Intracellular pH shifts in cultured kidney (A6) cells: effects on apical Na\textsuperscript{+} transport

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Zeiske, Wolfgang, Ilse Smets, Marcel Ameloot, Paul Steels, and Willy Van Driessche. Intracellular pH shifts in cultured kidney (A6) cells: effects on apical Na\textsuperscript{+} transport. Am. J. Physiol. 277 (Cell Physiol. 46): C469–C479, 1999.—We report, for the epithelial Na\textsuperscript{+} channel (ENaC) in A6 cells, the modulation by cell pH (pH\textsubscript{c}) of the transepithelial Na\textsuperscript{+} current (I\textsubscript{Na}), the current through the individual Na\textsuperscript{+} channel (i), the open Na\textsuperscript{+} channel density (N\textsubscript{o}), and the kinetic parameters of the relationship between I\textsubscript{Na} and the apical Na\textsuperscript{+} concentration. The i and N\textsubscript{o} were evaluated from the Lorentzian I\textsubscript{Na} noise induced by the apical Na\textsuperscript{+} channel blocker 6-chloro-3,5-diaminopyrazine-2-carboxamide. pH\textsubscript{c} shifts were induced, under strict and volume-controlled experimental conditions, by apical/basolateral NH\textsubscript{4}Cl pulses or basolateral arrest of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger (Na\textsuperscript{+} removal; block by ethylisopropylamiloride) and were measured with the pH-sensitive probe 2,7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein. The changes in pH\textsubscript{c} were positively correlated to changes in I\textsubscript{Na} and the apically dominated transepithelial conductance. The sole pH\textsubscript{c}-sensitive parameter underlying I\textsubscript{Na} was N\textsubscript{o}. Only the saturation value of the I\textsubscript{Na} kinetics was subject to changes in pH\textsubscript{c}. pH\textsubscript{c}-dependent changes in N\textsubscript{o} may be caused by influencing P\textsubscript{o}, the ENaC open probability, or/and the total channel number, N\textsubscript{T} = N\textsubscript{o}/P\textsubscript{o}.

Noise analysis; single-channel current; epithelial sodium channel; ammonium; cell volume

CELL pH (pH\textsubscript{c}) is under strict control (8, 15). Unexpectedly, cytosolic acidification, resulting from a lack of oxygen, appeared, at least at short term, beneficial against major cell damage from ischemia (39). Prior treatment of several cell types including kidney cells with salines of pH < 7 reduced or prevented cell damage, such as leak of enzymes or complete lysis during anoxia. Interestingly, analogous cytoprotective effects were obtained by treatment with glycine and alanine during ischemia. So far, no mechanisms of action underlying either type of cytoprotection could be settled (32, 39).

Recently, pH\textsubscript{c} has been discussed to represent another cytosolic second messenger, together with Ca\textsuperscript{2+}, cAMP, ATP, and other signaling molecules (18, 19). For instance, in tight epithelia such as frog skin or the cultured distal kidney cell line A6 from the clawed toad, Xenopus laevis, pH\textsubscript{c} was found to influence apical and basolateral cation permeabilities such that a concert up- and downregulation of apical Na\textsuperscript{+} (P\textsubscript{Na}) and basolateral K\textsuperscript{+} (P\textsubscript{K}) permeabilities (so-called “cross talk”) occurred. Within a comparably narrow range of pH\textsubscript{c} (7.4 to 7.0), P\textsubscript{Na} as well as P\textsubscript{K} became negligible upon cell acidification (18, 19). This might shed some light on the mechanism of the protective effect of protons. A closure of epithelial cation channels by cellular acidification could prevent, after ATP depletion in an anoxic state, the accumulation of cell Na\textsuperscript{+}, the parallel loss of cell K\textsuperscript{+}, and a gain in cell NaCl and, consequently, of water followed by cell disruption.

To study the dependence of plasma membrane ion permeability on pH\textsubscript{c}, the so-called “NH\textsubscript{4} pulse” method has become a popular means to alter pH\textsubscript{c}. Usually, when more than millimolar concentrations of NH\textsubscript{4}Cl salts are added to the extracellular saline, an alkalinization of the cytosol due to hydrolysis of the easily permeant NH\textsubscript{3} has been observed (9, 10, 21). Extracellular NH\textsubscript{4} removal would in turn acidify the cell (“NH\textsubscript{4} prepulse” method (6)). The rate of the subsequent realkalization will reflect the activities of pH\textsubscript{c}-regulating transporters, such as the Na\textsuperscript{+}/H\textsuperscript{+} exchanger or primary active H\textsuperscript{+} pumps. If otherwise K\textsuperscript{+}-permeable entrance pathways for NH\textsubscript{4}Cl (10, 23) are in parallel to the lipid or, as discussed (34), a possible aquaporin permeation route of NH\textsubscript{3}, cytosolic pH drops may also be caused by intracellular NH\textsubscript{4}Cl hydrolysis, which counteracts the alkalinization from NH\textsubscript{3}. When tissue pH changes are evoked by simple addition (6, 10, 21, 31) of 10–30 mM NH\textsubscript{4}Cl salts to saline, without being balanced osmotically, cell volume changes may as well influence ion channel permeabilities (11, 40).

In the context of using A6 cells as model epithelium for the study of the consequences of anoxia and the protective effects of protons, we set out to investigate NH\textsubscript{4}Cl addition to, as well as its removal from, NaCl-Ringer solutions without or with osmotic control. For each method, eventual alterations in cell volume were recorded. pH\textsubscript{c} was monitored using a membrane-permeant derivative of the pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF; see Refs. 6 and 9). To inspect transepithelial Na\textsuperscript{+} uptake, we monitored transepithelial conductance (G\textsubscript{T}) and the short-circuit current (I\textsubscript{sc}) carried by Na\textsuperscript{+}.

To establish whether pH\textsubscript{c}-dependent changes in apical P\textsubscript{Na} are related to changes in single-channel current (i) or channel density, we conducted fluctuation analysis with the Na\textsuperscript{+} channel blocker 6-chloro-3,5-diaminopyrazine-2-carboxamide (CDPC; see Ref. 35). Finally, the influence of pH\textsubscript{c} changes on Na\textsuperscript{+} current (I\textsubscript{Na}) kinetics was evaluated and compared with the results obtained from noise analysis.
Depending on the side of application, and the tonicity of the NH4Cl-containing saline, pHc and I_{Na} changed in a complex manner. Under strict conditions, however, changes in pHc affected only N_{Na}, the number of open apical Na⁺ channels, whereas the i and also the blocker kinetics appeared invariant.

**MATERIALS AND METHODS**

**Cell Culture**

A6 cells (passages 110–113) obtained from Dr. J. P. Johnson (University of Pittsburgh, PA) were cultured as described earlier (11). Polarized monolayers obtained after 15–30 days of culture on permeable tissue culture inserts (25 mm diameter; Nunc Anopore) were used to perform experiments. For the volume measurements, before seeding cells, the Anopore membranes were coated with fluorescent microspheres of 1 µm diameter (L5081; Molecular Probes, Eugene, OR) embedded in a thin gelatin layer.

**Cell Volume Measurements**

This method was previously described in detail (38). Briefly, cell thickness (T_c) was used as an index for cell volume of confluent monolayers. The apical (upper) side of the monolayer was labeled with fluorescent biotin-coated microbeads. Focussing of the microbeads was automatically performed with a piezoelectric focussing device (PIFOC; Physik Instrumente, Waldbronn, Germany). T_c is defined as the vertical distance between the basolateral and apical beads. Measured T_c values were corrected for the diameter of the fluorescent microbeads by subtracting 1 µm.

**Electrical Measurements**

Transepithelial direct current measurements. Epithelial monolayers were mounted in an Ussing-type chamber (chamber opening 0.7 cm²) designed to eliminate edge damage and were continuously superfused (3–5 ml/min) on both sides with Ringer solutions. The A6 tissues were short-circuited previously described in detail (35). Briefly, Lorentzian noise was induced with the uncharged amiloride analog CDPC (50 µM). Noise analysis methods have been previously described in detail (35). Briefly, Lorentzian noise was induced with the uncharged amiloride analog CDPC (50 µM). Fourier analysis of the fluctuation in current results in power density spectra (PDS; cf. Fig. 6A) normalized to the membrane area. The analysis of the PDS yields the Lorentzian parameters S_0 (plateau) and f_c (corner frequency). Assuming that Na⁺ channel blockage by CDPC fulfills pseudo-first-order kinetics (35) it follows

\[ 2\pi f_c = k_{on}[B] + k_{off} \]

with k_{on} and k_{off} being blocking and unblocking rate constants, respectively, and [B] the blocker concentration. These parameters, together with I_{Na}, the amiloride (0.1 mM)-blockable I_{sc}, served to calculate N_{Na} and the i using a two-state model for the apical Na⁺ channel block by apically applied CDPC (35) according to

\[ i = S_0\pi^2f_c^2/(Um_k[B]) \]

\[ N_0 = \frac{I_{Na}}{I} \]

\[ i = \gamma(V_c - E_Na) \]

where \( \gamma \) is the single-channel conductance, \( V_c \) is the cellular potential in short-circuit conditions, and \( E_Na \) is the apical Nernst potential for Na⁺.

**Fluorometric Measurement of pHc**

Confluent A6 tissues were mounted in an Ussing-type chamber (see Transepithelial direct current measurements) and were loaded from the apical side with BCECF (Molecular Probes) by exposure to a final concentration of 10 µM of the AM form of the dye (stock solution 5 mM in DMSO). Loading was performed for 60 min, at room temperature, in control NaCl Ringer with continuous perfusion at the basolateral side. After loading, excessive dye in the apical solution was washed out for at least 20 min.

The fluorescence measurements were performed with an own-built microfluorimeter under computer control. We used an inverted microscope (TMD 35; Nikon, Tokyo, Japan) in epifluorescence mode, equipped with a 32/0.40 objective (Leitz, Wetzlar, Germany). Excitation light of a 100-W Xe lamp (Nikon) was filtered at 440 and 490 nm (440DF20 and 490DF20, Omega Optical). Switching of the interference filters was done with a computer-controlled filter wheel (Lambda-10; Sutter Instrument, Novato, CA). The intensity of the source was reduced by neutral density filters inserted between the microscope and the filter wheel. The fluorescence emission was monitored at 535 nm (interference filter 535DF25; Omega Optical). The fluorescence was detected with a photomultiplier tube (9124A; Thorn-EMI, Middlesex, England) operating in photon counting mode. The pulses were transferred to the computer through a counter/timer board C 660 (Thorn-EMI). The data were collected with a photomultiplier tube (9124A; Thorn-EMI, Middlesex, England) operating in photon counting mode. The fluorescence intensity was corrected for the dead time of the counting system. The background due to scattering and autofluorescence was subtracted subsequently from each of the signals.

At the end of each experiment, calibration of the BCECF fluorescence (F) ratio \( R = F_{490\text{nm}}/F_{440\text{nm}} \) versus a given pHc was performed, using the nigericin-high K⁺ technique (36). Cells were clamped at three different pHc values (6.6, 7.3, and 8.0) using calibration solutions containing 13 µM nigericin and 137 mM K⁺. This K⁺ concentration closely approximates the reported cytosolic K⁺ concentration in A6 cells (27). Figure 1 shows the resulting linear fit through data of 16 in vivo calibrations on different A6 monolayers. When individual tissue calibration at the end of the experiment failed, we used the pooled calibration curve \[ \text{pH}c = [R + 19.0(± 0.6)]/3.45(± 0.08) \] to evaluate the experimental data. Figure 1 shows that in A6 cells the calibration procedure was quite reproducible.

**Statistics**

Mean values from N experiments (different monolayers) are given ± SE.

**Solutions and Chemicals**

NaCl Ringer solution had the composition (in mM) 70 NaCl, 3 KCl, 1 CaCl₂, 40 sucrose, 5 glucose, and 10 HEPES.
NH₄Cl, so-called “isosmotic swelling” due, e.g., to net osmotic sucrose in the saline is isosmotically replaced with followed by an obligatory water flow. Even when NaCl cell shrinkage will be counteracted by solute influx the NH₃, or NH₄⁺ cytosolic pH was investigated, high concentrations of A6 cells where hypertonic NaCl Ringer abolishes Na⁺ transport as for instance in

In many reports in which the influence of NH₄⁺ on cytosolic pH was investigated, high concentrations of the NH₄⁺ salt were added to the bath, giving rise to a noticeable increase in osmolality. In a number of tight epithelia, hyperosmotic cell shrinking strikingly reduces transepithelial Na⁺ transport as for instance in A6 cells where hypertonic NaCl Ringer abolishes Na⁺ absorption. On the other hand, due to the permeability of the cell membrane for NH₃, or NH₄⁺ with Cl⁻, cell shrinkage will be counteracted by solute influx followed by an obligatory water flow. Even when NaCl or sucrose in the saline is isosmotically replaced with NH₄Cl, so-called “isosmotic swelling” due, e.g., to net NH₄Cl entrance into the cells, could occur. Such a phenomenon has already been described for A6 cells when basolateral NaCl was replaced by KCl or glycerol.

This result confirms previous observations that cell volume does not respond to apical anisotonicity as the apical membrane of A6 cells is known to be quite impermeable to water. We also see that the expected isosmotic volume increase does not occur. This may indicate that there is no massive influx of solute/water or that the volume regulation occurs as fast as the osmotic disturbance. Regarding the high osmotic sensitivity of the basolateral side, putatively pH-c related transport changes that result from NH₄⁺ exposure must therefore be studied in the absence of any osmotic imbalance. Moreover, the sidedness of the application of a pH-c-shifting agent must be under strict control. Only isosmotic experiments are reported below. Also, a strictly unilateral treatment with pH-c-influencing substances was employed.
pHc and INa Changes During Apical or Basolateral Isoosmotic NH4Cl Exposure

Figure 3, A and B, depicts recordings (from two different epithelia) of pHc and Isc, respectively, when tissues were isosmotically exposed to apical NH4Cl-containing NaCl saline. Typically, a very similar time course in the change of both parameters is observed; apical NH4+, as predicted, alkalinizes the cells, which raises INa. In the presence of apical amiloride, Isc remains close to zero despite the change in pHc (not shown). Figure 3D shows, for eight tissues, the rise in D apical NH4Cl course in the change of both parameters is observed; containing NaCl saline. Typically, a very similar time that an increase in pHc correlates with an increase in 

In Fig. 4, A-D, the situation is considerably more complex. Figure 4, A and B, shows simultaneously recorded traces of pHc and Isc, respectively, from the same tissue. In Fig. 4A, during the first phase after NH4+ addition, pHc rises similarly with apical NH4+. However, this pHc rise is quickly reversed into a marked drop. In addition, another typical feature is observed in the time course of Isc only; right after introduction of basolateral NH4+ before pHc moves, a sharp and immediate current drop occurs followed by a gradual increase that occurs synchronously with pHc, first increasing and then dropping below the control value. Hence, the initial fall in Isc cannot be related to pHc changes, whereas the subsequent changes in Isc and pHc are quite similar. Figure 4, C and D, summarizes the long phase drop in pHc (N = 5) and INa (N = 10). At this point, we may state that a probably causal relationship exists between pHc and the magnitude of INa, disregarding for a moment the initial “blip” event in Isc obtained with basolateral NH4+. Cell alkalinization and current rise occur simultaneously with NH4+ on either side (basolaterally only in the beginning). Cell acidification takes place in the late phase with basolateral NH4+. Below we investigate, by means of noise analysis, which apical factor(s) determines the parameters of Na+ transport that are influenced by pHc.

Evaluation of Na+ Channel Blocker Noise

Influence of pHc on i and No

NH4+ pulses. To generate a Na+ channel blocker noise in Isc, we employed CDPC, a noncharged amiloride analog, rather than amiloride itself (35). Because simultaneous measurements of pHc and noise analysis could not be done, we assume, for the experiment depicted in Fig. 5 and the following ones, that the respective alterations of pHc due to isosmotic NH4Cl exposure are analogous to those reported (e.g., Fig. 3) in the absence of an inhibitor. A typical protocol for noise analysis is shown in Fig. 5A. In the presence of 50 µM CDPC, apical sucrose replacement by NH4Cl led to the already known rise in INa. A similar behavior of Gt, mainly determined by the apical membrane resistance, is under control of pHc. Interesting here, but occurring only in a minority of cases, is the observation that the alkalinization (causing the INa rise) seems subsequently to be counteracted even in the presence of NH4+, probably by a regulatory mechanism such as Cl-/HCO3 exchange (9). This would tend to acidify the cells, just like the basolateral action of NH4+. After elimination of apical NH4Cl, we see a slight undershoot of the parameters before return to control values; this may be related to an additional and well-known (6, 21) cell acidification after NH4+ removal (see also Fig. 5B).

Fig. 3. Effect of NH4+ on pHc and short-circuit current (Isc). A: typical time course of changes in pHc when 20 mM NH4Cl is added isosmotically (replacement of 40 mM sucrose) to the apical (ap) NaCl saline and after subsequent withdrawal. B: typical time course of changes in (mainly Na+-carried) Isc for another tissue treated as in A. C: comparison (5 tissues) of steady-state pHc before (control) isosmotic apical NH4Cl addition with pHc at maximal point of cell alkalinization. D: comparison of Na+ current (INa) before and at maximal response to apical isosmotic treatment with NH4Cl. Shown are the data for 8 tissues.
INTRACELLULAR pH AND Na⁺ TRANSPORT

When the agent is applied basolaterally, we see again the same features for \( I_{\text{Na}} \) as shown in Fig. 4B for \( I_{\text{sc}} \), accompanied by an almost completely proportional behavior of \( G_i \) with the notable exception of the negative initial blip characteristic for \( I_{\text{sc}} \). After removal of basolateral NH₄Cl, both \( G_i \) and \( I_{\text{Na}} \) show a marked negative overshoot. The protocol (Fig. 5A) ends with exposure to apical amiloride to determine the Na⁺-specific part in \( I_{\text{sc}} \) (35). The reason for the current undershoot after basolateral NH₄⁺ removal becomes clear from Fig. 5B: NH₄⁺ removal leads to a considerable further \( \text{pH}_c \) drop and \( I_{\text{Na}} \) decrease (Fig. 5C), which are followed, like here when serosal Na⁺ is present (Fig. 5, B and C), by their slow recovery. When this protocol is repeated, however, in the absence of basolateral Na⁺ at the end of the experiment (Fig. 5C), this \( I_{\text{sc}} \) recovery does not occur, which strongly suggests the involvement of a serosal Na⁺/H⁺ antiport (6) in the backregulation of \( \text{pH}_c \) and, consequently, of \( I_{\text{Na}} \) and \( G_i \).

We used noise analysis of the CDPC-induced fluctuation in \( I_{\text{sc}} \) at various points in time in which stable current values had been attained. The period of the initial putative \( \text{pH}_c \) rise after serosal NH₄⁺ was too short to perform data recording for noise analysis. The results from this experiment are given in Table 1. \( \text{pH}_c \) had no influence on the \( f_i \) of the CDPC-induced Lorentzian noise (Fig. 6A). However, the \( S_o \) magnitude was altered dramatically and reflected the direction of changes in \( G_i \) and \( I_{\text{Na}} \) (cf. Fig. 5). Analysis on the basis of the two-state channel model for interaction with the blocker clearly revealed (Table 1) that \( i \) remained unaffected by changes in \( \text{pH}_c \). Therefore, \( \text{pH}_c \) exerts a direct control on Na⁺ channel activity as expressed by \( N_o \). A rise in \( \text{pH}_c \) augments \( G_i \) by means of Na⁺ channel opening, and vice versa (internal channel \( \text{pH}_c \) “titration”). Figure 6, B and C, summarizes such results for eight tissues. To compare different epithelia with sometimes much different transport capacities, we plotted, in Fig. 6B, the relative magnitude of the \( S_o \) (ratio of \( S_o \) in the presence of NH₄⁺ over \( S_o \) in the absence of NH₄⁺) and the function of the respective relative currents. These parameters turned out to be strictly proportional for a number of different conditions (see legend for Fig. 6). In the framework of the equations used for noise analysis (see MATERIALS AND METHODS), we thus conclude that, as already suggested by the typical experiment depicted in Table 1 and Fig. 5A, only \( N_o \) is responsible for the observed \( \text{pH}_c \)-dependent alterations in \( I_{\text{Na}} \). Consistently, the corresponding relationship between \( I_{\text{Na}} \) and \( N_o \) is roughly linear (Fig. 6C) for the cumulated data, which impressively underlines that, for all the investigated tissues, the individual \( i \) (solid line in Fig. 6C equals averaged \( i \) values; see Eq. 3) are of comparable magnitude and remain unchanged during shifts of \( \text{pH}_c \). Thus, in this sort of experiment, the apical \( P_{\text{Na}} \) reflects the \( N_o \), and no attention must be given to \( i \) or blocker kinetics as revealed in \( f \).

Arrest of the basolateral Na⁺/H⁺ exchanger.

BASELATERAL Na⁺ OMISSION. Casavola et al. (6) previously reported for A6 cells the existence of an Na⁺/H⁺ antiport exclusively in the basolateral membrane. Presumably, removal of Na⁺ from the basolateral saline could acidify the cytosol. We tested this by measuring \( \text{pH}_c \) when serosal Na⁺ was replaced by Tris or choline. In Fig. 7 we show an experiment with Tris (also representative for choline), and it can be seen (A) that,
just-established apical to basolateral Na\(^+\) concentration ([Na\(^+\)]) gradient, since, in experiments in which the Na\(^+\)/proton exchanger was, in the presence of basolateral Na\(^+\), stopped with EIPA (see below), this phenomenon could not be seen. On the other hand, control experiments (not shown) in which basolateral Na\(^+\) was omitted in the absence of apical Na\(^+\) still exhibited such an overshoot that excludes Na\(^+\) as origin. Furthermore, because the apical Cl\(^-\) channel blocker (29) NPPB also had no influence on this (not shown), a contribution from Cl\(^-\) secretion (12, 29) is unlikely, and the origin of this phenomenon remains obscure.

Noise analysis (a typical experiment is shown in Fig. 7C, see legend for details) led for the case of serosal Na\(^+\) substitution by Tris to exactly the same finding as for the cell acidification during the late phase of basolateral NH\(_4\)\(^+\) treatment (Fig. 5A); only channel density was reduced by Na\(^+\) removal, whereas \(i\) and CDPC kinetics remained unaffected. Figure 8 summarizes for eight tissues the outcome of this type of experiment. Again, as outlined in Fig. 6 for the effects of apical or basolateral NH\(_4\)\(^+\), a linear relationship was found not only between the relative \(S_0\) and the relative \(I_{Na}\) (Fig. 8A) but also for the dependence of \(I_{Na}\) on \(N_o\) (Fig. 8B).

**NA\(^+\)/H\(^+\) EXCHANGE BLOCKING BY EIPA.** To check our above hypothesis that omission of basolateral Na\(^+\) brings the serosal Na\(^+\)/H\(^+\) exchanger to a halt, we tried to inhibit it directly (6), in the presence of Na\(^+\), with 50 µM EIPA in the serosal bath. In Table 2, we show results from noise analysis of three tissues that were studied under these conditions, before and after at least 15 min treatment with basolateral EIPA. Again, the results fully mirror those obtained above with Na\(^+\) removal in that \(N_o\) was the sole parameter affected by the EIPA-dependent arrest of the exchanger; these results point to an EIPA-induced drop in pH\(_c\).

### Cytosolic pH and Macroscopic \(I_{Na}\) Kinetics

Most tight epithelia display a saturating dependence of Na\(^+\) uptake on [Na\(^+\)]\(_{ap}\). This is also the case with A6 cells, and we could recently (35) elucidate that two

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**Table 1.** Lorentzian parameters, \(i\), and \(N_o\) from the CDPC \(I_{Na}\) noise at the time periods indicated by numbers for the experiment shown in Fig. 4A

<table>
<thead>
<tr>
<th>(f_o) (Hz)</th>
<th>(S_0) (10(^{-9})A/cm(^2))</th>
<th>(i) (pA)</th>
<th>(N_o) (10(^8) channels/cm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>101.9</td>
<td>9.50</td>
<td>0.20</td>
</tr>
<tr>
<td>2</td>
<td>99.9</td>
<td>10.3</td>
<td>0.18</td>
</tr>
<tr>
<td>3</td>
<td>101.3</td>
<td>8.45</td>
<td>0.18</td>
</tr>
<tr>
<td>4</td>
<td>109.2</td>
<td>7.26</td>
<td>0.22</td>
</tr>
<tr>
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<td>110.2</td>
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<td>0.24</td>
</tr>
<tr>
<td>6</td>
<td>109.5</td>
<td>3.13</td>
<td>0.18</td>
</tr>
<tr>
<td>7</td>
<td>108.2</td>
<td>6.30</td>
<td>0.22</td>
</tr>
</tbody>
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\(f_o\), Corner frequency; \(S_0\), Lorentzian plateau; \(i\), single-channel current; \(N_o\), open-channel density; CDPC, 6-chloro-3,5-diaminopyrazine-2-carboxamide; \(I_{sc}\), short-circuit current. The \(i\) was calculated according to Eq. 2. Because \(f_o\) was independent of cell pH (pH\(_i\)), we chose the parameter value (see Eq. 2) \(k_0\) (blocking rate constant) = 6.48 µM\(^{-1}\) s\(^{-1}\) from our previous publication (38).
the fairly Michaelis-Menten-like saturation of \( I_{Na} \) with, however, an apparent "macroscopic" \( K_m \) of \( \sim 5 \) mM. Our present findings from noise analysis suggest \( N_o \) to play the decisive role in determining \( \text{pH}_c \)-regulated \( I_{Na} \). In the DISCUSSION, we assume that this conclusion may be extended to conditions in which no channel blocker is present.

Because both the \( \text{pH}_c \)-independent \( i \) and the \( \text{pH}_c \)-dependent \( i \) are a function (both in hyperbolic but opposite ways) of \([Na^+]_{ap}\), the question arises about the manner in which \( \text{pH}_c \) influences \( N_o \), i.e., by changing the channel density or rather the \( K_m \) of its \([Na^+]_{ap} \) dependence (or both). For instance, for a rise in \( \text{pH}_c \), an increase of \( N_o \) should become visible as an increase in maximal \( I_{Na} \) when plotting current-saturation

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**Figure 6.**

A: power density spectrum of the \( I_{sc} \) noise in the presence of 50 µM apical CDPC. The control spectrum (●) is calculated for position 1, and the lower spectrum (bl + NH₄Cl; ▲) is calculated for position 6 as indicated in Fig. 5A. Fitted and calculated parameters are listed in Table 1. B: ratio (normalized to control) of the CDPC-\( I_{sc} \) noise Lorentzian \( S_o \) at 50 µM CDPC plotted vs. the analogous relative \( I_{Na} \) for different tissues and various treatments. According to theoretical considerations, this function must intercept both abscissa and ordinate at 0; the finding of a linear relationship \( S_o/S_o^{CTR} = (i/i^{CTR} \cdot (I_{Na}/I_{Na}^{CTR})) \) suggests that the relative \( i \) (\( i^{CTR} \)) remains fairly constant over all tissues and \( \text{pH}_c \) conditions. □ Control; ■ NaCl in the presence of apical NH₄Cl (cell alkaline); ○, after removal of apical NH₄Cl; ●, in the presence of basolateral NH₄Cl (cell acidic); △, after removal of basolateral NH₄Cl; ●, removal of basolateral NH₄Cl combined with basolateral Na⁺ replacement by Tris⁺. C: \( I_{Na} \) plotted as a function of \( N_o \) for the same tissues and for the same conditions (cf. symbols) depicted in B. According to theory (cf. MATERIALS AND METHODS), the slope of the resulting linear relationship for the pooled data (solid line) equals the average \( i \) (mean \( i = 0.196 \pm 0.005 \) pA).

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**Figure 7.**

A: effect of basolateral replacement of Na⁺ by Tris⁺ on \( \text{pH}_c \). B: same experiment as in A for another tissue; shown, however, is the time course of \( I_{Na} \). C: time course of \( I_{Na} \) and \( G_t \) when 50 µM CDPC was first added apically followed by basolateral Na⁺ replacement by Tris⁺ (CDPC present), plus 0.1 mM apical amiloride, and basolateral cation reversal (presence of amiloride). Parameters from CDPC noise at times indicated: arrow 1: \( f_c = 93.4 \) Hz, \( S_o = 4.23 \times 10^{-20} \text{A}^2 \cdot \text{s/cm}^2 \), \( i = 0.17 \) µA, \( N_o = 38.17 \times 10^6 \) channels/cm²; arrow 2: \( f_c = 97.9 \) Hz, \( S_o = 0.91 \times 10^{-20} \text{A}^2 \cdot \text{s/cm}^2 \), \( i = 0.16 \) µA, \( N_o = 10.42 \times 10^6 \) channels/cm².
the case of 20 mM basolateral NH$_4^+$ obtained exactly the same result that is exemplified for after basolateral NH$_4^+$ the I

ments in which pH$_c$ was increased by apical NH$_4^+$ the apparent macroscopic coefficient of one, and we did not observe any change of fit a hyperbola (solid lines) to the data using a Hill Tris mixtures, is displayed. As shown in Fig. 9, we could shifting the maximal current level downward. There-

NaCl-saline on INa, i, and No for 3 tissues

Table 2. Effect of 50 µM EIPA in the basolateral NaCl-saline on INa, i, and No for 3 tissues

<table>
<thead>
<tr>
<th>Tissue No.</th>
<th>I$_{Na}$ (µA/cm$^2$)</th>
<th>i (nA)</th>
<th>N$_{Na}$ 10$^6$ channels/cm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>18.2</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>Basolateral + EIPA</td>
<td>8.5</td>
<td>0.15</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>7.9</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Basolateral + EIPA</td>
<td>3.0</td>
<td>0.11</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>3.5</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Basolateral + EIPA</td>
<td>2.7</td>
<td>0.18</td>
</tr>
</tbody>
</table>

EIPA, ethylisopropylamiloride; INa, Na$^+$ current.

Fig. 8. Relationship between the relative $S_0$ magnitudes vs. the relative $I_{Na}^{CTR}$ (A) and linear function $I_{Na}$ vs. N$_{0}$ (B) from experiments in which basolateral Na$^+$ (CTR, ×) was replaced by Tris$^+$ (●). For further details, see Fig. 6, B and C. From the slope in B we obtain a mean pH$_c$-independent i = 0.178 ± 0.003 pA.

kinetics (Fig. 9). An alternative would be a shift of the K$_m$ (K$_{Na}$), the half-maximal [Na$^+$]. For many experiments in which pH$_c$ was increased by apical NH$_4^+$ or decreased by basolateral NH$_4^+$ or Na$^+$ withdrawal, we obtained exactly the same result that is exemplified for the case of 20 mM basolateral NH$_4^+$ in Fig. 9 in which the I$_{Na}$ saturation function, obtained with apical Na$^+$-Tris mixtures, is displayed. As shown in Fig. 9, we could fit a hyperbola (solid lines) to the data using a Hill coefficient of one, and we did not observe any change of the apparent macroscopic K$_m$. The cell acidification after basolateral NH$_4^+$ had only one effect, namely, shifting the maximal current level downward. Therefore, we do not deal with a Na$^+$-competitive but rather with an allosteric block of I$_{Na}$ by cytosolic H$^+$.

DISCUSSION

A6 cells possess the mechanisms for active and cAMP- and Ca$^{2+}$-controlled Na$^+$ reabsorption (36) and also Cl$^-$ secretion (12, 29). The virtual absence of apical water channels (11) renders the cells sensitive to basolateral osmotic disturbances only. There is much evidence (18) that second messengers not only operate on the apical, rate-limiting barrier for ion transport processes but also are able to couple properties of apical and basolateral membranes in concert for optimal transcellular ion movements. A novel coupling agent may be pH$_c$; in A6 and other tight epithelia (18, 19, 21), pH$_c$ regulates apical P$_{Na}$ and basolateral P$_K$ in much the same manner and within a narrow pH$_c$ range, with maximal permeabilities at pH > 7.5 but vanishing permeabilities at pH < 7.1. In the present paper, we explored which apical parameters are responsible for the pH$_c$-dependent modulation of apical Na$^+$ channels.

So far, we may conclude that, whatever means are used to modify pH$_c$, 1) blocker-induced channel fluctuations appear to be unaffected by pH$_c$; 2) with an invariant i, pH$_c$-invariant single-channel conductance and electrochemical apical driving force seem reasonable assumptions; and 3) N$_{0}$ available for interaction with apical CDPC is the only parameter that plays a role in the pH$_c$-regulated Na$^+$ transport in the A6 cell line.

Methodical Aspects

The electrical techniques used here, including the simultaneous recording of T$_c$ have been described and discussed at length previously (11, 35, 38). Especially with respect to the Na$^+$ kinetics, as well as the CDPC noise analysis in A6 cells, using a two-state model, we refer the reader to Smets et al. (35).

To evaluate pH$_c$-dependent parameter shifts when using either hyper- or isosmotic NH$_4$Cl-containing solutions, we used Ringer (70 mM NaCl) in which NH$_4$Cl had been added (hyperosmotic) or replaced isosmotic 40 mM sucrose. Anisotonic addition of NH$_4$Cl is commonly used (6, 10, 21) to evoke pH$_c$ changes. The inherent dangers are clear, and earlier data show that
hypertonic salines as such do already decrease cell volume and \(N_o\) (40). This prompted us to establish conditions of zero volume changes for the use of NH4Cl (Fig. 2).

For unknown reasons, successful cell loading with BCECF-AM could not be achieved sufficiently often; quick washout from the cells after fast dye entrance also occurred frequently. Thus, in many experiments in which individual \(\text{pH}_c\) calibration at the end of an experiment with a given tissue was impossible, calibration had to be performed, as done by others (6, 9), using data from pooled experiments designed for the construction of a calibration curve only (Fig. 1).

Complexities Arising From Attempts to Shift \(\text{pH}_c\) with NH4 Pulses

With extracellular media containing NH4+, cell alkalinization is a direct consequence of ammonia entry; also, acidification of the cytosol follows withdrawal of external NH4+. It was mostly ignored and only rarely (10, 21) appreciated that NH4+ entering cells in a nonnegligible quantity through normally \(\text{K}^+\)-permeable pathways (24, 50), may lead to direct cell acidification due to intracellular hydrolysis of NH4+. Moreover, because NH4+ will then compete with \(\text{K}^+\), it may contribute to otherwise typically \(\text{K}^+\)-dependent phenomena (cation transporter fluxes, membrane polarization, or channel currents), or else, impede \(\text{K}^+\) transporters (42). Therefore, effects additional to those from \(\text{pH}_c\) shifts may be expected, e.g., changes in basolateral membrane \(\text{K}^+\) channel resistance and ensuing hyper- or depolarization of a normally \(\text{K}^+\)-dependent membrane potential difference. In A6 cells at short circuit, a change so achieved in the negative intracellular electrical potential \(V_{sc}\) (see Eq. 4) would immediately affect the net apical driving force for Na+ entry. If, as in other tissues (10, 21), basolateral permeability of \(\text{K}^+\) channels for \(\text{NH}_4^+\) is finite, the addition of \(\text{K}^+\)-mimicking NH4+ to the basolateral side could cause a \(V_{sc}\) depolarization that would impede \(\text{Na}^+\) influx (Eq. 4). Such a mechanism could explain the transient initial blip-like current reduction as seen in Fig. 4B or Fig. 5A, although i, and thus \(V_{sc}\) as part of the driving force, seem unchanged at steady-state conditions (Table 1).

There are also hints for cellular \(\text{pH}\) backregulation after an externally provoked \(\text{pH}_c\) shift. As can be seen, e.g., in Fig. 5A, a more transient \(\text{pH}_c/\text{Na}^+\) rise during apical \(\text{Na}^+\) may be the consequence of the activation of the basolateral Cl−/\(\text{HCO}_3^-\) exchanger that has been established in A6 cells (9). On the other hand, the ubiquitous Na+/H+ antiport that exists basolaterally in A6 cells (6, 9) must mediate realkalinization (Fig. 5, B and C) after \(\text{NH}_4^+\) removal-induced acidification, an effect only observed in the presence of basolateral Na+ and in the absence of EIPA. A block of the Na+/K+ antiporter with EIPA excludes that a putative stop of the basolateral Na+/\(\text{Ca}^{2+}\) antiport (2) with subsequent rise in cell \(\text{Ca}^{2+}\), which has been discussed to inhibit \(P_{\text{Na}}\) (1, 16, 30), is responsible for the \(I_{\text{Na}}\) drop after \(\text{Na}^+\) omission. In contrast, augmented cell calcium, e.g., after hormones that enhance \(\text{Na}^+\) transport such as vasopressin, has recently been shown to have the dominant function in the stimulatory hormone action on \(I_{\text{Na}}\) in A6 cells (22). In fact, Lyall et al. (25) suspected that a number of \(\text{Na}^+\) uptake-activating hormones exert their effects via cell alkalinization.

As it is generally assumed (6) that the \(\text{Na}^+\)/H+ antiport starts to work only after a certain degree of cell acidification, the question of why our methods that putatively stop the exchanger cause an immediate fall in \(\text{pH}_c\) and \(I_{\text{Na}}\) arises. One reason could be that metabolism produces enough protons, and another reason could be that ion channels allow a constant “leak” of protons into the cells (24), so that the exchanger is permanently active. This is the case, e.g., in frog skin (20).

In some reports on A6 cells, the above discussed points and problems arising from the choice of ill-defined experimental conditions [e.g., simple bilateral NH4Cl exposure, including bilateral isosmotic NaCl replacement by NH4Cl to study the Na+/H+ exchanger (6) or \(I_{\text{Na}}\) kinetics (9)] have been ignored. Such studies of \(\text{pH}_c\)-related transport activities are then suited to yield erroneous interpretations, such as claiming a “mixed competition” (6) of intracellular protons with extracellular \(\text{Na}^+\).

Parameters of Apical \(\text{Na}^+\) Transfer

Our data provide strong evidence that the kinetic parameters, i.e., \(K_{\text{Na}}\) with respect to apical \(\text{Na}^+\) (see Fig. 9) and \(f_c\) with respect to CDPC block (Figs. 5 and 7), are unaffected by maneuvers that change \(\text{pH}_c\). With a \(\text{pH}_c\)-independent \(i\), both single-channel conductance and the net apical driving force (see Eq. 4) are virtually \(\text{pH}_c\)-independent: 1) at Ringer-[Na+]ap, the apical Nernst potential for \(\text{Na}^+\) is stable as the rate of the basolateral \(\text{Na}^+-\text{K}^+\)-ATPase is not a function of \(\text{pH}_c\) at least not above \(\text{pH} 6.9\) (13); and 2) the practically indistinguishable \(\text{pH}_c\)-titration curves of apical \(P_{\text{Na}}\) and basolateral \(P_{\text{K}}\) (18, 19) ensure that \(\text{pH}_c\) changes both permeabilities always by the same factor, therefore yielding constant fractional membrane resistances and constant \(V_{sc}\).

With respect to “spontaneous” open-closed conformational changes and our inference that \(N_o\) is \(\text{pH}_c\)-dependent whether CDPC is present or not, the “inherent” (when blocker is absent) ENaC open probability (\(P_o\)) could be subject to \(\text{pH}_c\), which would result in a change in \(N_{o'}\), being the product of \(P_o\) and the total number (\(N_{o'}\)) of \(\text{Na}^+\) channels (open plus closed). Indeed, a most recent report (7) demonstrated for the \(\alpha\)-subunit of ENaC, expressed in Xenopus oocytes or reconstituted in planar lipid bilayers, that cytosolic-side acidification reduced \(P_o\) (approaching 0 at \(\text{pH} < 7\)) and mean open time while increasing the mean closed time, with unaffected single-channel conductance. In addition, \(N_T\) could vary if a fraction of channels, as a consequence of acidification, disappeared, either by becoming permanently closed or by endocytotic removal. Indeed, a drop in \(\text{pH}_c\) is known to result in apical exocytosis of \(\text{H}^+\).
pumps in some tight epithelia (4, 17). Moreover, influences of pHc on vesicle traffic have been described (14), and the role of exocytotic events underlying the stimulatory action of several hormones on Na+-transport in tight epithelia is heavily discussed (16) as is the INa stimulation by cell volume increase, which can be prevented by interaction with cytoskeleton-directed drugs (28). It has been reported that cytoskeletal elements, such as small actin filaments, induce Na+-channel activity in A6 cells (5). We are presently unable to decide whether pHc affects only P0 or also NT.

Modeling of the pHc Dependence of PNa

Figure 9 suggests that we deal with an allosteric site where the interaction of internal protons should be noncompetitive with external Na+. This may also explain, even when pHc changes, the constancy of the parameter i (and thus single-channel conductance; see Eq. 4), which is under dominant influence of external Na+. With respect to the findings on the pHc sensitivity of patch-clamped ENaC-type epithelial Na+-channels (18, 19), reversible vesicle fusion may seem less likely than reversible allosteric opening-closing (by (de)protonation) of permanently resident apical Na+-channels, e.g., by affecting P0. At present, most recent publications about presumable structures of the ENaC (26, 33) do not yet provide conclusive hints for a tentative identification of the titrated allosteric intracellular site(s). However, according to the published pHc–titration curve of the A6 cells (18, 19) as well as for the cloned (7) Na+-channel, the apparent pK1 range (7.2–7.5) may point to a histidine as titrated group. For instance, His-94 in the α1-ENaC has been discussed by Chalfant et al. (7) to be a proton target during intracellular titration.

Summary and Perspectives

With respect to our data, we arrive at the following conclusions. 1) The apical A6 cell membrane permits little, if any, apical entry of NH4+ but rather NH3, which causes a cytosolic alkalization (sometimes followed by pHc “backregulation”). The concomitant rise in Gt and INa is exclusively due to a rise of Nw probably caused by allosteric opening of apical Na+-channels. All other parameters of apical Na+-transfer remain unaffected. 2) Basolateral NH4+ first increases pHc (and therefore Gt and INa) due to effects identical to those discussed for apical NH4+-exposure. Secondarily, however, NH4+ as pointed out also by other groups for other tissues (10, 21), enters the cells. This occurs probably (21, 23, 41) via otherwise K+-permeable conductive pathways, since we see an immediate rise in Gt in parallel to a drop in INa. This can easily be understood if, initially, NH4+ as imitator of K+, depolarizes the cell negative short-circuit potential that reduces the net driving force for apical Na+ entry. Subsequent H+ release from entered NH4+ ions would decrease apical PNa, which is possibly due to a shortened open time and a prolonged closure (Chalfant et al. (7)), thus leading to a decrease in time-averaged N0. Subsequent NH4+ withdrawal will tend to further acidify the cell, and, depending on the activity of basolateral Na+/H+ exchange, pHc will recover.

A variety of messenger roles for intracellular protons have been proposed to date, including the concerted cross-talk modulation of apical Na+ and basolateral K+-permeabilities in tight, Na+-transporting epithelia. Another possible consequence of a rise in cell H+ concentration could be a liberation of cell Ca2+ from storage proteins or vesicles, so that Ca2+ would be the final messenger (30), although cell Ca2+ has recently been shown to be stimulatory rather than inhibitory (20).

Alkali ion channels deliver protons to the cytosol (25) that will pile up in the cell when energy supply is short as in the condition of ischemia or anoxia, and glycolysis and ATP splitting without regeneration will add even more to cell acidification which, in the end, would tend to deregulate cell life. This adverse, positive-feedback reaction chain may, however, be brought to a halt if the influx pathway for protons, the alkali ion channels, is closed down in a negative-feedback loop by cytosolic protons so that the cells would neither lose K+ basolaterally nor gain Na+ apically and, consequently, Cl- and water. That may prevent cell volume increase and rupture. Such mechanisms may, at least in part, account for the observed protective effects of internal H+ when they are derived from the “therapeutic” acidification of the extracellular bath. It is most interesting that EPA and similar drugs that stop the Na+/H+ exchange have been shown to be protective in conditions of cardiac ischemia (3, 34).

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