Na\(^+\)/H\(^+\) exchangers in the human eccrine sweat duct

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Granger, D., M. Marsolais, J. Burry, and R. Laprade. Na\(^+\)/H\(^+\) exchangers in the human eccrine sweat duct. Am J Physiol Cell Physiol 285: C1047–C1058, 2003. First published July 2, 2003; 10.1152/ajpcell.00581.2002.—Using an anti-NHE1 antibody, we demonstrate the presence of a Na\(^+\)/H\(^+\) exchanger of isoform 1 (NHE1) in the human eccrine sweat duct. A strong staining was observed at the basolateral membrane of the outer cell layer (NHE1\(_{\text{basal}}\)), at the junction between inner and outer cells layers (NHE1\(_{\text{inter}}\)), and along the lateral membranes (NHE1\(_{\text{lateral}}\)) of all cells of the duct. At the luminal membrane, no staining was demonstrated either for NHE1 or NHE3. To investigate Na\(^+\)/H\(^+\) mediated proton transport, straight sweat duct portions were isolated and perfused in vitro under HCO\(_3\)\(^-\)-free conditions. In the presence of basolateral 5-ethyl-N-isopropyl amiloride (EIPA), an acidification of 0.29 ± 0.03 pH units was observed, whereas no effect was observed with luminal EIPA. Bath sodium removal generated a stronger acidification (0.41 ± 0.09 pH units). Removal of luminal sodium (in the absence or presence of basolateral EIPA), or low luminal chloride, led to an alkalization, presumably due to a decrease in intracellular sodium, strongly suggesting functional activity of NHE1\(_{\text{inter}}\). We therefore conclude that in the sweat duct, NHE1 plays a major role in intracellular pH regulation.

5-ethyl-N-isopropyl amiloride; chloride; intracellular pH measurements; immunolocalization; epithelial cells

The human eccrine sweat gland is a tubular structure consisting of two morphologically and functionally distinctive regions: 1) a deep subdermal-coiled secretory portion and 2) a ductal portion that passes through the dermis and epidermis of the skin. In contrast to the secretory coil, which is a single epithelial cell layer of three principal cell types, the sweat duct presents two cell layers: an inner or luminal cell layer surrounded by an outer or peripheral cell layer. Gap junctions and desmosomes have been demonstrated among the interdigitations between inner and outer cells by electron microscopy (13). It was also demonstrated that extensive dye and electrical coupling exists between the inner and outer cells in the isolated sweat duct, suggesting intracellular communications and a syncytial relationship between these two cell layers.

Functionally, the secretory portion of the eccrine sweat gland generates a fluid called the primary secretion. The composition of this isotonic fluid is similar to an ultrafiltrate of plasma with a pH of 7.4. However, as it flows through the ductal portion of the gland, it is modified by the reabsorption of solutes such as NaCl, lactate, and bicarbonate, leaving a hypotonic sweat (9, 37). The pH of final sweat is a function of the sweat rate. When the sweat rate is low, the pH can reach values as acidic as 5, but it increases with increasing sweat rate (9). Intracellular voltage recordings have been used to study electrolyte transport in the isolated microperfused human eccrine sweat duct (38, 41–44). The apical membrane of the sweat duct possesses high sodium (epithelial Na\(^+\) channel; ENaC) and chloride (cystic fibrosis transport regulator; CFTR) conductances that play a role in the reabsorption of NaCl (10, 41, 45, 46). At the basal membrane, a CFTR channel (43), a Na\(^+\)/K\(^+\)ATPase (39), and a K\(^+\) channel have also been identified (44).

Because the sweat duct has the ability to acidify sweat, proton-excreting mechanisms must exist at the luminal membrane. Recently, a vacuolar-type H\(^+\)ATPase has been demonstrated at this membrane by immunolocalization and intracellular pH (pHi) measurements (11, 18). Interestingly, at the basolateral membrane, bicarbonate-independent, sodium-coupled acid-base transport has been reported (3), therefore suggesting the possible involvement of a Na\(^+\)/H\(^+\) exchanger. Indeed, the reabsorptive role of the sweat duct suggests a parallel with other transporting epithelia. More precisely, the isoform 1 of Na\(^+\)/H\(^+\) exchanger (NHE1) has been found at the basolateral membrane of most epithelial cells, such as those of the intestine (54) and the kidney (7, 26), and is involved in the regulation of pHi and cellular volume. At the apical membrane of the intestine (25), proximal tubule of kidney (24), and excretory duct of the mandibular salivary gland (15), the Na\(^+\)/H\(^+\) exchanger is rather involved in the transepithelial reabsorption of NaCl and proton secretion. Recently, the presence of NHE3 has been demonstrated at the apical membrane of rat epididymis (2, 36), where the luminal acidic environment is primordial for sperm maturation. Na\(^+\)/H\(^+\) exchangers are generally classified pharmacologically according to their sensitivity to amiloride and its various analogs. Apical membrane Na\(^+\)/H\(^+\) exchangers (principally NHE2 and NHE3) are more resistant in general to amiloride and its derivatives than the basolateral isoform (NHE1) (19, 33).

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The purpose of this study was therefore to investigate the presence, distribution (apical and/or basolateral), isoform type, and functional activity of Na+/H+ exchangers in the human eccrine sweat duct. To achieve these goals, we have used immunofluorescence and a combination of microperfusion and pH measurements.

**MATERIALS AND METHODS**

**Immunocytochemistry**

**Tissue fixation.** Specimens of human healthy skin (female) were taken after esthetic surgery, with consent from the patients and ethical committee approval. Skin was conserved at 4°C in Dulbecco's phosphate-buffered saline containing CaCl₂ and MgCl₂ from Sigma Chemical (St. Louis, MO) until used. Sections of human skin were fixed for 1 h in 2% paraformaldehyde, 75 mM lysine, and 10 mM sodium periodate (PLP) at room temperature, and slices of 3 mm were cut with scissors. All sections were fixed overnight at 4°C in fresh PLP. The tissue was moved in phosphate buffer containing 1.9 mM NaH₂PO₄, 8.1 mM Na₂HPO₄, 0.9% NaCl, and 0.02% NaN₃, pH 7.4 (PBS), until the cryostat step. Rats were killed by exsanguination via the aorta. Blood was washed out of the vasculature with 20 ml of a solution of 0.5% NaNO₂, 10 U/ml heparin in 0.5% NaN₃, pH 7.4 (PBS), followed by 20 ml of PLP. The tissue was moved in phosphate buffer containing 0.5% NaN₃, pH 7.4 (PBS), until the cryostat step. Rats were sublethally anesthetized with 0.5 ml of Somnotol (MTC Pharmaceuticals, Cambridge, ON, Canada) and then cannulated via the aorta. Blood was washed out of the vasculature with 20 ml of a solution of 0.5% NaN₃, 10 U/ml heparin in 0.5% PBS, followed by 20 ml of PLP. The fixed kidney was removed and cut in sections of 3 mm before being fixed as described above.

**Cryostat sectioning.** Skin and kidney sections were cryoprotected in PBS-30% sucrose for 12 h before the cryostat step. Once placed onto slides, the tissue was covered with a drop of Tissue-Tek (Sakura, Torrance, CA) embedding medium on a cryostat chuck before being frozen in liquid nitrogen. Sections were cut at a thickness of 5 μm on a cryostat, using disposable knives at a chamber temperature of −25°C. Sections were picked up onto Fisher Superfrost Plus charged glass slides and stored at −20°C until immunofluorescence staining.

**Cell culture.** The Caco-2 cell line (ATCC) originally derived from colonic adenocarcinoma was used after 36 and 39 passages. The cells were grown in DMEM supplemented with 1% nonessential amino acids, 2 mM glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum in a 5% CO₂-humidified incubator at 37°C.

**Antibodies.** Two primary antibodies were tested against Na⁺/H⁺ exchangers: anti-NHE1 and anti-NHE3 antibodies. The NHE1 antibody used is a rabbit antiserum, named N1P1, raised against the last 157 amino acids of the cytoplasmic domain of human NHE1 (49) purified by affinity. The anti-NHE3 used was a commercial monoclonal antibody produced against the COOH-terminal 131 amino acids of rabbit NHE3 (Chemicon International, Temecula, CA) (8). Controls, including omission of primary antibodies, and preimmune serum N1P1 were conducted. NHE1 antibody was used at 1/100 of the supplied concentration, whereas NHE3 antibody was used at 0.01 and 0.1 μg/ml. Secondary antibodies, a goat anti-rabbit IgG-FITC for NHE1 and a goat anti-mouse IgG-FITC for NHE3 (ImmunoResearch, West Grove, PA) were used at 7.5 μg/ml.

**Incubation procedure for sweat duct and kidney.** The incubation procedures were based on the protocol described by Brown and coworkers (14). The standard procedure and also the antigen unmasking procedure in which an incubation with 1% SDS was performed were done (14). The incubation of sections with antibodies was carried out overnight at 4°C for the primary antibody and for 2 h at room temperature for the secondary antibody.

**Photography.** Tissues were viewed at ×200 or ×400 magnification with a Nikon Fluor 40/1.3 objective in a Nikon epifluorescence microscope with fluorescein filters (450- to 490-nm excitation, 510-nm barrier). Sections were photographed in black and white with a Nikon FX-35DX camera on Kodak TMax 400 film at 1600 ASA.

**Localization of NHE1 and NHE3 on Caco-2 cells.** Labeling of NHE1 and NHE3 was performed as described in detail previously (32). Briefly, cells were grown on coverslips at confluency for 17 days. After being washed with PBS, cells were fixed as described above. Cells were then left for 10 min at room temperature. Fixative solution was removed, and cells were rinsed twice with PBS. PBS containing 50 mM NH₄Cl (Fluka, Ronkonkoma, NY) and 0.2% gelatin (Sigma Chemical) for 5 min at room temperature. Monolayers were then incubated with anti-NHE3 monoclonal antibody or anti-NHE1 antibody (N1P1) in buffer PBS-BSA-gelatin for 1 h at room temperature, followed by washing with PBS. Cells were then washed five times with PBS and incubated for 60 min at room temperature with FITC-conjugated secondary antibody. Finally, cells were washed five times with PBS, mounted with polyvinyl alcohol (Gel Vatol), and examined under epifluorescent illumination with excitation-emission filters for fluorescein. Image were visualized using a Nikon microscope and photographed with digital camera Nikon Coolpix995.

**Measurements of pH.**

**Isolation and microperfusion of sweat duct.** Eccrine sweat glands were isolated using a previously described shearing technique (28). Portions of reabsorptive ducts were dissected using sharpened forceps and set up in vitro between glass pipettes on a conventional microperfusion apparatus on an inverted microscope (Olympus). Bath solution temperature was kept at 37°C, and the flow of the bath solution (flow rate > 1 ml/min) was aimed directly at the sweat duct as described by Macri and collaborators (29). The luminal perfusion rate was high enough to eliminate axial changes in the luminal fluid composition.

**pH.** pH was measured in perfused sweat ducts as previously described (4) using the fluorescent probe 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). The ace- toxymethyl ester form of the dye (BCECF-AM) was added to the bath solution at a final concentration of 5 μM, and loading was allowed in the nonperfused bath for 5–10 min at 25°C. After loading, the bath was perfused with control solution for at least 5 min before an experiment was begun. The duct was alternately excited at 450 and 500 nm, and the emitted fluorescence was monitored at 530 nm with a photomultiplier-based spectrofluorimeter (PTI D104; Photon Technology International, London, ON, Canada) linked to a computer. The fluorescence ratio (F₅₃₀/F₄₅₀) corrected for autofluorescence was calculated and converted to pH₅ at the end of each experiment by calibration with the high-K⁺ nigericin method (52).

**Solutions and chemicals products.** Sodium nitrate was purchased from Anachemica Chemicals (Montreal, Canada).
and heparin from Leo Laboratories (Ajax, Canada). BDH Laboratories (Poole, England) supplied L-lysine monohydrochloride. The HCO3⁻/H11002⁻-free control solution used to perfuse and bathe the sweat ducts contained (in mM) 114 NaCl, 25 sodium gluconate, 2.5 K2HPO4, 1 MgCl2, 1 CaCl2, 5 glucose, and 4 sodium lactate. Osmolality was adjusted to 300 mosmol/kgH2O with mannitol, and pH was adjusted to 7.4 with Tris-HEPES. For experiments designed to investigate the effects of low luminal chloride on pHi, NaCl was replaced with equimolar sodium gluconate. For experiments designed to investigate the effects of zero luminal or peritubular sodium on pHi, sodium lactate and NaCl were replaced with N-methyl-D-glucamine (NMDG)-lactate and -chloride, respectively.

BCECF-AM was obtained from Molecular Probes (Eugene, OR) and stored in a freezer as a 1 mM stock in ethanol 95%. 5-Ethyl-N-isopropyl-amiloride (EIPA) was obtained from Research Biochemicals International (Natick, MA), stored at 4°C as a 100 mM solution in DMSO, and used at a final concentration of 10 or 50 μM. The final concentration of DMSO in any solution did not exceed 0.1% and had no effect on pHi. All other chemicals products were purchased from Sigma Chemical.

Statistics. Results are presented as means ± SE. Statistical significance was analyzed using the paired or unpaired Student’s t-test. Significance was accepted at P ≤ 0.05.

RESULTS

Distribution of Na⁺/H⁺ Exchangers in the Sweat Duct

Immunostaining of NHE1. To determine the cellular localization of NHE1 in the human eccrine sweat gland, we used the anti-NHE1 antibody (27, 49), on cryosections of PLP-fixed human skin. Sweat duct cells showed a strong staining at the basal membrane (NHE1basal) of the cells of the outer layer, at the junction between the inner and outer cell layers (NHE1inter), as well as all along the lateral junction between adjacent cells from both the inner and outer layers (NHE1later) (Fig. 1A and C). However, staining was never detectable at the luminal membrane, even with the 1% SDS epitope-unmasking technique (14). The same pattern of staining (at all membranes except luminal membrane) was also obtained in the secretory portion of the gland (Fig. 1D). Control incubation using preimmune rabbit serum gave no detectable staining.

Immunostaining of NHE3. Previous immunochemical studies have shown that NHE3 is an apical Na⁺/H⁺ exchanger in many epithelial cells (1, 2, 6, 47). To

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Fig. 1. Immunofluorescence staining of the eccrine sweat gland, using a purified anti-NHE1 antibody (N1P1) raised against the last 157 amino acids of the cytoplasmic domain of human Na⁺/H⁺ exchanger isoform 1 (NHE1) (27, 49), on 5-μm cryosections of 2% paraformaldehyde, 75 mM lysine, and 10 mM sodium periodate (PLP)-fixed human skin. A and C: NHE1 immunolocalization on sweat ducts portions. B: phase-contrast microscopy of image in A. The Na⁺/H⁺ exchangers at the basolateral membrane (NHE1basal), at the membrane junction between 2 cell layers (NHE1inter), and at the lateral membrane of cells (NHE1later) are indicated (C). D: NHE1 immunolocalization on the secretory coil portion. Bars, 16 μm.
investigate for its presence in the sweat duct, we tested
an anti-NHE3 monoclonal antibody directed against
the rabbit NHE3 (8) (Fig. 2A). No staining was seen
with or without the 1% SDS epitope-unmasking techni-
que (14). As a positive control, we tested this anti-
body on cryosections of PLP-fixed rat kidney. The latter
has been shown to possess a large amount of NHE3 at
the brush border membranes of proximal tubules (1).
As expected, the apical plasma membrane of the proxi-
mal tubule showed a strong staining (Fig. 2C). To
confirm that our anti-NHE3 antibody is able to recog-
nize human NHE3, we tested it on Caco-2, an intesti-
nal human cell line. These cells are known to possess
an apical NHE3 and show standard and flower pat-
terns of staining (22, 32). An identical staining pattern
was obtained, demonstrating that our NHE3-antibody
is indeed able to recognize human NHE3 (Fig. 2E).
This staining can be compared with a typical basolat-
eral staining observed for NHE1 on these same cells
(Fig. 2F) (22, 32). Both positive controls, combined with
the absence of staining for NHE3 on sweat duct,
strongly suggest that this isoform is not present on the
sweat duct.

Functional Na⁺/H⁺ Exchange Activity

In the following series of experiments, we measured
pH changes in the isolated and perfused eccrine sweat
duct in response to EIPA and sodium concentration
changes. To simplify the interpretation of the results,
HCO₃⁻/CO₂-free solutions (bath and lumen) were used
to minimize the influence of bicarbonate-dependent
transport systems on pH. Under these conditions with
control solution, we measured a baseline pH of 7.33 ±
0.03, n = 41 (Table 1).

Effect of EIPA. To test for the presence of Na⁺/H⁺-
mediated proton transport activity at the luminal and
basal membranes, we measured pH changes in re-
sponse to lumen and bath EIPA, a highly selective
Na⁺/H⁺ exchanger inhibitor (34). A representative
trace showing the effect of bath and luminal EIPA (10
μM) on pH, is presented in Fig. 3A. Individual results
together with means ± SEs are presented in Fig. 3B.

Fig. 2. Immunofluorescence staining of NHE3. The an-
ti-NHE3 antibody used was a monoclonal antibody pro-
duced against the COOH-terminal 131 amino acids of
rabbit NHE3 (8). A: immunofluorescence light micros-
copy on 1% SDS-treated 5-μm cryosections of PLP-fixed
human skin. B: phase-contrast image corresponding
with A (antibody concentration, 0.1 μg/ml; time expo-
sure, 80 and 2 s, respectively; bars, 30 μm). C: positive
control for immunolocalization of NHE3 at the apical
membrane of the proximal tubule of rat kidney. D:
phase-contrast image corresponding with C (antibody
concentration, 0.01 μg/ml; time exposure, 12 and 2 s,
respectively; bars, 50 μm). E: positive human control
for immunolocalization of NHE3 at the apical mem-
brane of Caco-2 cells (antibody concentration, 0.1 μg/
ml; time exposure, 1 s; bar, 20 μm). F: immunolocaliza-
tion of NHE1 at the basolateral membrane on the same
cells in E (antibody dilution, 1:100; time exposure, 1 s;
bar, 20 μm).
Bath EIPA led to a decrease in pH from pH 7.30 ± 0.04 to 7.01 ± 0.03 (n = 36), with a recovery to 7.23 ± 0.09 (n = 5). In another series of experiments, bath EIPA was increased to 50 μM after pH had reached a stable value following an initial dose of 10 μM. In these experiments (n = 5), no additional acidification was observed (results not shown).

In contrast, luminal EIPA had no effect on pH at either 10 or 50 μM (results not shown). To further investigate for the presence of any Na/H activity at the apical membrane of the inner cells, we conducted an additional series of experiments. In these, we measured pH recovery after an acid load with bath EIPA, in the absence or presence of luminal EIPA (50 μM) (Fig. 4). We have previously shown that this pH recovery is largely due to an apical proton pump, which, in the absence of sodium, is nearly completely abolished by luminal concanamycin A (18). As shown in Fig. 4, no significant difference was observed between the slope in the presence or absence of luminal EIPA (0.09 ± 0.02 vs. 0.08 ± 0.03 pH units/min, respectively, n = 4). These results clearly indicate the absence of any significant Na/H transport activity at the apical membrane of inner cells, in agreement with the immunolocalization results.

Effect of bath sodium removal. As expected from the reversal of the basolateral exchanger, bath sodium removal produced a strong and reversible decrease in pH. A representative trace is presented in Fig. 5A. Individual results and means ± SEs are presented in Fig. 5B, where pH is seen to decrease from pH 7.19 ± 0.07 to 6.78 ± 0.09, with a recovery to 7.15 ± 0.06 (n = 9). The acidification generated by this treatment is stronger than that produced by bath EIPA (0.41 ± 0.09 vs. 0.29 ± 0.03 pH units).

We conducted an additional series of experiments in which bath sodium removal followed a period of bath

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**Table 1. pH measurements on isolated human eccrine sweat ducts perfused in vitro**

<table>
<thead>
<tr>
<th>Experimental Maneuver</th>
<th>Change in pH</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>EIPA (B)</td>
<td>-0.29 ± 0.03</td>
<td>36</td>
</tr>
<tr>
<td>EIPA (L)</td>
<td>No change</td>
<td>13</td>
</tr>
<tr>
<td>0 Na⁺ (B)</td>
<td>-0.41 ± 0.15</td>
<td>9</td>
</tr>
<tr>
<td>0 Na⁺ (L)</td>
<td>+0.27 ± 0.04</td>
<td>23</td>
</tr>
<tr>
<td>Low Cl⁻ (L)</td>
<td>+0.20 ± 0.02</td>
<td>16</td>
</tr>
<tr>
<td>EIPA (B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+0 Na⁺ (L)</td>
<td>+0.25 ± 0.09</td>
<td>4</td>
</tr>
<tr>
<td>+Low Cl⁻ (L)</td>
<td>+0.18 ± 0.05</td>
<td>5</td>
</tr>
<tr>
<td>+0 Na⁺ (B)</td>
<td>-0.15 ± 0.05</td>
<td>9</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of experiments. EIPA, 5-ethyl-N-isopropyl amiloride; pH, baseline intracellular pH; (L), luminal; (B), basolateral. Control pH, was 7.33 ± 0.03 (n = 41). During the removal of luminal sodium, NaCl and sodium lactate were replaced with equimolar N-methyl-D-glucamine (NMDG)-chloride and NMDG-lactate, respectively. During the low luminal chloride period, NaCl was replaced with equimolar sodium gluconate for a final chloride concentration of 4 mM. All solutions used for pH; measurements were bicarbonate free.
incubation with 50 μM EIPA. As shown in Fig. 6, after the acidification that was observed in Fig. 5 following EIPA addition, an additional acidification was observed, indicating an incomplete blockade of basolateral Na⁺/H⁺ exchange. Individual results and means ± SEs are presented in Fig. 6B. Bath EIPA led to an initial acidification of 0.39 ± 0.05 pH units, n = 9 (from a pHi of 7.27 ± 0.05 to 6.88 ± 0.05), followed by an additional acidification of 0.15 ± 0.05 pH units, n = 9 (to 6.73 ± 0.06) due to bath sodium removal (Table 1), with a recovery of 0.11 pH units, n = 7 (to 6.81 ± 0.05).

Effect of luminal sodium removal. Considering that the inner and outer cells are coupled and reasoning that NHE1basal, NHE1inter, and NHE1later should respond to changes in intracellular sodium, sodium was removed from the luminal solutions. As expected, removal of luminal sodium, which should decrease intracellular sodium, through the apical sodium channels led to a reversible alkalinization (Fig. 7A and Table 1). Figure 7B shows individual results and means ± SE for this alkalinization from pH 7.13 ± 0.04 to 7.40 ± 0.04 (n = 23), with a recovery to 7.11 ± 0.05 (n = 13), for a mean alkalinization of 0.27 ± 0.04 (n = 23) (Table 1). However, in some ducts, no response was observed after removal of luminal sodium. At the present time, the most probable explanation for this different behavior would be an axial heterogeneity through the ductal portion of the sweat gland (18). We then repeated the same experiment, but in the presence of bath EIPA. Figure 8A shows a typical trace for this experiment. As in previous experiments, 10 μM bath EIPA produced an acidification (Fig. 3 and Table 1). Surprisingly, a quite comparable alkalinization was observed upon removal of luminal sodium, indicating that under these conditions, blockade of basolateral Na⁺/H⁺ exchange is even less efficient than for bath sodium removal. Individual results and means ± SEs are presented in Fig. 8B; pHi increased from 7.06 ± 0.05 to 7.31 ± 0.07 with a recovery to 7.04 ± 0.09, for a mean alkalinization of 0.25 ± 0.09 (n = 4) compared with 0.27 ± 0.04 in absence of EIPA (Fig. 7 and Table 1).

Effect of low luminal chloride. From the above results and considering that sodium and chloride transport is electrically coupled at the luminal membrane of Fig. 6. Effect of 50 μM bath EIPA and removal of extracellular sodium on pHi of perfused sweat ducts. A representative trace (A) and individual results as well as means ± SEs (B) are presented. During the removal of bath sodium, NaCl was replaced with equimolar N-methyl-D-glucamine (NMDG)-chloride and NMDG-lactate, respectively. C1, control before sodium removal; 0Na (B), zero bath sodium; C2, control after sodium removal.

Fig. 5. Effect of bath sodium removal. A representative trace (A) and individual results as well as means ± SEs (B) are presented. During the period indicated by horizontal bar in A, NaCl and sodium lactate were replaced with equimolar N-methyl-D-glucamine (NMDG)-chloride and NMDG-lactate, respectively. C1, control before sodium removal; 0Na (B), zero bath sodium; C2, control after sodium removal.
the inner cells (46), we attempted to decrease intracellular sodium through a reduction in extracellular chloride. As expected, decreasing the luminal chloride concentration from 118 to 4 mM led to an alkalinization similar to that obtained with luminal sodium removal (Fig. 9A). Individual results and means ± SEs are shown in Fig. 9B. The mean alkalinization was $0.20 \pm 0.02$ pH units ($n = 16$) (Table 1) from a pH$_i$ of 7.18 ± 0.03 to 7.39 ± 0.04, with recovery to 7.18 ± 0.04. Also, similar to zero luminal sodium experiments, a few ducts did not show alkalinization after reduction of luminal chloride concentration.

As for sodium removal, we repeated the same protocol in the presence of bath EIPA. Under these conditions and similar to the sodium removal experiments, an alkalinization of comparable amplitude was observed after the decrease in luminal chloride concentration (Fig. 10A and Table 1). Individual results and means ± SEs are presented in Fig. 10B. The mean alkalinization was $0.18 \pm 0.05$ pH units ($n = 5$) compared with $0.20 \pm 0.02$ in the absence of EIPA (Table 1) from a pH$_i$ of 7.05 ± 0.06 to 7.23 ± 0.09, with a recovery to 7.04 ± 0.07.

The major aim of this study was to investigate the distribution, the isofrom type of the Na$^+/H^+$ exchangers, and their possible role in pH regulation and proton transport in human eccrine sweat duct. We used immunolocalization to localize the Na$^+/H^+$ exchangers and microperfusion, which allows rapid and independent luminal and bath fluid exchange, together with pH$_i$ measurements, to study their functional activity.

**Basolateral and Apical Membranes of the Sweat Duct**

The localization of a Na$^+/H^+$ exchanger at the basal and lateral membranes of the outer cells of the duct by immunolocalization (Fig. 1, A and C) confirms a previous report showing basolateral bicarbonate-independent, sodium-coupled acid-base transport in the sweat duct (3). The NHE1 isoform that we have identified in the present study, often referred to as the ubiquitous or "housekeeping" form, also corresponds to the isoform found at the basolateral membrane of most epithelial cells. Marked inhibition of the NHE1 exchanger by EIPA was maximal at 10 μM, because it could not be
increased further by an additional 40 μM, which is compatible with the published values of $K_{0.5}$ for NHE1 exchangers at low (0.02 μM) (12, 33) as well as high (0.06–0.6 μM) sodium concentration (27, 35). Nevertheless, sodium removal after bath EIPA led to a further decrease in pH of 0.15 pH unit (Fig. 6). This suggests incomplete blockade of the NHE1 exchangers despite EIPA concentrations well beyond the $K_{0.5}$ values. We cannot exclude the presence of a putative EIPA-resistant isoform such as NHE4 (16). However, it also should have been largely inhibited at 50 μM EIPA. Therefore, it would appear that EIPA can produce only a partial inhibition of basolateral Na+/H+ activity. This interpretation would be compatible with the fact that bath sodium removal led to a more important acidification (0.41; Fig. 5) compared with bath EIPA (0.29; Fig. 3). The incomplete blockade of the NHE1 exchanger might be due to the fact that the full EIPA concentration is unable to reach the intercellular regions most remote from the basal side. Such a possibility is suggested by the observation that ouabain binding to the sweat duct Na+/K+-ATPase, whose localization is very similar to that seen for NHE1 in the present study, takes 2 h to reach steady state (39). A likely explanation could be the convective flux ($J_c$), due to reabsorption that would oppose the diffusion flux of the inhibitor ($J_d$), in the very narrow and tortuous intercellular spaces between cells and cell layers, thus preventing the full EIPA concentration from reaching the deepest regions of the epithelial intercellular spaces. Indeed, given an in vitro reabsorption rate of the order of 1 nl·min⁻¹·mm⁻¹ (30) and reasonable values for the fraction of the area of epithelium occupied by the intercellular spaces (5, 48), one obtains comparable values for convective and diffusion fluxes,¹ therefore predict-

1 An estimate of the relative importance of convective vs. diffusive fluxes is given by $J_c/J_d = h/V/D$ (51), where $h$ is the effective length of the intercellular spaces, $V$ is the velocity of the reabsorbate in the intercellular spaces, and $D$ is the diffusion coefficient of the inhibitor in these spaces. Given reabsorption rates of $-1$ nl·min⁻¹·mm⁻¹ for the sweat duct (30) and an external diameter of 40 μm (50), and estimating from the kidney the area of intercellular space per cm² of duct surface to be 0.004 (5, 48), we obtain $3.33 \times 10^{-3}$ cm/s for $V$. Next, with a value for epithelium thickness of 15 μm (50), a tortuosity factor $\alpha$ for the intercellular spaces, and an estimate of $10^{-5}$ cm²/s for $D$, the EIPA diffusion coefficient, we obtain $J_c/J_d = 0.5\alpha$. The intercellular spaces of the sweat duct are very tortuous (13, 20), so $\alpha$ and $J_c/J_d$ must be much larger than 1.

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**Fig. 9.** Effect of low luminal chloride on pH of perfused sweat ducts. A representative trace (A) and individual results as well as means ± SEs (B) are presented. During the period indicated by horizontal bar in A, NaCl was replaced with equimolar sodium gluconate. C1, control before chloride reduction; Low Cl (L), 4 mM luminal chloride; C2, control after chloride return to normal.

**Fig. 10.** Effect of low luminal chloride in the presence of bath EIPA (10 μM) on pH of perfused sweat ducts. A representative trace (A) and individual results as well as means ± SEs (B) are presented. During the period indicated by shaded horizontal bar in A, NaCl was replaced with equimolar sodium gluconate. C1, control before chloride reduction; Low Cl (L), 4 mM luminal chloride; C2, control after chloride return to normal.

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ing a concentration gradient of EIPA in these spaces. From this, it can be argued that the convective flux is very likely to reduce substantially the EIPA concentration in the intercellular spaces most remote from the basal side and therefore prevent full blockade of the exchangers in these areas. As a consequence, the observation of only a partial blockade of the NHE1 exchanger by bath EIPA would suggest that some of the NHE1\textsubscript{inter} and NHE1\textsubscript{later} exchangers are functional.

Despite the absence of NHE1 at the apical membrane, we may have expected to find an Na\textsuperscript{+}/H\textsuperscript{+} exchanger at this membrane because epithelial cells, like those of proximal tubule, intestine, epididymis, and salivary duct, possess an apical proton-extruding NHE that contributes to luminal NaCl or HCO\textsubscript{3}-\textsuperscript{-} (re)absorption (15, 24, 25, 47). To test for the presence of such an Na\textsuperscript{+}/H\textsuperscript{+} exchanger, we used an anti-NHE3 antibody because 1) NHE3 is the predominant epithelial isoform in apical membranes of numerous epithelia (1, 2, 36), and 2) Northern analysis has detected NHE3 message in many other human tissues such as kidney, small intestine, testis, ovary, colon, prostate, thymus, peripheral leukocyte, brain, spleen, and placenta, unlike the rabbit or rat, for which NHE3 message was restricted to kidney, intestine, stomach, brain (12), and more recently to epididymis (2, 36). However, no staining was observed (Fig. 2A) on sweat duct even with the SDS-unmasking technique (14), in contrast with rat proximal tubule and Caco-2 cells, our positive controls (Fig. 2, C and E). The fact that the anti-rabbit-NHE3 antibody recognized apical NHE3 from human-derived Caco-2 cells excludes the possibility that subtle structural differences between human and rabbit NHE3 protein, which present 88% amino acid identity (12), may be responsible for the absence of staining.

Thus our immunolocalization results clearly indicate the absence of both NHE1 and NHE3 isoforms at the apical membrane of the sweat duct. It still remained possible that other isoforms might contribute to apical Na\textsuperscript{+}/H\textsuperscript{+} exchange. For example, NHE2 which is found in gastrointestinal and renal epithelial tissues, also participates in transepithelial NaCl transport (17, 21, 53, 55). However, functional data using pH\textsubscript{i} measurements provided us with further arguments to conclude that Na\textsuperscript{+}/H\textsuperscript{+} exchange is absent from the apical membrane of the sweat duct. First, luminal EIPA has no effect on steady state pH\textsubscript{i} (Fig. 3) even at a dose of 50 \(\mu\text{M}\), while the IC\textsubscript{50} value for the most resistant isoform (NHE3) varies between 2.4 and 8 \(\mu\text{M}\) (12, 19, 33). Second, removal of luminal sodium caused an increase in pH\textsubscript{i} of sweat duct cells (Fig. 7), instead of the decrease observed in epithelial cells possessing apical NHE such as the rabbit proximal tubule (31), the main excretory duct of the mouse mandibular salivary gland (15), and the rat epididymis (2). Finally, the absence of effect of luminal EIPA on pH\textsubscript{i} recovery following an acid load strongly suggests the absence of any significant Na\textsuperscript{+}/H\textsuperscript{+} exchange activity at the apical membrane of the inner cells layer. However, we cannot exclude the presence of an EIPA-insensitive exchanger at this membrane. Therefore, proton transport at this mem-

brane would be largely, if not exclusively, attributable to the recently reported concanamycin A-sensitive V-ATPase (Fig. 4) (11, 18).

**Lateral and Interlayer NHE1**

An unexpected finding of this study is the distribution of NHE1 at the junction between inner and outer cell layers. The sweat duct has been generally described as composed of two distinct cell types (inner and outer cells) functioning as a single layer of cells. With Lucifer yellow, both dye-coupling and electrocoupling have been observed between neighboring cells in a given layer as well as between neighboring cells in the two layers, demonstrating the presence of intracellular communications between the two cell types and the syncytial nature of the epithelium. Therefore, based on the assumption that the inner and outer cell layers work as a syncytium, the majority of previously published studies have considered that the sweat duct behaves as a single cell layer epithelium, like the kidney tubule. The results of the present study showing NHE1 at the junction between inner and outer cell layers underline the complex architecture of the sweat duct and point to a possible role of NHE1\textsubscript{inter} in pH\textsubscript{i} regulation and proton transport.

Indeed, transmission electron microscopy images of the human eccrine sweat duct (13, 20) clearly show extensive lateral interdigitations between neighboring cells, in both the inner and outer layers, associated with a well-developed intercellular space. The same is true between the basal membrane of the inner cells and the apical membrane of the outer cells. This strongly suggests that these intercellular spaces act as pathways for transport from the cell to the basal side of the duct, in a way similar to the sodium transport mediated by the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase in the kidney (reviewed in Ref. 23). Figure 11 incorporates these features together with the transport systems reported to date and the immunolocalization findings of the present study. Figure 11 therefore shows that, in addition to the basal side of the outer cells, NHE1 is present at the lateral junctions of inner and outer cells and therefore covers the lateral perimeter of these cells in both layers (NHE1\textsubscript{inter}). NHE1 is also present at the junction between the basal side of the inner cells and the apical side of the outer cells (NHE1\textsubscript{inter}). From our images, we cannot distinguish whether only one or both membranes are stained. The intensity of staining at this junction, which is similar to that seen at the lateral junctions (Fig. 1, A and C), where two membranes are necessarily stained, and brighter than that seen at the single basal membrane of the outer cells may suggest that both membranes are stained. However, because NHE1 is systematically observed at basolateral membranes, in Fig. 11 we represent it only at the basal membrane of the inner cell layer.

The question remains as to the functionality of NHE1\textsubscript{inter} and NHE1\textsubscript{inter}. However, as discussed above, the incomplete blockade of the exchanger by EIPA may provide a clue. Indeed, possibly due to fluid
reabsorption that would oppose its diffusion, EIPA may not reach, or reach only at a lower concentration, the most remote spaces between cells, particularly those between inner and outer cell layers, so that some of the exchangers in these remote locations would not be completely inhibited. Also, the observation that the alkalinization produced by luminal sodium (or chloride) removal is minimally altered by bath EIPA, and the fact that it is the cells of the inner layer in contact with these most remote regions that first see the reduction in intracellular sodium would reinforce this possibility. Clearly, more work will be needed to establish definitively the contributions of NHE1inter and NHE1later to the Na+/H+ exchange activity of sweat duct.

Chloride Involvement in pH i Regulation

Previous studies have demonstrated that low luminal sodium leads to a decrease in intracellular chloride concentration, likely consequent to the preservation of electroneutrality, due to the high chloride conductance at the apical membrane of the inner cells (45). Indeed, no evidence is available indicating the presence of an apical NaCl or Na-K-2Cl cotransporter in sweat duct (44). More recently, the same authors have demonstrated that activation of the ENaC requires CFTR chloride channel function (41).

In the present study, low luminal chloride led to a reversible alkalinization of 0.20 ± 0.02 (n = 16, Fig. 9), similar to that observed with luminal sodium removal. This result strongly suggests that the increase in pH i is caused by the same mechanism as that for luminal sodium removal, i.e., the reduction in intracellular sodium concentration resulting from the electrogenic driving force produced by chloride exit. Also, as expected from the apical sodium removal experiments, some ducts did not respond to chloride removal, and an alkalinization following low luminal chloride was still observed in the presence of basolateral EIPA (Fig. 10). The presence of a Cl-/OH- exchanger at the luminal membrane may represent an alternative explanation. However, our experiments with an anti-AE2 antibody (affinity-purified rabbit antiserum raised against the carboxy-terminal amino acids nucleotides 1224–1237 of mouse AE2) identical in sequence to this region of human AE2 showed no staining of human sweat duct, even after epitope unmasking with SDS (14). This antibody has wide species cross-reactivity and recognizes human AE2 immunostains in multiple tissues (Alper SL, personal communication). We cannot exclude the possibility that other Cl-/OH- isoforms may be present. However, our experiments with an anti-AE2 antibody showed no staining of human sweat duct, even after epitope unmasking with SDS (14). This antibody has wide species cross-reactivity and recognizes human AE2 immunostains in multiple tissues (Alper SL, personal communication). We cannot exclude the possibility that other Cl-/OH- isoforms may be present. However, their importance could only be minor in the light of the alkalinization observed upon sodium removal, because the presence of a Cl-/OH- would predict an acidification consequent to intracellular chloride reduction (45).

It should be pointed out that Reddy and coworkers (40) also found that pH i is coupled to luminal chloride...
in sweat duct cells. However, contrary to our findings, they observed a decrease of cytoplasmic pH following luminal chloride removal (substitution with impermeable anion gluconate). At this point, we do not have a clear explanation for this opposite behavior. One element might be their conditions, where potassium completely replaced sodium for bath perfusion. Indeed, it has been shown (33) that potassium can strongly inhibit NHE1 in the absence of sodium, so the acidification may be linked to another mechanism.

Role of the Na+/H+ Exchangers in the Sweat Duct

The NHE1 exchanger is active at resting cell pH, because both EIPA causes a cellular acidification (Figs. 3, 6, 8, and 10). This response indicates that the transporter operates in a direction opposite to sweat acidification but in a manner similar to that of basolateral NHE1 expressed in many nephron segments (proximal tubule, thick ascending limb, principal cells of cortical collecting duct, medullary collecting duct), which operate in a direction opposite to urine acidification (26). This basolateral exchanger does not contribute to NaCl absorption but likely plays a role in pH3 or volume regulation as proposed for basolateral NHE1 in the S3 segment of the rabbit proximal straight tubule (26).

Conclusion

In the present study, we have demonstrated that NHE1 is present at all membranes of human eccrine sweat duct, except the apical membrane of the inner or luminal cells, and is likely active at all these membranes. The NHE1 exchanger is also distributed in a similar way in the secretory coil. No significant Na+/H+ activity could be detected at the apical membrane of the inner cell layer, indicating that proton transport at this membrane would be largely, if not exclusively, attributable to a concanamycin A-sensitive proton pump (18). Finally, these findings underline the complex architecture of the sweat duct and point to a possible role of the NHE1 exchangers in cellular pH homeostasis and proton transport.

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REFERENCES


