Pflügers Arch - Eur J Physiol (2000) 441:341–350
DOI 10.1007/s004240000430

ORIGINAL ARTICLE

Angelos-Aristeidis Konstas · Dimitrios Mavrelos
Christoph Korbmacher

Conservation of pH sensitivity in the epithelial sodium channel (ENaC) with Liddle's syndrome mutation

Received: 14 June 2000 / Received after revision: 1 August 2000 / Accepted: 3 August 2000 / Published online: 25 October 2000

Abstract Gain-of-function mutations of the epithelial Na^+ channel (ENaC) cause a rare form of hereditary hypertension, Liddle's syndrome. How these mutations lead to increased channel activity is not yet fully understood. Since wild-type ENaC (wt-ENaC) is highly pH-sensitive, we wondered whether an altered pH-sensitivity of ENaC might contribute to the hyperactivity of ENaC with Liddle's syndrome mutation (Liddle-ENaC). Using *Xenopus laevis* oocytes as an expression system, we compared the pH-sensitivity of wt-ENaC ($\alpha\beta\gamma$rENaC) and Liddle-ENaC ($\alpha\beta_{R564stop}\gamma$rENaC). Oocytes were assayed for an amiloride-sensitive (2 μM) inward current (ΔI_{ami}) at –60 mV holding potential and cytosolic pH was altered by changing the extracellular pH in the presence of 60 mM sodium acetate. Alternatively, cytosolic acidification was achieved by proton loading the cells using a proton-coupled oligopeptide transporter (PepT-1) co-expressed in the oocytes together with ENaC. Cytosolic but not extracellular acidification substantially reduced ΔI_{ami} while cytosolic alkalinisation had a stimulatory effect. This pH-sensitivity was largely preserved in oocytes expressing Liddle-ENaC. The inhibition of wt-ENaC and Liddle-ENaC by cytosolic acidification was independent of so-called sodium-feedback inhibition, since it was not associated with a concomitant increase in intracellular Na^+ concentration estimated from the reversal potential of ΔI_{ami}. In addition C-terminal deletions in the α or γ subunits or in all three subunits of ENaC did not abolish the inhibitory effect of cytosolic acidification. We conclude that ENaC's pH-sensitivity is not mediated by its cytoplasmic C-termini and that an altered pH-sensitivity of ENaC does not contribute to the pathophysiology of Liddle's syndrome.

A.-A. Konstas · D. Mavrelos · C. Korbmacher (✉)
University Laboratory of Physiology, Parks Road,
Oxford OX1 3PT, UK
e-mail: christoph.korbmacher@physiol.ox.ac.uk
Tel.: +44-1865-282175,
Fax: +44-1865-282174 or +44-1865-272488

Keywords Amiloride · Cytosolic pH · Epithelial sodium channel (ENaC) · Na^+ transport · Proton-coupled oligopeptide transporter (PepT-1) · *Xenopus laevis* oocyte

Introduction

The epithelial sodium channel (ENaC) is the rate-limiting step for sodium absorption in the renal collecting duct and in other sodium-absorbing epithelia [17, 19]. Its function and appropriate regulation are critically important for the maintenance of body sodium balance and consequently for the regulation of arterial blood pressure. Direct evidence that molecular dysfunction of ENaC affects blood pressure regulation has come from the analysis of two human genetic diseases, Liddle's syndrome and pseudohypoaldosteronism type 1 (PHA-1). Liddle's syndrome is caused by gain-of-function mutations of ENaC which result in increased sodium absorption and an early onset of severe arterial hypertension [35], while loss-of-function mutations of ENaC cause urinary sodium loss and low blood pressure in patients with PHA-1 [7]. These findings demonstrate that the appropriate molecular function of ENaC is important for long-term control of blood pressure.

The mutation identified in the affected patients of the original Liddle's syndrome kindred is a C→T substitution that results in the introduction of a stop codon at Arg-564 of the β subunit ($\beta_{R564stop}$). This mutation leaves the second transmembrane domain intact but removes most of the downstream cytoplasmic C-terminal portion of the protein [35]. Meanwhile, a number of additional pedigrees with Liddle's syndrome have been studied, and all the mutations map to a very specific part of the protein, namely the proline-rich PXY motif in the C-terminus of either the β or the γ subunit [29]. The amiloride-sensitive Na^+ whole-cell currents in *Xenopus laevis* oocytes expressing ENaC with Liddle's syndrome mutation are considerably larger than the currents observed in oocytes expressing wild-type ENaC, consistent with a gain-

of-function mutation causing increased renal sodium reabsorption in vivo [30, 31].

How the gain-of-function mutations cause increased ENaC activity in Liddle's syndrome is not yet fully understood. While Liddle's syndrome mutations do not affect the single-channel conductance of ENaC [30, 36], they have been reported to increase both the number of channels expressed at the cell surface (*N*) [36] and the open probability (P_o) of the expressed channels [14]. Recent evidence suggests that ENaC density is regulated by modulating the rate of channel retrieval from the plasma membrane and the rate of degradation of the channel protein [37]. Using a yeast-two hybrid screen and in vitro binding studies, the ubiquitin protein ligase Nedd4 was identified as a binding partner of ENaC with Nedd4-WW domains binding to the PXY motifs of the channel [38]. Moreover, it was shown that the α and γ subunits of ENaC are ubiquitinated and are thereby targeted for degradation [39]. Nedd4 and channel ubiquitination are likely to regulate ENaC stability at the cell surface, and in Liddle's syndrome defective binding of mutant ENaC to Nedd4 is thought to be responsible for reduced channel retrieval. The PXY motif may also serve as an internalisation signal responsible for clathrin-mediated endocytosis of the channel, and hence its deletion/mutation in Liddle's syndrome may lead to an accumulation of channels at the cell surface due to defective internalisation [33]. Taken together these findings indicate that defective regulation of the surface expression of ENaC is likely to play a major role in the pathophysiology of Liddle's syndrome. On the other hand, it has been demonstrated that the number of channels expressed at the cell surface cannot completely account for the increase in macroscopic current, and that a change in channel gating leading to a significantly higher P_o is also an important factor in increasing overall channel activity [14]. The underlying mechanisms responsible for this increased P_o of Liddle's syndrome ENaC are not yet known.

In native tissues or cultured cells, single-channel patch-clamp recordings have revealed that ENaC P_o is highly variable, ranging from 0.05 to 0.9 with an average value of about 0.5 in the cortical collecting duct (CCD) of salt-depleted rats with high plasma aldosterone levels [28]. A large number of factors have been reported to affect the P_o of ENaC [17]. One well recognised factor is pH, which has been shown to influence amiloride-sensitive sodium absorption in various epithelia [15, 16, 18, 22, 25, 27, 44]. A reduction in cytosolic pH (pH_i) reduces the Na^+ permeability of the mucosal membrane of frog skin [18, 44], toad bladder [26], and principal cells in rabbit CCD [41]. Indeed, the pH-mediated regulation of ENaC is thought to play a role in epithelial "cross-talk", to co-ordinate sodium entry and exit via the apical and basolateral membranes [18]. Patch-clamp experiments using excised inside-out patches of microdissected rat CCD have demonstrated a marked reduction of ENaC's P_o upon cytosolic acidification [27]. A recent study of *Xenopus laevis* oocytes confirmed this inhibitory effect of acidification on heterologously expressed ENaC. Furthermore, the study demonstrated that ENaC activity is affected by changes in pH_i but not by changes in extracellular pH (pH_o) [6].

Mutations of ENaC may change its pH-sensitivity and therefore may be responsible for the altered activity of mutated channels. For example, a decreased sensitivity to intracellular acidification may contribute to the increased channel activity of ENaC with Liddle's syndrome mutation. Therefore, the aim of the present study was to investigate whether the pH-sensitivity of ENaC is affected in Liddle's syndrome. Using the *Xenopus laevis* oocyte expression system, we compared the pH-sensitivity of wild-type ENaC with that of mutated ENaCs. To confirm the pH-sensitivity of wild-type ENaC we used two different methods of manipulating the pH_i of the oocytes, the well-established acetate technique [6, 8, 42] and a novel approach involving co-expression of the proton-coupled oligopeptide transporter PepT-1 [12]. In addition to the original Liddle's syndrome $\beta_{R564stop}$ mutation we studied ENaC mutants with corresponding C-terminal truncations in the α and γ subunits to explore the possible contribution of their C-termini to ENaC's pH-sensitivity.

Materials and methods

cRNA preparation

Plasmids containing cDNAs encoding the three subunits of wild-type (wt) rat ENaC (α, β, γrENaC) [5] or encoding the truncated rENaC subunits $\alpha_{P646stop}$, $\beta_{R564stop}$, and $\gamma_{F606stop}$ [31] were cloned into the pSD5 vector and were a generous gift from Professor Bernard C. Rossier and Professor Laurent Schild (Lausanne, Switzerland). The vectors containing the wt and the truncated α and β subunits were linearised with *Bgl*II, the vectors containing the wt and the truncated γ subunits were linearised with *Pvu*II and *Not*I respectively. The linearised plasmids were used as templates for cRNA synthesis using either T7 or SP6 RNA polymerases (mMessage mMachine, Ambion, Austin, Tex., USA). Using the cDNA clone of the rabbit intestinal proton-coupled peptide transporter (PepT-1) [12] cRNA was prepared as described previously [24] and was kindly provided by Dr. Corinna Swords (Department of Human Anatomy and Genetics, Oxford University, Oxford UK).

Isolation of oocytes and injection of cRNA

The experimental procedures for obtaining and handling the oocytes were as described previously [3]. Oocytes were isolated from ovarian lobes of adult *Xenopus laevis* by enzymatic digestion using type-V collagenase from *Cl. histolyticum* with an activity of 490 units/mg (Sigma, Poole, UK). Oocytes were incubated at 18–20°C for 4–6 h with 1 mg/ml collagenase. Then the oocytes were rinsed twice with Ca^{2+}-free NaCl solution to remove the collagenase and debris and twice with modified Barth's saline (MBS) containing (in mM): 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.3 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 15 HEPES adjusted to pH 7.6 with Tris. Oocytes were stored at 18–20°C in MBS supplemented with 10 units/ml sodium penicillin and 10 µg/ml streptomycin sulphate to prevent bacterial overgrowth. Defolliculated stage V-VI oocytes were injected with one of the following cRNA combinations (1 ng of each subunit): (1) αβγ rENaC (wild-type ENaC), (2) $\alpha\beta_{R564stop}\gamma$ rENaC (Liddle's syndrome mutant), (3) $\alpha_{P646stop}\beta\gamma$ rENaC, (4) $\alpha\beta\gamma_{F606stop}$ rENaC, and (5) $\alpha_{P646stop}\beta_{R564stop}\gamma_{F606stop}$ rENaC. cRNAs were dissolved in RNase-free water and the total volume injected

was 50.6 nl (Nanoject automatic injector, Drummond, Broomall, Pa., USA). Oocytes were studied 16–24 h after injection using the two-electrode voltage-clamp technique. In one set of experiments oocytes were first injected with 23 ng PepT-1 cRNA, re-injected 3 days later with αβγ rENaC (1 ng of each subunit), and measured the following day.

Two-electrode voltage-clamp experiments

Oocytes were placed in a small experimental chamber of about 200 µl volume and were constantly superfused with solutions at a rate of 3–12 ml/min. Experiments were performed at room temperature. Both intracellular electrodes were filled with 3 M KCl and had resistances of 0.2–3.0 MΩ and 0.2–1.5 MΩ for the voltage and current electrodes, respectively. The transmembrane potential was measured differentially between the intracellular voltage electrode and a second bath electrode positioned close to the oocyte. The bath electrodes consisted of Ag-AgCl pellets. After impalement with the two intracellular electrodes, oocytes were held continuously at a holding potential of –60 mV (unless stated otherwise). *I–V* plots were obtained from voltage-step protocols using consecutive 500-ms step changes of the clamp potential from –60 mV to –120 mV up to +60 mV in 20-mV increments. For *I–V* plots the average current values reached during the last 100 ms of the voltage steps were used. Amiloride-sensitive whole-cell current traces were obtained by subtracting the whole-cell currents in the presence of amiloride (2 µM) from the corresponding whole-cell currents prior to the addition of amiloride. Using these subtracted *I–V* plots, the reversal potential (E_{rev}) of the amiloride-sensitive whole-cell current (ΔI_{ami}) was monitored during different phases of the experiments. The apparent intracellular Na^+ concentration ($[Na^+]_{i\text{-}app}$) was calculated from E_{rev} using the Nernst equation:

$$[Na^+]_{i\text{-}app}=[Na^+]_o\, e^{-E\,rev[(zF)/(RT)]}$$

where z is valency, F the Faraday constant, R the Gas constant, T the absolute temperature (295 K), and $[Na^+]_o$=110 mM.

The clamp potential of the two-electrode voltage-clamp amplifier (OC-725C, Warner, Conn., USA) was controlled using the program "Pulse and PulseFit" (HEKA Electronic, Lambrecht, Germany) in conjunction with an ITC-16 interface (Instrutech, Long Island, N.Y., USA). Pulsed data were filtered at 200 Hz and directly written to hard disk at a sample rate of 1 kHz. Experiments were also continuously recorded on video tape using a VR10B Digital Data Recorder (Instrutech, Long Island, N.Y., USA). The data were analysed using the Patch program (Dr. Bernd Letz, HEKA Electronic, Lambrecht, Germany). Data are given as mean values ±SEM, and significance was evaluated by the appropriate version of Student's *t*-test.

Solutions and chemicals

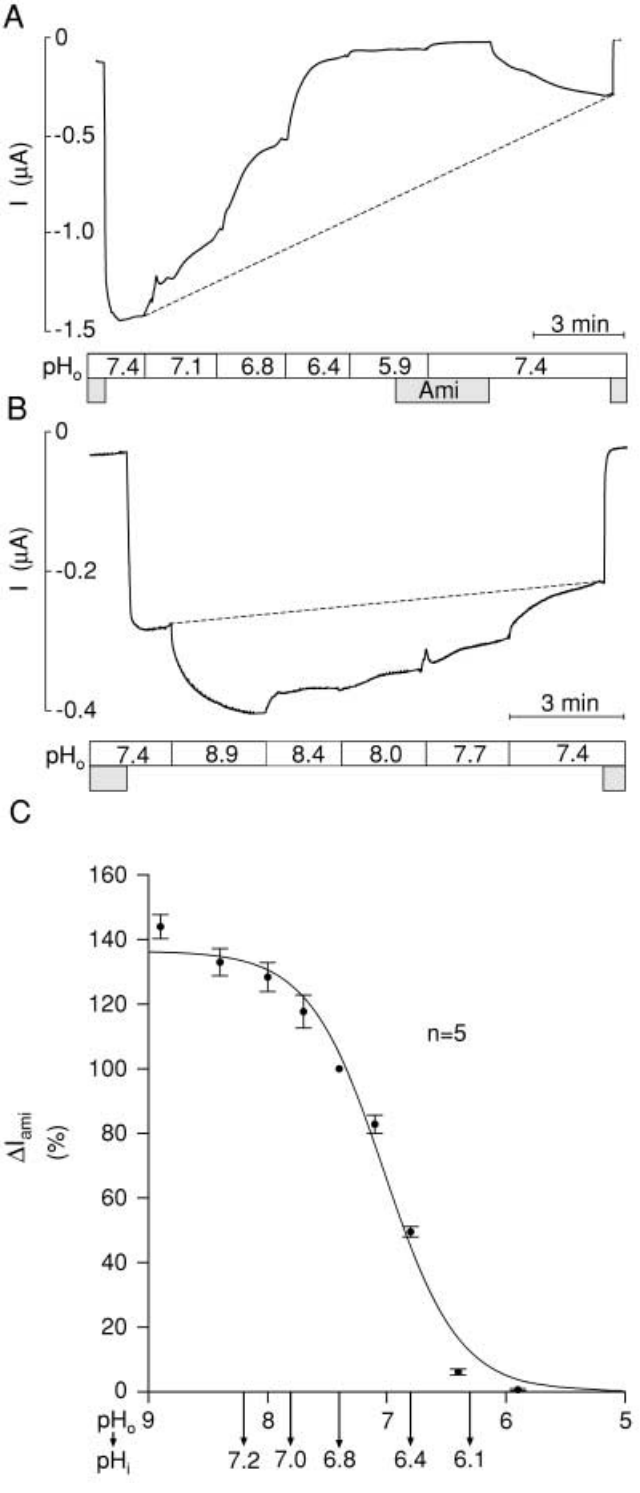
During the experiments oocytes were bathed either in NaCl solution (in mM: 95 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES) or in sodium acetate solution (in mM: 50 NaCl, 60 sodium acetate, 2 KCl, 0.2 $CaCl_2$, and 1.0 $MgCl_2$). The pH of these solutions was titrated to values ranging from 5.9 to 8.9 using HCl or Tris where appropriate. The dipeptide Leu–Leu (L–L isomer) and amiloride hydrochloride were purchased from Sigma (Poole, UK). Aqueous 1 mM amiloride stock solution was freshly prepared on the day of the experiment and was kept at 4°C in the dark. Immediately prior to use, an aliquot of the stock solution was added to the appropriate bath solution to give a final concentration of 2 µM.

Results

pH sensitivity of wild-type ENaC

In an initial set of experiments we investigated the response of wild-type ENaC currents to changes in pH_i. To modify pH_i we used a bath solution containing 60 mM sodium acetate. In its non-dissociated protonated form, acetic acid is lipophilic and can readily transfer protons across cell membranes. Thus, changing extracellular pH in the presence of acetate induces corresponding pH_i changes in *Xenopus laevis* oocytes [8, 42]. The effect of intracellular acidification was tested in experiments as shown in Fig. 1A. The whole-cell current was continuously monitored at a holding potential of –60 mV. Initially the bath solution contained 2 µM amiloride and had a pH of 7.4. Washout of amiloride resulted in an increase of the inward current revealing an amiloride-sensitive inward current (ΔI_{ami}) and thereby confirming ENaC expression. Stepwise acidification caused a stepwise reduction of the inward current. At an extracellular pH of 5.9 the inward current had declined to its initial level in the presence of amiloride and application of amiloride essentially had no additional inhibitory effect. The inhibitory effect of acidification was partially reversible upon returning bath pH to 7.4. The incomplete recovery may partly be due to spontaneous "run-down" of ENaC currents, a well-known phenomenon in oocytes which is possibly related to sodium-feedback inhibition [1, 20]. Figure 1B shows an experiment in which the effect of intracellular alkalinisation was investigated. In the presence of acetate, changing extracellular pH from pH 7.4 to 8.9 resulted in an increase of the inward current consistent with ENaC activation upon intracellular alkalinisation. A stepwise return to pH 7.4 was accompanied by a corresponding stepwise decrease of the inward current which eventually reached a level below the initial value in pH 7.4 in the absence of amiloride. Re-application of amiloride demonstrated that ΔI_{ami} had declined during the experiment, consistent with partial "run-down" of ENaC. Note that the rate of "run-down" (indicated by the dotted lines in Fig. 1) was less pronounced in oocytes subjected to alkalinisation than in oocytes subjected to acidification, averaging 14.5±6.0% (n=5) and 45.6±4.9% (n=5; P<0.05) within 8 min, respectively. Control experiments (data not shown) demonstrated that in the absence of acetate ENaC currents were essentially resistant to extracellular pH changes that are known to have little effect on pH_i of oocytes [42] which have a negligible H^+ conductance [4]. Thus, our findings are consistent with previous reports demonstrating that ENaC expressed in oocytes is sensitive to intracellular but not extracellular pH changes [6]. Figure 1C summarises the results from five acidification and five alkalinisation experiments as shown in Fig. 1A and 1B, respectively. Normalised ΔI_{ami} is plotted versus pH. In addition to extracellular pH values, estimates of pH_i are given. These estimates are based on pH_i measurements with pH-sensitive microelectrodes made from *Xenopus laevis* oocytes exposed to

extracellular pH changes in the presence of acetate under similar conditions as used in the present study [8]. Fitting the data with the Hill equation revealed a Hill coefficient of about 1.4. This suggests that more than one proton binding site may be involved in mediating the sensitivity of ENaC to pH_i. Moreover, the figure illustrates that ENaC currents have a steep pH-dependence over a physiologically relevant range.

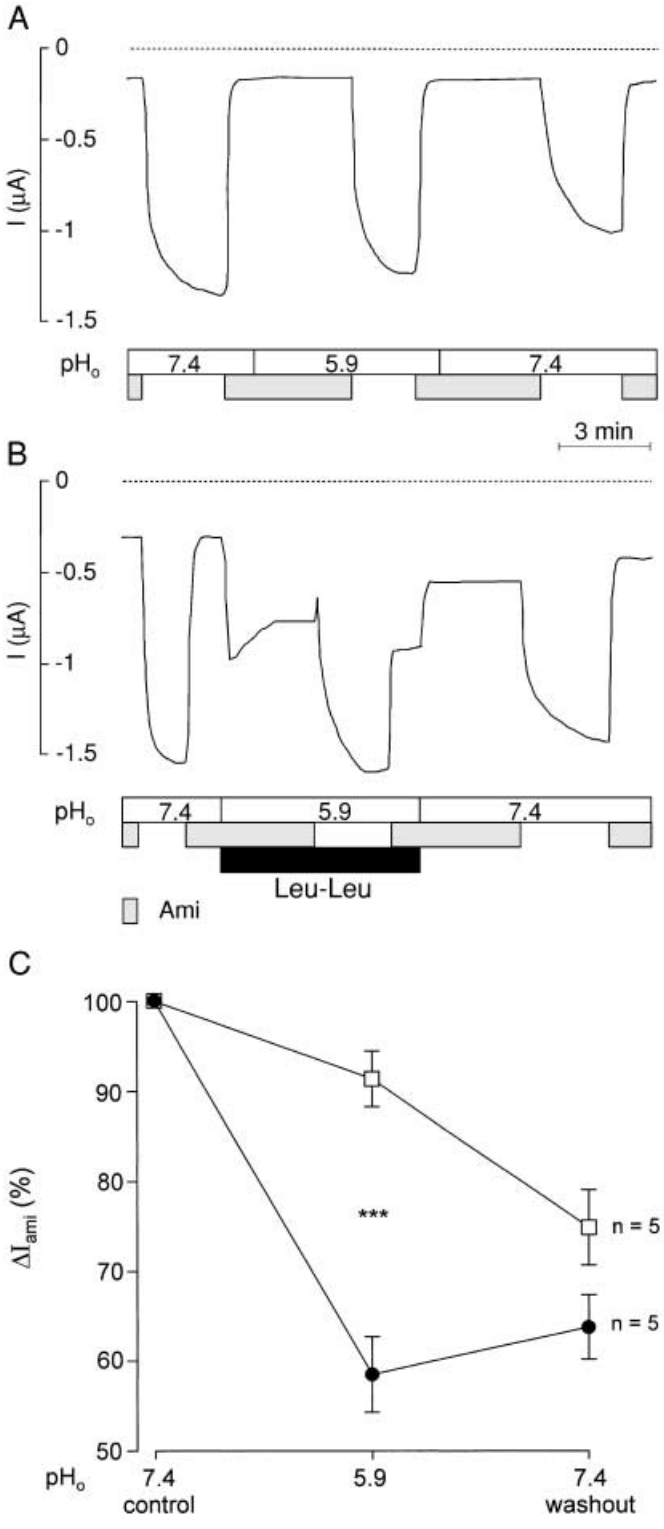


Intracellular acidification via PepT-1 also inhibits ENaC currents

To confirm that the effects observed in the presence of acetate are mediated by pH_i changes and not by non-specific effects of acetate on ENaC, we used an alternative strategy to produce an intracellular acidification. It has been shown that a substantial intracellular acidification (0.2–0.3 pH units) occurs when oocytes expressing the proton-coupled oligopeptide transporter PepT-1 are exposed to extracellular peptides in the presence of a low extracellular pH [12, 40].Therefore, PepT-1 was used as a tool to achieve intracellular acidification. In oocytes co-expressing PepT-1 and wild-type rENaC, changing extracellular pH from 7.4 to 5.9 in the absence of extracellular peptides did not change steady-state inward current (Fig. 2A). This is consistent with the reported lack of significant conductive proton "slippage" via PepT-1 in the absence of co-transported peptides [13]. The experiment shown in Fig. 2A also confirms that extracellular acidification per se does not affect ΔI_{ami} which was monitored by repeated amiloride washout and re-addition. During the course of the experiment ΔI_{ami} displayed the usual degree of run-down independent of extracellular pH changes. In contrast (Fig. 2B), changing pH_o to 5.9 while simultaneously applying the neutral dipeptide Leu–Leu (3 mM) resulted in a significant increase of the inward current by 0.60±0.14 μA (n=5; P<0.001). This is consistent with electrogenic co-transport of protons and peptide into the oocyte, resulting in intracellular acidification. Under these conditions ΔI_{ami} was reduced by 41.5±4.2% (n=5). This inhibition was significantly larger than the spontaneous run-down (Fig. 2A) which averaged 8.6±1.4% (n=5; P<0.001) in matched control experiments from the same batch of oocytes. Note that in the experiment shown in Fig. 2B ΔI_{ami} showed substantial recovery upon changing back to peptide-free bath solution with pH 7.4. However, a similar partial recovery was only

Fig. 1A–C Wild-type epithelial sodium channel (*ENaC*) is inhibited by acidification and stimulated by alkalinisation. Whole-cell currents (*I*) were recorded at a holding potential of –60 mV from oocytes expressing wild-type ENaC. To modify cytosolic pH a bath solution containing 60 mM sodium acetate was used and extracellular pH (pH_o) was varied as indicated. The amiloride-sensitive current was assessed by periodic application of 2 μM amiloride (*Ami*). Representative experiments are shown illustrating the effects of stepwise acidification (**A**) and alkalinisation (**B**). The *dotted lines* indicate the estimated rate of spontaneous run-down of ENaC activity. In **C** the results of five acidification and five alkalinisation experiments as shown in **A** and **B** are summarised. Currents were normalised to ΔI_{ami} measured at pH_o 7.4. Spontaneous run-down of ENaC (as illustrated by the *dotted lines*) was taken into account for estimates of ΔI_{ami} at different extracellular pH (pH_o). Average ΔI_{ami} (%) values (mean ±SEM) are plotted versus pH_o and estimated values for cytosolic pH (pH_i) are also given. Data were fitted using the following equation: $\Delta I_{ami}=(\Delta I_{ami})_{max}/\{1+([H^+]_o/K)^n\}$ where $[H^+]_o$ is the extracellular proton concentration, K is the constant for half-maximal inhibition and n is the Hill coefficient. The fit revealed a K of 0.095 μM and an n of 1.38

observed in three out of five experiments. This is probably due to delayed pH_i recovery and the confounding effect of ENaC run-down. Results are summarised in Fig. 2C. Taken together theses co-expression studies confirm the inhibitory effect of intracellular acidification on ENaC.



Inhibition by intracellular acidification is preserved in the Liddle's syndrome mutant of ENaC

We investigated whether rENaC containing a $\beta_{R564stop}$ subunit is similarly affected by intracellular acidification as the wild-type channel. The $\beta_{R564stop}$ mutation results in a C-terminally truncated β subunit and corresponds to the mutation causing Liddle's disease in the original Liddle's kindred [30]. Intracellular acidification was achieved by changing the pH of the acetate bath solution from 7.4 to 6.4, which corresponds to an pH_i decrease from about 6.8 to 6.2 [8]. Periodic washout and re-application of 2 µM amiloride was used to monitor the effect of intracellular acidification on ΔI_{ami}. The current trace shown in Fig. 3A was obtained from an oocyte expressing wild-type αβγ rENaC while Fig. 3B shows a similar recording from an oocyte expressing the Liddle's syndrome mutant (α, $\beta_{R564stop}$, γ rENaC). Initial ΔI_{ami} was significantly larger in oocytes injected with Liddle's syndrome mutant than in oocytes from the same batch injected with wild-type ENaC, averaging 14.0±1.7 µA/oocyte (n=7) and 1.8±0.2 µA/oocyte (n=28; P<0.001), respectively, consistent with a hyperactive mutant channel [30]. Changing bath pH to 6.4 caused a rapid and substantial decline of ΔI_{ami} in oocytes expressing wild-type ENaC (Fig. 3A) and also in oocytes expressing the Liddle's syndrome mutant (Fig. 3B). The inhibition of ΔI_{ami} caused by acidification was partially reversible upon returning to an extracellular pH of 7.4. Figure 3C and D summarise results from similar experiments as shown in Fig. 3A and B, respectively. On average, intracellular acidification inhibited ΔI_{ami} by 92±3% (n=28; P<0.001) in oocytes expressing wild-type ENaC and by 82±2% (n=7, P<0.001) in oocytes expressing Liddle's syndrome mutant. The minor difference in the apparent degree of inhibition (92% versus 82%) is probably due to a lower rate of spontaneous run-down of the mutant channel [20], consistent with its more complete recovery upon returning to a bath pH of 7.4 (Fig. 3B). The main conclusion from these experiments is that the inhibitory effect of intracellular acidification is essentially preserved in Liddle's syndrome mutant.

Fig. 2A–C Intracellular acidification by proton-coupled oligopeptide transporter (*PepT-1*) reduces ΔI_{ami}. Oocytes co-expressing wild-type ENaC and the proton-coupled oligopeptide transporter (*PepT-1*) were continuously clamped at –100 mV. Bath pH (pH_o) was changed from 7.4 to 5.9 in the absence (**A**) or presence (**B**) of extracellular neutral dipeptide Leu–Leu (3 mM), a known substrate of PepT-1. ΔI_{ami} was monitored by repeated applications of 2 µM amiloride (*Ami*). Note the increase of inward current upon application of Leu–Leu consistent with proton-coupled oligopeptide transport causing intracellular acidification. **C** Summary of data from five similar experiments as shown in **A** and **B**. *Open squares* and *filled circles* represent mean data from experiments with and without Leu–Leu, respectively. ΔI_{ami} was normalised to its value at the beginning of the experiment in pH_o 7.4. *Vertical error bars* indicate SEM values. At pH_o 5.9 the average ΔI_{ami} was significantly reduced in the presence of Leu–Leu as compared to ΔI_{ami} in the absence of Leu–Leu (***P<0.001)

C-terminally truncated ENaC subunits do not affect the responsiveness to intracellular acidification

In addition to the classic Liddle's syndrome mutant with a C-terminally truncated β subunit, we also investigated ENaCs lacking the corresponding parts of the C-termini of the α or γ subunit. As shown in Fig. 4 expression of $\alpha_{P646stop}$,β,γ rENaC or of α,β,$\gamma_{F606stop}$ rENaC produced average ΔI_{ami} values similar to those achieved with expression of the Liddle's syndrome mutant and significantly larger than those observed with wild-type ENaC. Co-expression of all three C-terminally truncated ENaC subunits ($\alpha_{P646stop}$,$\beta_{R564stop}$,$\gamma_{F606stop}$) resulted in an average ΔI_{ami} which was about 27 times larger than that observed in oocytes expressing wild-type ENaC (Fig. 4). This suggests a synergism of the three C-terminal truncations in activating ENaC [9].

Figure 5 summarises the results of experiments in which the effect of intracellular acidification was tested on ENaCs with C-terminal truncations in either the α subunit, the γ subunit, or in all three subunits using a similar experimental protocol as shown in Fig. 3. In all cases the inhibitory effect of intracellular acidification was preserved, averaging 92±2% (n=14; P<0.001), 95±1% (n=7; P<0.001), and 87±4% (n=7; P<0.001), respectively. These findings suggest that the C-termini are not essential for mediating the inhibitory effect of intracellular acidification.

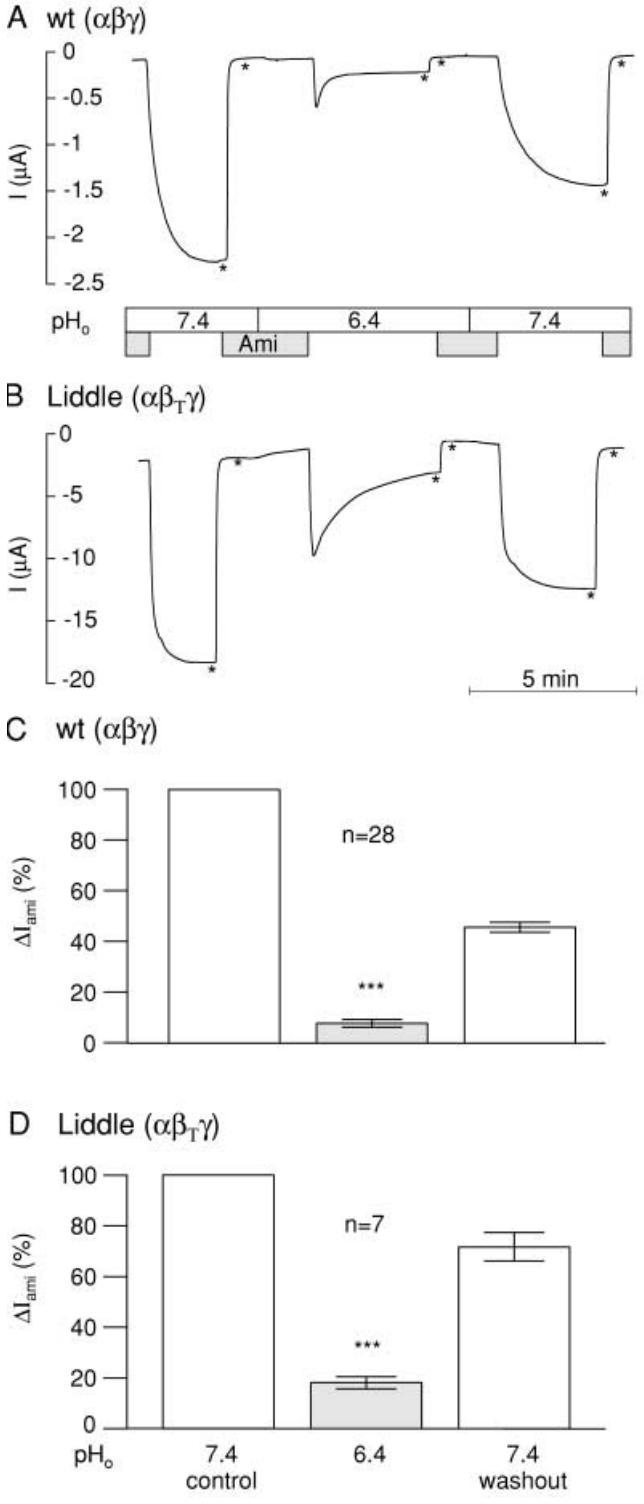


Fig. 3A–D Inhibition by intracellular acidification is preserved in Liddle's syndrome mutant ENaC. Representative whole-cell current (*I*) recordings are shown from oocytes clamped at –60 mV holding potential expressing **A** wild-type ENaC [*wt(αβγ)*] or **B** Liddle's syndrome mutant ENaC [*Liddle(αβ$_T$γ)*]. The pH of the sodium acetate bath (*pH$_o$*) was changed from 7.4 to 6.4 and back to 7.4, as indicated. To monitor ΔI_{ami} 2 μM amiloride (*Ami*) was repeatedly applied. Voltage-step protocols (see Materials and methods) were performed at times indicated by *asterisks* (*) but the resulting current responses were omitted from the trace for clarity. In **C** and **D** the average inhibition of wild-type ENaC [*wt(αβγ)*] and Liddle's syndrome mutant ENaC [*Liddle(αβ$_T$γ)*] by intracellular acidification is shown, respectively. *Bar diagrams* summarise data from experiments as shown in **A** and **B**. For each experiment ΔI_{ami} was normalised to its value in pH$_o$ 7.4 at the beginning of the experiment. In both groups of oocytes ΔI_{ami} (%) was significantly (***P<0.001) reduced in pH$_o$ 6.4

The inhibitory effect of intracellular acidification is not caused by changes of $[Na^+]_i$

There is evidence that an increase in $[Na^+]_i$ may inhibit ENaC via so-called feedback inhibition [2, 10, 17]. The mechanisms involved in this feedback inhibition are not fully understood and may involve soluble cytosolic factors and a putative intracellular Na^+ receptor [1, 21]. A defect in feedback inhibition may be the reason for the hyperactivity of the Liddle's syndrome mutant [20]. A decrease in pH_i may cause a rise of $[Na^+]_i$ via activation of the Na^+/H^+ exchange mechanism. To investigate the possibility that the observed inhibitory effect of intracellular acidification is mediated by an increase in $[Na^+]_i$ we performed voltage-step protocols in the presence and absence of amiloride to determine the *I–V* relationship of ΔI_{ami} and to estimate the apparent intracellular sodium concentration ($[Na^+]_{i\text{-}app}$) from its reversal potential (E_{rev}) during the different phases of experiments (see Materials and methods). In 17 oocytes expressing wild-

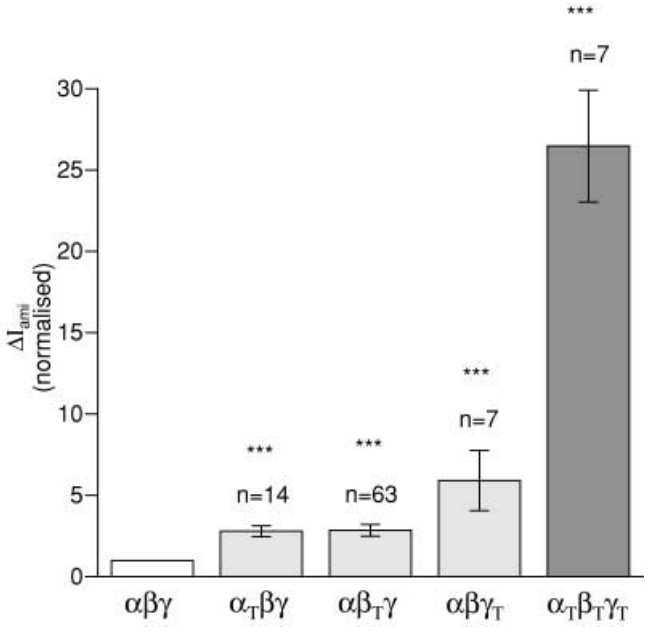


Fig. 4 C-terminal truncations of ENaC subunits increase ΔI_{ami}. In oocytes expressing $\alpha_{P646stop}\beta\gamma$rENaC ($\alpha_T\beta\gamma$), $\alpha\beta_{R564stop}\gamma$rENaC ($\alpha\beta_T\gamma$), $\alpha\beta\gamma_{F606stop}$rENaC ($\alpha\beta\gamma_T$), or $\alpha_{P646stop}\beta_{R564stop}\gamma_{F606stop}$ rENaC ($\alpha_T\beta_T\gamma_T$), ΔI_{ami} was determined by application of 2 µM amiloride at a holding potential of –60 mV in the presence of pH_o 7.4. *Bar diagrams* represent currents normalised to ΔI_{ami} measured from oocytes (same batch) expressing wild-type αβγrENaC ($\alpha\beta\gamma$). *Asterisks* (***) indicate average currents that are significantly (P<0.001) different from currents in wild-type αβγrENaC-expressing oocytes

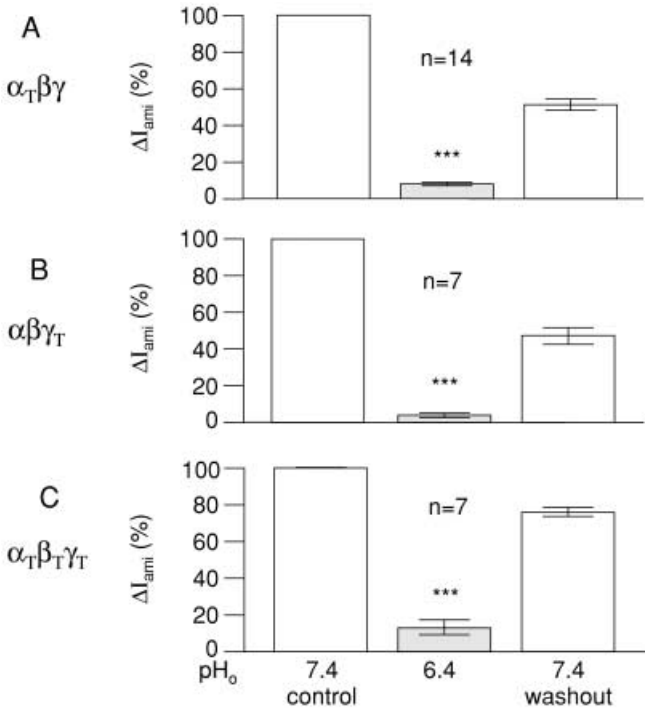


Fig. 5A–C Inhibition by intracellular acidification is preserved in ENaC with C-terminally truncated subunits. Using the same experimental protocol as shown in Fig. 3 the effect of intracellular acidification on ΔI_{ami} was tested in oocytes expressing **A** $\alpha_{P646stop}\beta\gamma$rENaC ($\alpha_T\beta\gamma$), **B** $\alpha\beta\gamma_{F606stop}$rENaC ($\alpha\beta\gamma_T$), or **C** $\alpha_{P646stop}\beta_{R564stop}\gamma_{F606stop}$ rENaC ($\alpha_T\beta_T\gamma_T$) as described in Fig. 4. For each experiment ΔI_{ami} was normalised to its value in pH_o 7.4 at the beginning of the experiment. In all three groups of oocytes ΔI_{ami} (%) was significantly (***P<0.001) reduced in pH_o 6.4

type ENaC the $[Na^+]_{i\text{-}app}$ averaged 101±1 mM, 99±3 mM, and 103±2 mM in the presence of pH 7.4, pH 6.4, and pH 7.4 washout, respectively. The corresponding values in seven oocytes expressing ENaC with Liddle's syndrome mutation were 144±8 mM, 132±7 mM, and 170±9 mM, respectively. These results demonstrate that the inhibitory effect of intracellular acidification is not mediated by an increase in $[Na^+]_{i\text{-}app}$ which suggests that feedback inhibition by intracellular Na^+ is not involved unless intracellular acidification largely increases the sensitivity of the Na^+ feedback mechanism.

Effect of intracellular alkalinisation on wild-type ENaC and Liddle's syndrome mutant

Experiments as shown in Fig. 6 were performed to test the effect of intracellular alkalinisation on ΔI_{ami} of oocytes expressing wild-type ENaC (Fig. 6A) or Liddle's syndrome mutant (Fig. 6B). After washing out amiloride (2 µM) to assess initial ΔI_{ami} in the presence of pH 7.4, the extracellular pH was changed to 8.9 in the presence of acetate to cause an intracellular alkalinisation. This caused an increase of the inward current and once a plateau was reached amiloride was re-applied to quantify ΔI_{ami} after stimulation by intracellular alkalinisation. On average, intracellular alkalinisation increased ΔI_{ami} by

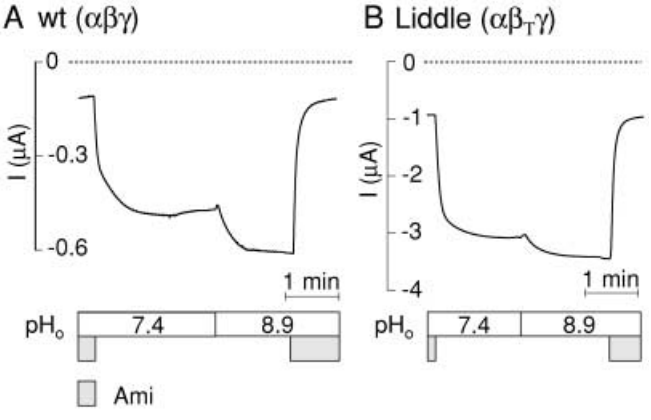


Fig. 6A, B Wild-type ENaC and ENaC with Liddle's syndrome mutation are stimulated by intracellular alkalinisation. Representative whole-cell current (I) recordings are shown from oocytes clamped at –60 mV holding potential expressing **A** wild-type ENaC [$wt(\alpha\beta\gamma)$] or **B** Liddle's syndrome mutant ENaC [$Liddle(\alpha\beta_T\gamma)$]. The pH of the sodium acetate bath (pH_o) was changed from 7.4 to 8.9 in order to assess the effect of intracellular alkalinisation. To monitor ΔI_{ami} 2 µM amiloride (*Ami*) was applied as indicated

28±5% (n=10; P<0.001) in oocytes expressing wild-type ENaC and by 15±4% (n=10; P<0.001) in oocytes expressing Liddle's syndrome mutant. Control experiments in which extracellular pH was increased from 7.4 to 8.9 in the absence of acetate in the bath solution confirmed that extracellular pH changes per se have no significant effect on ΔI_{ami}. Interestingly, the average stimulatory effect of intracellular alkalinisation was smaller in the Liddle's syndrome mutant compared to wild-type ENaC (P<0.05). This cannot be explained by a reduced run-down of the channels with Liddle's syndrome mutation, since a lack of run-down would tend to overestimate the stimulatory effect of alkalinisation in the Liddle's syndrome mutant. Nevertheless, the results demonstrate that qualitatively the stimulatory effect of intracellular alkalinisation is preserved in ENaC with Liddle's syndrome mutation.

Discussion

This study confirms that cytosolic acidification inhibits ENaC activity regardless of how the cytosolic acidification is achieved. It also demonstrates that ENaC's pH-sensitivity is steep within a physiologically relevant range of pH_i. Moreover, inhibition of ENaC by cytosolic acidification was found to be independent of so-called sodium-feedback inhibition, since it is not associated with a concomitant increase in $[Na^+]_i$. The main finding of the study is that the inhibitory effect of cytosolic acidification is preserved in ENaC with Liddle's syndrome mutation ($\beta_{R564stop}$). Our results are in good agreement with preliminary findings mentioned in a recently published study of cut-open oocytes which also found that pH-sensitivity was preserved in Liddle's syndrome mutation [1]. In addition, our study demonstrates that ENaC with C-terminal deletions in the α or γ subunits, or ENaC with C-terminal deletions in all three subunits remained pH-sensitive.

A lowering of pH_i has been shown to markedly reduce currents through a variety of ion channels. The mechanism by which lowering the pH modulates ion channels may involve direct interaction of protons with the channel pore or may be mediated by proton-induced conformational changes of the pore-forming membrane proteins. Alternatively, pH-sensitive regulatory proteins of the channel may mediate the effect. Finally, heteromeric channels may exist, in which one of the subunits acts as a pH-sensor as recently shown for the inwardly rectifying potassium channel Kir5.1/Kir4.1 [43].

Single-channel recordings from the microdissected collecting duct suggest that the rapid and reversible inhibitory effect of cytosolic acidification on ENaC activity is mainly mediated by a reduction of P_o and does not involve channel retrieval from the membrane. In microdissected rat CCD, lowering pH_i from 7.4 to 6.4 reduced P_o of ENaC by more than 80% from 0.41 to 0.05 [27]. The magnitude of this inhibitory effect is consistent with the degree of inhibition observed in the present study. Thus, a reduction of P_o appears to be sufficient to explain the reduction of the amiloride-sensitive whole-cell currents in ENaC-expressing oocytes upon intracellular acidification. Moreover, the rapid onset of the inhibitory action of cytosolic acidification is consistent with an effect on P_o rather than an effect mediated by channel retrieval.

The inhibitory effect of intracellular acidification was partially but not fully reversible upon changing back to the initial bath pH of 7.4. As discussed before the lack of complete recovery back to initial ΔI_{ami} values may be due to concomitant spontaneous run-down of channel activity during the course of the experiment. However, the different degree of apparent run-down in oocytes exposed to acidification as compared to oocytes exposed to alkalinisation suggests that the inhibitory effect of intracellular acidification per se may not be fully reversible. This is consistent with the observation that complete closure of ROMK channels at low pH_i for more than 45 s seems to interfere with reactivation upon return to a pH 7.4 bath solution. The reason for this irreversible inhibition is not yet understood [8].

The steep pH-dependence of ENaC activity with a Hill coefficient of 1.4 suggests that more than one proton binding site is involved in ENaC inhibition. These findings are similar to those reported for the pH-sensitivity of ROMK [8]. Histidine is generally a good candidate for pH-sensitive residues. The original Liddle's syndrome mutation ($\beta_{R564stop}$) deletes one histidine residue (His 583) by causing a C-terminal truncation. However, since pH-sensitivity is preserved in ENaC with Liddle's syndrome mutation, a major role for this histidine residue in pH-sensitivity can be ruled out. Similarly, our results rule out a pH-sensing role for histidine residue 648 in the α subunit. Moreover, the finding that the C-termini of all three subunits can be deleted without a major effect on ENaC's pH-sensitivity suggests that putative phosphorylation sites [34] and binding sites for regulatory proteins in the C-termini are irrelevant for ENaC's pH-sensitivity.

In particular, our results demonstrate that a regulatory interaction of Nedd4 at the PXY motifs of the three subunits [31, 38] is not required to mediate the pH-sensitivity of the channel. This is consistent with our conclusion that sodium-feedback inhibition, which is thought to involve Nedd4 [10] and to be altered in Liddle's syndrome [20], does not contribute to the pH effect. A contribution of sodium-feedback inhibition is unlikely since in our experiments the intracellular Na^+ concentration of the oocytes was already very high at the beginning of the experiments and did not further increase upon intracellular acidification. The $[Na^+]_{i\text{-}app}$ of about 100 mM or more estimated in our present study is surprisingly high but consistent with previously reported findings [1, 20]. Indeed, it is well known that ENaC-expressing oocytes kept in the presence of a high extracellular Na^+ concentration will become severely sodium loaded [5]. Moreover, it should be pointed out that the values calculated from the E_{rev} of ΔI_{ami} probably reflect the Na^+ concentra-

tion in a cytosolic compartment close to the plasma membrane and not necessarily the bulk Na^+ concentration inside the cell. Hence, during the voltage-clamp experiments, entry of Na^+ through ENaC influences the apparent Na^+ concentration in the unstirred compartment close to the membrane and this may result in an overestimation of $[Na^+]_{i\text{-}app}$. Nevertheless, we can probably assume that the intracellular sodium concentration in the oocytes under investigation was sufficiently high so that sodium-feedback inhibition had occurred and had essentially been completed prior to the start of the pH experiments. Interestingly, the finding that ΔI_{ami} is significantly smaller in oocytes expressing wild-type ENaC than in oocytes expressing ENaC with Liddle's syndrome mutation has been attributed to the lack of sodium-feedback inhibition of the mutated channel [20]. Since the wild-type and the mutated channel are similarly affected by cytosolic acidification, the pH sensitivity of ENaC seems to be independent of sodium-feedback inhibition of the channels. Our data are also in good agreement with a recent study of cut-open oocytes, in which dialysis of the oocytes resulted in a loss of sodium-feedback inhibition while the pH sensitivity of ENaC was preserved [1]. Thus, while sodium-feedback inhibition is likely to involve a soluble cytosolic factor or sodium receptor [21], the pH-mediated inhibition is probably a direct effect on the channel protein.

The lack of ENaC sensitivity to extracellular pH changes is in good agreement with previous studies [6]. This indicates that the six, seven, and eight histidine residues in the extracellular loops of the α, β, and γ-subunit, respectively, do not contribute to the pH sensitivity of ENaC. The high sensitivity of ENaC to internal but not to external pH is reminiscent of the properties of ROMK [42]. Recently it was discovered that a single lysine residue N-terminal to the first hydrophobic segment (M1) is necessary for conferring the pH_i sensitivity of ROMK [8, 11]. Two arginine residues in the distant N- and C-termini are located in close spatial proximity to the lysine, shifting its pK_a into the neutral pH range. Structural disturbance of this triad as a result of a number of point mutations found in patients with antenatal Bartter's syndrome shifts the pK_a of the lysine residue off the neutral pH range and results in channels being permanently inactivated under physiological conditions [32]. Thus, the tertiary structure of ENaC is likely to be an important factor in determining which residues may contribute to its pH sensitivity.

In conclusion, our studies make it unlikely that the C-termini of the ENaC subunits contain an important pH-sensing amino acid residue and suggest that the main pH-sensitive sites are located in the cytoplasmic N-terminus of ENaC. The histidine residues in the N-terminus are possible candidates for mediating the channel's pH-sensitivity. Importantly, our present study demonstrates that a reduced sensitivity to cytosolic acidification cannot provide an explanation for the increased ENaC activity in Liddle's syndrome. In fact, the preserved inhibitory effect of cytosolic acidification and the slightly reduced activation of the mutated channels by cytosolic alkalinisation strongly argue against a role for ENaC's pH-sensitivity in the pathophysiology of Liddle's syndrome.

Acknowledgements We thank Dr. Stephen J. Tucker for helpful discussion and Dr. C. A. Richard Boyd for his support and advice regarding the PepT-1 experiments. Supported by the Wellcome Trust and the National Kidney Research Fund (NKRF). Part of this work was previously published in abstract form [23].

References

1. Abriel H, Horisberger JD (1999) Feedback inhibition of rat amiloride-sensitive epithelial sodium channels expressed in *Xenopus laevis* oocytes. J Physiol (Lond) 516:31–43
2. Awayda MS (1999) Regulation of the epithelial Na^+ channel by intracellular Na^+. Am J Physiol 277:C216–C224
3. Bielfeld Ackermann A, Range C, Korbmacher C (1998) Maitotoxin (MTX) activates a nonselective cation channel in *Xenopus laevis* oocytes. Pflügers Arch 436:329–37
4. Burckhardt BC, Kroll B, Frömter E (1992) Proton transport mechanism in the cell membrane of *Xenopus laevis* oocytes. Pflügers Arch 420:78–82
5. Canessa CM, Schild L, Buell G, Thorens B, Gautschi I, Horisberger JD, Rossier BC (1994) Amiloride-sensitive epithelial Na^+ channel is made of three homologous subunits. Nature 367:463–7
6. Chalfant ML, Denton JS, Berdiev BK, Ismailov, II, Benos DJ, Stanton BA (1999) Intracellular H^+ regulates the alpha-subunit of ENaC, the epithelial Na^+ channel. Am J Physiol 276: C477–C486
7. Chang SS, Grunder S, Hanukoglu A, Rosler A, Mathew PM, Hanukoglu I, Schild L, Lu Y, Shimkets RA, Nelson Williams C, Rossier BC, Lifton RP (1996) Mutations in subunits of the epithelial sodium channel cause salt wasting with hyperkalaemic acidosis, pseudohypoaldosteronism type 1. Nature Genet 12:248–53
8. Choe H, Zhou H, Palmer LG, Sackin H (1997) A conserved cytoplasmic region of ROMK modulates pH sensitivity, conductance, and gating. Am J Physiol 273:F516–F529
9. De La Rosa DA, Zhang P, Naray Fejes-Toth A, Fejes-Toth G, Canessa CM (1999) The serum and glucocorticoid kinase sgk increases the abundance of epithelial sodium channels in the plasma membrane of *Xenopus* oocytes. J Biol Chem 274: 37834–37839
10. Dinudom A, Harvey KF, Komwatana P, Young JA, Kumar S, Cook DI (1998) Nedd4 mediates control of an epithelial Na^+ channel in salivary duct cells by cytosolic Na^+. Proc Natl Acad Sci USA 95:7169–7173
11. Fakler B, Schultz JH, Yang J, Schulte U, Brandle U, Zenner HP, Jan LY, Ruppersberg JP (1996) Identification of a titratable lysine residue that determines sensitivity of kidney potassium channels (ROMK) to intracellular pH. EMBO J 15: 4093–4099
12. Fei YJ, Kanai Y, Nussberger S, Ganapathy V, Leibach FH, Romero MF, Singh SK, Boron WF, Hediger MA (1994) Expression cloning of a mammalian proton-coupled oligopeptide transporter. Nature 368:563–566
13. Fei YJ, Romero MF, Krause M, Liu J-C, Huang W, Ganapathy V, Leibach FH (2000) A novel H^+-coupled oligopeptide transporter (OPT3) from *Caenorhabditis elegans* with a predominant function as a H^+ channel and an exclusive expression in neurons. J Biol Chem 275:9563–9571
14. Firsov D, Schild L, Gautschi I, Merillat AM, Schneeberger E, Rossier BC (1996) Cell surface expression of the epithelial Na channel and a mutant causing Liddle syndrome: a quantitative approach. Proc Natl Acad Sci USA 93:15370–15375
15. Garty H, Asher C, Yeger O (1987) Direct inhibition of epithelial Na^+ channels by a pH-dependent interaction with calcium, and by other divalent ions. J Membr Biol 95:151–162

16. Garty H, Civan ED, Civan MM (1985) Effects of internal and external pH on amiloride-blockable Na^+ transport across toad urinary bladder vesicles. J Membr Biol 87:67–75
17. Garty H, Palmer LG (1997) Epithelial sodium channels: function, structure, and regulation. Physiol Rev 77:359–396
18. Harvey BJ, Thomas SR, Ehrenfeld J (1988) Intracellular pH controls cell membrane Na^+ and K^+ conductances and transport in frog skin epithelium. J Gen Physiol 92:767–791
19. Horisberger JD (1998) Amiloride-sensitive Na channels. Curr Opin Cell Biol 10:443–449
20. Kellenberger S, Gautschi I, Rossier BC, Schild L (1998) Mutations causing Liddle syndrome reduce sodium-dependent downregulation of the epithelial sodium channel in the *Xenopus* oocyte expression system. J Clin Invest 101:2741–2750
21. Komwatana P, Dinudom A, Young JA, Cook DI (1998) Activators of epithelial Na^+ channels inhibit cytosolic feedback control. Evidence for the existence of a G protein-coupled receptor for cytosolic Na^+. J Membr Biol 162:225–232
22. Leaf A, Keller A, Dempsey EF (1964) Stimulation of sodium transport in toad bladder by acidification of mucosal medium. Am J Physiol 207:547–552
23. Mavrelos D, Konstas AA, Korbmacher C (2000) Cytosolic acidification inhibits wild-type epithelial sodium channel (ENaC) and Liddle's mutant. Pflügers Arch 439 [Suppl. 6]:R307
24. Meredith D, Boyd CA, Bronk JR, Bailey PD, Morgan KM, Collier ID, Temple CS (1998) 4-Aminomethylbenzoic acid is a non-translocated competitive inhibitor of the epithelial peptide transporter PepT-1. J Physiol (Lond) 512:629–634
25. Palmer LG (1984) Voltage-dependent block by amiloride and other monovalent cations of apical Na channels in the toad urinary bladder. J Membr Biol 80:153–165
26. Palmer LG (1985) Modulation of apical Na permeability of the toad urinary bladder by intracellular Na, Ca, and H. J Membr Biol 83:57–69
27. Palmer LG, Frindt G (1987) Effects of cell Ca and pH on Na channels from rat cortical collecting tubule. Am J Physiol 253:F333–F339
28. Palmer LG, Frindt G (1996) Gating of Na channels in the rat cortical collecting tubule: effects of voltage and membrane stretch. J Gen Physiol 107:35–45
29. Rossier BC (1997) 1996 Homer Smith Award Lecture. Cum grano salis: the epithelial sodium channel and the control of blood pressure. J Am Soc Nephrol 8:980–992
30. Schild L, Canessa CM, Shimkets RA, Gautschi I, Lifton RP, Rossier BC (1995) A mutation in the epithelial sodium channel causing Liddle disease increases channel activity in the *Xenopus laevis* oocyte expression system. Proc Natl Acad Sci USA 92:5699–5703
31. Schild L, Lu Y, Gautschi I, Schneeberger E, Lifton RP, Rossier BC (1996) Identification of a PY motif in the epithelial Na channel subunits as a target sequence for mutations causing channel activation found in Liddle syndrome. EMBO J 15:2381–2387
32. Schulte U, Hahn H, Konrad M, Jeck N, Derst C, Wild K, Weidemann S, Ruppersberg JP, Fakler B, Ludwig J (1999) pH gating of ROMK (K(ir)1.1) channels: control by an Arg-Lys-Arg triad disrupted in antenatal Bartter syndrome. Proc Natl Acad Sci USA 96:15298–15303
33. Shimkets RA, Lifton RP, Canessa CM (1997) The activity of the epithelial sodium channel is regulated by clathrin-mediated endocytosis. J Biol Chem 272:25537–25541
34. Shimkets RA, Lifton R, Canessa CM (1998) In vivo phosphorylation of the epithelial sodium channel. Proc Natl Acad Sci USA 95:3301–3305
35. Shimkets RA, Warnock DG, Bositis CM, Nelson Williams C, Hansson JH, Schambelan M, Gill JR Jr, Ulick S, Milora RV, Findling JW, Canessa CM, Rossier BC, Lifton RP (1994) Liddle's syndrome: heritable human hypertension caused by mutations in the beta subunit of the epithelial sodium channel. Cell 79:407–414
36. Snyder PM, Price MP, McDonald FJ, Adams CM, Volk KA, Zeiher BG, Stokes JB, Welsh MJ (1995) Mechanism by which Liddle's syndrome mutations increase activity of a human epithelial Na^+ channel. Cell 83:969–978
37. Staub O, Abriel H, Plant P, Ishikawa T, Kanelis V, Saleki R, Horisberger JD, Schild L, Rotin D (2000) Regulation of the epithelial Na^+ channel by Nedd4 and ubiquitination. Kidney Int 57:809–815
38. Staub O, Dho S, Henry P, Correa J, Ishikawa T, McGlade J, Rotin D (1996) WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na^+ channel deleted in Liddle's syndrome. EMBO J 15:2371–2380
39. Staub O, Gautschi I, Ishikawa T, Breitschopf K, Ciechanover A, Schild L, Rotin D (1997) Regulation of stability and function of the epithelial Na^+ channel (ENaC) by ubiquitination. EMBO J 16:6325–6336
40. Steel A, Nussberger S, Romero MF, Boron WF, Boyd CA, Hediger MA (1997) Stoichiometry and pH dependence of the rabbit proton-dependent oligopeptide transporter PepT-1. J Physiol (Lond) 498:563–569
41. Tabei K, Muto S, Furuya H, Sakairi Y, Ando Y, Asano Y (1995) Potassium secretion is inhibited by metabolic acidosis in rabbit cortical collecting ducts in vitro. Am J Physiol 268: F490–F495
42. Tsai TD, Shuck ME, Thompson DP, Bienkowski MJ, Lee KS (1995) Intracellular H^+ inhibits a cloned rat kidney outer medulla K^+ channel expressed in *Xenopus* oocytes. Am J Physiol 268:C1173–C1178
43. Tucker SJ, Imbrici P, Salvatore L, D'Adamo MC, Pessia M (2000) pH-dependence of the inwardly rectifying potassium channel, Kir5.1, and localization in renal tubular epithelia. J Biol Chem 275:16404–16407
44. Ussing HH, Zerahn K (1951) Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Acta Physiol Scand 23:110–127