Extracellular Protons Regulate Human ENaC by Modulating Na\(^+\) Self-inhibition*  

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The epithelial Na\(^+\) channel, ENaC, is exposed to a wide range of proton concentrations in the kidney, lung, and sweat duct. We, therefore, tested whether pH alters ENaC activity. In Xenopus oocytes expressing human α-, β-, and γENaC, amiloride-sensitive current was altered by protons in the physiologically relevant range (pH 8.5-6.0). Compared with pH 7.4, acidic pH increased ENaC current, whereas alkaline pH decreased current (pH\(_{50}\) = 7.2). Acidic pH also increased ENaC current in H441 epithelia and in human primary airway epithelia. In contrast to human ENaC, pH did not alter rat ENaC current, indicating that there are species differences in ENaC regulation by protons. This resulted predominantly from species differences in γENaC. Maneuvers that lock ENaC in a high open-probability state (“DEG” mutation, proteolytic cleavage) abolished the effect of pH on human ENaC, indicating that protons alter ENaC current by modulating channel gating. Previous work showed that ENaC gating is regulated in part by extracellular Na\(^+\) (“Na\(^+\) self-inhibition”). Based on several observations, we conclude that protons regulate ENaC by altering Na\(^+\) self-inhibition. First, protons reduced Na\(^+\) self-inhibition in a dose-dependent manner. Second, ENaC regulation by pH was abolished by removing Na\(^+\) from the extracellular bathing solution. Third, mutations that alter Na\(^+\) self-inhibition produced corresponding changes in ENaC regulation by pH. Together, the data support a model in which protons modulate ENaC gating by relieving Na\(^+\) self-inhibition. We speculate that this may be an important mechanism to facilitate epithelial Na\(^+\) transport under conditions of acidosis.

The epithelial Na\(^+\) channel, composed of three homologous subunits (α-, β-, and γENaC), functions as a pathway for Na\(^+\) reabsorption across epithelia in the kidney collecting duct, lung, distal colon, and sweat duct (for review, see Refs. 1 and 2). In this role the channel is critical for the maintenance of Na\(^+\) homeostasis and to control the composition and quantity of the fluid on the apical membrane of these epithelia. ENaC mutations and defects in its regulation cause inherited forms of hypertension and hypotension (3) and may contribute to the pathogenesis of lung disease in cystic fibrosis (4).

As a member of the DEG/ENaC family of ion channels, ENaC shares common structural and functional features with channels that are gated by diverse stimuli. All DEG/ENaC subunits share a common overall structure, with two transmembrane domains and relatively short cytoplasmic N and C termini, leaving the majority of the protein exposed as a large extracellular domain (5). Differences in these extracellular domains between DEG/ENaC family members result in dramatic functional diversity, from mechanosensitive ion channels in Caenorhabditis elegans (6) to ligand-gated channels such as the peptide (FMRF-amide)-gated FaNaCh channel in mollusks (7) and proton-gated ASIC channels (8, 9).

Although no analogous ligand has yet been identified for ENaC, it is clear that the extracellular domain is important in modulating ENaC activity. For example, proteolytic cleavage at two sites in the extracellular domains of α- and γENaC convert channels from an inactive to an active state (10–12). One population of channels is cleaved by furin in the Golgi complex (11). A second population reaches the cell surface in an uncleaved state and is susceptible to cleavage and activation by proteases at the cell surface and in the extracellular fluid (12–15). Other molecules in the extracellular fluid also modulate ENaC gating. Extracellular Na\(^+\) inhibits ENaC activity through a process called Na\(^+\) self-inhibition, which serves as a negative feedback mechanism to regulate Na\(^+\) transport (16–18). Two observations implicate a role for the extracellular domain in Na\(^+\) self-inhibition. First, mutations of conserved histidine residues in the extracellular domains of α- and γENaC alter Na\(^+\) self-inhibition (18). Second, proteolytic cleavage of the extracellular domain prevents Na\(^+\) self-inhibition (19). Divalent cations including Zn\(^{2+}\) and Ni\(^{2+}\) are also thought to alter ENaC activity by binding to the extracellular domain (20, 21).

These findings suggest that the extracellular domain might function as a sensor to allow a variety of signals in the extracellular environment to modulate ENaC activity. In this regard protons are a strong candidate to regulate ENaC. In its location at the apical membrane of epithelia, ENaC is exposed to extremes of pH. For instance, urine pH in the collecting duct can vary from 4.5 to 8 in response to metabolic acidosis and alkalosis as well as with changes in diet and volume status (22). Normal airway surface liquid pH is slightly alkaline (pH 7.8–8.1) (23) but can become highly acidic (pH 4–6) with lung disease (24). Sweat pH can fall to <6 when the production rate is low but becomes neutral as production increases (25).

Although ENaC appears poised to respond to changes in pH, previous studies investigating this possibility have been conflicting. External pH < 5 decreased short-circuit current in frog...
skin (26) but increased Na\(^+\) transport in toad bladder (27). In cultured *Xenopus* collecting duct cells (A6), acidification of the extracellular medium (pH 6.4 and 5.4) produced a slow increase in amiloride-sensitive current that peaked at ~30 min (28). More recent work investigated the role of external pH in regulation of rat α-, β-, and γENaC expressed in *Xenopus* oocytes. These studies reported a very small transient increase (28), small decrease (pH 4.0) (29), or no change in current (30, 31). When α- and βENaC were coexpressed without γENaC, acidic pH inhibited the channel (which has an open-probability close to 1.0) (30). Moreover, an additional subunit, δENaC, was activated by acidic pH (29, 32), although this subunit is not expressed in renal epithelia. Together the data suggest that pH modulates epithelial Na\(^+\) transport, but the magnitude and direction of the regulation may vary in different tissues and species. To further explore this possibility and to understand the mechanisms by which pH regulates Na\(^+\) transport, we tested the effect of protons on the activity of human ENaC.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—cDNAs for human and rat α-, β-, and γENaC in pMT3 were cloned as previously described (33, 34). Mutations in αENaC (H255R) and γENaC (H233R) were generated by site-directed mutagenesis (QuikChange; Stratagene) and sequenced in the University of Iowa DNA Core.

**Expression and Whole-cell Electrophysiology in Xenopus Oocytes**—Oocytes were harvested from albino *Xenopus laevis* females and manually defolliculated after a 1-h treatment with 0.75 mg/ml type IV collagenase (Sigma) in Ca\(^{2+}\)-free ND-96 (96 mm NaCl, 2 mm KCl, 1 mm MgCl\(_2\), 5 mm HEPES, pH adjusted to 7.4 with NaOH). After nuclear injection of cDNAs encoding human or rat α-, β-, and γENaC (0.02 μg/μl each), cells were incubated at 18 °C in modified Barth’s saline (88 mM NaCl, 1 mm KCl, 0.4 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM HEPES, 2.4 mM NaHCO\(_3\), 10 mM HEPES, 10 μg/ml gentamycin sulfate, 10 μg/ml sodium penicillin, 10 μg/ml streptomycin sulfate, pH adjusted to 7.4 with NaOH) for 20–24 h before the study. Oocytes were voltage-clamped (two-electrode voltage clamp), and currents were amplified with an Oocyte Clamp OC-725C (Warner Instruments), digitized with a MacLab/200 interface (ADInstruments), and recorded and analyzed with Chart software (ADInstruments). Unless otherwise noted, recordings were done at -60 mV in a 116 mM NaCl solution (116 mM NaCl, 2 mM KCl, 0.4 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM HEPES, pH adjusted to 7.4 with NaOH). Low Na\(^+\) solutions (0 or 1 mM NaCl, 116 or 115 mM N-Methyl-D-glucamine chloride) were used as indicated in the figure legends (Figs. 5 and 6). The pH of test solutions (8.5–5.5) was adjusted with HCl or NaOH. Amiloride-sensitive current was determined by adding 10 μM amiloride to the bathing solution. The pH-induced change in amiloride-sensitive current was calculated as the fold increase/decrease relative to the pH 7.4 base line just before each test solution application. This was done to reduce the effect of time-dependent current run-down. The resulting data were plotted and fit to the Hill equation using IGOR Pro software (WaveMetrics Inc.). Na\(^+\) self-inhibition was measured by rapidly changing the bathing solution from low sodium (1 mm NaCl) to high sodium (116 mM NaCl) and quantitated as (peak current (I\(_p\)) – steady state current (I\(_ss\)))/peak current (I\(_p\)).

**RESULTS**

**pH Changes Alter ENaC Current**—To determine whether changes in extracellular pH alter ENaC current, we expressed human α-, β-, and γENaC in *Xenopus* oocytes and recorded Na\(^+\) current at a holding potential of -60 mV. In Fig. 1A, we found that current was reduced when we shifted the extracellular pH from 7.4 to 8.5. Conversely, pH 6.5 increased current. The changes in current were rapidly reversible on returning to pH 7.4. When ENaC was blocked by amiloride, changes in pH did not alter current, indicating that pH regulated ENaC and not an endogenous oocyte current. Also consistent with this conclusion, pH changes from 8.5 to 5.5 did not significantly alter current in un.injected oocytes (not shown). Fig. 1B shows the pH dose-response relationship for amiloride-sensitive ENaC current (relative to current at pH 7.4). Current was maximal at pH 6.0 (I\(_{p}\)/I\(_{ss}\) = 1.4 ± 0.148) and minimal at pH 8.5 (I\(_{p}\)/I\(_{ss}\) = 0.8 ± 0.01), with a pH\(_{50}\) of 7.2. Thus, human ENaC

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**FIGURE 1.** Extracellular protons modulate human ENaC current. A, representative trace of current versus time recorded in *Xenopus* oocyte expressing human αβγENaC at holding potential of -60 mV. The extracellular bath was changed from pH 7.4 (open bars) to pH 8.5 and 6.5 (black bars) with or without 10 μM amiloride (black bars) as indicated. B, amiloride-sensitive current for human ENaC (relative to current at pH 7.4) with bathing solution pH varied from 8.5 to 5.5 (mean ± S.E., n = 37; error bars are hidden by the symbols). Data are fit to the Hill equation (R\(^2\) = 0.9991).
current is regulated by pH within the range found in the kidney collecting duct and other epithelia.

In Figs. 2, A and B, we tested the effect of pH on amiloride-sensitive current in epithelia that express endogenous ENaC. In a human lung carcinoma cell line (H441), amiloride-sensitive current was larger at pH 6.5 than at pH 7.4 (Fig. 2, A and C). Results were similar in primary cultures of human airway epithelia (Fig. 2, B and C). pH 6.5 increased ENaC current in both epithelia to an extent similar to that when ENaC was expressed in Xenopus oocytes (Fig. 2C). These data indicate that shifts in pH alter ENaC current not only in heterologous cells but also in Na⁺-transporting epithelia.

Species Specificity of pH Regulation—In contrast to human ENaC, rat ENaC currents were not altered by pH changes over the range 8.5–6 in Xenopus oocytes (Fig. 3, A and B). This finding suggests that pH regulates ENaC in a species-specific manner. To determine which subunit(s) underlies this species specificity, we substituted one of the human ENaC subunits in the channel complex with a rat subunit. Channels composed of human α- and γENaC with rat βENaC had a pH dose-response relationship similar to human ENaC (Fig. 3B). In contrast, pH failed to alter current when rat γENaC was expressed with human α- and βENaC (Fig. 3B). When rat αENaC was expressed with human β- and γENaC, pH altered ENaC current, although the effect of pH was reduced (Fig. 3B). Together the data indicate that sequence differences in γENaC (and perhaps αENaC) underlie the observed species variation in ENaC regulation by protons.

pH Regulates ENaC Gating—To determine the biophysical mechanism by which pH alters ENaC current, we took advantage of a strategy that prevents changes in ENaC gating. Covalent modification of a cysteine introduced at the DEG position in the external pore (β$_{5220}$C) locks ENaC in an open conformation (open probability 0.96) (36). When β$_{5220}$C was coexpressed with α- and γENaC in Xenopus oocytes, pH altered current to a similar extent as wild-type ENaC (Fig. 4, A and D). In contrast,
after modification of the channel with [2-(trimethylammonium)ethyl]methanethiosulfonate bromide (MTSET), changes in pH had a minimal effect on ENaC current (Fig. 4, B and D). As a second approach to address this question, we activated channels at the cell surface by proteolytic cleavage with trypsin, which dramatically increases ENaC open probability (12). After incubation with trypsin, changes in pH failed to alter ENaC current (Fig. 4, C and D). Together the data suggest that pH regulates ENaC by altering channel gating and argues against effects of pH on single channel conductance or channel number.

**Extracellular pH Modulates Na\(^+\) Self-inhibition**—Previous work indicates that extracellular Na\(^+\) regulates ENaC gating, a mechanism known as Na\(^+\) self-inhibition. The representative traces in Fig. 5A illustrate this regulation. A shift in extracellular Na\(^+\) from 1 to 116 mM induced a peak inward current (\(I_p\)) that rapidly decreased to a lower steady state level (\(I_{ss}\)). This decline in current reflects inhibition of ENaC by Na\(^+\). Proteolytic cleavage activates ENaC by preventing Na\(^+\) self-inhibition (19). Because we found that cleavage abolished the effect of pH on ENaC, we hypothesized that pH might regulate ENaC gating by altering Na\(^+\) self-inhibition. To test this hypothesis, we varied the extracellular pH and quantitated the fraction of ENaC current that was inhibited by Na\(^+\) after a shift from 1 to 116 mM Na\(^+\). We found that Na\(^+\) self-inhibition was pH-dependent. At acidic pH (6.5), a smaller fraction of amiloride-sensitive current was inhibited by Na\(^+\) than at alkaline pH (8.5), resulting in an increase in steady state current at pH 6.5 (Fig. 5, A and B). In contrast to its effect on steady state current, pH had no effect on the peak current induced by the shift in Na\(^+\) concentration (Fig. 5, A and C).

Protons could inhibit Na\(^+\) self-inhibition by reducing binding of Na\(^+\) to ENaC. To investigate this possibility, we determined dose-response relationships for Na\(^+\) self-inhibition at pH 8.5, 7.4, and 6.5 (fit to the Hill equation). In Fig. 5D we shifted the extracellular Na\(^+\) from 0 mM to concentrations between 1 and 116 mM. At pH 7.4 Na\(^+\) produced a dose-dependent increase in self-inhibition; half-maximal inhibition \((K_I)\) occurred at 44.9 ± 1.6 mM Na\(^+\), and the maximum fraction of current inhibited by Na\(^+\) was 0.57 ± 0.01. pH 6.5 decreased the maximal effect of Na\(^+\) (0.49 ± 0.03), but it had no effect on the sensitivity of ENaC to Na\(^+\) \((K_I = 44.7 ± 4.8)\). pH 8.5 had the opposite effect, increasing the maximal effect of Na\(^+\) (0.66 ± 0.05) with no effect on sensitivity \((K_I = 43.5 ± 5.4)\). Thus, pH modulates Na\(^+\) self-inhibition by altering the maximal effect of Na\(^+\) on ENaC without affecting its sensitivity to Na\(^+\).

**Regulation by Extracellular pH Requires Sodium**—If pH regulates ENaC by modulating Na\(^+\) self-inhibition, then the pH effect should be dependent on the presence of Na\(^+\) in the extracellular solution. To test this prediction, we measured outward amiloride-sensitive currents (holding potential +30 mV) with 1 or 116 mM Na\(^+\) in the bathing solution. In 1 mM Na\(^+\), pH had a negligible effect on ENaC current (Fig. 6, A and C). In contrast, in 116 mM Na\(^+\), pH 8.5 reduced and pH 6.5 increased outward ENaC current (Fig. 6, B and C), similar to the effect of pH on inward currents with 116 mM Na\(^+\) (Fig. 6C, compare black bars to open bars). Thus, extracellular Na\(^+\) is required for pH to regulate ENaC. In addition, these results indicate that pH regulates ENaC independent of voltage and the direction of Na\(^+\) movement.

**Mutations That Alter Na\(^+\) Self-inhibition also Affect pH Regulation**—Previous work identified two ENaC histidine residues critical for Na\(^+\) self-inhibition (18). Mutation of His-239 in mouse γENaC (His-233 in human) abolished self-inhibition, whereas mutation of the equivalent residue in αENaC (mouse His-282, which is His-255 in human) increased self-inhibition. To test if these mutations alter the effect of pH on ENaC current, we expressed \(\alpha_{255R}\)βENaC or \(\alpha_{255R}\)γ\(_{233R}\) ENaC in Xenopus oocytes. \(\gamma_{123R}\) nearly abolished the effect of pH on ENaC current (Fig. 7, A and C). Conversely, \(\alpha_{255R}\) increased the degree of ENaC activation by acidic pH (compared with wild-type ENaC), although it had no effect at alkaline pH (Fig. 7, B and C). Thus, mutation of γENaC abolished both Na\(^+\) self-
inhibition and ENaC regulation by pH, whereas both properties were increased by mutation of αENaC. This correlation together with our other data supports a model in which pH alters ENaC current by modulating Na⁺ self-inhibition.

**DISCUSSION**

Our data indicate that the activity of human ENaC is modulated by changes in extracellular pH; acidic pH increased activity, whereas alkaline pH reduced activity. pH changes altered ENaC gating; by relieving Na⁺ self-inhibition, protons increased ENaC open-probability.

How does proton alter Na⁺ self-inhibition? We hypothesize that protons titrate one or more ENaC residues that alter the response to Na⁺. This could occur through two general mechanisms. First, it is possible that protons reduce Na⁺ binding to the extracellular domain. However, our finding that pH changes did not alter the $K_i$ for Na⁺ self-inhibition argues against this model, as it indicates that protons do not alter the affinity of Na⁺ for ENaC. Thus, it is unlikely that protons titrate residues that form the Na⁺ binding site. Second, pH could function downstream of Na⁺ binding, altering the transduction of Na⁺ binding into changes in ENaC gating. Consistent with this model, we found that protons increased the maximal effect of Na⁺ on ENaC current.

What residues are titrated by protons? We found that the effect of pH on ENaC current was independent of voltage and on the direction of current flow, which indicates that protons do not alter ENaC current by titrating residues in its pore. Thus, it is likely protons titrate one or more residues in extracellular domain. The recent crystal structure of ASIC1 identified H⁺ binding sites located at the interface between the finger and thumb domains of this related channel (37). Movement of the thumb domain relative to the finger domain was proposed to underlie channel gating. Although the ASIC1 H⁺ binding residues are not well conserved in ENaC, we speculate that the interface between the finger and thumb domains has a conserved role in modulating channel activity and participates in H⁺ binding to ENaC. Consistent with this idea, residues that modulate ENaC function are located at this interface. For example, proteolytic cleavage activates ENaC by removal of part of the finger domain. Moreover, the α- and γENaC histidines important for Na⁺ self-inhibition (αH233R and γH255R) are located at the finger-thumb interface. However, it seems unlikely that protons regulate ENaC by titrating these histidines, as the αH233R and γH255R mutations had opposite effects on pH modulation of ENaC current. In addition, these histidines are conserved in rat ENaC, which did not respond to changes in pH.

As a related question, which ENaC subunits mediate the pH sensitivity of ENaC? Do protons bind to all three subunits or only a subset of subunits? Our data implicate a role for γENaC, as substitution of rat in place of human γENaC abolishes channel regulation by protons. Substitution of the α subunit had a partial effect on pH regulation, suggesting that residues in αENaC may also contribute. In contrast, substitution of βENaC did not alter ENaC regulation by protons. This situation is somewhat analogous to ENaC regulation by proteolytic cleavage, where ENaC is regulated by cleavage of the extracellular domains of α- and γENaC but not βENaC (11). However, our current data do not exclude a role for βENaC; it is possible that human and rat βENaC share conserved H⁺ binding sites. The species difference in pH regulation suggests a strategy to identify residues that contribute to this regulation. The extracellular domain of human γENaC contains a large number of potential targets for protons that are not present in rat ENaC (14 histi-
dines and acidic residues), and there are 10 in αENaC. Based on analogy to ASIC1 (37, 38), we speculate that titration of multiple residues contributes to pH regulation of human ENaC.

How can we reconcile our current results with previous conflicting data in the literature? First, there appear to be species differences in ENaC regulation by pH. Although pH altered human ENaC current, it had no effect on rat ENaC in the range we tested, consistent with previous work. From a comparative physiology perspective, we do not yet know why pH regulates ENaC in some species but not others. In humans, differences in diet can produce wide variations in urine pH. For example, consumption of meat produces an acidic urine, whereas vegetarians have an alkaline urine. Perhaps rats lacked selective pressure to develop or retain the pH response because of a less varied diet. Conflicting data may also arise from differences in the proteolytic cleavage state of ENaC in various experimental systems. We found that proteolytic cleavage (trypsin) abolished the effect of pH on human ENaC. Thus, the response to pH is influenced by the cleavage state of the channel. Under conditions where the majority of ENaC is fully cleaved, the response to pH will be blunted.

ENaC is exposed to extreme changes in pH. Coupled with our data showing that pH alters ENaC current, this suggests that pH may be an important regulator of epithelial Na⁺ transport. What is the physiological role of this regulation? In the kidney-collecting duct, it is well described that there is an interdependence between Na⁺ absorption and H⁺ secretion; Na⁺ absorption generates a transmembrane voltage which increases H⁺ secretion (39, 40). Perhaps activation of ENaC by protons facilitates secretion of an acid load. In this regard it is interesting that the Na⁺-absorbing principal cells are located next to intercalated cells, which are responsible for H⁺ secretion. This suggests a potential model in which protons function as a paracrine signal to regulate Na⁺ absorption. In the lung it is possible that acidic airway fluid stimulates ENaC to remove liquid from the lung in conditions such as pneumonia and pulmonary edema.

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