_i change.

DISCUSSION

The purpose of the experiments described above was to examine one aspect of the double-antiport hypothesis of apical membrane NaCl entry, i.e., whether an anion exchanger exists at this site. Because of the uncertainties associated with the use of isolated membrane vesicle preparations, a test of this hypothesis in an intact epithelium is necessary. To rule out results dependent on the composition of the bathing media, whenever possible the experiments were carried out in both HEPES- and HCO_3^- -Ringer's.

Demonstration of Apical Membrane Cl^-/HCO_3^- Exchange

We conclude that an anion antiport (Cl^-/HCO_3^- or similar process) exists at the apical membrane from three main experimental arguments.

(a) Anion fluxes across the apical membrane contribute to the changes in mucosal solution pH observed upon stopping superfusion. As shown in Fig. 1 and Table I, blocking the Na^+/H^+ antiport with amiloride and then removing this agent caused the sequence of changes of extracellular pH to be expected from a parallel anion antiport that normally transports base equivalents into the lumen. The effects of Na^+ removal from the lumen (Table I) were also in

agreement with this hypothesis, as were the changes of luminal pH observed upon reversing the Cl^- gradient across the apical membrane (Fig. 2 and Table II).

Even though these results are consistent with the anion antiport hypothesis, they might also be explained qualitatively by other mechanisms, such as an H^+ -conductive pathway (or equivalent transporter) in parallel with both the Na^+/H^+ exchanger and a putative NaCl symport. However, a sizable H^+ conductance can be ruled out because although the apical membrane depolarizes when luminal pH is lowered, its resistance increases rather than decreases (Reuss et al., 1981). Other results show that the effect of luminal pH on membrane potential is due to titration of K^+ -conductive apical membrane sites (Reuss et al., 1981). In addition, three results in the current series of experiments are not easily reconciled with this cation antiport/ H^+ leak model. First, SITS reduced the luminal acidification observed immediately after Cl^- removal; second, removal of luminal Cl^- resulted in larger pH_i changes in HCO_3^- -Ringer's than in HEPES-Ringer's, whereas in the H^+ leak model the opposite would have been expected; third, the pH_i changes produced by Cl^- removal were reduced by SITS.

During exposure to HEPES-Ringer's on both sides, with NaCl absorption proceeding at a steady state rate, both apical membrane exchangers should operate at the same rate. The observation of luminal acidification when superfusion is stopped suggests that Cl^- is exchanged with HCO_3^- , not OH^- . Equivalent transport of H^+ and OH^- into the luminal solution should not change its pH, whereas equivalent net fluxes of H^+ and HCO_3^- should cause acidification if CO_2 is allowed to accumulate.

(b) The second experimental argument in favor of an apical Cl^-/HCO_3^- antiport is that removing Cl^- from the lumen causes increases of pH_i (Figs. 4 and 5). The pH_i changes are larger in HCO_3^- -Ringer's than in HEPES-Ringer's (Table III), and are inhibited by SITS. The time course of the effect of Cl^- removal on pH_i is inconsistent with the possibility of an indirect effect of Cl^- removal on pH_i , e.g., by activating Na^+/H^+ exchange. As shown in Figs. 4 and 5, pH_i starts changing within seconds of Cl^- removal, whereas the fall of aNa_i is not measurable during the first 2 min after Cl^- removal (S. A. Weinman and L. Reuss, unpublished observations).

The pH_i changes observed are large, which suggests a high rate of exchange, particularly in HCO_3^- -Ringer's. A quantitative analysis of these rates, in relation to fluid transport, will be presented elsewhere.

(c) There is a third argument in favor of the existence of an anion antiport at the apical membrane. Changes in pH_i produced by exposure to sodium propionate or ammonium chloride cause the expected aCl_i changes (Fig. 7 and Table IV). Particularly significant is the fact that aCl_i increases during exposure to NH_4Cl , when pH_i is elevated, and decreases after removal of NH_4Cl , when pH_i falls to a value more acid than control (Figs. 6 and 7; see also Boron and De Weer, 1976; Boron and Boulpaep, 1983a). These results can best be explained from the changes of intracellular pH and HCO_3^- concentration upon entry of propionic acid or ammonia, with resulting changes of both the Cl^- flux across the apical membrane and aCl_i . Similar observations have been made by Guggino

et al. (1983) in studies of basolateral Cl^- transport in *Necturus* proximal tubule. However, since in our experiments it was not directly proven that the net Cl^- flux across the apical membrane changed, this argument is less conclusive than the preceding ones.

In conclusion, both our pH_i and $a\text{Cl}_i$ measurements under various conditions are in harmony with the hypothesis of an apical anion antiport.

Characteristics of Apical Membrane $\text{Cl}^-/\text{HCO}_3^-$ Exchange

As shown in Figs. 4 and 5 (see also Petersen and Reuss, 1983; Garcia-Diaz et al., 1983), Cl^- removal from the mucosal bathing solution results in apical and basolateral membrane hyperpolarization, i.e., a change in membrane potential in the direction opposite to that predicted for Cl^- electrodiffusion across the apical membrane. This observation, in the light of the $\text{Cl}^-/\text{HCO}_3^-$ antiport demonstrated above, could indicate that its operation is electrogenic, i.e., that the coupling ratio ($J_{\text{HCO}_3^-}/J_{\text{Cl}^-}$) is >1.0 . Two observations argue against this conclusion. First, changes in mucosal solution HCO_3^- concentration (at constant pH or at constant PCO_2) do not cause the cell membrane potential changes expected for a conductive HCO_3^- transport mechanism across the apical mem-

TABLE IV
Effects of Intracellular Acidification (Propionate) or Alkalinization (Ammonium) on Intracellular Cl^- Activity

| | Control | Propionate | Control | Ammonium |
|----------------------------|---------|------------|---------|----------|
| HEPES-Ringer's | 17±2 | 14±3 | 19±2 | 24±2 |
| Δ | | 3±1 | | -5±1 |
| HCO_3^- -Ringer's | 17±2 | 15±2 | 18±3 | 22±4 |
| Δ | | 3±1 | | -4±1 |

Shown are mean (\pm SEM) $a\text{Cl}_i$ values under steady state conditions after 3 min (propionate) or 2 min (ammonium) of exposure to a mucosal bathing medium in which NaCl was partially (20%) substituted with Na propionate or NH_4Cl , at constant pH_o . Data shown under each pair are the differences (means \pm SEM). All experimental values differed significantly from the control ones. In HEPES-Ringer's, $n = 4$ (propionate) and 5 (ammonium); in HCO_3^- -Ringer's, $n = 6$ in both series.

brane (K.-U. Petersen and L. Reuss, unpublished observations); second, the changes in cell membrane potentials observed upon Cl^- removal are much slower than those observed during Na^+ substitutions with K^+ (at comparable superfusion rates), which suggests that they are caused by an indirect effect of Cl^- removal (Petersen and Reuss, 1983). The most likely explanation for these cell membrane potential changes is an increase in apical (and probably basolateral) K^+ permeability that is secondary to intracellular alkalinization (Petersen and Reuss, 1983; Garcia-Diaz et al., 1983).

The data obtained from the Na^+ and Cl^- removal experiments indicate that the cation and anion exchangers can operate independently, and hence that they are not obligatorily linked. In other preparations, in which pH_i regulation involves transport of Na^+ , Cl^- , and HCO_3^- (Thomas, 1974; see also Boron, 1983), the anion fluxes are uphill processes. In our experiments, both cation and anion

fluxes through the apical membrane exchangers appeared to be downhill under all experimental conditions.

Effects of Sodium Propionate and Ammonium Chloride on Cell Membrane Potentials

Significant cell membrane potential changes were also observed in the ammonium chloride and sodium propionate experiments. During exposure to ammonium chloride, moderate depolarization was observed in most cases, in both HEPES-Ringer's and HCO_3^- -Ringer's, but in some experiments there was hyperpolarization. In all cases, transient hyperpolarization was observed upon removal of NH_4Cl . The changes during exposure to NH_4Cl are probably the result of several factors, including (a) NH_4^+ permeation of the K^+ channel (Hille, 1975), (b) NH_4^+ block of the K^+ channel, a mechanism supported by the consistent observation of an increase in apparent cell membrane resistance ratio (data not shown), and (c) pH_i changes altering P_K (see above). Upon removal of NH_4Cl , the hyperpolarization could be due at least in part to an outwardly directed NH_4^+ gradient. The transient hyperpolarization produced by sodium propionate is due to an increase in P_K at both membranes (Petersen and Reuss, 1982). The mechanism of this effect is unknown.

Significance of the Apical Cl^-/HCO_3^- Exchange

We have demonstrated the existence of an anion antiport at the apical membrane of *Necturus* gallbladder epithelial cells, which appears to be electroneutral and can be dissociated from the cation (Na^+/H^+) antiport previously demonstrated in the same membrane (Weinman and Reuss, 1982, 1984). This transport process is active when the tissue is exposed either to HEPES- or HCO_3^- -Ringer's, i.e., in the absence of experimental perturbations such as cell shrinkage. Our observations are in general agreement with two recent preliminary reports by Machen and Zeuthen (1983) and Zeuthen and Machen (1984), although there are some quantitative differences between their results and ours. Furthermore, Cremaschi et al. (1983) have recently carried out tracer flux kinetic analyses in rabbit gallbladder which support the idea of a double exchanger at the apical membrane, but also raise the intriguing possibility of alternative modes of operation of the same carrier proteins, i.e., a shift from the double exchange to the symport mode. In contrast with these results and interpretations, Spring and co-workers (Ericson and Spring, 1982a, b; Larson and Spring, 1983; Spring and Ericson, 1982) have concluded that the apical membrane antiports, which would be inactive under control conditions, are incorporated into the membrane and/or activated by perturbations such as osmotic shrinkage of the cells, and that transepithelial NaCl transport is entirely ascribable to a bumetanide-sensitive apical membrane NaCl symport. The reasons for the discrepancies between their results and ours are presently unclear. Our data suggest strongly that the main mechanism of NaCl entry in this epithelium could well be the simultaneous but independent operation of Na^+/H^+ and Cl^-/HCO_3^- antiports. Further support for this hypothesis will require experiments designed to estimate the rates at which these exchanges take place, and their relationship to the rates of transepithelial

transport under the same conditions, and to identify the mechanisms by which the two antiports are synchronized to provide equal Na^+ and Cl^- influxes across the apical membrane.

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