Role of Na⁺/H⁺ Exchange in the Control of Intracellular pH and Cell Membrane Conductances in Frog Skin Epithelium

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ABSTRACT Ion-sensitive microelectrodes and current-voltage analysis were used to study intracellular pH (pHi) regulation and its effects on ionic conductances in the isolated epithelium of frog skin. We show that pHi recovery after an acid load is dependent on the operation of an amiloride-sensitive Na⁺/H⁺ exchanger localized at the basolateral cell membranes. The antiporter is not quiescent at physiological pHi (7.1–7.4) and, thus, contributes to the maintenance of steady state pHi. Moreover, intracellular sodium ion activity is also controlled in part by Na⁺ uptake via the exchanger.

Intracellular acidification decreased transepithelial Na⁺ transport rate, apical Na⁺ permeability (P_{Na}) and Na⁺ and K⁺ conductances. The recovery of these transport parameters after the removal of the acid load was found to be dependent on pH i regulation via Na⁺/H⁺ exchange. Conversely, variations in Na⁺ transport were accompanied by changes in pH i. Inhibition of Na⁺/K⁺ ATPase by ouabain produced covariant decreases in pH i and P_{Na}, whereas increases in Na⁺ transport, occurring spontaneously or after aldosterone treatment, were highly correlated with intracellular alkalinization.

We conclude that cytoplasmic H⁺ activity is regulated by a basolateral Na⁺/H⁺ exchanger and that transcellular coupling of ion flows at opposing cell membranes can be modulated by the pH i-regulating mechanism.

INTRODUCTION

Changes in ion permeability at one epithelial cell membrane are often associated with modifications in ion transport at the opposing membrane ("cross-talk") (Schultz, 1981; Diamond, 1982). The nature of the cellular signal for "cross-talk" is not known, however, in a companion paper (Harvey et al., 1988) we have shown that sodium and potassium conductances in frog skin epithelium are very sensitive to slight variations in intracellular pH (pHi). This raises the interesting possibility that the mechanism(s) regulating pH i could also influence transepithelial ion transport. An understanding of how this epithelium maintains and regulates cell H⁺ is, therefore, essential in interpreting changes in pH i and their electrical effects. Baso-
lateral Na⁺/H⁺ exchange may be involved in pH_i control since recently we found that an intracellular acid load produced a stimulation of basolateral Na⁺ uptake into the cell (Ehrenfeld et al., 1987). In this paper we used double-barreled Na⁺- and H⁺-sensitive microelectrodes to determine whether pH_i is controlled by a Na⁺/H⁺ exchanger and if there is a link between Na⁺ transport and pH_i regulation.

**MATERIAL AND METHODS**

The experiments were performed on the short-circuited isolated epithelium of *Rana esculenta* ventral skin mounted in a modified Ussing chamber.

**Solutions**

The normal control Ringer solution was HCO₃⁻-free, gassed in air, buffered in 10 mM MES to pH 7.4, and contained (in millimolar) 83 NaCl, 2.5 KCl, 2 CaCl₂, 11 Na₂SO₄, 2 MgSO₄, 1.2 KH₂PO₄, 2.5 Na₂HPO₄, and 11 glucose. Intracellular acid-base disturbances were produced at constant external pH by modifying the control Ringer as described in our previous paper (Harvey et al., 1988). Ringer solution buffered in CO₂:HCO₃ containing 24 mM HCO₃ gassed in 5% CO₂ in place of NaCO₃ and MES in the control Ringer. Solutions of various Na⁺ concentrations were produced by equimolar replacement of choline for Na⁺. In Na⁺-free solutions KCl and Na₂HPO₄ were replaced by 2.5 mM K₂HPO₄. Amiloride was purchased from Merck, Sharp & Dohme (West Point, PA), and ouabain and aldosterone were purchased from Sigma Chemical Co. (St. Louis, MO). Aldosterone was made up as a stock solution of 0.01 M in methanol and used at a 1:5,000 dilution in Ringer solution. The electrical arrangements for recording current-voltage curves (I-V) were similar to that described in our companion paper. Apical membrane Na⁺ conductance (gₐ) and permeability (Pₐ₉) were calculated from the best-fit Goldman-Hodgkin-Katz flux equation applied to the amiloride-sensitive I-V relations of the apical cell membranes obtained before and after block of the short-circuit current by apical amiloride (50 μM).

The slope conductance of the basolateral membrane (mainly due to K⁺) was calculated by linear regression analysis of the amiloride-sensitive I-V relations of the basolateral cell membrane. The I-V relations of apical and basolateral membranes were determined simultaneously and details of their analysis are given in the companion paper.

**Double-barreled Ion-sensitive Microelectrodes**

Intracellular Na⁺ activity and pH were measured using double-barreled ion-sensitive microelectrodes constructed from micropipettes in the following way. Two separate tubes of borosilicate glass (Schott, Mainz, FRG) 9-mm od, 7-mm id; and 8-mm od, 5-mm id (the latter tube contained an internal solid glass capillary 1-mm diam) were fused in a fish-tail burner, coiled around each other into a plait, and drawn out to a total external diameter of 3 mm. These double-barreled capillaries were then pulled in a Kopf-puller to produce micropipettes with tip diameters of 0.5 μm in the wider barrel (WB) and 0.3 μm in the narrow barrel (NB). The WB later served as the ion-sensitive electrode and the NB was used to measure membrane potential. Selective silanization of the inner tip wall of the WB was achieved by modification of a technique described by Harvey and Kernan, 1984.

The micropipette blanks were heated in an oven for 1 h at 110°C, after which the NB was backfilled to the tip with distilled water to protect it from silanization. The entire tip was then dipped in a 2% solution of trimethyl dimethyl silyl amine (Fluka, Buchs, Switzerland) in toluene under microscopic control (×300; Diavert Wild Leitz, Heerbrugg, Switzerland). When the column of silane mixture in the WB reached a height of 50–100 μm, the micropi-
pette was withdrawn and the evaporation of the silane from the WB and the stability of the water column in the NB were verified under the microscope. The micropipette was then placed tip upwards in an oven and baked for 30–90 min at 110°C. These silanized micropipettes were stable for 1 wk before filling if they were stored in a dry, dust-free atmosphere. The NB was backfilled to the tip with 1 M KCl and the entire micropipette tip was then dipped in the appropriate liquid ion exchanger (LIX): 10% Swiss Federal Institute of Technology (ETH) 227 (Fluka, 0.5% Na⁺ tetrabenzylboron in 89.5% wt/wt o-nitrophenyl octyl ether for Na⁺ electrodes (double-barrel [DB] Na⁺ LIX), and proton ionophore tridecylamine (82500; Fluka) equilibrated with CO₂ overnight for pH electrodes (DB H⁺ LIX). Dipping lasted 5–10 min to obtain a column of LIX 50–100 µm in length in the tip of the WB, which was then backfilled with a solution of 0.1 M NaCl and 0.5 M KCl for Na⁺ electrodes, and with a solution of 0.1 M NaCl buffered to pH 6.0 with sodium citrate for H⁺-sensitive electrodes.

The Na⁺- and pH-sensitive barrels had a tip resistances of 5 × 10¹⁰ Ω and 10¹³ Ω, respectively, when measured in Ringer solutions, while that of the reference tip was 80 M Ω. The electrical outputs from the WB and NB were fed via Ag/AgCl wires to a high impedance dual differential amplifier (FD 223; World Precision Instruments [WPI], New Haven CT). The electrodes were shielded using a feedback circuit (FC 23; WPI) connecting the differential output of the FD 223 amplifier to a sheet of aluminium wrapped around the double-barreled microelectrode. This shielding greatly reduced noise interference and increased the response time of the electrodes. The DB Na⁺ LIX has a 90% response time (t₀₉) of 0.4 s measured by adding 1 ml of 1 M NaCl to 9 ml of 10 mM NaC1 solution, under vigorous stirring. The t₀₉ of DB H⁺ LIX was 1 s when measured by adding 1 ml of 1 N HCl to 9 ml of 0.1 M NaCl lightly buffered to pH 7.4 with 4 mM imidazole.

**Calibration**

The drift in electromotive force of both types of DB LIX was < 1 mV/d and calibration curves were reproducible at the beginning and end of experiments lasting up to 8 h. The DB Na⁺ LIX were calibrated in "mixed" solutions of 1, 10, and 100 mM NaCl, each containing 100 mM KCl or 2 mM CaCl₂ to provide a constant background K⁺ or Ca²⁺ interference, respectively. Selectivity coefficients were determined from the Nicolsky equation:

\[
K_j = \frac{a'_i - a''_j \cdot \exp z_i \cdot \Delta\psi}{(a_jz_i/z_j(\exp z_i \cdot \Delta\psi - 1)}
\]

where Δψ = (V' - V")F/RT, V' and V" are the potentials in case ' and " and zᵢ and zⱼ are the valencies of ion i and j, a is the ionic activity, and Kⱼ is the selectivity coefficient.

Measured activity coefficients (γ) for Na⁺ and K⁺ in normal Ringer were γNa⁺ = 0.78, γK⁺ = 0.76. In the concentration range 1–100 mM NaCl with a background of 100 mM KCl, γK⁺ = 0.71 and γNa⁺ = 0.77 and with a background of 2 mM CaCl₂ γCa₂⁺ = 0.54 at 100 mM NaCl and γNa⁺ = 0.91 at 10 mM NaCl and 0.79 at 100 mM NaCl. Calculated selectively coefficients were KNa⁺ = 0.014 and KNaCa₂⁺ = 0.63. Although the DB Na⁺ LIX is poorly selected for Na⁺ over Ca²⁺, the intracellular Ca²⁺ activity is expected to be < 10⁻⁷ M and its interference with aNa⁺ measurements will be negligible. For the case of K⁺ interference, a decrease in aNa⁺ of 50 mM would cause an error of only 0.7 mM in the measurement of aNa⁺. The aNa⁺ was, therefore, determined with reference to the calibration curves with fixed background K⁺ interference. The DB Na⁺ LIX gave a slope of 55 ± 2 mV/decade change in Na⁺ activity (n = 12). The DB H⁺ LIX is virtually free from interference from Na⁺, K⁺, Cl⁻, HCO₃⁻, and Ca²⁺ and had a slope of 54–58 mV/pH unit when tested in Ringer solution buffered to pH values between 5 and 8. The DB Na⁺ and H⁺ LIX were stable for up to 1 wk after construction if stored at 6°C.
Apparent $H^+$ Efflux and $Na^+$ Influx Across the Basolateral Cell Membranes

The apparent $H^+$ efflux rate $\Delta[H^+]_i$, expressed in neq $\cdot$ h$^{-1}$ $\cdot$ cm$^{-2}$, was defined as the product of the amiloride-sensitive pHi recovery rate ($dpHi/dt$) after an intracellular acid load, the total intracellular buffering power ($\beta_i$), and the epithelial volume-to-surface ratio ($6.18 \pm 0.31$ $\mu$l $\cdot$ cm$^{-2}$, $n = 6$). The latter was measured from differences in wet and dry tissue weights expressed per unit exposed chamber area. $dpHi/dt$ was determined from the slope of the line drawn through the pen recording of the initial phase of pHi recovery from an acid load, in the absence and presence of basolateral amiloride (0.5 mM) at various levels of Na$^+$ concentration in the basolateral bath.

$\beta_i$ is the sum of intrinsic ($\beta_i$) and CO$_2$ ($\beta_{CO_2}$) buffering power. We used a $\beta$ value of 35 meq H$^+$/$pH$ unit determined previously under similar experimental conditions (Harvey et al., 1988). $\beta_{CO_2}$ was calculated for each initial phase of pHi recovery; assuming that the intracellular and extracellular pCO$_2$ are similar, $\beta_{CO_2} = \ln [HCO_3^-]$. The [HCO$_3^-$] was calculated from the Henderson-Hasselbach equation using the pHi value at the peak acid load.

The basolateral $^{22}Na$ uptake ($Na^+$ flux from serosal solution, $J_{Na^+}$) was determined in isolated epithelia, different from those used to calculate $\Delta[H^+]_i$, by a technique described in detail in a previous paper (Ehrenfeld et al., 1987). Briefly, isolated epithelia were bathed in beakers in the presence of phenamil (10$^{-5}$ M) to block apical Na$^+$ conductive entry, and then they were loaded for 2 min with $^{22}Na$ (10 $\mu$Ci/ml), essentially through the basolateral cell membranes. The cellular component of $^{22}Na$ flux was determined from a double-exponential curve fit to the flux data. The $J_{Na^+}$ was then measured in control conditions (standard CO$_2$-free Ringer) and after return to standard Ringer, directly after an acid load produced in epithelia at constant external pH by incubating in 15 mM NH$_4$Cl for 15 min or at variable external pH by equilibration for 1/2 h in Ringer gassed with 5% CO$_2$ (pH 6.2). The proportion of $J_{Na^+}$ passing via Na$^+$/H$^+$ exchange was calculated from the difference between the $J_{Na^+}$ values calculated in the absence or presence of amiloride ($10^{-4}$ M) or ethyl isopropyl amiloride (EIPA) ($10^{-3}$ M), a specific inhibitor of Na$^+$/H$^+$ exchange.

Results are expressed as mean $\pm$ SEM, and the number of experiments are given in parentheses.

RESULTS

Effects of Serosal Amiloride on pH, and Na$^+$

In a recent paper (Ehrenfeld et al., 1987) we showed that the uptake of $^{22}Na$ across the basolateral cell membranes into isolated epithelia was inhibited by high concentrations of amiloride or low doses of EIPA. Here the effects of serosal amiloride on $a_{Na^+}^i$ and pHi were measured using double-barreled ion-sensitive microelectrodes. All microelectrode impalements were made from the basolateral side of the isolated epithelium.

In Fig. 1 it can be seen that the application of amiloride (5 $\times$ 10$^{-4}$ M) to the basolateral side of an isolated epithelium caused a reversible fall in intracellular Na$^+$ activity. In eight epithelia the $a_{Na^+}^i$ decreased from 10.8 $\pm$ 1 to 6.5 $\pm$ 0.8 mM in the presence of basolateral amiloride. It could, however, be argued that amiloride at such a high concentration enters the cells to block apical Na$^+$ conductance at the cytoplasmic side of the opposing apical cell membrane and thus bring about the fall in $a_{Na^+}^i$ and $I_c$. We therefore repeated the experiment in the presence of amiloride on the apical side at a concentration (50 $\mu$M) sufficient to inhibit $I_c$ to 3 $\pm$ 2 $\mu$A $\cdot$ cm$^{-2}$.
and to increase the fractional resistance of the apical membrane to 0.97 ± 0.02 (n = 4). Under these conditions, when apical Na⁺ entry was inhibited, the addition of amiloride (5 × 10⁻⁴ M) to the basolateral side still produced a fall in $d_{Na}$ (Fig. 1) from 2.6 ± 0.2 to 1.1 ± 0.2 mM (n = 4). These results show that an amiloride-sensitive Na⁺ leak pathway directed into the cell exists at the basolateral cell membranes, which contributes to the intracellular Na⁺ transport pool under spontaneous transepithelial Na⁺ transport conditions and in the absence of apical Na⁺ entry.

When amiloride (5 × 10⁻⁴ M) was added to the basolateral Ringer solution, the pH₁ measured under steady state short-circuit conditions was reversibly decreased (Fig. 2) from 7.30 ± 0.03 to 7.22 ± 0.03 (n = 6). These amiloride-induced changes in $d_{Na}$ and pH₁ are in a direction compatible with those expected for the inhibition of a Na⁺/H⁺ exchanger, which is not quiescent under steady state conditions (at normal pH₁ values) and which plays a role in maintaining pH₁, as well as contributing to the cytoplasmic Na⁺ transport pool. The inhibitory effect of basolateral amiloride on transepithelial Na⁺ transport rate ($I_{oc}$) (Figs 1 and 2) may be explained by the decreased apical Na⁺ conductance and permeability (Table I). This effect "at a distance" of serosal amiloride on apical membrane transport properties is expected as a consequence of the fall in pH₁ (as discussed in Harvey et al., 1988).
Regulation of pHi by Na\(^+\)/H\(^+\) Exchange

To determine whether Na\(^+\)/H\(^+\) exchange can regulate pHi, we investigated the sensitivity of pHi recovery after an intracellular acid load to high doses of amiloride or low Na\(^+\) concentrations in the basolateral Ringer solution.

An intracellular acid load was produced at constant external pH by switching from control Ringer's on the apical side to one buffered in 5% CO\(_2\) and 24 mM NaHCO\(_3\). This maneuver produced a large and prolonged intracellular acidosis that was completely reversible upon return to the CO\(_2\)-free control Ringer solution (Fig. 3, A and C). The recovery of pHi was substantially slowed when Na\(^+\) was absent.

**TABLE 1**

| Condition                        | \(P_{Na}\) (\(\text{cm} \cdot \text{s}^{-1} \times 10^{-7}\)) | \(g_s\) (\(\text{mS} \cdot \text{cm}^{-2}\)) | \(g_b\) (\(\text{mS} \cdot \text{cm}^{-2}\)) | \(FR_s\) |
|----------------------------------|-------------------------------------------------------------|---------------------------------------------|---------------------------------------------|---------|
| Control (n = 12)                 | 8.58 ± 0.7                                                  | 0.729 ± 0.086                               | 0.80 ± 0.07                                 | 0.52    |
| Basolateral amiloride (n = 8)    | 6.74 ± 0.54                                                | 0.554 ± 0.042                               | 0.61 ± 0.005                                | 0.52    |
| Basolateral Na\(^+\) free (n = 8)| 5.69 ± 0.51                                                | 0.432 ± 0.039                               | 0.47 ± 0.04                                 | 0.52    |
| Acid load (n = 12)               | 2.65 ± 0.11                                                | 0.156 ± 0.021                               | 0.20 ± 0.02                                | 0.56    |
| Recovery in presence of amiloride| (n = 6)                                                     | 3.15 ± 0.27                                 | 0.292 ± 0.032                               | 0.32 ± 0.05 | 0.52 |
| Recovery in absence of amiloride | (n = 6)                                                     | 8.25 ± 0.65                                 | 0.705 ± 0.082                               | 0.77 ± 0.08 | 0.52 |

Apical \(P_{Na}\) and \(g_s\) were determined from the fit of the GHK equation to amiloride-sensitive (apical side 50 \(\mu\)M) I-V relations of apical cell membranes. Simultaneous recording of the I-V relations of the basolateral cell membranes permitted the determination of the slope conductance of these membranes (\(g_b\)). In the first section of the Table the effects of amiloride (5 \(\times\) 10\(^{-4}\) M) application on the basolateral side, or Na\(^+\) removal from the basolateral Ringer solution are presented. In the lower half of the Table we present data for the effects of an intracellular acid load (CO\(_2\) on apical side) and the subsequent return to control Ringer in the presence or absence of amiloride (5 \(\times\) 10\(^{-4}\) M) on the basolateral side. The \(FR_s\) is the apical membrane resistance divided by transcellular resistance (\(FR_s = R_a/R_t \div R_b\)).
from the basolateral Ringer solution (Fig. 3 B) or when amiloride (5 x 10⁻⁴ M) was present in this solution (Fig. 3 D).

The rate of pHᵢ recovery may be expressed in terms of an apparent H⁺ efflux (Δ[H⁺]ᵢ) obtained from the product of the total intracellular buffering power (βᵢ) and the rate of change in intracellular H⁺ concentration as described in the Methods. We examined the dependence of Δ[H⁺]ᵢ on the concentration of Na⁺ in the basolateral Ringer solution and found that this relationship, shown in Fig. 4, could be described by simple Michaelis-Menten kinetics with a Kᵢₘ for Na⁺ of 18 ± 2 mmol/liter and a Vₘₐₓ for H⁺ extrusion of 680 ± 50 neq · h⁻¹ · cm⁻² (n = 8).

We also measured unidirectional ²⁸Na influx across the basolateral cell membranes (J⁺bas) of isolated epithelia bathed in control Ringer solution, and immediately after return to this Ringer after an intracellular acid load as described in the Methods. Control J⁺bas was 806 ± 101 neq · h⁻¹ · cm⁻² (n = 15), which agrees well with previous findings of a large basolateral Na⁺ "leak" pathway (Stoddard and Helman, 1985; Ehrenfeld et al., 1987). Of this total J⁺bas a flux of 280 neq · h⁻¹ · cm⁻² was inhibited by amiloride (10⁻⁴ M) or EIPA (10⁻⁵ M) (Fig. 5). An intracellular acidification created by a CO₂ or NH₄⁺ load increased the basal amiloride- (or EIPA-) sensitive J⁺bas by 125% and 50%, respectively (Fig. 5). The maximum value of acid-stimulated J⁺bas = 620 neq · h⁻¹ · cm⁻² is very similar to the Vₘₐₓ for apparent H⁺ efflux 0 neq · h⁻¹ · cm⁻²), which indicates a 1:1 stoichiometry for Na⁺/H⁺ exchange.

Reversibility of the Na⁺/H⁺ Exchanger

The normal function of the Na⁺/H⁺ exchanger of extruding H⁺ is dependent on concentration of Na⁺ on the basolateral side, then lowering [Na⁺]₀ should rease the activity of the exchanger and could even cause the reversal of its nor-operation. Such a possibility was tested by recording the response of aᵣᵢ, Iᵢₑ, and to suddenly lowering [Na⁺]₀ to 5 mM (choline replacement) under non-acid conditions. This maneuver was accompanied by a rapid fall in aᵣᵢ that was ed down in the presence of amiloride (5 x 10⁻⁴ M) on the basolateral side (Fig. 6). In this particular situation of low basolateral Na⁺ concentration, ~60% of the cell Na⁺ loss occurred via an amiloride-sensitive pathway, the remainder of Na⁺ leaving the cell may be transported by the Na⁺/K⁺ ATPase, whose activation energy is lowered due to the reduced [Na⁺]₀. If the Na⁺ loss into low [Na⁺]₀ solutions is mediated by reversal of a Na⁺/H⁺ exchanger then one would expect net uptake of H⁺ into the cell under these conditions and a fall in Iᵢₑ due to the low pHᵢ. This result was observed and was especially marked upon complete removal of Na⁺ from the basolateral Ringer solution (choline replacement), which produced a reversible decrease in pHᵢ (Fig. 7) from 7.27 ± 0.05 to 7.05 ± 0.05 (n = 4). These results provide additional evidence that H⁺ extrusion is dependent on a favorable inwardly directed Na⁺ chemical gradient across the basolateral cell membrane.

Effect of Inhibition of the Na⁺/H⁺ Exchanger on Cell Membrane Conductances

In the companion paper (Harvey et al., 1988) we reported that apical membrane Na⁺ conductance and basolateral membrane K⁺ conductance were very sensitive to changes in pHᵢ. Since here we provide evidence that pHᵢ is maintained and regu-
Figure 3
Relationship between the initial rate of H⁺ extrusion (Δ[H⁺]) determined (as described in Methods) from the initial rate of pHi recovery from an intracellular acid load as a function of the [Na⁺] in the basolateral Ringer solution. The relationship was fit by the Michaelis-Menten equation (solid line) yielding a $K_m$ of $18 \pm 2$ mM and a $V_{max}$ of $680 \pm 50$ neq · h⁻¹ · cm⁻² ($n = 8$).

lated by Na⁺/H⁺ exchange, we looked at the effects of modifying the activity of the antiporter on cell membrane conductances.

In four isolated epithelia, where $g_b$ was determined approximately every 10 s from $I_b$-$V_b$ relations, while pHi was measured simultaneously with a double-barreled H⁺-sensitive microelectrode, complete removal of Na⁺ from the basolateral Ringer solution (choline replacement) caused a reversible decrease in basolateral membrane conductance (Fig. 7 and Table I). The changes in $g_b$ were covariant with the fall in pHi, which occurred upon decreasing basolateral Na⁺ concentration. This response could not be mimicked by a low concentration of amiloride ($5 \times 10^{-6}$ M) added to the basolateral Ringer solution (Fig. 7). Such a dose of amiloride is sufficient to cause > 50% block of apical membrane Na⁺ conductance. This result would appear to eliminate the possibility of a Na⁺ electrodiffusive pathway in the basolateral cell membrane. Moreover, the response of the basolateral cell membrane potential to lowering [Na⁺]ₙ (depolarization) is in a direction opposite to that expected for the reduction of an inward Na⁺ current. Thus the response of $g_b$ to Na⁺ removal is unlikely to be due to changes in a Na⁺-conductive transport pathway, but to result from pHi effects on K⁺ conductance as discussed in the companion paper. There is also the possibility that Na⁺ replacement by the impermeant

Figure 4. (Opposite) Double-barreled pH-sensitive microelectrode recording of an intracellular acid load produced by switching from standard Ringer to a 5% CO₂:24 mM HCO₃⁻-buffered Ringer solution on the apical side. The pHi recovered upon return to control Ringer solution (A and C) but was retarded if the basolateral Ringer solution was Na⁺-free (choline replacement) (B) or contained $5 \times 10^{-4}$ M amiloride (D). Subsequent readmission of Na⁺ or removal of amiloride accelerated pHi recovery.
cation choline, may induce cell shrinkage, a phenomenon that is reported to reduce K⁺ conductance in tight epithelia (Davis and Finn, 1982). This possibility was not investigated here.

Complete removal of basolateral Na⁺ also produced a fall in apical membrane Na⁺ conductance and permeability (Table I). The decrease in gₘ was found to be covariant with the variations in pHᵢ shown in Fig. 7. Thus Na⁺ removal from the

![Activity of the basolateral Na⁺/H⁺ exchanger](image)

**Figure 5.** Activity of the basolateral Na⁺/H⁺ exchanger expressed as the basolateral ²²Na uptake inhibited by amiloride (10⁻⁴ M) EIPA (10⁻⁵ M) measured in 15 isolated epithelia under normal acid-base status and during the initial recovery phase after an intracellular acid load, which was produced by exposure to 5% CO₂ for 1½ h or to 15 mM NH₄Cl for 15 min.

The basolateral side can "act at a distance" to reduce apical Na⁺ entry. The contemporaneous effects of Na⁺-free serosal solutions on gₘ and gₙ is highlighted by the near constancy of the fractional resistance (FRₘ = Rₙ/Rₙ + Rₘ) (Table I).

Amiloride in high concentrations is known to inhibit Na⁺⁺/H⁺ exchange and we have seen that, when applied from the basolateral side, amiloride caused a decrease in pHᵢ under spontaneous transepithelial Na⁺ transport conditions. The effects of
FIGURE 6. Reversal of the normal function of the amiloride-sensitive Na\(^+\)/H\(^+\) exchanger. Intracellular Na\(^+\) activity (\(a_{\text{Na}}\)) was measured with a double-barreled Na\(^+\)-sensitive microelectrode and was observed to decrease when the concentration of Na\(^+\) in the basolateral Ringer solution was lowered from 115 to 5 mM (choline replacement). The decrease in \(a_{\text{Na}}\) was reduced by \(-60\%\) when amiloride (5 \(\times\) 10\(^{-4}\) M) was present on the basolateral side. Lowering of Na\(^+\) in the basolateral bath also reduced the \(I_{\text{sc}}\).

FIGURE 7. Effects of complete removal of Na\(^+\) (choline replacement) from the basolateral Ringer solution on \(I_{\text{sc}}\) (Δ), basolateral membrane potential (Ψ), and conductance (Gb), and \(I_{\text{sc}}\) (---). Na\(^+\)-free Ringer caused a fall in \(I_{\text{sc}}\) of 50% and in pH\(_i\) of \(-0.2\) units and a fourfold decrease in \(G_b\) as measured from circuit analysis. The effects of Na\(^+\)-free Ringer are unlikely to be due to changes in a Na\(^+\) conductive pathway since basolateral application of amiloride (5 \(\mu\)M) had no significant effect on \(V_b\) or \(G_b\). Amiloride at this low concentration is a known blocker of epithelial Na\(^+\) channels. Note the covariance between the variations in pH\(_i\) and \(G_b\).
adding amiloride (5 x 10^-4 M on the basolateral side) on \(g_a\) and \(g_b\) were determined by simultaneously recording \(I-V\) relations of apical and basolateral cell membranes. Basolateral amiloride caused an immediate and simultaneous decrease in the slope conductance of both membranes (Fig. 8, A and B). Both \(g_a\) and \(g_b\) were decreased by approximately the same amount since the \(FR_a\) remained almost constant. The trans-epithelial Na\(^+\) transport rate (\(I_n\)) and apical Na\(^+\) permeability (obtained from the GHK fit to \(I_n-V_n\) relations) were also decreased (Table I and Figs. 1 and 2). These experiments demonstrate that amiloride block of Na\(^+\)/H\(^+\) exchange decreases basolateral membrane conductance and can also produce an inhibition "at a distance" of apical Na\(^+\) conductance, and, thus, ultimately on Na\(^+\) transport rate. This phenomenon can be observed under normal steady state transport conditions.

The recovery of \(g_a\) and \(g_b\) after cell acidification was blocked by serosal amiloride (Fig. 9, A and B). The maximum decrease of \(g_a\) and \(g_b\) occurred when pH\(_i\) was lowered to 6.5; in the presence of amiloride this inhibition was maintained despite the removal of the source of the acid load (NH\(_4\)Cl or CO\(_2\)).

![Figure 8](image_url)

**Figure 8.** Amiloride-sensitive \(I-V\) relations of the apical cell membranes (A) and basolateral cell membranes (B), in control conditions (+), in the presence of amiloride (5 x 10^-4 M) on the basolateral side (○), and during an intracellular acid load (△) produced after washout of 15 mM NH\(_4\)Cl which had been present in the basolateral solution for 15 min.

![Figure 9](image_url)

**Figure 9.** \(I-V\) relations of apical (A) and basolateral (B) cell membranes fit by the GHK equation (solid lines) in control (○) and acid-loaded (△) conditions, and upon the return to control in the presence of 5 x 10^-4 M basolateral amiloride (×); and after the washout of amiloride (○). The GHK equation parameter values and basolateral membrane slope conductance for these experiments are given in Table I.
This raises the interesting possibility that Na\textsuperscript{+}/H\textsuperscript{+} exchange could modulate Na\textsuperscript{+} and K\textsuperscript{+} conductances and transepithelial Na\textsuperscript{+} transport rate.

*Covariance between Transepithelial Na\textsuperscript{+} Transport and pH\textsubscript{i} in Conditions of "Cross-Talk"

We tested the hypothesis that pH\textsubscript{i} may mediate transcellular coupling of Na\textsuperscript{+} and K\textsuperscript{+} conductances, by investigating whether pH\textsubscript{i} changes occur during spontaneous variations in Na\textsuperscript{+} transport and during two classical conditions of "cross-talk" (inhibition or stimulation of $I_{\text{m}}$ by ouabain or aldosterone, respectively).

One of the original examples of cross-talk in epithelia is that described for ouabain inhibition of Na\textsuperscript{+}/K\textsuperscript{+} ATPase followed by a subsequent decrease in apical Na\textsuperscript{+} permeability ($P_{\text{Na}}$) and conductance (MacRobbie and Ussing, 1968; Helman et al., 1979). We tested whether this phenomenon could be related to changes in pH\textsubscript{i} in the isolated epithelium by monitoring pH\textsubscript{i} with double-barreled H\textsuperscript{+}-sensitive microelectrodes and determining apical Na\textsuperscript{+} permeability and [Na\textsuperscript{+}], approximately every 2 min from $I_{\text{m}}$-$V_{\text{m}}$ relations in the presence of ouabain (10\textsuperscript{-4} M) in the basolateral Ringer solution (Fig. 10). In the first 10 min after ouabain, the $P_{\text{Na}}$ decreased slightly while $I_{\text{m}}$ fell to 55% of control values. During this time the calculated [Na\textsuperscript{+}], showed a steady increase, presumably as a result of the inhibition of Na\textsuperscript{+}/K\textsuperscript{+} ATPase and of unrestricted entry of Na\textsuperscript{+} across the apical cell membrane. After 10 min, the pH\textsubscript{i} and $P_{\text{Na}}$ began to decrease simultaneously and [Na\textsuperscript{+}], increased significantly. At 24 min $I_{\text{m}}$ was 10% of control, [Na\textsuperscript{+}], was increased fivefold, while $P_{\text{Na}}$ was

**Figure 10.** Effect of ouabain (10\textsuperscript{-4} M) addition to the basolateral Ringer solution in the isolated epithelium on apical $P_{\text{Na}}$ (○) (measured from the fit of the GHR equation to $I_{\text{m}}$-$V_{\text{m}}$ relations), $I_{\text{m}}$ (▲) and on intracellular Na\textsuperscript{+} (▲) on log scale and pH\textsubscript{i} (△) (measured with double-barreled ion sensitive microelectrodes). Note the covariance between the reduction in pH\textsubscript{i} and $P_{\text{Na}}$.
half that of control. The close correlation found between short-circuit current and pH_i in the presence of ouabain (Figs. 10 and 11), and our previous observations (Harvey et al., 1988) of the dependence of P_{Na} and g_a on pH_i, indicate that intracellular H^+ may provide the link between ouabain block of Na^+/K^+ ATPase and subsequent "action at a distance" on apical membrane Na^+ permeability.

Transcellular coupling of g_a and g_b has also been described in the presence of aldosterone. After long term exposure (overnight) to the hormone, Nagel and Crabbé (1980) reported that both apical and basolateral cell membrane resistances were decreased in amphibian skin. We found that in isolated epithelia the pH_i increased by 0.21 ± 0.03 units (n = 11) within 30–90 min of exposure to aldosterone (5 μM) from the basolateral side. This effect was covariant with the stimulation in amiloride (apical)–sensitive transepithelial Na^+ transport rate (I_a) (Fig. 11).

It is of physiological importance that we found a strong covariance between the spontaneous Na^+ transport rate and pH_i (Fig. 11). Epithelia showing high rates of Na^+ transport were associated with an elevated pH_i, whereas low transporters had a more acidic pH_i. Thus, hormonal, diuretic, and spontaneous modifications of Na^+ transport rates are accompanied by changes in pH_i, which are in the right direction to mediate parallel changes in Na^+ and K^+ conductances. These results suggest a physiological role for pH_i in the control of transepithelial Na^+ transport.

**DISCUSSION**

**Basolateral Na^+/H^+ Exchange**

The aim of this study was to determine the mechanism of pH_i regulation after an acid load, and its possible role in controlling cell membrane conductances in the classical model epithelium of frog skin. The results demonstrate that an amiloride-sensitive Na^+/H^+ exchanger is present at the basolateral cell membranes that participates in the maintenance of pH_i under steady state spontaneous Na^+ transport conditions. The antiporter is essential for the recovery of pH_i after an experimentally induced acid load. Besides its pH_i regulation function we found that the Na^+/H^+
The Na+/H+ exchanger contributes to the cell Na+ transport pool both under spontaneous Na+ transport conditions and when apical Na+ entry was blocked by low concentrations of amiloride (applied from the apical side). This result is an agreement with our recent finding (Ehrenfeld et al., 1987) that high concentrations of ethyl isopropyl amiloride, a specific inhibitor of Na+/H+ exchange, caused a decrease in both basolateral 32Na+ influx and Na+ transport pool.

**Driving Forces for Na+/H+ Exchange**

Falls in pHo and ∆Na+ were produced by lowering the serosal Na+ concentration below 10 mmol/liter. If Na+/H+ exchange is driven solely by the free energy (∆Gnet) contained in the transmembrane chemical gradient for Na+ and H+ then

\[
\Delta G_{Na} = RT \ln \frac{[Na^+]}{[Na^+]o},
\]

\[
\Delta G_{H} = RT \ln \frac{[H^+]}{[H^+]o},
\]

\[
\Delta G_{net} = RT \ln \frac{[Na^+]}{[Na^+]o} \cdot \frac{[H^+]}{[H^+]o}.
\]

Under normal steady state conditions pHo = 7.4, pHl = 7.27, [Na+]o = 115 mM, [Na+]l = 11 mM, giving ∆GNa = −2.35 RT, ∆GH = −0.30 RT, and the exchanger extrudes cell H+. At [Na+]o = 5 mM, ∆Gnet = 0.49 RT, and the exchanger is predicted to acidify the cell, as was observed.

**The Role of the Na+/H+ Exchanger in the Control of Cell Membrane Conductances**

Inhibition of the Na+/H+ exchanger by amiloride was accompanied by a depolarization of the basolateral membrane potential. This should not necessarily be construed to mean that the antiporter is electrogenic since the change in Vb may be explained by the effect of lowering pHl to decrease K+ conductance and reduce outward K+ current across this membrane.

We found that the operation of the Na+/H+ exchanger is essential for the recovery of cell membrane conductances after an intracellular acid load. An analogous result has recently been found in the frog proximal tubule in which amiloride inhibition of Na+/H+ exchange produced a fall in apparent K+ conductance (Oberleithner et al., 1986).

Net extrusion of protons via the exchanger was dependent on the maintenance of a favorable inward Na+ chemical gradient across the basolateral cell membrane. This gradient is itself a function of the balance between apical Na+ entry and basolateral Na+ exit. Thus the activity of the Na+/H+ exchanger is influenced to some extent by the rate of transepithelial Na+ transport and on the equilibrium (or cross-talk) between ion transfer at apical and basolateral cell membranes. The regulation of pHl and transepithelial Na+ transport may, therefore, be interrelated (Fig. 12), especially since natriferic hormones (insulin, aldosterone) also affect Na+/H+ exchange (Moore, 1986; Oberleithner et al., 1987).

Ouabain causes an increase in intracellular Na+ activity (Harvey and Kernan, 1984), and the operation of the Na+/H+ exchanger may be reduced under such conditions. This may be the mechanism for the observed intracellular acidification and the subsequent fall in PN. We also found that ouabain decreases gb with the
same time course as the intracellular acidification; this is most likely due to the effects of lowering pH$i$ on K$^+$ conductance. Ouabain has also been reported to reduce apparent K$^+$ conductance in frog renal proximal tubules (Messner et al., 1985).

Aldosterone caused an alkaline shift in pH$i$, which became apparent in the relatively short time of 30–90 min after hormonal treatment. Since Na$^+$ transport rate and Na$^+$ and K$^+$ conductances are all increased by a rise in pH$i$, it is possible that the intracellular alkalinization is in part responsible for (or reinforces) the effects of aldosterone on Na$^+$ and K$^+$ transport. A recent report has provided electrophysiological evidence that aldosterone enhances pH$i$ regulation via activation of Na$^+$/H$^+$ exchange in giant fused cells from proximal tubule (Oberleithner et al., 1987).

We also found a direct correlation between the amiloride-sensitive spontaneous short-circuit current and pH$i$. A high rate of Na$^+$ transport is characterized by a high apical membrane Na$^+$ conductance and often, but not always, by an elevated basolateral membrane conductance (Davis and Finn, 1982; Thomas et al., 1983; Nagel, 1985). The dependence of $g_a$ and $g_b$ on pH$i$ is such that, under spontaneous Na$^+$ transport conditions, the degree of coupling between apical and basolateral cell membranes will be influenced by changes in intracellular proton concentration.

\[ \text{pH}_i \text{ and [Ca$^{2+}$], in Cross-Talk} \]

Up until now many of the investigations of transepithelial coupling of ion flows in epithelia have concentrated on the role of intracellular calcium to signal this phenomenon (Gristein and Erlij, 1978; Taylor and Windhager, 1979; Chase and Al-Awqati, 1981; Windhager et al., 1986). Changes in [Ca$^{2+}$], have been proposed to be coupled to transepithelial Na$^+$ transport rate via the effect of the transbasolateral Na$^+$ chemical gradient on a Na$^+$/Ca$^{2+}$ exchanger localized at the basolateral cell membranes. However, there is as yet no firm evidence that changes in the Na$^+$ transport rate can be translated into a change in [Ca$^{2+}$]. In fact, many of the arguments used to support the regulation of cross-talk via Na$^+$/Ca$^{2+}$ exchange can also be used to consolidate the role of Na$^+$/H$^+$ exchange in this phenomenon. Recently, problems have arisen with regard to the Ca$^{2+}$ hypothesis in that maneuvers designed to change [Ca$^{2+}$] in frog skin epithelium were without the predicted effects on Na$^+$ transport (Erlij et al., 1986) and cell membrane conductances (Nagel, 1987). Moreover, Palmer and Frindt (1987) found an effect of pH but not of Ca$^{2+}$ on the activ-
inity of Na\(^+\) channels in excised patches of apical cell membranes from cultured renal cortical collecting tubules. In a recent study on cross-talk phenomena in the Necturus urinary bladder, Demarest and Finn (1987) were unable to involve the Ca\(^{2+}\) hypothesis to account for their results. However, the experiments of Garty et al., (1987) on vesicles from toad bladder indicated that [Ca\(^{2+}\)], inhibits the amiloride-sensitive Na\(^+\) flux and that pH\(_i\) has an effect on the inhibitory potency.

The relationship between pH\(_i\) and [Ca\(^{2+}\)], in epithelia is not yet known for certain. However, recent studies in our laboratory (Harvey and Thomas, 1987) using the intracellular Ca\(^{2+}\) chelator MAPTAM [bis-(2-amino-5-methyl-phenoxy)-ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester] and the pH\(_i\) buffer procaine provide evidence that intracellular hydrogen ions act directly on the Na\(^+\) channel without requiring changes in [Ca\(^{2+}\)].

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REFERENCES

Chase, H. S., and Q. Al-Awqati. 1981. Regulation of the sodium permeability of the luminal border of toad bladder by intracellular sodium and calcium. Role of sodium-calcium exchange in the basolateral membrane. *Journal of General Physiology.* 81:643–665.

Davis, C. W., and A. L. Finn. 1982. Sodium transport inhibition by amiloride reduces basolateral membrane K conductance in tight epithelia. *Science.* 216:525–527.

Demarest, J. R., and A. L. Finn. 1987. Interaction between basolateral K\(^+\) and apical Na\(^+\) conductances in Necturus urinary bladder. *Journal of General Physiology.* 89:563–580.

Diamond, J. M. 1982. Transcellular cross-talk between epithelial cell membranes. *Nature.* 300:683–685.

Ehrenfeld, J., E. J. Cragoe, and B. J. Harvey. 1987. Evidence for a Na\(^+\)/H\(^+\) exchanger at the basolateral membranes of the isolated frog skin epithelium: effect of amiloride analogues. *Pflügers Archiv.* 409:200–207.

Erlij, D., L. Gersten, G. Sterba, and H. F. Schoen. 1986. Role of prostaglandin release in the response of tight epithelia to Ca\(^{2+}\) ionophores. *American Journal of Physiology.* 250:C629–C636.

Garty, H., C. Asher, and O. Yeger. 1987. Direct inhibition of epithelial Na\(^+\) channels by a pH-dependent interaction with calcium and by other divalent cations. *Journal of Membrane Biology.* 95:151–162.

Grinstein, S., and D. Erlij. 1978. Intracellular calcium and the regulation of sodium transport in the frog skin. *Proceedings of the Royal Society of London.* 202:353–360.

Harvey, B. J., and R. P. Kernan. 1984. Intracellular ionic activities in relation to external sodium and effects of amiloride and/or ouabain. *Journal of Physiology.* 349:501–517.

Harvey, B. J., and R. C. Thomas. 1987. Intracellular pH and calcium effects on sodium conductance and transport in isolated frog skin epithelium. *Journal of Physiology.* 394:92p. (Abstr.)

Harvey, B. J., S. R. Thomas, and J. Ehrenfeld. 1988. Intracellular pH controls cell membrane Na\(^+\) and K\(^+\) conductances and transport in frog skin epithelium. *Journal of General Physiology.* 92:767–791.
Helman, S. I., W. Nagel, and R. Fisher. 1979. Ouabain on active transepithelial Na transport by frog skin: studies with microelectrodes. *Journal of General Physiology*. 74:105–127.

MacRobbie, E. A. C., and H. H. Ussing. 1961. Osmotic behaviour of the epithelial cells of frog skin. *Acta Physiologica Scandinavica*. 53:348–365.

Messner, G., W. Wang, M. Paulmichl, H. Oberleithner, and F. Lang. 1985. Ouabain decreases apparent potassium conductance in proximal tubules of the amphibian kidney. *Pflügers Archiv*. 405:131–137.

Moore, R. P. 1986. The role of intracellular pH in insulin action and in diabetes mellitus. *Current topics in membranes and transport*. 26:263–290.

Nagel, W. 1985. Basolateral membrane ionic conductance in frog skin. *Pflügers Archiv*. 405(Suppl.):S39–S43.

Nagel, W. 1987. On the origin of transport inhibition after omission of serosal sodium. *American Journal of Physiology*. 252:C528–C529.

Nagel, W., and J. Grabbé. 1980. Mechanism of action of aldosterone in frog skin. *Pflügers Archiv*. 385:181–187.

Oberleithner, H., G. Munich, A. Schwab, and P. Dietl. 1986. Amiloride reduces potassium conductance in frog skin via inhibition of Na/H exchange. *American Journal of Physiology*. 251:F66–F73.

Oberleithner, H., H. Weight, H. J. Westphale, and W. Wang. 1987. Aldosterone activates Na+/H+ exchange and raises cytoplasmic pH in target cells of the amphibian kidney. *Proceedings of National Academy of Sciences*. 84:1464–1468.

Palmer, L. G., and G. Frindt. 1987. Effects of cell Ca and pH on Na channels from rat cortical collecting tubule. *American Journal of Physiology*. 253:F333–F339.

Schultz, S. G. 1981. Homocellular regulatory mechanisms in sodium transporting epithelia avoidance of extinction by "flushthrough." *American Journal of Physiology*. 241:F579–F590.

Stoddard, J. S., and S. I. Helman. 1985. Dependence of intracellular Na+ concentration on apical and basolateral membrane Na+ influx in frog skin. *American Journal of Physiology*. 249:F662–F671.

Taylor, A., and E. E. Windhager. 1979. Possible role of cytosolic calcium and Na-Ca exchange in regulation of transepithelial sodium transport. *American Journal of Physiology*. 236:F505–F512.

Thomas, S. R., Y. Suzuki, S. M. Thompson, and S. G. Schultz. 1983. Electrophysiology of *Necturus* urinary bladder. I. Instantaneous current-voltage relations in the presence of varying mucosal sodium concentrations. *Journal of Membrane Biology*. 73:157–175.

Windhager, E., G. Frindt, J. M. Yang, and C. O. Lee. 1986. Intracellular calcium ions as regulators of renal tubular sodium transport. *Klinische Wochenschrift*. 64:847–852.