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ORIGINAL ARTICLES

Effect of Cytosolic pH on Epithelial Na^+ Channel in Normal and Cystic Fibrosis Sweat Ducts

M. M. Reddy · X. F. Wang · P. M. Quinton

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Abstract The activities of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel and the amiloride-sensitive epithelial Na^+ channel (ENaC) are acutely coordinated in the sweat duct. However, the mechanisms responsible for cross-talk between these ion channels are unknown. Previous studies indicated that luminal pH of sweat ducts varies over 3 pH units and that the cytoplasmic pH affects both CFTR and ENaC. Therefore, using basolaterally α-toxin-permeabilized apical membrane preparations of sweat ducts as an experimental system, we tested the hypothesis that the cytosolic pH may mediate the cross-talk between CFTR and ENaC. We showed that while luminal pH had no effect, cytosolic pH acutely affected ENaC activity. That is, acidic pH inhibited, while basic pH activated, ENaC. pH regulation of ENaC appears to be independent of CFTR or endogenous kinase activities because basic pH independently stimulated ENaC (1) in normal ducts even when CFTR was deactivated, (2) in CF ducts that lack CFTR in the plasma membranes and (3) after blocking endogenous kinase activity with staurosporine. Considering the evidence of Na^+/H^+ exchange (NHE) activity as shown by the expression of mRNA and function of NHE in the basolateral membrane of the sweat duct, we postulate that changes in cytosolic Na^+ ($[Na^+]_i$) may alter cytosolic pH (pH_i) as salt loads into the cell during electrolyte absorption. These changes may play a role in coordinating the activities of ENaC and CFTR during transepithelial salt transport.

Keywords Sweat duct · CFTR · ENaC · pH · Electrolyte transport · Cystic fibrosis

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Introduction

The epithelial Na channel (ENaC) is expressed in the apical plasma membranes of numerous epithelial cells including salivary gland ducts, sweat duct, kidney, distal colon and airways. Abnormalities in channel function can be life-threatening in diseases such as Liddle syndrome, pseudohypoaldosteronism (PHA), cystic fibrosis (CF) and cardiovascular pathologies (Benos et al. 1995; Schwiebert et al. 1999). ENaC in the kidney is largely responsible for Na^+ absorption, which maintains extracellular volume, blood pressure and indirectly K^+ and H^+ homeostasis (Benos et al. 1995; Schwiebert et al. 1999). In the lung, ENaC and the CF transmembrane conductance regulator (CFTR) activity seems necessary to maintain airway surface fluid composition and volume (Boucher 2001; Boucher et al. 1991). The final composition of the fluids excreted by secretory organs such as salivary glands, sweat glands and the colon are modified by the concerted activities of ENaC and CFTR in hypertonically absorbing luminal NaCl (Benos et al. 1995; Quinton 1999). Possibly due to the relative inaccessibility of human tissues expressing ENaC and CFTR, little is known about the

physiological properties and mechanisms of controlling these channels in human epithelia despite the vital roles they play in health and disease.

The human sweat gland has two morphologically and physiologically distinct regions. The secretory coil first secretes isotonic sweat into the lumen, and the sweat duct then reabsorbs NaCl from the primary secretion so that salt-depleted sweat reaches the skin surface for evaporative cooling. The accessibility of the sweat duct renders a model to study the regulation of epithelial salt absorption (Quinton and Reddy 1992; Reddy and Quinton 2006). The two significant ion channels, CFTR and ENaC, expressed abundantly in the apical membrane of the native duct, provide a view of in vivo mechanisms that may regulate these channels under near physiological conditions (Reddy et al. 1999).

Studies on ex vivo systems expressing ENaC have implicated the role of PKA, ATP and G proteins in regulating ENaC (Berdiev et al. 1997; Bubien et al. 2001; Ismailov et al. 1997; Stutts et al. 1995; Stutts et al. 1997). However, no such regulation of ENaC by any of these agonists has been observed in the native sweat duct (Reddy and Quinton 2005). Except for recent observations that ENaC and CFTR channels appear to be functionally coupled (Reddy et al. 1999), little is known about the mechanisms responsible for coordinating the activities of these channels during physiological transport activity. It is well known that ENaC operates under wide changes in luminal pH conditions in epithelia (Bijman and Quinton 1987; Lingueglia et al. 1997; Waldmann et al. 1997a, b), which varies from as low as 4.5 to as high as 7.8 in sweat (Bijman and Quinton 1987). Furthermore, a number of studies involving heterologous expression systems as well as endogenous ENaC-expressing cell lines have shown that both cytosolic as well as luminal pH could regulate ENaC (Chalfant et al. 1999; Lingueglia et al. 1997; Snyder et al. 1994; Waldmann et al. 1997a, b). Based on these observations, the objective of this study was to further define the intracellular mechanisms that may not only regulate the activity of ENaC but also contribute to coordinating its activity with that of CFTR in this native epithelium.

Even though pH is well known to regulate ion channel activities in epithelial cells (Cook et al. 1984; Duffey and Devor 1990; Reddy et al. 1998; Stampe and Vestergaard-Bogind 1985), the physiological significance of such regulation is unclear. These studies suggest insights into mechanisms that may coordinate apical salt transport activity mediated by ENaC and CFTR to changes in cytosolic pH. Herein, we present evidence of Na^+/H^+ exchange (NHE) activity in the sweat duct that could play a significant role in changing cytosolic pH in response to apical Na^+ influx through ENaC.

We find that cytosolic, but not luminal, pH controls ENaC activity in sweat duct. Acidic pH inhibits and basic pH activates ENaC. Unlike pH regulation of CFTR through control of its phosphorylation state, the pH regulation of ENaC appears to be direct and independent of CFTR and phosphorylation process. Based on our present and previous studies, we present a simplified model of pH-mediated control of transepithelial salt transport as a part of negative feedback control of cellular salt absorption.

Methods

Tissue Acquisition

Sweat glands were obtained from adult male volunteers who gave informed consent. Full-thickness skin biopsies (3 mm diameter) were taken over the scapula and stored in Ringer solution. Individual sweat glands were isolated from the biopsy in cold Ringer solution by dissection with fine-tipped tweezers visualized at 80× magnification. The isolated glands were transferred to a dissection cuvette with Ringer solution cooled with a peltier block to 4°C, where the segments of reabsorptive duct (>1 mm in length) were separated from the secretory coil of the sweat gland. The sweat duct was transferred to a perfusion chamber containing Ringer solution at 35 ± 2°C.

Selective Permeabilization of the Basolateral Membrane

The basolateral membrane of the sweat duct was selectively permeabilized with the pore-forming agent α-toxin (1,000 units/ml) in cytoplasmic Ringer solution containing 140 mm KGlu (potassium gluconate) and 5 mm ATP. α-Toxin was applied to the basolateral surface of the microperfused sweat duct for 15–30 min. α-Toxin forms pores, which pass molecules of 3,500–5,000 mw (Quinton and Reddy 1992), so that the concentration of intracellular molecules such as cAMP and ATP as well as intracellular pH (pH_i) could be clamped by their concentration in the extracellular bath solution.

pH_i

Qualitative changes in pH_i in response to luminal Cl^- substitution were measured using a pH-sensitive dye intracellularly, acetoxymethyester of 2′,7′-bis-(2-carboxyethyl)-(5,6)-carboxyfluorescein (BCECF)/AM, by fluorescent ratiometry (Reddy et al. 1998). Changes in pH_i were monitored while perfusing the lumen at relatively high rates (>50 nl/min) to insure that the composition of the perfusate remained virtually constant in the duct lumen. In this way, the

compositions of both the luminal and the bath solutions were known and controlled.

RT-PCR

Sweat ducts were freshly isolated from sweat glands and immediately processed for total RNA extraction. Total RNA was extracted using the Absolutely Nanoprep Kit (Stratagene, La Jolla, CA). RNA was reverse-transcribed using the Sensiscript RT Kit (Qiagen, Valencia, CA). The resulting first-strand cDNA was directly used for PCR amplification (TaqPCR Core Kit, Qiagen). The conditions for PCR were 3 min at 94°C (initial melt); 35 cycles of 1 min at 94°C, 1 min at 55–60°C, 1 min at 72°C and 10 min at 72°C (final extension). For the negative control, RT-PCR was performed in the absence of RT. PCR products were analyzed by agarose gel electrophoresis stained with ethidium bromide. Primers were constructed on the basis of the published cDNA sequences of different NHEs from GenBank. The pairs of primers for NHE1–NHE5 are listed in Table 1.

Electrical Measurements

After cannulating the lumen of the sweat duct with a double-lumen cannula made from theta glass (1.5 mm diameter; Clark Electromedical Instruments, Reading, UK), a constant current pulse of 50–100 nA for a duration of 0.5 s was injected through one barrel of the cannulating pipette containing NaCl Ringer solution. The other barrel of the cannulating pipette served as an electrode for measuring transepithelial potential (V_t) with respect to the contraluminal bath and as a cannula for perfusing the lumen of the duct with selected solutions. V_t was monitored continuously using one channel of a WPI-700 dual electrometer referenced to the contraluminal bath. Since the sweat duct is a tubular epithelium whose lumen is a conductive core surrounded by an insulating epithelium, we measured the transepithelial specific conductance (G_t) as described earlier from the amplitude of transepithelial voltage deflections in response to 50–100 nA transepithelial constant current pulse using a cable equation (Greger 1981; Helman et al.1971; Quinton and Reddy 1992; Reddy et al. 1999).

CFTR Cl^- conductance (gCFTR) and ENaC Na^+ conductance (gENaC) were calculated using the following formulae: $G_t = gENaC + gX$ and $G_t^* = gCFTR + gENaC^* + gX$, where G_t and G_t^* are total transepithelial conductances before and after stimulating gCFTR (with cAMP + 5 mm ATP in the cytoplasmic bath), respectively; gX is a nonspecific shunt conductance measured after blocking gENaC (with luminal amiloride) and gCFTR (by removing cAMP and ATP from cytoplasmic bath); and gENaC and gENaC* represent ENaC conductance before and after activating CFTR with cyclic nucleotides, respectively, as mentioned above. The aforementioned formulae used to calculate the magnitude of gCFTR and gENaC are based on the fact that CFTR and ENaC provide the predominant (only) Cl^- and Na^+ conductance pathways in this tissue (Reddy et al. 1999).

Solutions

The luminal perfusion Ringer solutions contained (in mm) NaCl (150), K (5), PO_4 (3.5), $MgSO_4$ (1.2), Ca^{2+} (1.0) and amiloride (0.01), pH. 7.4. The cytoplasmic/bath solution contained K (145), gluconate (140), PO_4 (3.5), $MgSO_4$ (1.2) and 260 μm Ca^{2+} buffered with 2.0 mm EGTA (Sigma, St. Louis, MO) to 80 nm free Ca^{2+}, pH 6.8. The effect of luminal or cytoplasmic pH on CFTR G_{Cl} was evaluated by directly manipulating bath (cytoplasmic) or luminal pH from 4.5 to 8.5 under activated (0.1 mm cAMP/5 mm ATP) and deactivated (with ATP but without cAMP, with cAMP but without ATP or without either ATP or cAMP) conditions.

Data Analysis

The data are presented as the mean ± SE (where n = number of ducts from at least four human subjects). Statistical significance was determined on the basis of Student's t-test for paired samples. $p < 0.05$ was chosen to indicate a statistically significant difference. The data

Table 1 Primers for NHE1–NHE5 and ß-actin

Gene	Sequence	BP
NHE1	CCCTTCAACTCCTCATTCACCA (forward) CTGGCGTCTCAACTGTCTCTA (reverse)	422
NHE2	GCAGATGGTAATAGCAGCGA (forward) CCTTGGTGGGGGCTTGGGTG (reverse)	310
NHE3	TGGACCTCCTCAAACACGGCCAG (forward) ACGTCCAGGACCCCTACATC (reverse)	460
NHE4	GGCTGGGATTGAAGATGTATGT (forward) GCTGGCTGAGGATTGCTGTAA (reverse)	501
NHE5	GTGTTTCACCTGTCTCGGAAAG (forward) GATGGCACCCAAGTTGTCAAAG (reverse)	250
ß-Actin	TTCAACTCCATCATGAAGAAGTGTGACGTG (forward) CTAAGTCATAGTCCGCCTAGAAGCATT (reverse)	312

presented as electrophysiological traces are representative of experiments repeated at least three times.

Results

Luminal pH Does Not Affect ENaC

We examined the effect of luminal pH on ENaC activity. Changing luminal pH between 4.5 and 8.5 while maintaining cytoplasmic bath pH constant (6.8) had very little effect (if anything, alkaline pH 8.5 may have had a slight inhibitory effect on ENaC activity as indicated by a small decrease in lumen-negative Na^+ diffusion potential, Fig. 1).

Cytoplasmic pH Affects ENaC

In contrast to the lack of effect of luminal pH, changing cytosolic pH had a marked effect on ENaC activity. Lowering the cytosolic pH deactivated and raising the pH stimulated ENaC. The effect of pH was reversible (Fig. 2)

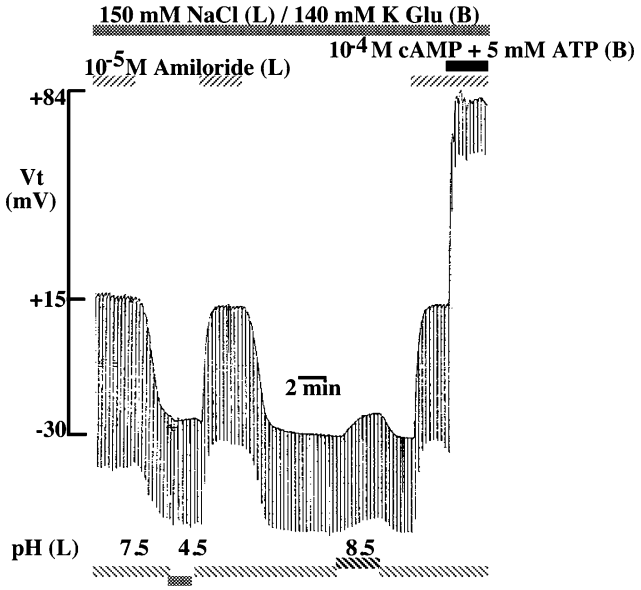


Fig. 1 No effect of luminal pH on ENaC activity. In this experiment, amiloride-sensitive Na^+ diffusion potentials and apical membrane conductance were measured at three luminal pH values. Acute acidification (4.5 pH) and alkalinization (7.5–8.5 pH) had little effect (with the exception that alkalinization had a slight inhibition) on ENaC conductance as indicated by lack of change in Na^+ diffusion potentials (150 mm Na^+ in the lumen and 150 mm K^+ in the cytoplasmic bath of the basolaterally α-toxin-permeabilized sweat duct). Also notice that application of cAMP + ATP caused significant activation of CFTR Cl^- conductance, as indicated by the large increase in lumen-positive Cl^- diffusion potential (150 mm Cl^- in the lumen and 140 mm Glu^- in the cytoplasmic bath) and the decrease in transepithelial voltage defections due to constant current (50 nA/20 mS) across the apical membrane

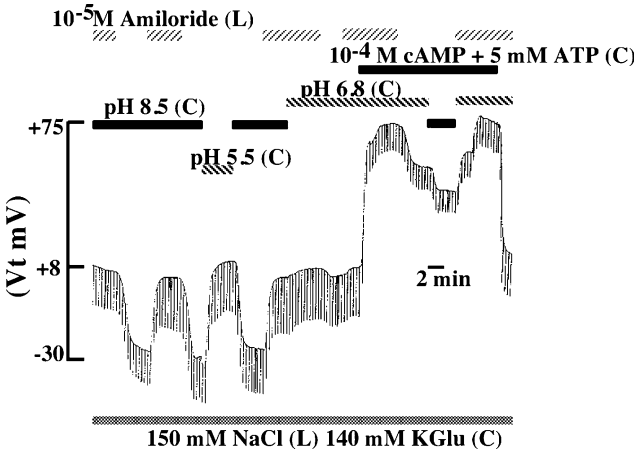


Fig. 2 Cytosolic pH regulates ENaC. Alkaline pH activates and acidic pH deactivates ENaC independently of CFTR activation by cAMP + ATP. Before activating CFTR (no cAMP), ENaC conductance was minimal at pH 5.5 (same as in the presence of amiloride at pH 8.5). At pH 6.8, CFTR activation by cAMP + ATP increased ENaC activity, which was further enhanced by alkaline pH. ENaC activity is indicated by the magnitude of lumen hyperpolarizing Na^+ diffusion potentials and increasing amiloride-sensitive apical membrane conductance

and dose-dependent (Fig. 3). The gENaC was close to zero at pH 5.5, with an inhibitory effect that mimicked that of amiloride (Fig. 2). Under these conditions, the remaining apical membrane conductance reflects a leak conductance of ~ 3 mS/cm^2, measured in the presence of amiloride in the lumen and without cAMP and ATP in the cytoplasmic bath. The leak conductance was subtracted to obtain the amiloride-sensitive conductance. In contrast, alkalinizing the cytosol activated gENaC to an apparent maximum at about pH 8.0. gENaC was only very slightly higher at pH 8.5 (Fig. 3). Therefore, we used pH 8.5 as a maximal stimulus, while comparing the stimulatory effects of alkaline pH on ENaC before and after activating CFTR in normal and CF ducts (Figs. 4, 5).

Activating CFTR Does Not Alter the Effect of pH on ENaC

Since ENaC normally activates along with CFTR (Reddy and Quinton 2006), we asked what additional effect pH might exert on ENaC. We found that in addition to the simultaneous activation of ENaC with CFTR (in the presence of cAMP + ATP), raising pH from 6.8 to 8.5 caused further activation of gENaC as indicated by a significant increase in Na^+ diffusion potentials ($\Delta 17.7 \pm 3.4$ mV, i.e., the apical membrane potential became less positive from 34.3 ± 4.3 to 16.7 ± 1.8 mV; $n = 5$ ducts) and gENaC ($\Delta 11.0 \pm 1.0$ mS/cm^2, i.e., from 31.7 ± 1.7 to 42.7 ± 1.5 mS/cm^2, $n = 5$) (Figs. 2, 4, 5). That is, the apical

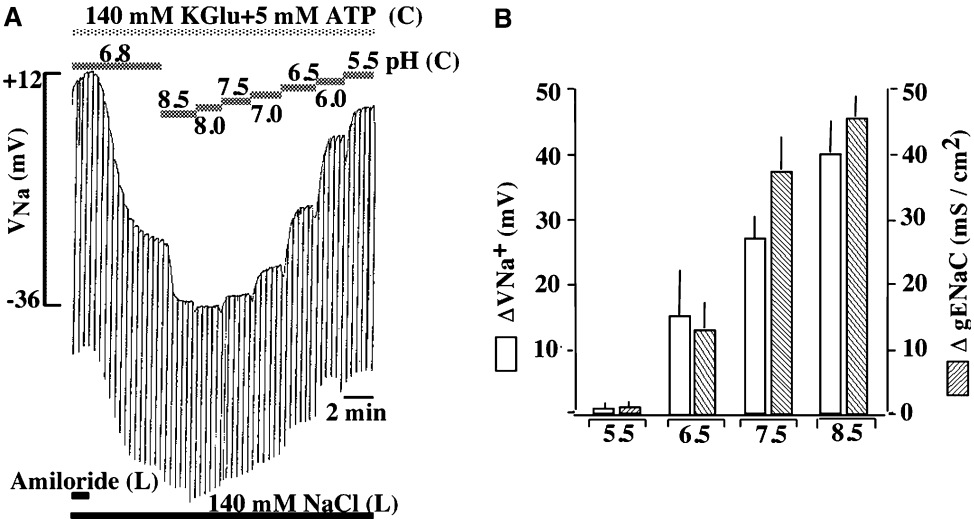


Fig. 3 pH dose-dependent ENaC activity. (**a**) Representative electrical trace showing dose-dependent responses of Na^+ diffusion potentials and conductances across the apical membrane. ENaC activity is clearly dependent on cytoplasmic pH, which is minimal at 5.5 and highest at 8.5. (**b**) Summary of data obtained with experiments similar to those in (**a**) that show the change in V_{Na+} (change in Na^+ diffusion potentials) and gENaC (change in Na^+ conductance) as a function of cytosolic pH

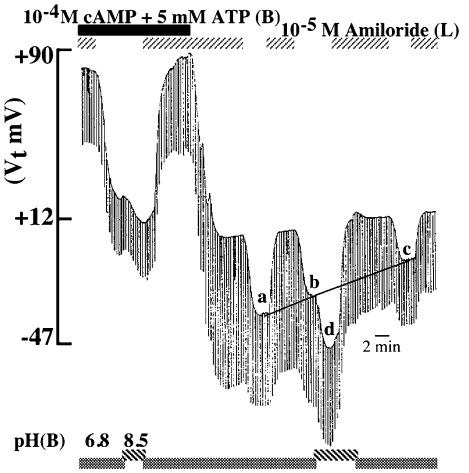


Fig. 4 Basic pH prevents ENaC rundown following deactivation of CFTR. Application of cAMP + ATP generated about +90 m*V* lumen-positive Cl^- diffusion potential when ENaC activity was blocked with amiloride. However, after removing amiloride inhibition of ENaC, the Na^+ diffusion potentials thus generated completely shunted the Cl^- diffusion potential in this example (not always the case), thereby taking the measured potentials to the junction potential (~11 m*V* for 140 mm KGlu in the cytoplasmic bath versus 150 mm NaCl in the lumen). Shunting of the Cl^- diffusion potential by the Na^+ diffusion potential occurred because the respective chemical driving forces for Cl^- and Na^+ are almost equal but opposite. These results indicated that the magnitude of ENaC activity must be near that of CFTR activity. Notice that following deactivation of CFTR by washing out cAMP and ATP, the Na^+ diffusion potentials after amiloride washout continued to decrease, which was reversed by alkalinizing the cytosol. The *oblique line joining the peaks* (denoted by peaks *a, b* and *c*) of lumen-negative Na^+ diffusion potentials at pH 6.8 after deactivating CFTR show continuous decay in ENaC activity that could be reversed by raising the pH to 8.5 (denoted by peak *d*)

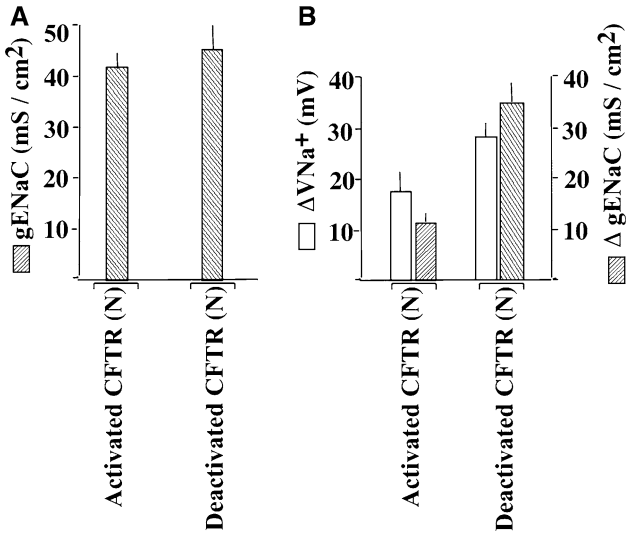


Fig. 5 Effect of cytosolic pH on the absolute (**a**) and relative (**b**) changes in ENaC conductance as a function of CFTR Cl^- conductance.(**a**) Summary of the data on the effect of basic pH on the absolute amiloride-sensitive ENaC conductance in the apical membrane in the presence and complete absence of CFTR Cl^- conductance (no cAMP + ATP). Notice that the absolute ENaC conductance at 8.5 pH is comparable between the two groups regardless of the CFTR functional status. (**b**) The effect of alkaline pH (8.5) on ENaC conductance before and after activating CFTR Cl^- channel function by cAMP + ATP. Notice that increasing cytosolic pH after activating CFTR caused relatively smaller changes (Δ) in ENaC diffusion potentials and conductance because ENaC was already partially activated following CFTR activation, as indicated by the fact that the absolute ENaC conductance at 8.5 pH (as shown in **a**) appears to be the same

membrane potential hyperpolarized (became less positive) and the amiloride-sensitive conductance increased as reflected by increased lumen to cell Na^+ diffusion potentials.

Effect of pH on gENaC in the Absence of CFTR Cl^- Channel Function

In order to test the effect of pH on ENaC activity independent of CFTR Cl^- channel function, we first deactivated CFTR by washing out cAMP and ATP in the

presence of luminal amiloride. The deactivation of CFTR was accompanied by a loss of the Cl^- diffusion potential and a reduction of apical membrane conductance to the level of the leak conductance (see "Methods"). We then washed out luminal amiloride, changed the cytosolic pH from 6.8 to 8.5 and measured amiloride-sensitive Na^+ diffusion potentials and conductance. We found that gENaC increased Na^+ diffusion potentials significantly ($\Delta 28.0 \pm 2.1$ m*V*, i.e., from -12 ± 2.1 to -40 ± 2.9 m*V*, $n = 6$) and gENaC ($\Delta 35.3 \pm 1.8$ mS/cm^2, i.e., from 10.3 ± 2.3 to 45.7 ± 3.8 mS/cm^2, $n = 6$). The change in gENaC induced by basic pH appeared relatively larger with deactivated CFTR than with activated CFTR (Fig. 5). However, the absolute magnitude of gENaC at 8.5 pH with both activated and deactivated CFTR conditions was not significantly different (Fig. 5).

ENaC Sensitivity to Cytosolic pH is Independent of CFTR Expression

Not only did alkaline pH stimulate ENaC before (in the absence of cAMP and ATP) and after (by application of cAMP + ATP in the cytoplasmic bath, Figs. 3–5) activation of CFTR, but high pH also enhanced gENaC in ducts from homozygous ΔF508 CF subjects who lack CFTR in the apical membrane (Fig. 6). Na^+ diffusion potentials increased from -14.3 ± 2.3 to -25 ± 5.0 m*V* ($n = 3$) and gENaC increased from 3.3 ± 0.7 to 37 ± 1.7 mS/cm^2 ($n = 3$) following the increase of cytosolic pH from 6.8 to 8.5.

pH Activation of gENaC is Insensitive to Staurosporine

Since alkaline pH is known to stimulate PKA phosphorylation activity in the sweat duct (Reddy et al. 1998), we determined whether activation of ENaC is caused by PKA phosphorylation. Our results show that inhibiting endogenous kinases with the general kinase inhibitor staurosporine (10^{-6} m) in the cytoplasmic bath did not prevent gENaC activation by high pH (Fig. 7).

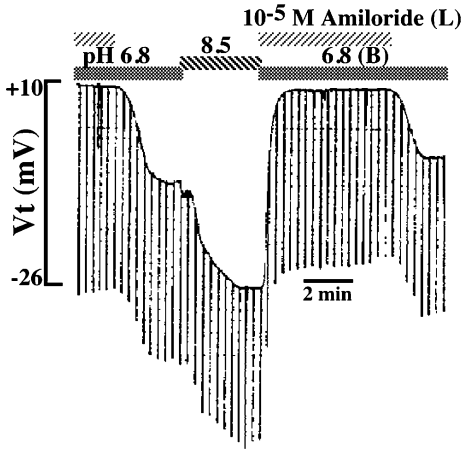


Fig. 6 pH regulation of ENaC is independent of CFTR. This experiment demonstrates that in the absence of CFTR in the apical membrane of sweat ducts from homozygous ΔF508 CF subjects, gENaC is activated by basic pH also

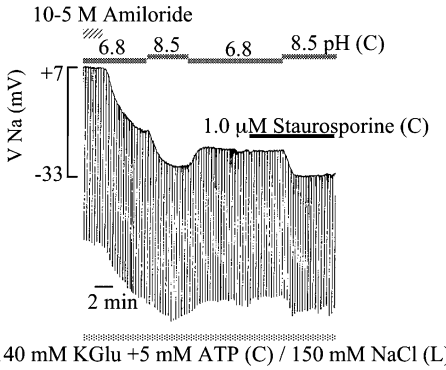


Fig. 7 Effect of pH on ENaC is independent of phosphorylation. In order to determine whether the endogenous kinase phosphorylation mediated the effect of pH on ENaC activity, we first inhibited kinases with a nonspecific kinase inhibitor, staurosporine, before raising the cytosolic pH. Notice that alkaline pH activates ENaC even after application of staurosporine, indicating that the cytosolic pH control of ENaC does not involve kinase phosphorylation

High pH Blocks ENaC Rundown

We showed earlier (Reddy et al. 1999) that gENaC activity gradually falls following deactivation of CFTR by washing out cAMP and ATP. Raising cytosolic pH to 8.5 prevented such rundown of ENaC activity (Fig. 4).

High Cytoplasmic Na^+ Blocks gENaC

Since the Na^+ gradient across the plasma membrane affects cytosolic pH via NHE activity, we investigated the effect of changing cytosolic Na^+ in basolaterally α-toxin-permeabilized ducts on ENaC activity. These results indicated that substituting cytosolic K^+ with Na^+ completely abolished the amiloride-sensitive conductance (Fig. 8).

NHE is Expressed and Functional in the Sweat Duct

Since changes in intracellular Na^+ alter pH_i via NHE, we assayed for NHE in the sweat duct. We found that human sweat ducts express mRNA for a series of NHEs including NHE1, NHE2, NHE3, NHE4 and NHE5. These exchangers are most likely in the basolateral membrane since recovery from acid loads was dependent on basolateral Na^+ (Fig. 9).

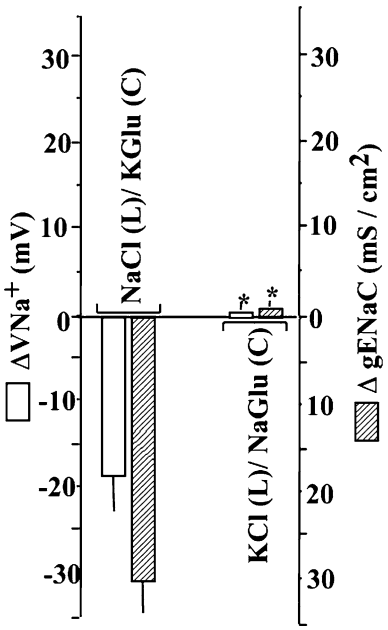


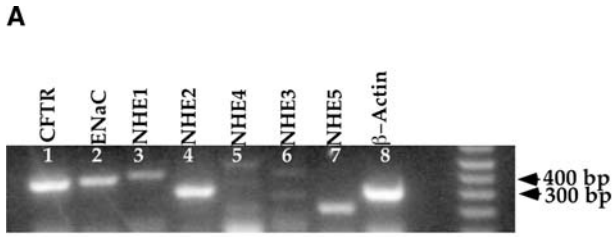
Fig. 8 Effect of cell Na^+ on ENaC activity. Since ENaC activity depends on the activation of CFTR, we activated CFTR with cAMP. Imposing a cation gradient by substituting luminal K^+ with an equimolar concentration of Na^+ (*left*) generated a significant and sustained Na^+ diffusion potential (no amiloride in the lumen). Reversing Na^+ gradient from cell to lumen (150 mm Na^+ in the cytosol and 150 mm K^+ in the lumen, *right*) caused almost complete loss of Na^+ diffusion potentials and gENaC. These results indicate that increasing cytosolic Na^+ blocks ENaC activity. Note: Since CFTR activity is also inhibited by cytosolic K^+ substitution with Na^+ by dephosphorylating CFTR, we irreversibly phosphorylated CFTR using ATP-γ-S as substrate in order to prevent CFTR deactivation during cytosolic K^+ substitution by Na^+ as previously described (Reddy et al. 1998)

Discussion

The Na^+ channel ENaC is composed of three subunits, α, β and γ, which play significant roles in the transepithelial electrogenic Na^+ absorption by a number of epithelia (Butterworth et al. 2005a; Garty and Palmer 1997; Lingueglia et al. 1993) including sweat duct (Reddy et al. 2005). Amiloride-sensitive Na^+ absorption has been shown to be acutely regulated by cytosolic pH (Garty et al. 1985; Garty and Palmer 1997; Harvey et al. 1988; Leaf et al. 1964; Lingueglia et al. 1993; Lyall et al. 1995; Ussing and Zerahn 1951). Recent reports indicated not only that ENaC possesses biophysical properties similar to those expressed in the apical membranes of epithelia whose amiloride-sensitive Na^+ absorption is pH-sensitive (Canessa et al. 1994; Garty and Palmer 1997) but also that the channel activity is inhibited by protons (Chalfant et al. 1999; Snyder et al. 1994). Therefore, we investigated the role of luminal as well as cytosolic pH in regulating ENaC activity in the apical membrane of native human sweat ducts.

gENaC is Insensitive to Changes in Luminal pH

Extracellular pH has been shown to regulate other members of the ENaC/FaNaC/DRASIC/degenerin superfamily (Lingueglia et al. 1997; Waldmann et al. 1997a, b). ENaC in the apical membrane of many native epithelia can be subjected to drastic changes in extracellular pH (Awayda et al. 2000; Bijman and Quinton 1987). In the sweat duct, where ENaC is richly expressed, luminal pH also undergoes extreme changes from alkaline (pH 7.8) to significantly acidic (pH $<$ 5.0) values (Bijman and Quinton 1987). Therefore, we sought to determine the role of luminal pH in regulating gENaC. We tested the effect of lowering luminal pH from 8.5 to 5.0 on ENaC. We found that such extreme changes in luminal pH had little effect on the magnitude of gENaC (Fig. 1). These results are consistent with the early observations on the heterologous systems expressing ENaC subunits, which indicated that



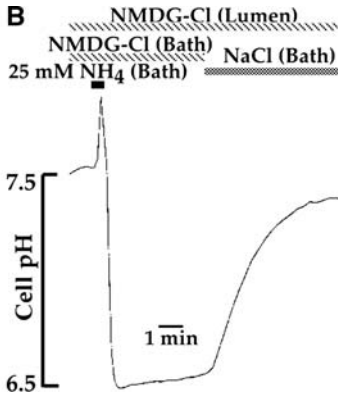


Fig. 9 **a** mRNA expression of CFTR, ENaC and NHE1-5 in isolated human sweat ducts. RT-PCR bands from primers specific for CFTR, ENaC, actin, NHE1, NHE2, NHE3, NHE4 and NHE5. **b** NHE activity in the basolateral membrane of human sweat duct. pH_i was monitored ratiometrically by the pH-sensitive fluorescent dye BCECF. The isolated duct was perfused first with NMDG-Cl (150 mm) bilaterally to remove Na^+. The cells were acid-loaded with a 30-s pulse of 25 mm NH_4Cl as shown by the rapid decrease of cell pH to about 6.5. After a brief period, Na^+ replaced $NMDG^+$ in the bath, at which point the cell immediately began to recover back to pH $\sim$7.3

changes in pH on the apical side had no effect on channel function (Chalfant et al. 1999). However, these findings are in contrast to observations on A6 kidney cells in which apical acidic pH was shown to enhance ENaC activity through changes in cytosolic Ca^{2+} activity (Awayda et al. 2000; Bijman and Quinton 1987). In fact, experimental manipulation of cytosolic Ca^{2+} in the permeabilized ducts did not have any effect on ENaC activity in sweat ducts (unpublished personal observations). Previously, we also reported that luminal pH does not affect CFTR Cl^- channel function, indicating that both these channels function efficiently to absorb salt regardless of wide changes in the luminal pH (Reddy et al. 1998).

gENaC is Sensitive to Changes in Cytosolic pH

Cytosolic pH is well known to regulate the activities of several proteins involved in transepithelial salt transport including Na^+/K^+ pump activity (Eaton et al. 1984; Hwang et al. 1994), basolateral K^+ conductance (Duffey and Devor 1990; Stame and Vestergaard-Bogind 1985) and epithelial Cl^- channels (Cuppoletti et al. 1993; Reddy et al. 1998). The Na^+ conductance of heterologously expressed ENaC was also affected by pH changes on the cytoplasmic surface (Chalfant et al. 1999). We have previously shown that the transport activity at the apical membrane causes changes in intracellular pH and that CFTR Cl^- channel may be acutely regulated by cytosolic pH control of phosphorylation of the channel (Reddy et al. 1998). Therefore, we investigated whether cytosolic pH could, in fact, regulate endogenous ENaC activity in a native epithelium and found that changes in cytosolic pH acutely control ENaC activity (Fig. 3). Acidic pH inhibits and basic pH activates ENaC. Furthermore, it is clear that this channel can withstand and respond reversibly to alterations in cytosolic pH (Fig. 3). Moreover, the apical membrane is devoid of K^+ conductance and pH has no effect on apical membrane conductance after blocking ENaC (with amiloride) and CFTR (by removing cAMP and ATP) (Reddy et al. 1998, 1999; Reddy and Quinton 2005). Further, if basic pH activated a K^+ channel, V_t would depolarize (not hyperpolarize as shown here) because the cell to lumen K^+ gradient would make the lumen more positive. Therefore, we conclude that ENaC is the only cation channel in the apical membrane (Reddy and Quinton 1991, 2006) and that the pH-induced changes in apical cation conductance are mediated by ENaC. It is also noteworthy that CFTR, the anion channel that transports the co-ion (Cl^-) to Na^+, displays a similar pH sensitivity (Reddy et al. 1998). Thus, the activities of these channels respond in parallel to changes in pH_i. Furthermore, removing luminal Cl^- significantly changed apical transport activity, which reversibly decreased pH_i (Cuppoletti et al. 1993; Reddy et al. 1998). Even though we did not quantify the magnitude of these changes in pH_i, they were qualitatively consistent with acidifying and deactivating ENaC.

pH Regulates ENaC Independent of CFTR

CFTR channel activation appears to be coupled with increases in ENaC activity in this tissue (Reddy et al. 1999). Conversely, inhibiting CFTR activity with DIDS or Cl^- removal was accompanied by significant inhibition of ENaC. These observations raised the question of whether pH activation of ENaC is also dependent on CFTR channel function. Therefore, we compared the magnitude of ENaC activation before and after activating CFTR-Cl^- channel function and found that alkaline pH always caused significant activation of ENaC independent of CFTR activation (Figs. 2, 4, 5). In fact, the relative increase in gENaC upon raising cytosolic pH from 6.8 to 8.5 was much larger before activating than after activating CFTR by cAMP + ATP in the cytoplasmic bath. However, the absolute magnitude of ENaC conductances was virtually the same at pH 8.5.

Further, we have previously reported that ENaC activity is severely depressed in CF ducts that lack CFTR in the apical plasma membrane. Application of cAMP + ATP failed to activate ENaC in CF ducts (Reddy et al. 1999, 2005; Reddy and Quinton 2005). We therefore asked if pH regulation of ENaC is also affected by lack of CFTR in the apical plasma membrane. Raising cytosolic pH also activated ENaC in these ducts (Fig. 6). These results suggest that, independent of CFTR function, ENaC activity is sensitive to pH and maximally activated by alkaline pH conditions.

pH Regulation of gENaC Appears to be Independent of Phosphorylation

We have previously reported that cytosolic pH regulates CFTR by controlling its phosphorylation state (Reddy et al. 1998) and PKA phosphorylation appears to stimulate ENaC in heterologous expression systems (Schwiebert et al. 1999; Stutts et al. 1995, 1997). We therefore asked whether cytosolic pH regulates ENaC and observed that (1) activation of pH is independent of the presence of ATP, an essential substrate in the phosphorylation reaction (Figs. 1, 2, 4, 6), and (2) alkaline pH activation of ENaC is unaffected by inhibition of endogenous kinase activity by the nonspecific kinase inhibitor staurosporine (Fig. 7). Thus, regulation of ENaC appears to be independent of phosphorylation.

Cytosolic Alkaline pH Prevents Spontaneous Decrease in gENaC

We observed that removing cAMP and ATP deactivated CFTR and led to progressive rundown of ENaC activity as

indicated by a gradual decrease in amiloride-sensitive Na^+ diffusion potentials and conductance (Fig. 4). cAMP stimulates sodium transport in tissues that express ENaC by increasing either the open probability (P_o) or ENaC channel density in the apical membrane (Butterworth et al. 2005a, b). Therefore, it seems possible that removing cAMP (to deactivate CFTR) could have similar inhibitory effects on ENaC trafficking, thereby reducing channel density. However, the fact that application of cAMP + ATP in the absence of CFTR function in normal ducts (no Cl^- in the lumen or cytosol or inhibitor blocked CFTR activity) or in the absence of CFTR in CF ducts failed to stimulate ENaC activity in the sweat duct (Reddy et al. 1999; Reddy and Quinton 2005) indicates that ENaC rundown following CFTR deactivation is not likely due to cAMP/ATP washout. Raising cytosolic pH prevented such rundown, as shown in Fig. 4. The fact that alkalinizing the cytosol acutely stimulates gENaC could indicate that in the native sweat duct cytosolic pH may be important in regulating ENaC activity. The mechanism by which alkaline pH prevents ENaC rundown is unclear but may involve activation of latent channels in the plasma membrane or through increased single-channel open probability and decreased closed time (Chalfant et al. 1999; Schwiebert et al. 1999). In fact, a decrease in cytosolic pH also caused a rapid inhibition of ENaC expressed in *Xenopus* oocytes (Chalfant et al. 1999; Schwiebert et al. 1999) and in planar lipid bilayers incorporating cloned ENaC. Thus, ENaC sensitivity to cytosolic pH may be a general property of its control.

Potential Mechanisms Involved in pH Regulation of ENaC Activity

We have previously shown that cytosolic cation composition is significantly altered as a function of transport activity through the apical membrane (Reddy and Quinton 1991, 2006). Na^+ entry through ENaC could increase Na^+ from about 10 mm in nontransporting cells to ~70 mm in transporting cells (Reddy and Quinton 2006). Such increases in cytosolic Na^+ inhibited ENaC in isolated mandibular gland cells (Komwatana et al. 1996; Palmer 1985). The present results also indicate that an increase in cytosolic Na^+ concentration causes significant inhibition of ENaC conductance in sweat duct, as shown in Fig. 8. Earlier reports suggested that the cytosolic Na^+ regulation of ENaC is not direct but mediated by changes in cytosolic pH and free $[Ca^{2+}]_i$ (Komwatana et al. 1996). However, as the present studies on the effect of cytosolic Na^+ on gENaC in permeabilized sweat duct cells were conducted under conditions of constant intracellular $[Ca^{2+}]_i$ and pH_i, we conclude that the effect of cytosolic Na^+ on ENaC must be independent of changes in $[Ca^{2+}]_i$ and pH_i. These observations do not rule out the possibility that secondary changes in cytosolic pH under physiological conditions play a significant role in regulating ENaC in the sweat duct. Fluctuations in cytosolic Na^+ activity should significantly affect NHE activity (which depends on the Na^+ gradient) and alter cytosolic pH. Activation of Na^+/K^+ activity should reduce levels of intracellular Na^+, leading to increases in cytosolic pH. Hence, we investigated whether NHE is expressed in the sweat duct. We found that the sweat duct cell expresses mRNA for NHE1, NHE2, NHE3, NHE4 and NHE5 (Fig. 9a). Moreover, intracellular recovery from acid loads was dependent on serosal Na^+. These results are consistent with the presence of NHE activity in the basolateral membrane (Fig. 9b) and with a recent report of NHE3 in the basolateral membrane of the sweat duct (Granger et al. 2003). Granger et al. (2003) also indicated that NHE activity in sweat duct is primarily confined to the basolateral membrane. These early studies (Granger et al. 2003) also indicated that inhibiting apical salt absorption either by removing Na^+ or its co-anion Cl^- (note: because of the requirement to maintain electroneutrality during transepithelial salt transport, removal of either Na^+ or Cl^- results in the cessation of absorptive transport in sweat duct) caused a significant increase in cytosolic pH. These results were shown to be consistent with the presence of a basolateral NHE because not only did the NHE inhibitor EIPA not have any effect on pH_i when applied to the apical surface of the duct but also the presence of an apical NHE would have acidified (not alkalinizes) the cytosol. These observations suggest that changes in cytosolic Na^+ induced by ENaC activity in the apical membrane alter the cytosolic pH by altering the Na^+ gradient for NHE activity in the basolateral membrane. Since the cytosolic pH has corresponding effects on CFTR activity as on ENaC, we surmise that transport activity may involve a negative feedback control on apical salt influx as a function of cytosolic pH, which stabilizes cytosolic salt content to match the capacity of the Na^+/K^+ pump.

Conclusions

Cytosolic, but not luminal, pH may participate in regulating ENaC activity, which appears to be independent of CFTR and phosphorylation. ENaC rundown following CFTR deactivation could be acutely reversed by raising cytosolic pH. Changes in cytosolic Na^+ as a function of ENaC activity in the apical membrane could alter cytosolic pH by affecting NHE function in the basolateral membrane. Such modulation of cytosolic pH by changing cellular Na^+ concentration may serve in negative feedback control of salt influx by regulating the activities of the anion and cation ion channels (CFTR and ENaC) for salt transport across the apical membrane. Thus, cytoplasmic

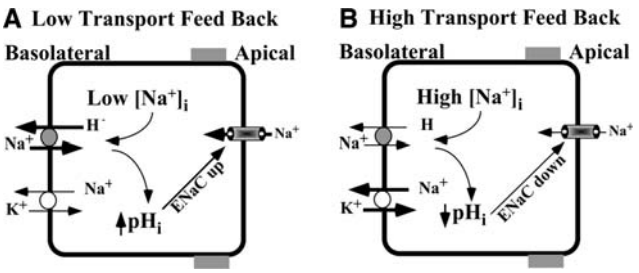


Fig. 10 A model depicting the role of cytosolic Na^+ and NHE in the feedback regulation of ENaC and CFTR in the human sweat duct. The model illustrates that as intracellular Na^+ concentration goes up as a function of ENaC activity, the chemical driving force for proton extrusion by the NHE in the basolateral membrane falls and allows cytosolic acidification that would negatively feed back and inhibit both ENaC and CFTR activity and limit salt entry across the apical membrane (**b**). The feedback inhibition of salt entry would relax as intracellular Na^+ is extruded by the Na^+/K^+ pump in the basolateral membrane (**a**). Preventing salt influx in excess of pump capacity would protect the cell from disruptive changes in cell volume during transepithelial salt absorption

pH regulation of epithelial salt absorption may imply a role in coordinating the apical transport activity with that of active transport in the basolateral membrane (Fig. 10).

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