Apical Membrane Na^+/H^+ Exchange in *Necturus* Gallbladder Epithelium

Its Dependence on Extracellular and Intracellular pH and on External Na^+ Concentration

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ABSTRACT Intracellular microelectrode techniques and extracellular pH measurements were used to study the dependence of apical Na^+/H^+ exchange on mucosal and intracellular pH and on mucosal solution Na^+ concentration ($[Na^+]_o$). When mucosal solution pH (pH_o) was decreased in gallbladders bathed in Na^+-containing solutions, aNa_i fell. The effect of pH_o is consistent with titration of a single site with an apparent pK of 6.29. In Na^+-depleted tissues, increasing $[Na^+]_o$ from 0 to values ranging from 2.5 to 110 mM increased aNa_i; the relationship was well described by Michaelis-Menten kinetics. The apparent K_m was 15 mM at pH_o 7.5 and increased to 134 mM at pH_o 6.5, without change in V_{max}. In Na^+-depleted gallbladders, elevating $[Na^+]_o$ from 0 to 25 mM increased aNa_i and pH_i and caused acidification of a poorly buffered mucosal solution upon stopping the superfusion; lowering pH_o inhibited both apical Na^+ entry and mucosal solution acidification. Both effects can be ascribed to titration of a single site; the apparent pK's were 7.2 and 7.4, respectively. Diethylpyrocarbonate (DEPC), a histidine-specific reagent, reduced mucosal acidification by 58 ± 4 or $39 \pm 6\%$ when exposure to the drug was at pH_o 7.5 or 6.5, respectively. Amiloride (1 mM) did not protect against the DEPC inhibition, but reduced both apical Na^+ entry and mucosal acidification by 63 ± 5 and $65 \pm 9\%$, respectively. In the Na^+-depleted tissues mean pH_i was 6.7. Cells were alkalinized by exposure to mucosal solutions containing high concentrations of nicotine or methylamine. Estimates of apical Na^+ entry at varying pH_i, upon increasing $[Na^+]_o$ from 0 to 25 mM, indicate that Na^+/H^+ exchange is active at pH_i 7.4. Intracellular H^+ stimulated apical Na^+ entry by titration of more than one site (apparent pK 7.1, Hill coefficient 1.7). The results suggest that external Na^+ and H^+ interact with one site of the Na^+/H^+ exchanger and that cytoplasmic H^+ acts on at least two sites. The external titratable group seems to be an imidazolium, which is apparently different from the amiloride-binding site. The dependence of Na^+ entry on pH_i supports the notion that the Na^+/H^+ exchanger is operational under normal transport conditions.

INTRODUCTION

The gallbladder epithelium absorbs salt and water in isosmotic proportions, concentrating the impermeant components of the bile. Apical NaCl entry is mediated by electroneutral carrier systems. Experimental evidence from two groups strongly supports the notion that parallel Na^+/H^+ and Cl^-/HCO_3^- exchangers account for most if not all NaCl entry (Baerentsen et al., 1983; Reuss, 1984, 1989; Weinman and Reuss, 1984; Reuss and Stoddard, 1987), although other investigators have proposed that entry across the apical membrane is mediated by NaCl cotransport (Ericson and Spring, 1982), or that both entry mechanisms coexist (Davis and Finn, 1985).

The presence of Na^+/H^+ exchange at the apical membrane of *Necturus* gallbladder has been conclusively demonstrated (Weinman and Reuss, 1982, 1984; Reuss, 1984). The epithelium is capable of acidifying a poorly buffered static mucosal fluid in the presence of Na^+ or Li^+ but not K^+, Rb^+, or Cs^+; this process is inhibited by 1 mM amiloride and has a K_m for mucosal Na^+ of 11 mM (Weinman and Reuss, 1982). Exposure to ouabain reduces luminal acidification, but cell Na^+ depletion followed by imposition of an inward Na^+ gradient in the continued presence of ouabain restores H^+ transport (Weinman and Reuss, 1982). The notion of a Na^+/H^+ exchanger operating under control conditions is supported by the effects of amiloride on ion transport, namely a fall in intracellular pH (pH_i), and reductions in intracellular Na^+ activity (aNa_i) and unidirectional apical $^{22}Na^+$ uptake, and also by the acidification of the mucosal solution observed immediately after stopping superfusion (Weinman and Reuss, 1982, 1984; Reuss, 1984). At least 70% of the apical Na^+ entry appears to be via Na^+/H^+ exchange (Reuss, 1984).

The existence of Na^+/H^+ exchange has been demonstrated in apical (Friedman and Andreoli, 1982; Weinman and Reuss, 1982, 1984; Boron and Boulpaep, 1983; Oberleithner et al., 1983; Baum, 1987) and basolateral membranes of epithelia (Boron and Boulpaep, 1983; Ehrenfeld et al., 1987; Harvey and Ehrenfeld, 1988). Although the characteristics of this exchanger have been extensively studied in apical membrane vesicles from epithelia and in nonpolar cells (Kinsella and Aronson, 1980; Montrose and Murer, 1988; Warnock and Pollock, 1988), studies in intact epithelia have been less complete. Accordingly, we sought to examine mechanisms of regulation of apical membrane Na^+/H^+ exchange in *Necturus* gallbladder epithelium, focusing on the effects of pH_i, mucosal pH (pH_o) and mucosal Na^+ concentration ($[Na^+]_o$). The results demonstrate that the K_m for mucosal Na^+ in Na^+-depleted tissues at pH_o 7.5 and pH_i 6.7 is 15 mM, and suggest that mucosal H^+ inhibits Na^+/H^+ exchange in a purely competitive way. The apparent pK of the external side of the exchanger and the effect of a specific histidine reagent support the notion that a functionally important imidazolium group of histidine is present on the external side of the Na^+/H^+ exchanger. The dependence of the Na^+/H^+ exchanger on pH_i indicates the presence of more than one titratable group and suggests that the exchanger is active at normal pH_i.

METHODS

Mudpuppies (*Necturus maculosus*) purchased from Kon's Scientific Co. (Germantown, WI) or Nasco Biologicals (Ft. Atkinson, WI) were kept in a large aquarium at 5–10°C. After the ani-

mals were anesthetized with tricaine methanesulfonate, gallbladders were removed and mounted horizontally, mucosal side up, in a modified Ussing chamber at room temperature as previously described (Reuss and Finn, 1975a, 1977). Na^+-Ringer's contained (in millimolar): 109 NaCl, 1.5 KCl, 1.0 $CaCl_2$, and 1.0 K^+-N-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid (HEPES). This solution was equilibrated with air. Low-Na^+ and nominally Na^+-free solutions were prepared by isomolar replacement of NaCl with tetramethylammonium (TMA^+) chloride (Weinman and Reuss, 1982). Solutions were titrated to the desired pH with NaOH, TMAOH, or HCl. The initial pH of the mucosal solution was 7.5 in all experiments. Experimental changes in pH_o were carried out on the mucosal solution alone, maintaining the serosal solution pH at 7.5. In most experiments, mucosal and serosal sides were bathed with nominally Na^+-free Ringer for 10–15 min to achieve a maximum reduction of aNa_i (Weinman and Reuss, 1984).

Amiloride was a generous gift from Merck, Sharp and Dohme (West Point, PA). Diethylpyrocarbonate, nicotine, and methylamine were purchased from Sigma Chemical Co. (St. Louis, MO).

Measurements of Electrical Potentials

Transepithelial (V_{ms}) and cell membrane voltages (V_{mc} – apical, V_{cs} – basolateral) were measured as previously described (Reuss and Finn, 1975a). The serosal reference electrode was a Ag-AgCl pellet connected to the solution by a short Ringer-agar bridge. The mocosal electrode was a calomel half-cell connected to the bathing solution by a saturated-KCl flowing bridge to minimize liquid junction potentials arising from changes in the ionic composition of the mucosal solution. To measure the transepithelial resistance (R_t) and the apparent ratio of apical (R_a) and basolateral (R_b) membrane resistances ($R_a/R_b = \Delta V_{mc}/\Delta V_{cs}$), transepithelial current pulses (50 $\mu A/cm^2$, 2-s duration and 20 or 30-s interval) were applied by means of Ag-AgCl electrodes. Appropriate corrections were made for series resistances.

Measurements of Intracellular Na^+ and H^+ Activities

Intracellular pH and aNa_i were measured with double-barrel microelectrodes constructed and calibrated as previously described (Weinman and Reuss, 1982, 1984; Reuss et al., 1983, 1987). With background [KCl] of 120 mM, Na^+-selective electrodes had slopes of 48–59 mV/decade at $aNa > 7.5$ mM, 15–30 mV/decade in the 0.75–1.5 mM range, and 20–45 mV/decade in the 1.5–4 mM range. Even at 2 mM [Na^+], the Na^+-selective microelectrodes were virtually pH insensitive (voltage change < 1 mV for a pH change from 7.5 to 6.5), and hence no corrections were necessary for changes in intracellular pH. Using small-tip electrodes (resistances of 150–400 GΩ in the Na^+-selective barrel and 80–120 MΩ in the 3 M KCl barrel) it was possible to collect results at different [Na^+]$_o$, pH_o, or pH_i values from long-lasting impalements in single cells. Intracellular pH electrodes had resistances of 100–200 GΩ (pH barrel) and slopes of 50–60 mV/decade.

Measurements of Mucosal Solution pH

Mucosal solution pH was measured as previously described (Weinman and Reuss, 1982). In brief, a glass pH electrode of bulb diameter 0.5–1 mm was positioned in the mucosal solution 100–200 μm from the epithelial surface; this position was maintained throughout the experiment. Tissues were first Na^+ depleted (see above) and then the [Na^+] of the mucosal superfusate was suddenly increased to 25 mM. After 10–15 s the mucosal superfusion was stopped while measuring pH_o continuously. In the experiments at pH_o values other than 7.5, the mucosal superfusate was changed from nominally Na^+-free Ringer (pH_o 7.5) to Na^+-free Ringer at the desired pH_o for 1 min, to allow for electrode stabilization at the new pH_o, before the addition of Na^+. In Na^+-free media and in the absence of a transepithelial pH gradient

there was no change in mucosal solution pH upon stopping superfusion. Imposition of a transepithelial pH gradient caused a passive, Na^+-independent H^+ flux. Therefore, the Na^+-dependent rate of mucosal acidification was estimated by subtracting the rate measured in the absence of Na^+, at the same pH_o. Because when $[Na^+]_o$ is elevated to 25 mM the transepithelial voltage changes by ~10 mV (mucosa negative) there is also an electrical driving force for H^+ secretion. However, the apparent transepithelial H^+ permeability (P_H) calculated from the alkalinization of the mucosal solution during a transepithelial pH gradient in the absence of Na^+ was low, suggesting that 10 mV could account for a rate of acidification of <1% of that measured at pH_o 7.5.

The rate of acidification of the fluid surrounding the electrode (ΔH^+) was estimated from:

$$\Delta H^+ = \beta\ (dpH/dt) \tag{1}$$

where dpH/dt is the initial rate of change in pH_o (measured in the interval from 10 to 30 s or from 15 to 30 s), and β is the buffering power of the solution. The values of β, determined by titration of each experimental solution, agreed well with those calculated by applying the equation of Koppel and Spiro (see Roos and Boron, 1981).

Diethylpyrocarbonate (DEPC) Experiments

A stock solution of the histidine reagent DEPC in absolute ethanol was prepared daily. Because DEPC is unstable in aqueous solution, especially at alkaline pH (Miles, 1977), and since the DEPC stock solution is alkaline, we used a 20 mM-HEPES Ringer. To enhance inhibition of Na^+/H^+ exchange, the tissues were exposed to DEPC in 109 mM Na^+ (Grillo and Aronson, 1986). Tissues were exposed to DEPC for 5 min and then the mucosal side was superfused for 15 min with Na^+-free Ringer to deplete cell Na^+. At the end of this period, mucosal solution acidification was measured as described above. Preliminary experiments gave reproducible results with 4.5 mM DEPC. Effects were also observed at lower concentrations (1, 2, and 3 mM) but they were variable and too small to be accurately measured. Ethanol (final concentration 0.3% wt/vol) had no effect on luminal solution acidification.

Base-loading Experiments

In Na^+-depleted tissues the steady-state pH_i is lower than in tissues perfused with normal Ringer (6.7 vs. 7.4). To study the pH_i dependence of Na^+ influx it was necessary to alkalinize the cells. Exposure to NH_4Cl (Boron and De Weer, 1976) initially alkalinizes the epithelial cells in this tissue (Reuss and Petersen, 1985), but NH_4^+ is transported by the cation exchanger with a similar or higher affinity than that for Na^+ (Kinsella and Aronson, 1981), making kinetic analysis of Na^+ entry difficult. Further, in contrast with the results of others (Grinstein et al., 1984; Chaillet and Boron, 1985), in our hands exposure of the apical and basolateral sides to nigericin (up to 200 μM) in the presence of high-K^+ Ringer (120 mM KCl or K^+-cyclamate) yielded a poor pH_i response to changes of pH_o. Nystatin (added to the serosal side at concentrations up to 30 μg/ml) was also ineffective; i.e., little change in pH_i was observed with extracellular alkalinization. Cells were alkalinized successfully by exposing the apical side of the epithelium to either of two structurally different permeant bases, nicotine and methylamine (pK's ~8.2 and 10.6, respectively). Both bases are known to permeate cell membranes and to alkalinize the cytoplasm (Adler, 1972; Boron and Roos, 1976; Roos and Boron, 1981). Nicotine was used at concentrations ranging from 3 to 40 mM, and methylamine at 30 or 60 mM. The required exposure times were <1 min for nicotine and 4–5 min for methylamine. Nicotine replaced TMACl mole by mole and the osmolality was adjusted with TMACl after titration to pH 7.5 with HCl. Methylamine hydrochloride replaced TMACl

mole by mole, the solution was titrated with TMAOH, and the osmolality was adjusted with TMACl.

Data Analysis

Results are presented as means ± SEM. Statistical comparisons were made by Student's t test on paired or nonpaired data as appropriate. A value of $P < 0.05$ was considered significant.

The initial rates of change of $a\mathrm{Na_i}$ and $\mathrm{pH_i}$ were estimated from the initial slopes (i.e., from ~5 to 20–30 s) of the differential voltages traces (ion-sensitive minus conventional barrel). Over this interval, the voltage change is nearly linear with time. The use of this measure to assess the initial rate of change of ion activities will be discussed below.

Most results were fit by nonlinear least-squares routines to the Michaelis-Menten or the Hill equation, or to the appropriate equation to determine the pK of a single titratable group.

RESULTS

Effect of pH_o on aNa_i

It is clear that $\mathrm{Na^+/H^+}$ exchange accounts for a major fraction of the $\mathrm{Na^+}$ influx across the apical membrane of *Necturus* gallbladder epithelium (Weinman and Reuss, 1982; Baerentsen et al., 1983). If acidification of the mucosal solution inhibits $\mathrm{Na^+}$ entry, then $a\mathrm{Na_i}$ should fall. To evaluate the change in $\mathrm{Na^+}$ entry across the apical membrane, we used the initial rate of decrease of $a\mathrm{Na_i}$. If under these conditions there are cell volume changes via perturbation of $\mathrm{Na^+}$ transport, their effects must be small, because removing mucosal $\mathrm{Na^+}$ causes a fall in cell volume of only 1% in the initial 30 s (Davis and Finn, 1985). If in addition basolateral $\mathrm{Na^+}$ extrusion is initially (0–30 s) unchanged by alterations in $\mathrm{pH_o}$, then the initial rate of change in $a\mathrm{Na_i}$ reflects the changes in apical $\mathrm{Na^+}$ entry, mediated primarily by $\mathrm{Na^+/H^+}$ exchange (Reuss and Stoddard, 1987).

As shown in Fig. 1, in tissues exposed on both sides to 110 mM $\mathrm{Na^+}$, mucosal solution acidification reduces $a\mathrm{Na_i}$ rapidly and reversibly from the control value of 11 ± 1 mM ($n = 5$). Decreasing $\mathrm{pH_o}$ in steps from 7.5 to 4.5 caused falls in $a\mathrm{Na_i}$ that were highly variable in magnitude. In three of eight experiments, the $a\mathrm{Na_i}$ change with the smaller $\mathrm{pH_o}$ steps were too small to be quantified accurately. These experiments were excluded from further analysis. Mucosal solution alkalinization has no effect on $a\mathrm{Na_i}$ (Fig. 2). Initial rates of change of $a\mathrm{Na_i}$ were normalized to that measured upon lowering $\mathrm{pH_o}$ from 7.5 to 4.5, and fit to Eq. 2 for determination of the pK of a single titratable group:

$$\mathrm{dNa_i'} = (\mathrm{dNa_i'})_{min} + (\mathrm{dNa_i'})_{max}[10^{\mathrm{pK-pH}}/(1 + 10^{\mathrm{pK-pH}})] \tag{2}$$

where $\mathrm{dNa_i'}$ is the normalized initial rate of change in $a\mathrm{Na_i}$, and $(\mathrm{dNa_i'})_{min}$ and $(\mathrm{dNa_i'})_{max}$ are the normalized minimal and maximal initial rates of change in $a\mathrm{Na_i}$, respectively. The fit (Fig. 2) yields an apparent pK of 6.29 ± 0.05, a $(\mathrm{dNa_i'})_{max}$ of 1.00 ± 0.02, and a $(\mathrm{dNa_i'})_{min}$ of -0.03 ± 0.02.

In addition to the decrease in $a\mathrm{Na_i}$, mucosal acidification produced changes in membrane voltages (V_{cs} traces in Fig. 1). An initial depolarization was observed at all
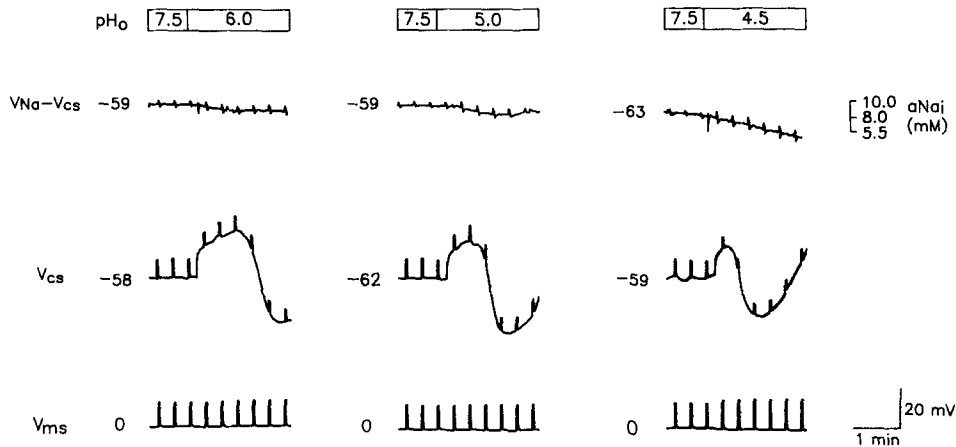
pH$_0$ [7.5   6.0]   [7.5   5.0]   [7.5   4.5]

V_{Na}–V_{cs} –59   –59   –63   ⎡10.0⎤ aNai
                                                                             ⎣ 8.0⎦ (mM)
                                                                              5.5

V_{cs} –58   –62   –59

V_{ms} 0   0   0   ⎤20 mV
                                                              ___⎦
                                                              1 min

FIGURE 1.   Effects of changes in mucosal pH (pH$_0$) on intracellular Na$^+$ activity (aNa$_i$), measured using a double-barrel Na$^+$-selective microelectrode. V_{Na}-V_{cs}, the difference between intracellular voltages measured by Na$^+$-sensitive and conventional barrels; V_{cs}, basolateral membrane voltage; V_{ms}, transepithelial voltage. Initial voltages are indicated at the beginning of the traces. The rapid deflections in the records are the result of transepithelial current pulses (2-s duration at 20-s intervals) applied to estimate the transepithelial resistance and the ratio of cell membrane resistances. All traces were obtained in the same cell. Reducing mucosal pH from 7.5 to 6.0, 5.0, or 4.5 decreased aNa$_i$. Reversibility is denoted by the baseline Na$^+$ activities preceding each pH change. Interval between traces, 15 min.

pH$_0$ values lower than 7.5. At pH$_0$'s 7.0 and 6.5 the depolarization was in most cases monotonic, but at lower pH$_0$'s it was followed by hyperpolarization and oscillations in membrane voltages. It has been shown that these voltage changes are due to effects of external pH on the electrodiffusive K$^+$ permeability (P_K) of the apical membrane (Reuss et al., 1981), i.e., a decrease in P_K by moderate mucosal solution acidification, or cyclic changes in K$^+$ conductance at both luminal and basolateral membranes, in phase with the voltage changes, at lower pH$_0$ (Reuss et al., 1981).

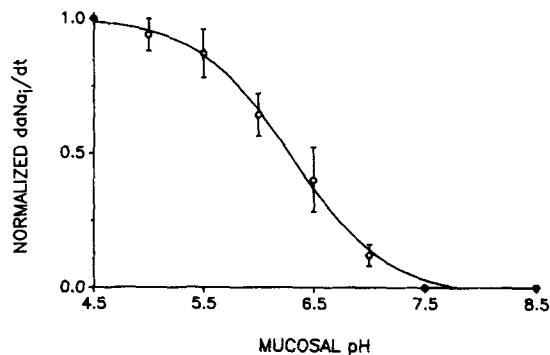FIGURE 2.   Effect of mucosal pH on the pH-sensitive initial rate of change in aNa$_i$ ($d a$Na$_i$/dt), measured from 5 to 20 s. Data are means ± SEM (n = 5), normalized to the value measured, in every experiment, when pH$_0$ was decreased from 7.5 to 4.5 (on average 5.1 ± 1.5 mM/min). Protocol as in Fig. 1. The SEM's of the pH values (not shown) were <0.02. The solid line corresponds to the least-squares fit of the data to Eq. 2 (see text). The apparent pK is 6.29.

(graph: NORMALIZED $d a$Na$_i$/dt vs MUCOSAL pH; y-axis 0.0–1.0, x-axis 4.5–8.5)

Effect of Mucosal Acidification on Relative V_{max} and Apparent K_m for Mucosal Na^+

To determine the dependence of apical Na^+ entry on $[Na^+]_o$, tissues were incubated in nominally Na^+-free Ringer on both sides for 10–15 min to achieve maximal reductions of aNa_i. Then, during impalement with a Na^+-sensitive double-barrel microelectrode, $[Na^+]_o$ was rapidly raised from nominally 0 to a value in the range of 2.5–110 mM and the initial change of aNa_i was measured. Fig. 3 shows records obtained in a single cell.

The validity of estimates of Na^+ entry from the initial rate of change of aNa_i depends on the characteristics of the response of the Na^+-selective microelectrodes at low aNa_i levels and requires constant cell volume and an initially negligible rate of Na^+ extrusion across the basolateral membrane. The slope of the voltage response
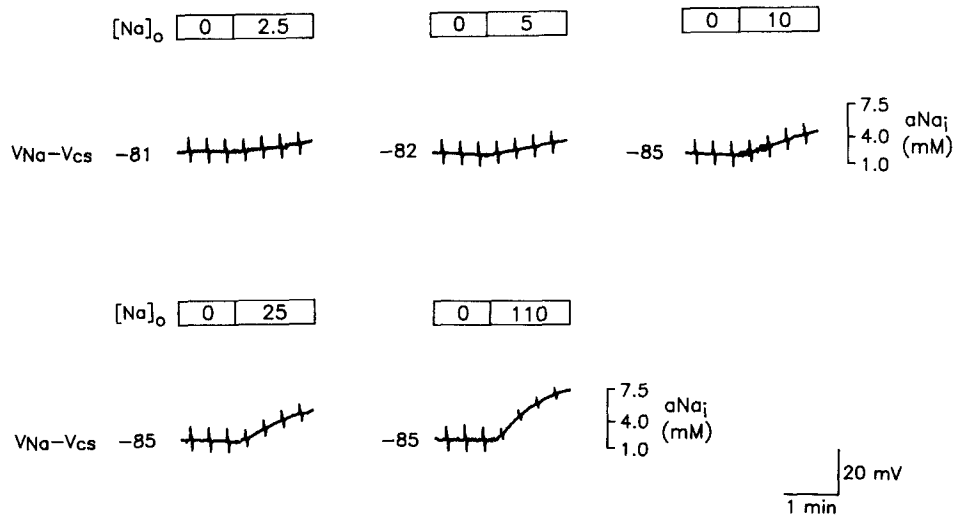
FIGURE 3. Changes in aNa_i upon increasing mucosal solution $[Na^+]$ ($[Na^+]_o$) in Na^+-depleted tissues at mucosal pH 7.5. The gallbladder was bathed in nominally Na^+-free Ringer (TMA^+ substitution) on both sides for 10–15 min, and than $[Na^+]_o$ was increased to 2.5, 5, 10, 25, 50, or 110 mM. During the intervals between panels, the tissue was depleted of Na^+ as described above. All data were obtained in the same cell. Symbols as in Fig. 1.

of the microelectrode to aNa was smaller at the lower activities, but in the range of interest (1–4 mM) the differences were small, and the error in the estimate of the rate of Na^+ entry was < 6%. Cell volume changes upon increasing $[Na^+]_o$ appear to be very small (Davis and Finn, 1985) and hence do not influence our calculations. Finally, significant Na^+ extrusion across the basolateral membrane during the first 20–30 s after elevating $[Na^+]_o$ is unlikely, because aNa_i is low and the activity of the Na^+ pump is reduced at the low pH_i (Eaton et al., 1984; Breitwieser et al., 1987). In these experiments, pH_i was on average 6.7 (see below). For these reasons, the initial rates of increase in aNa_i seem to yield good estimates of the apical Na^+ entry.

A separate question is whether during the measurement of the change in aNa_i there are changes in pH_i, which, if present, would cause errors in the estimation of

the initial entry rate. To test the effect of pH_o changes on pH_i, we measured pH_i with double-barrel microelectrodes upon elevating pH_o from 7.5 to 8.5 or 9.0 in the absence of Na^+ (TMA^+ replacement). In 30 s, there was no significant change in pH_i ($\Delta = 0.03 \pm 0.02$, $n = 3$). In another group of tissues, pH_i was lowered from 8.0 or 7.5 to 6.0 also in the absence of Na^+ (K^+ replacement). In 30 s, again there was no significant change in pH_i ($\Delta = 0.01 \pm 0.01$, $n = 3$). A related problem is whether the imposition of the inward Na^+ gradient results in cell alkalinization, which would influence significantly Na^+ entry. Upon increasing mucosal solution Na^+ to 25 mM at pH_o 7.5, the alkalinization rate was 0.14 ± 0.03 during the initial 30 s, remaining linear for at least 1 min (see Results). The rate of alkalinization should be $\sim$30% greater when mucosal $[Na^+]$ is elevated to 110 mM (see Fig. 4). From these data, the mean change in pH_i during the measurement of Na^+ uptake is 0.09, yielding an underestimate of Na^+ influx of at most 5% (see Fig. 13).

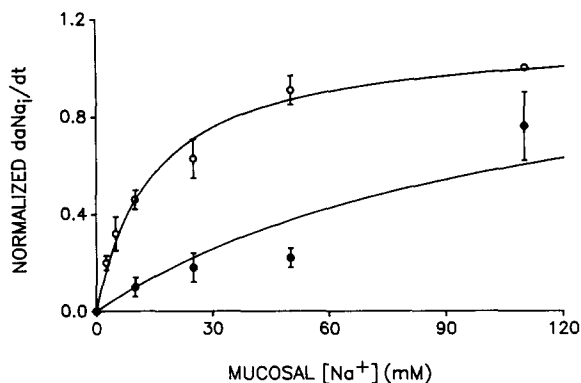The data obtained in six experiments (normalized to the rate of increase of aNa_i

FIGURE 4. Initial rates of increase of aNa_i ($daNa_i/dt$) at pH_o 7.5 (*open symbols*) and at pH_o 6.5 (*filled symbols*). Protocol as in Figs. 3 and 5. Each point is the mean ± SEM of six or five measurements (pH_o 7.5 and 6.5, respectively). All values were normalized to $daNa_i/dt$ elicited, in each experiment, by elevating $[Na^+]_o$ from 0 to 110 mM at pH_o 7.5 (on average 9.7 ± 2.9 mM/min). The lines are the least-squares fits to the Michaelis-Menten equation. K_m was 15 mM at pH 7.5 and 134 mM at pH 6.5.

produced by elevating $[Na^+]_o$ to 110 mM) are summarized in Fig. 4. The results fit well to the Michaelis-Menten equation, with relative V_{max} and apparent K_m of 1.12 ± 0.06 and 15 ± 3 mM, respectively. Although the preparation was exposed to nominally Na^+-free Ringer for 10–15 min, the apparent aNa_i was 1.4 ± 0.1 mM, and remained unchanged during continued superfusion with Na^+-free Ringer for up to 1 h. Since the Na^+-sensitive microelectrodes detect other cations (Dagostino and Lee, 1982), the real aNa_i must be even lower than the above estimate.

To ascertain the kinetics of inhibition of Na^+ entry by mucosal solution acidification, initial changes in aNa_i were measured upon elevating $[Na^+]_o$ from nominally 0 to values ranging from 10 to 110 mM, at pH_o 6.5. As shown in Fig. 5, larger elevations in $[Na^+]_o$ result in higher rates of Na^+ entry across the apical membrane, but the rates of entry are substantially reduced at pH_o 6.5, compared with pH_o 7.5. The inhibitory effect of luminal acidification is due to an increase in K_m from 15 ± 3 to 113 ± 34 mM, without significant change in relative V_{max}, which was estimated to be 1.22 ± 0.11 (Fig. 4). Because K_m increases without a change in V_{max}, we tentatively conclude that the effect of pH_o is purely competitive. However, a mixed effect can-

not be definitively excluded because it was not possible to obtain the apparent V_{max} directly from the measurements and because there was substantial scatter of the data at pH_o 6.5.

It may be argued that the data at pH_o 6.5 could be fit by a straight line, and hence that Na^+ entry at this pH_o could be electrodiffusive. However, this possibility is unlikely. First, the electrodiffusive Na^+ permeability (P_{Na}) of the apical cell membrane is very low under control conditions (Reuss and Finn, 1975*b*; Van Os and Slegers, 1975; Graf and Giebisch, 1979), and second, external solution acidification in the range used in these experiments causes only a small change in P_{Na} in other systems, whereas cell acidification substantially reduces P_{Na} (Leaf et al., 1964; Reuss, 1981; Park and Fanestil, 1983).

Effect of pH_o on Apical Na^+ Entry

To further characterize the effect of pH_o on Na^+ entry via Na^+/H^+ exchange it is necessary to perform kinetic experiments varying external pH while the initial Na^+
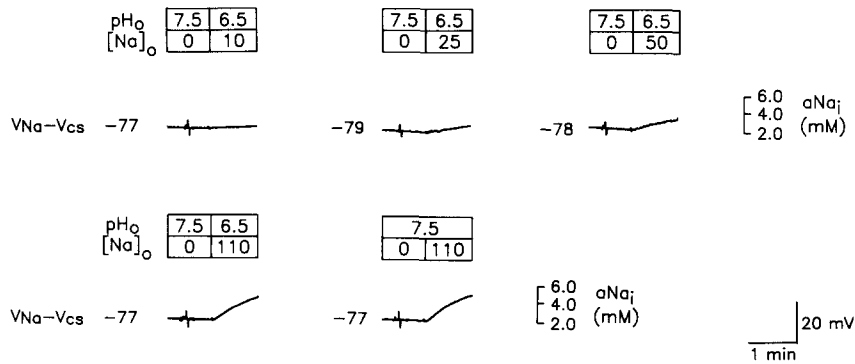

FIGURE 5.   Changes in aNa_i upon increasing $[Na^+]_o$ in Na^+-depleted tissues at mucosal pH 6.5. $[Na^+]_o$ was increased from 0 to the values indicated at mucosal pH's of 6.5 or 7.5. Symbols as in Fig. 1. For protocol details see legend to Fig. 3.

chemical potentials are constant. The simplest protocol is a zero-*trans* experiment at a nonsaturating $[Na^+]_o$. We incubated the tissues in nominally Na^+-free Ringer for 10–15 min, and then rapidly changed the mucosal solution to one containing 25 mM Na^+ at a pH_o ranging from 9.0 to 4.5, measuring the initial rate of change in aNa_i. Upon increasing $[Na^+]_o$ to 25 mM, the initial rates of rise of aNa_i are easily measurable. In addition, since the apparent K_m for luminal Na^+ is ~15 mM, the external Na^+ transport site is not saturated, facilitating the study of the effect of external $[H^+]$. Fig. 6 illustrates the effect of pH_o on Na^+ entry across the apical membrane, assessed with a single impalement. The results from eight such experiments are summarized in Fig. 7. The differences between the initial rates of change of aNa_i at a given pH_o and at pH_o 4.5 were normalized to the measured pH-sensitive rate of change of aNa_i (pH_o's 8.5–4.5) and fit to Eq. 3:

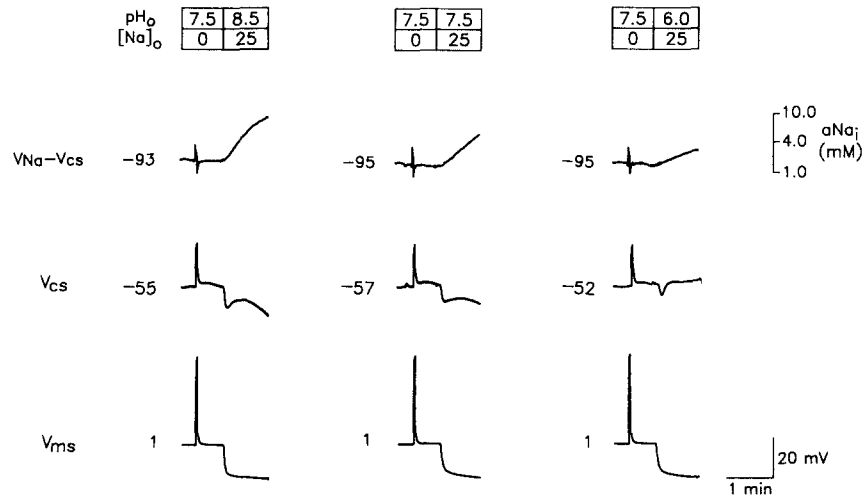$$dNa''_i = (dNa''_i)_{min} + (dNa''_i)_{max}[10^{pH-pK}/(1 + 10^{pH-pK})] \qquad (3)$$

pHₒ | 7.5 | 8.5 | | 7.5 | 7.5 | | 7.5 | 6.0 |
[Na]ₒ | 0 | 25 | | 0 | 25 | | 0 | 25 |

VNa–Vcs   −93   −95   −95   ⎡−10.0
                                                                            ⎢−4.0 aNaᵢ
                                                                            ⎣−1.0 (mM)

Vcs   −55   −57   −52

Vms   1   1   1
                                                                        |20 mV
                                                                        ⎯⎯⎯⎯
                                                                        1 min

FIGURE 6. Effect of mucosal pH on apical Na⁺ entry. The gallbladders were superfused with nominally Na⁺-free Ringer, pHₒ 7.5, and mucosal [Na⁺] was increased to 25 mM at pH values between 4.5 and 9.0. Four representative traces obtained in the same cell are shown. Symbols as in Fig. 1.

where dNa''ᵢ is the normalized initial rate of change in aNaᵢ at a given pHₒ, and (dNa''ᵢ)min and (dNa''ᵢ)max are the minimal and maximal normalized initial rates of change in aNaᵢ, respectively.

The apparent pK was 7.21 ± 0.16, (dNa''ᵢ)max was 1.06 ± 0.07, and (dNa''ᵢ)min was 0.11 ± 0.04. The pH-sensitive initial rate of change in aNaᵢ was 80 ± 2% of the value measured at pHₒ 8.5.

Raising [Na⁺]ₒ produced a two-phase hyperpolarization of Vcs. The rapid phase was completed in <15 s, and the slower phase started at 30–60 s. These Vcs changes are opposite in the direction to those expected from electrodiffusive Na⁺ entry. We

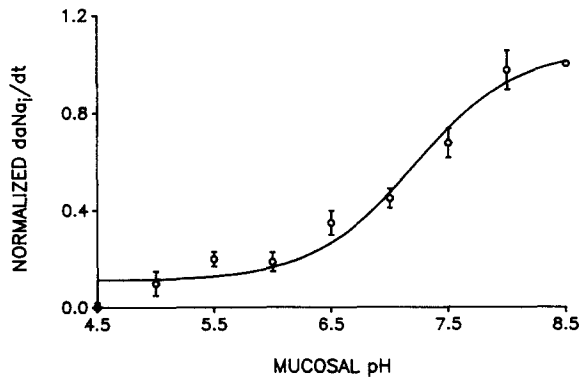[Graph: NORMALIZED daNaᵢ/dt vs MUCOSAL pH, x-axis 4.5 to 8.5, y-axis 0.0 to 1.2, sigmoidal curve]

MUCOSAL pH

FIGURE 7. Effect of mucosal pH on the pH-sensitive initial rate of increase in aNaᵢ (daNaᵢ/dt), expressed as a fraction of the difference between the rates at pHₒ's 4.5 and 8.5, measured in every experiment (on average 6.1 ± 1.0 mM/min). Values are mean ± SEM of determinations in five to eight tissues, except for pHₒ 8.0 (n = 2). The pH-sensitive fraction was 80 ± 2% of the total initial rate of increase of aNaᵢ. Protocol as in Fig. 3. The SEM's in the pH axis (not shown) were <0.02. The solid line corresponds to the least-squares fit of the data to Eq. 3 (see text). The apparent pK is 7.21.

attribute the rapid hyperpolarization to the change in intraepithelial current brought about by the Na⁺-TMA⁺ paracellular biionic potential. The differences in the magnitude of the hyperpolarization are consistent with differences in apparent ratio of membrane resistances (data not shown). The slower hyperpolarization could be due to activation of the Na⁺ pump via either an increase in pump current or a reduction in [K⁺] at the external surface of the basolateral membrane.

Effect of pH₀ on Mucosal Solution Acidification

Previous results from this laboratory have shown that the gallbladder acidifies a poorly buffered, static mucosal fluid, and that this effect is due to Na⁺ gradient—
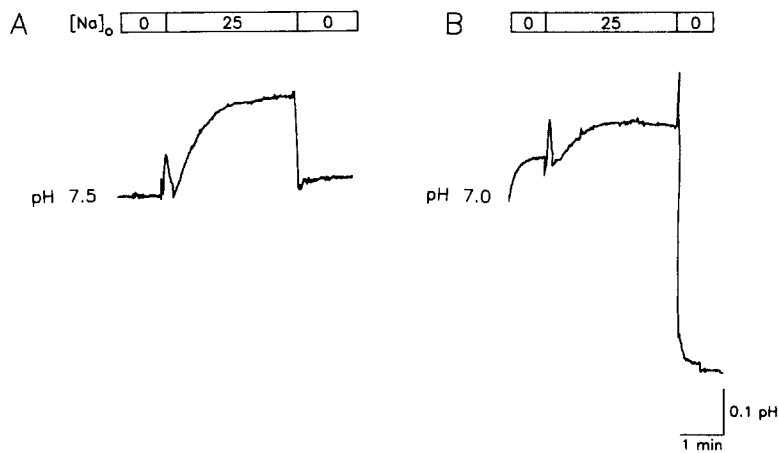
FIGURE 8. Mucosal solution acidification upon increasing $[Na^+]_o$ from 0 to 25 mM. (A) The gallbladder was superfused with nominally Na⁺-free Ringer on both sides, $[Na^+]_o$ was raised to 25 mM at pH_o 7.5, and after 10–15 s the mucosal superfusion was stopped for 3 min. The mucosal pH was measured at 100–200 μm from the cell surface with a glass pH electrode (see Methods). Finally, the superfusion was restarted with nominally Na⁺-free Ringer. (B) In the same preparation, the pH's of the Na⁺-free and 25 mM Na⁺ mucosal solutions were decreased to 7.0 from 1 min before raising $[Na^+]_o$. Upward voltage deflections denote acidification.

driven apical membrane Na⁺/H⁺ exchange (Weinman and Reuss, 1982). The preceding results demonstrate that apical Na⁺ entry is inhibited by decreasing pH_o, probably via inhibition of the exchanger. To test further this hypothesis, we studied the effect of pH_o on luminal acidification. Fig. 8 illustrates an experiment in which the gallbladder was first incubated in Na⁺-free Ringer and then $[Na^+]_o$ was suddenly raised to 25 mM at two different pH_o values. After 10–15 s, the superfusion was stopped and the changes in pH_o were recorded. As shown in the figure, the Na⁺-dependent ΔH^+ (ΔH_{Na}^+, see Methods) is reduced at low pH_o. Fig. 9 summarizes results from five such experiments. The data (normalized to the difference between the values at pH_o's 7.5 and 4.5) were well described by Eq. 4:

$$\Delta H_{Na}^+ = (\Delta H_{Na}^+)_{min} + (\Delta H_{Na}^+)_{max}[10^{pH-pK}/(1 + 10^{pH-pK})] \qquad (4)$$

where ΔH^+_{Na} is the normalized rate of mucosal acidification at a given pH_o, and $(\Delta H^+_{Na})_{min}$ and $(\Delta H^+_{Na})_{max}$ are the minimal and maximal normalized rates of mucosal acidification, respectively. The apparent pK was 7.39 ± 0.20, $(\Delta H^+_{Na})_{max}$ was 1.85 ± 0.37, and $(\Delta H^+_{Na})_{min}$ was 0.01 ± 0.02.

Amiloride (1 mM) reduced ΔH^+ at pH_o 7.5 by $65 \pm 9\%$, and inhibited apical Na^+ entry by a similar amount, i.e., $63 \pm 5\%$. This agreement is consistent with apical membrane Na^+/H^+ exchange mediating both Na^+ entry and H^+ efflux.

The pH_o dependency of apical Na^+ entry and mucosal acidification were also similar, but at pH_o 4.5 apical Na^+ entry was ~20% of the maximal rate, whereas mucosal acidification, at the same pH_o, was only ~4% of the estimated maximal rate. Although contributions of electrodiffusive Na^+ entry (Reuss and Finn, 1975b) and/ or electroneutral Na^+ entry via either NaCl or NaKCl cotransport are possible (Cremaschi and Hénin, 1975; Cremaschi et al., 1983; Larson and Spring, 1983; Davis and Finn, 1985), the most plausible explanation for this difference is that our mea-
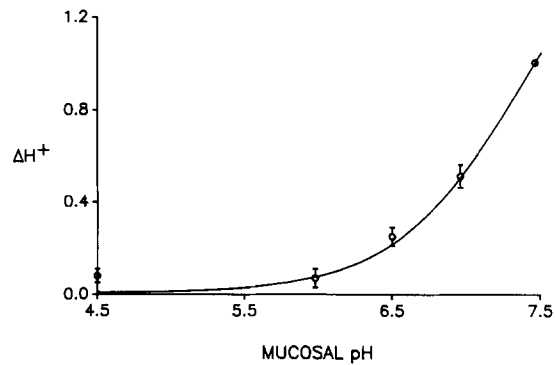

FIGURE 9. Effects of mucosal pH on the pH-sensitive and Na^+-dependent rate of mucosal acidification (ΔH^+). ΔH^+ was determined from the Na^+-dependent change of mucosal solution pH (during the interval from 10 to 30 s) and the buffering power of the solution, determined by titration of each solution (see text). Data are means $\pm$ SEM ($n = 5$), normalized to the difference between the values at pH_o's 7.5 and 4.5, measured in every experiment (on average 243 ± 34 mmol·liter^{-1}·min^{-1}). The SEM's of the pH values (not shown) were <0.04. The solid line corresponds to the least-squares fit of the data to Eq. 3 (see text). The apparent pK is 7.39.

surements of ΔH^+ underestimate the real rate of mucosal acidification (Weinman and Reuss, 1982). The ΔH^+ underestimates the H^+ efflux across the apical membrane for two main reasons: (a) HCO_3^-, even in its nominal absence from the bathing media, is transported from cell to mucosal solution (Reuss and Costantin, 1984), neutralizing H^+ and increasing the solution buffering power. (b) The CO_2 formed in the mucosal compartment is partly lost to the air, and can also diffuse into the cells and across the epithelium. Therefore, we cannot assess the absolute H^+ fluxes, but the paired determinations of ΔH^+ reflect appropriately the experimentally induced changes (Weinman and Reuss, 1982).

Effect of Increasing Mucosal Solution [Na⁺] on pH₍ᵢ₎

The preceding results show that elevating mucosal solution $[Na^+]$ from 0 to 25 mM increases aNa_i and acidifies the mucosal solution. To assess the effect on pH_i we used a protocol similar to that used to study apical Na^+ entry, but pH_i was measured

instead of $a\text{Na}_i$. The initial pH_i was 6.7, which was significantly lower than the value in the presence of Na^+ ($\sim$7.4). Increasing mucosal [Na^+] to 25 mM alkalinized the cell at an initial rate of 0.27 ± 0.06 min^{-1} ($n = 4$). The rate of alkalinization was linear for at least 1 min. This result demonstrates that the (Na^+-dependent) mucosal acidification elicited by elevating [Na^+]$_o$ occurs pari passu with intracellular alkalinization, as expected if the transport mechanism is Na^+/H^+ exchange.

Effect of DEPC on Mucosal Solution Acidification

The apparent pK's for Na^+ entry and mucosal solution acidification strongly suggest that the imidazolium group of histidine is the titratable group on the external side of the exchanger, since it is the only protein group with a nearly neutral pK. Accordingly, we tested the effect of the most specific histidine reagent available,

TABLE I

Effect of DEPC on Mucosal Solution Acidification (ΔH^+)

	ΔH^+	Δ	n
	$mmol \cdot liter^{-1} \cdot min^{-1}$	%	
Control	0.46 ± 0.09		
DEPC (pH 7.5)	0.20 ± 0.11	-58 ± 4*	6
Control	0.41 ± 0.08		
DEPC (pH 6.5)	0.24 ± 0.04	-39 ± 6[‡]	7
Control	0.55 ± 0.16		
DEPC (pH 7.5) + 1 mM amiloride	0.32 ± 0.10	-49 ± 7*	6

Tissues were bathed with nominally Na^+-free Ringer buffered with 1 mM HEPES (pH 7.5) for 15 min, then mucosal Na^+ concentration was increased to 25 mM and 15 s later superfusion was stopped and dpH/dt was measured. From this value and the buffering power of the solution ΔH^+ was calculated. Exposure to 4.5 mM DEPC lasted 5 min and was at the pH values indicated in parentheses. In some experiments amiloride was present together with DEPC. The measurements of ΔH^+ were made in the absence of drugs, after mucosal superfusion with nominally Na^+-free Ringer for 15 min.
*Significantly different from control.
[‡]Significantly different from both control and DEPC (pH 7.5).

DEPC (Miles, 1977). As explained in Methods, exposure to DEPC lasted 5 min and was followed by a 15-min period of Na^+ depletion, after which mucosal solution acidification was measured as described above. Mucosal solution acidification was measured after removal of DEPC, but its effect persisted for at least 60 min (data not shown). Table I shows that ΔH^+ decreased by $\sim$60% when the exposure was made at pH_o 7.5. The inhibition was less than when the exposure to DEPC was at pH_o 6.5, which is compatible with DEPC inactivation of imidazolium groups since the reaction is with the unprotonated form (Miles, 1977). The rate of hydrolysis of DEPC is higher in alkaline media (Miles, 1977), which would make the difference in its effects at pH 7.5 and 6.5 even greater than that shown in Table I. Previous studies in renal brush border vesicles have shown that DEPC inhibits Na^+/H^+ exchange and that amiloride protects against that inhibition (Grillo and Aronson, 1986). In

contrast, in our hands amiloride (1–4 mM) did not prevent the effect of DEPC. The 1 mM amiloride data are shown in Table I.

Cell Alkalinization by Nicotine and Methylamine

The time courses of the effects of nicotine and methylamine on pH_i and membrane voltages are illustrated in Fig. 10. Exposure of the apical surface of the tissue to
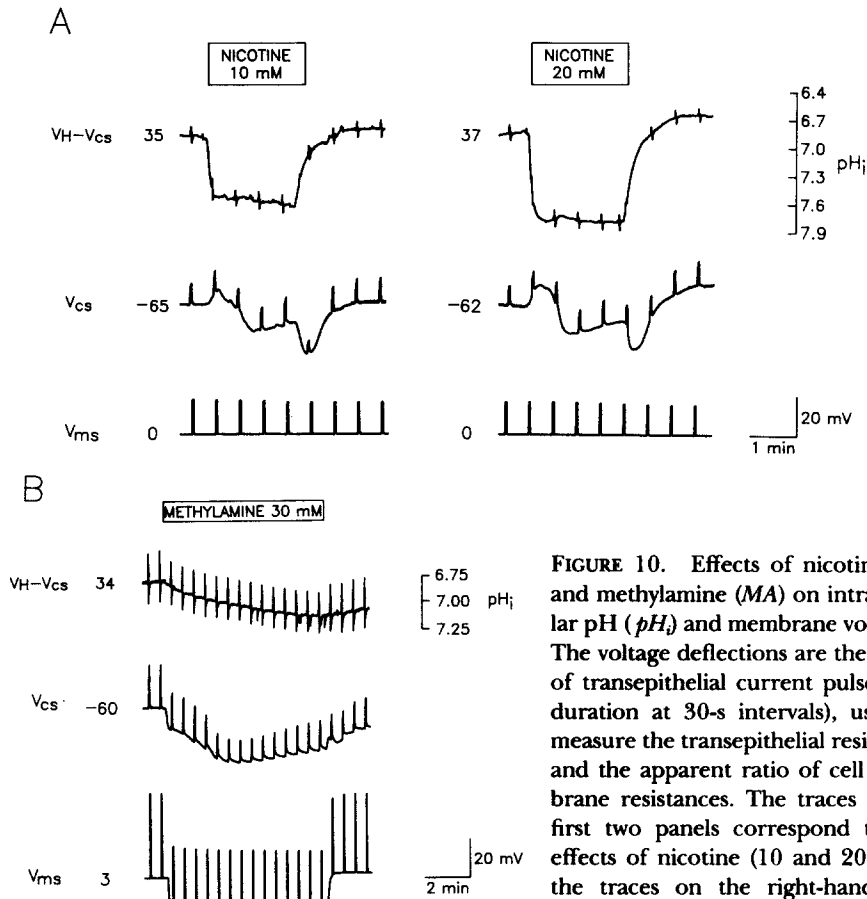
FIGURE 10. Effects of nicotine (N) and methylamine (MA) on intracellular pH (pH_i) and membrane voltages. The voltage deflections are the result of transepithelial current pulses (2-s duration at 30-s intervals), used to measure the transepithelial resistance and the apparent ratio of cell membrane resistances. The traces in the first two panels correspond to the effects of nicotine (10 and 20 mM); the traces on the right-hand side panel depict the effects of 30 mM methylamine. V_H-V_{cs}, difference between intracellular voltages measured with H$^+$-sensitive and reference barrels. See Fig. 1 for other symbols. The effects of both bases were reversible (not shown for methylamine).

nicotine produced a rapid elevation of pH_i which was complete in <1 min and remained stable for at least 2 min. Although the initial cell membrane voltage response was variable (i.e., depolarization or hyperpolarization), the steady-state V_{cs} was increased. The mechanism of these voltage changes was not studied in detail, but the lack of changes in V_{ms} or in the apparent ratio of cell membrane resistances suggests effects on conductive pathways at both apical and basolateral membranes.

Such effects could be in part secondary to the change in pH_i, but the hyperpolarization was already maximal at the lowest nicotine concentration used (3 mM). In addition to the cell alkalinization, the hyperpolarizing effect of nicotine may involve receptor activation, as in other tissues (Roberts et al., 1978).

With methylamine, the increase in pH_i was slower than with nicotine, taking place in 4–5 min. In addition to the rise in pH_i, there were changes in membrane voltages. The mucosa-negative V_{ms} change and the decrease in R_t indicate that the paracellular pathway has a higher permeability for methylammonium than for TMA^+. The hyperpolarization of V_{cs}, slower than with nicotine, was concentration independent, as in the nicotine experiments. The apparent ratio of cell membrane resistances did not change significantly, again suggesting effects at both cell membranes.

Fig. 11 summarizes the pH_i values obtained at several concentrations of nicotine and methylamine. Note that the degree of cell alkalinization is concentration dependent.
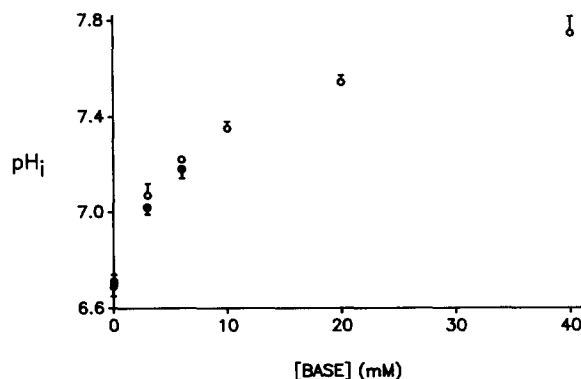

FIGURE 11. Effects of nicotine (*open symbols*) and methylamine (*filled symbols*) on pH_i. Abscissa denotes [nicotine] and one-tenth of [methylamine]. Experimental protocols as in Fig. 10. Values are means ± SEM of determinations in four to six gallbladders, at 2 or 7 min after exposure to nicotine or methylamine, respectively.

Effect of pH_i on Apical Na^+ Entry

Fig. 12 A shows the effects of exposure to nicotine (3 and 6 mM) on the rate of increase of aNa_i upon elevating $[Na^+]_o$ from 0 to 25 mM. The tissues were first Na^+-depleted and then exposed for 1–2 min to Na^+-free Ringer with nicotine.[1] After the cells were alkalinized, $[Na^+]_o$ was rapidly increased to 25 mM, while maintaining the mucosal nicotine concentration constant, and the changes of aNa_i were recorded. The rate of increase of aNa_i decreased with increasing nicotine concentrations. A similar result was obtained when the cells were alkalinized with methylamine (Fig. 12 B).

The results obtained with both bases are summarized in Fig. 13. The data, normalized to the pH_i in the absence of nicotine and methylamine, are not compatible with a single titratable site (fit not shown). The fit to the Hill equation gave a relative

[1] During impalement, exposure to nicotine at concentrations of 10 mM or higher caused concentration-dependent positive shifts in the Na^+ electrode voltage output, by 1–4 mV. However, nicotine had no effect on the extracellular response of the electrodes to $[Na^+]$. The mechanism of this small voltage change is unknown. The signal described was subtracted from the baseline. The correction in the apparent aNa_i was always <1.5 mM.

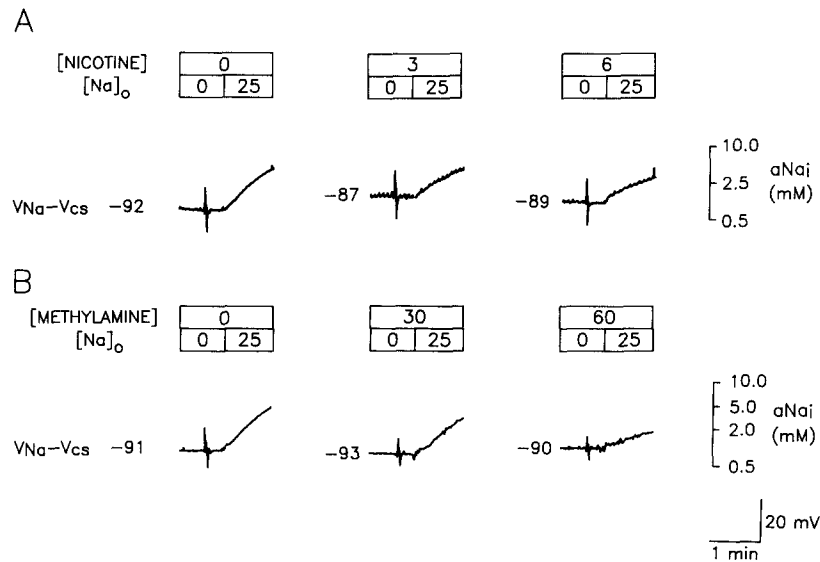FIGURE 12.   Increases of $a\mathrm{Na_i}$ elicited by elevating $[\mathrm{Na^+}]_o$ to 25 mM in $\mathrm{Na^+}$-depleted, base-loaded tissues. The preparations were first superfused with $\mathrm{Na^+}$-free Ringer on both sides and then mucosal $[\mathrm{Na^+}]$ was increased. (A) Nicotine was present in the $\mathrm{Na^+}$-free and in the 25 mM $\mathrm{Na^+}$ solutions, starting 2 min before elevating $[\mathrm{Na^+}]_o$ ($\mathrm{pH_o}$ 7.5), at the concentrations indicated (in millimolar). (B) Methylamine was added 7 min before increasing $[\mathrm{Na^+}]_o$ ($\mathrm{pH_o}$ 7.5), at the concentrations indicated (in millimolar). Symbols as in Fig. 1. Each panel consists of records obtained in a single cell.

V_{max} of 1.27 and an apparent pK of 7.10, with a Hill coefficient of 1.7. This coefficient indicates that the effect of internal $\mathrm{H^+}$ on $\mathrm{Na^+}$ influx is mediated by titration of more than one site, as found in other systems (Aronson et al., 1982; Grinstein et al., 1984; Montrose and Murer, 1988).
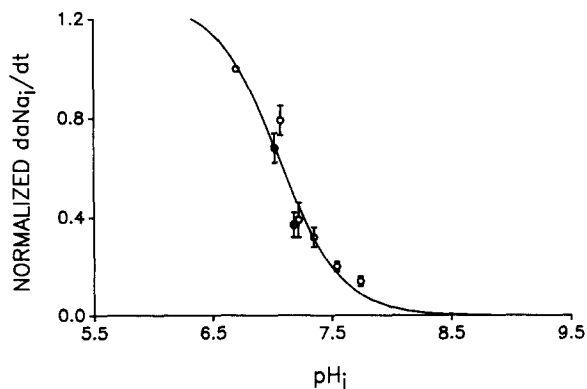

FIGURE 13.   Effect of $\mathrm{pH_i}$ on the normalized initial rate of increase of $a\mathrm{Na_i}$ ($da\mathrm{Na_i}/dt$). $\mathrm{pH_i}$ was increased by using nicotine (*open symbols*) or methylamine (*filled symbols*) as indicated in Fig. 11. Initial rates of increase of $a\mathrm{Na_i}$ were normalized to the value measured at the control $\mathrm{pH_i}$ in each experiment (on average 4.9 ± 1.9 and 5.7 ± 2.6 mM/ min, for nicotine and methylamine experiments, respectively). Data are means ± SEM ($N = 4$–6 gallbladders). The solid line is the least-squares fit to the Hill equation. The apparent pK is 7.1, and the Hill coefficient is 1.7.

It is conceivable that nicotine and/or methylamine may alter the properties of the Na^+/H^+ exchanger by mechanisms independent of the pH_i changes. An argument against this possibility is that the reduction in the initial rates of increase of $a Na_i$ was similar with both agents, for comparable alkalinizations, suggesting that they do not affect the pH_i dependence of the Na^+/H^+ exchanger. The possibility that methylamine is transported by the exchanger was tested by comparing the mucosal acidification rates (Eq. 1) in Na^+-free medium, upon stopping mucosal superfusion in the absence and in the presence of 60 mM methylamine. Four paired experiments were performed. In the absence of methylamine, ΔH^+ was 0.002 ± 0.002 $mmol \cdot liter^{-1} \cdot min^{-1}$. With 60 mM methylamine, the mucosal solution acidified at a rate of 0.059 ± 0.004 $mmol \cdot liter^{-1} \cdot min^{-1}$, which was not affected by 1 mM amiloride ($\Delta H^+ = 0.058 \pm 0.009$ $mmol \cdot liter^{-1} \cdot min^{-1}$). These results suggest that there is no detectable methylammonium/H^+ exchange. The methylamine-induced acidification is likely to result from transepithelial diffusion of the uncharged species, down its concentration gradient, across the cell membranes (see Fig. 10).

The cells are expected to swell during exposure to high concentration of weak permeant bases. However, calculations for nicotine assuming equilibrium of the permeant form, and taking into account the pH_i and the pK, indicate a maximum swelling of 8% at 40 mM, which would lead to a small underestimation of the rate of Na^+ entry. Cell swelling could also inhibit Na^+/H^+ exchange, but reducing the mucosal solution osmolality by 20% has no effect on mucosal solution acidification. In three paired experiments, the initial rates of mucosal solution acidification upon elevating mucosal solution $[Na^+]$ from nominally 0 to 25 mM (in $mmol \cdot liter^{-1} \cdot min^{-1}$) were 0.420 ± 0.041 and 0.416 ± 0.040, in control medium and in hyposmotic solution, respectively.

DISCUSSION

The present results provide further evidence for the presence of Na^+/H^+ exchange at the apical membrane of *Necturus* gallbladder, and reveal some of the properties of the external and internal sites of the exchanger. Several lines of evidence suggest that apical Na^+ entry and mucosal acidification, as assessed in these studies, represent Na^+/H^+ exchange: (a) both events are inhibited by 1 mM amiloride by nearly the same degree. The percent inhibition was less than previously reported for mucosal solution acidification (Weinman and Reuss, 1982), which is probably due to the lower pH_i in the present experiments. Although the effect of pH_i on the inhibition of Na^+/H^+ exchange by external amiloride has not been determined, interactions between internal and external sites have been demonstrated (Jean et al., 1985; Green et al., 1988). (b) The effects of pH_o on Na^+ influx and mucosal acidification are similar. For instance, the apparent pK's for inhibition of Na^+ entry and mucosal solution acidification are 7.2 and 7.4, respectively. (c) Na^+ addition to the mucosal side of previously Na^+-depleted tissues produces increases of $a Na_i$ and pH_i.

The interpretation of many of our results is based on the contention that the initial rate of change of $a Na_i$ is a reliable index of the initial rate of Na^+ entry via Na^+/H^+ exchange. The correctness of our analysis is supported by (a) the demonstration of coupling between apical membrane Na^+ and H^+ fluxes, and of similar amiloride sensitivities of Na^+ entry and H^+ secretion, (b) the fact that the Na^+-con-

ductive pathway across the apical membrane is minor (Reuss and Finn, 1975b) and amiloride insensitive (Reuss, 1984), and (c) that other Na^+ transport pathways (basolateral membrane Na^+/H^+ exchange or $NaHCO_3$ cotransport) are either nonexistent or small (Reuss and Petersen, 1985; Stoddard and Reuss, 1989). Underestimation of the initial rate of Na^+ entry by rapid activation of the pump is likely to be a minor problem (see Results). Finally, the possibility that our measurements reflect activation of volume-regulatory pathways because of cell shrinkage secondary to bilateral Na^+ removal was ruled out by control experiments in which the mucosal solution osmolality was reduced to increase cell volume (see Results).

The fit of the Na^+ entry data to the Michaelis-Menten equation suggests that external Na^+ interacts with the exchanger at a single site. It was previously estimated that the activation of mucosal acidification by external Na^+ has a K_m of 11 mM (Weinman and Reuss, 1982). The K_m for Na^+ influx estimated in the present experiments, i.e., 15 mM, is in good agreement with that result, as well as with values reported for other preparations (Kinsella and Aronson, 1980; Montrose and Murer, 1988).

Effect of pH_o on Na^+/H^+ Exchange

In the present experiments, decreasing pH_o reduced both Na^+ entry and H^+ efflux across the apical membrane. Together with other results, these observations indicate that pH_o modifies Na^+/H^+ exchange with an apparent pK of ~7.2, at $[Na^+]_o = 25$ mM, apparent $aNa_i = 1.4$ mM, and pH_i 6.7. This value is in the range reported for cortical renal membrane vesicles and intact cells of several systems (Aronson et al., 1983; Paris and Pouyssegur, 1983; Green et al., 1988). Our results support the idea that the effect of external H^+ on Na^+ influx is purely competitive, which is in agreement with most of the studies in other systems (Aronson et al., 1983; Paris and Pouyssegur, 1983), although a mixed effect of H^+ on Na^+/H^+ exchange has also been proposed (Ives et al., 1982, Vigne et al., 1982; Jean et al., 1985).

Studies in synaptosomes and osteoblasts (Jean et al., 1985; Green et al., 1988) have revealed interactions between the internal and external titratable sites of the Na^+/H^+ exchanger. For instance, it was found that increasing pH_o by 0.9 units increases the apparent pK of the internal site by about the same amount and that nearly the same change in the apparent pK of the external site occurs with pH_i is modified (Jean et al., 1985). Since the possibility of trans effects was not explored in our experiments, the apparent external pK does not necessarily reflect H^+ effects on the external site alone.

In the present experiments we found a lower apparent external pK in normal HEPES-Ringer, i.e., in 110 mM Na^+, compared with Na^+-depleted tissues, although pH_i values were lower in the latter condition (6.7 vs. 7.4) (see above). The lower apparent pK could explain the lack of effect of mucosal solution alkalinization on aNa_i in 110 mM Na^+. Although we have no definite explanation for the lower apparent pK in the presence of Na^+, in the tissues in which mucosal Na^+ was increased from 0 to 25 mM the external Na^+ site is not saturated with Na^+ and a high apparent affinity for H^+ would be expected. Of course, Na^+-depleted tissues also have a lower aNa_i (1 vs. 11 mM). If internal Na^+ competes with internal H^+ (Green et al., 1988), then the decrease in apparent pK of the internal site would also

reduce the apparent pK of the external site. We cannot rule out possible roles of other factors such as intracellular Ca^{2+} activity (Muldoon et al., 1985), which could be elevated by removal of external Na^+, but there is no agreement on the effect of intracellular Ca^{2+} on the Na^+/H^+ exchanger (Smith and Rozengurt, 1978; Hesketh et al., 1985; Muldoon et al., 1985).

Effect of DEPC

The apparent external pK (~7.2) suggests that an imidazolium group is involved in some way in Na^+/H^+ exchange. DEPC inhibits Na^+/H^+ exchange in cortical renal brush border vesicles (Grillo and Aronson, 1986) and in human lymphocytes (Grinstein et al., 1984). Our results show that DEPC inhibits mucosal acidification and hence support the idea of the involvement of the imidazolium residue of histidine in Na^+/H^+ exchange. It has been suggested that the histidine residue is the external cationic-binding site and the amiloride-binding site of the exchanger, since in renal brush border membrane vesicles amiloride protects against the inhibition by DEPC (Grillo and Aronson, 1986). In the present experiments, amiloride did not alter the effect of DEPC, although it was used at a concentration known to inhibit Na^+/H^+ exchange at 110 mM Na^+ (Weinman and Reuss, 1982; Reuss, 1984). Thus, our results suggest that the groups that react with amiloride and DEPC are distinct and not close enough to show interaction between the two inhibitors.

Effect of pH_i on Apical Na^+ Entry

Increases in pH_i were obtained using two different bases, nicotine and methylamine. Since there is no detectable methylammonium/H^+ exchange, and at comparable pH_i values both bases had similar effects on Na^+ entry, the possibility of pH_i-independent effects on apical Na^+ influx is unlikely. The main results from these experiments indicate, first, that at least under the present conditions the apical Na^+/H^+ exchanger is active at physiological pH_i, and second, that internal $[H^+]$ has a more pronounced effect on the exchanger than external $[H^+]$, indicating that internal protons interact with more than one titratable group.

With respect to the first conclusion, it is clear from our results that at the normal pH_i the exchanger operates at ~30% of the rate measured at pH_i 6.7, i.e., that it accounts for a sizable Na^+ entry rate. Measurable rates of Na^+/H^+ exchange have been found in apical or basolateral membranes of epithelial cells under physiologic conditions (Friedman and Andreoli, 1982; Ehrenfeld et al., 1987; Miller and Pollock, 1987). In the present work we have extended these studies to an apical membrane Na^+/H^+ exchanger that subserves transepithelial Na^+ transport in a leaky epithelium. These results are in contrast with the proposals that, at physiological pH_i, the exchanger is nearly inactive (studies in renal cortical membrane vesicles, see Aronson et al., 1982) or inactive (results in a number of nonepithelial cells, e.g., Grinstein et al., 1984; Green et al., 1988).

Although cooperative effects of pH_i on Na^+/H^+ exchange have been extensively documented in several cell types and membrane preparations (Aronson et al., 1982; Montrose and Murer, 1988), this is the first demonstration in an intact epithelium. In a previous publication from our laboratory, pH_i recovery was studied in the pres-

ence of external Na^+ and using the NH_4^+ loading technique to acidify the cells. The recovery of the pH_i, which is due to Na^+/H^+ exchange, obeyed simple saturation kinetics, suggesting interaction with a single site (Reuss and Petersen, 1985). In the earlier experiments pH_i was measured without estimation of intracellular buffering power at different pH_i's. If the effect of pH_i on Na^+/H^+ exchange is not affected (directly or indirectly) by extracellular $[Na^+]$, the previous and current results suggest that the intracellular buffering power increases between pH_i 7.5 and 6.5, as shown by others in several cell types (Boron et al., 1979; Renner et al., 1989; Simchowitz and Roos, 1985).

Physiologic Significance of the Results

Although the mechanism of Na^+ transport across the apical membrane of *Necturus* gallbladder epithelium remains somewhat controversial (Ericson and Spring, 1982; Cremaschi et al., 1983), results from Zeuthen et al. (Baerentsen et al., 1983; Zeuthen and Machen, 1984) and from this laboratory (Weinman and Reuss, 1982, 1984; Reuss; 1984; Reuss and Constantin, 1984) indicate that Na^+/H^+ exchange is present at this membrane, operates under normal fluid-transporting conditions in parallel with Cl^-/HCO_3^- exchange, and appears to be the main or sole pathway for Na^+ entry. In the experiments reported in this paper aNa_i fell on average by 1.6 mM/min when we decreased pH_o from 7.5 to 6.5. The gallbladder acidifies liver bile from pH 7.5 to ~7.0 (Diamond, 1968), and because of poor mixing of luminal fluid in vivo, the pH near the cell surface may be considerably lower. Since the external apparent pK for inhibition of apical Na^+/H^+ exchange is ~6.3, pH_o may play a role in the regulation of NaCl absorption. In addition, changes in pH_o and pH_i under transporting conditions could play significant roles in matching the fluxes via the apical exchangers, contributing to maintain the high rates of NaCl reabsorption characteristic of the gallbladder and other leaky epithelia. The relative importances of pH_o and pH_i in controlling Na^+/H^+ exchanger activity in *Necturus* gallbladder epithelium may vary, depending on a number of factors. However, inasmuch as the cytosolic apparent pK of the exchanger is 7.1 and the dependence of the exchange is steeper for pH_i than for pH_o, pH_i may have a larger role in the control of salt movements across the apical membrane.

Experiments in a variety of nonepithelial cells and in membrane vesicles obtained from renal cortex suggest that the Na^+/H^+ exchanger is quiescent or nearly inactive at the normal pH_i (Montrose and Murer, 1988). However, there is abundant evidence in support of the notion that Na^+/H^+ exchange operating in parallel with a Cl^-/HCO_3^- or another anion exchanger accounts for salt entry across the apical membrane of leaky epithelia (Karniski and Aronson, 1985; Baum, 1987; Preisig and Rector; 1988). Our results in the Na^+-depleted intact epithelium of *Necturus* gallbladder are consistent with the view that Na^+/H^+ exchange is active at the normal cell pH (Weinman and Reuss, 1982; Reuss, 1984).

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