Identification of Extracellular Domain Residues Required for Epithelial Na\textsuperscript{+} Channel Activation by Acidic pH

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Background: The epithelial Na\textsuperscript{+} channel (ENaC) functions as a pathway for Na\textsuperscript{+} absorption across epithelia.

Results: Seven acidic residues in the extracellular domain of γENaC and one in βENaC are required for regulation by acidic pH.

Conclusion: The ENaC extracellular domain functions as sensors to detect changes in extracellular pH.

Significance: These findings provide new insights into mechanisms that regulate Na\textsuperscript{+} homeostasis and blood pressure.

A growing body of evidence suggests that the extracellular domain of the epithelial Na\textsuperscript{+} channel (ENaC) functions as a sensor that fine tunes channel activity in response to changes in the extracellular environment. We previously found that acidic pH increases the activity of human ENaC, which results from a decrease in Na\textsuperscript{+} self-inhibition. In the current work, we identified extracellular domain residues responsible for this regulation. We found that rat ENaC is less sensitive to pH than human ENaC, an effect mediated in part by the γ subunit. We identified a group of seven residues in the extracellular domain of γENaC (Asp-164, Gln-165, Asp-166, Glu-292, Asp-335, His-439, and Glu-455) that, when individually mutated to Ala, decreased proton activation of ENaC. γE455 is conserved in βENaC (Glu-446); mutation of this residue to neutral amino acids (Ala, Cys) reduced ENaC stimulation by acidic pH, whereas reintroduction of a negative charge (by MTSES modification of Cys) restored pH regulation. Combination of the seven γENaC mutations with βE446A generated a channel that was not activated by acidic pH, but inhibition by alkaline pH was intact. Moreover, these mutations reduced the effect of pH on Na\textsuperscript{+} self-inhibition. Together, the data identify eight extracellular domain residues in human β- and γENaC that are required for regulation by acidic pH.

The epithelial Na\textsuperscript{+} channel (ENaC)\textsuperscript{4} is composed of three homologous subunits (α-, β-, and γENaC). Each subunit has relatively short cytoplasmic N and C termini, leaving the bulk of the protein exposed to the extracellular environment. ENaC functions as a pathway for Na\textsuperscript{+} reabsorption across epithelia in the kidney collecting duct, lung, distal colon, and sweat duct (1, 2). The channel is critical for the maintenance of Na\textsuperscript{+} homeostasis and control of the composition and quantity of fluid on the apical membrane of these epithelia. ENaC mutations, and defects in its regulation, cause inherited forms of hypertension and hypotension (1, 3) and cause lung disease with features similar to cystic fibrosis (4–6).

The large extracellular domain of ENaC is exposed to highly varied environments. A growing body of evidence suggests that this domain detects concentration changes in a number of molecules, producing compensatory changes in ENaC gating. As a complement to long term regulation by changes in channel abundance, this may provide a more rapid, fine-tuning of ENaC activity in response to a relentless assault of diverse extracellular challenges. For example, the concentrations of Na\textsuperscript{+} and Cl\textsuperscript{−} in the urine change dynamically with shifts in volume state. Under conditions of volume depletion, Na\textsuperscript{+} and Cl\textsuperscript{−} concentrations are very low (<10 mM) as a result of decreased delivery to the distal nephron (7). Conversely, Na\textsuperscript{+} and Cl\textsuperscript{−} concentrations increase in response to volume excess (~150 mM). Under these conditions, Na\textsuperscript{+} (via “Na\textsuperscript{+} self-inhibition” (8–10)) and Cl\textsuperscript{−} (11, 12) inhibit ENaC gating, providing a negative feedback pathway to limit Na\textsuperscript{+} absorption.

In previous work, we found that ENaC activity is also regulated by changes in extracellular pH. In the kidney collecting duct, pH varies between 4.5 and 8 in response to metabolic acidosis and alkalosis as well as with changes in diet and volume status (7). In the lung, acidic airway surface liquid may contribute to the pathogenesis of cystic fibrosis (13). We found that pH modulates the activity of human ENaC. Acidic pH increased activity by reducing Na\textsuperscript{+} self-inhibition, whereas alkaline pH reduced ENaC activity (14). Acidic pH also has a second opposing effect on ENaC activity; by titrating a Cl\textsuperscript{−} binding site, acidic pH enhances ENaC inhibition by extracellular Cl\textsuperscript{−} (11).

In the current work, we explored the molecular mechanism by which acidic pH stimulates ENaC. We took advantage of species differences to identify extracellular domain residues that contribute to this regulation.

EXPERIMENTAL PROCEDURES

DNA Constructs—cDNAs for human α-, β-, and γENaC in pMT3 were cloned as previously described (15, 16). Mutations were generated by site-directed mutagenesis (QuikChange II XL; Agilent Technologies) and sequenced in the University of Iowa DNA Core.

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\textsuperscript{4}The abbreviation used is: ENaC, epithelial Na\textsuperscript{+} channel; MTSES, sodium (2-sulfonatoethyl)limethanethiosulfonate.

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Expression and Whole Cell Electrophysiology in Xenopus Oocytes—Oocytes were harvested from albino *Xenopus laevis* females and manually deloculated following a 1-h treatment with 640 units/ml type IV collagenase (Worthington Biochemical Corporation) in Ca²⁺-free ND-96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH adjusted to 7.4 with NaOH). Following nuclear injection of cDNAs encoding α₁-, β₁-, and γENaC (0.02 µg/µl each), cells were incubated at 18 °C in modified Barth’s saline (88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 2.4 mM NaHCO₃, 10 mM HEPES, 50 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 prior to study. Oocytes were voltage clamped (two-electrode voltage clamp), and currents were amplified with an Oocyte Clamp (ADInstruments), and recorded and analyzed with Chart software (ADInstruments). As noted, recordings were performed at −60 mV in a 58 mM Na₂SO₄ solution (58 mM Na₂SO₄, 58 mM d-mannitol, 2 mM KCl, 0.4 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH adjusted to 7.4 with NaOH). High Na⁺ (116 mM NaCl, 2 mM KCl, 0.4 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH adjusted to 7.4 with NaOH) and low Na⁺ solutions (1 mM NaCl, 115 mM N-methyl-d-glucamine, 2 mM KCl, 0.4 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH adjusted with HCl) were used as indicated in the figure legends to measure Na⁺ self-inhibition. Amiloride-sensitive current was determined by adding 10 µM amiloride to the bathing solution.

pH-induced changes in amiloride-sensitive current were calculated as the -fold increase/decrease relative to the baseline current in pH 7.4 Na₂SO₄ Ringer’s just prior to each test solution application. This was done to reduce the effect of time-dependent current rundown.

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 − steady-state current)/peak current). Cl⁻ inhibition was measured by rapidly changing the bathing solution from high chloride (116 mM NaCl) to low chloride (58 mM Na₂SO₄) and quantitated as -fold change in amiloride-sensitive current.

In all experiments, to account for day-to-day variation, values were normalized to values recorded from cells expressing wild-type ENaC on the same day. All values are reported as averages ± S.E. Statistical significance was calculated using a two-tailed Student’s t test (p < 0.05).

RESULTS

**Human γENaC Is Necessary for pH Regulation of Channel Activity**—To test whether human γENaC is sufficient for the pH response, we expressed rat α₁- and β₁ENaC with human γENaC. The pH dose-response relationship for channels expressing this combination of two rat and one human subunit was very similar to channels expressing only rat αβENaC (Fig. 2). This demonstrates that although human γENaC was required, it was not sufficient to restore pH sensitivity to rat ENaC. Therefore, the data suggest that more than one subunit is involved in the pH response seen in human ENaC.

**Identification of Extracellular Residues in γENaC That Contribute to Regulation by Acidic pH**—We hypothesized that sequence differences between the extracellular domains of human and rat γENaC contributed to differences in pH regulation. Fig. 3 shows a partial sequence alignment of the extracellular domain of human and rat γENaC. In total, 50 residues differ between the two species in the extracellular domains of γENaC (indicated by boxes). Of these, we focused on 15 residues that H⁺ might titrate within the physiologically relevant acidic pH range (acidic residues and histidines, Fig. 3, gray boxes). We mutated each of these residues to Ala and tested the effect of pH changes on ENaC current. Fig. 4 shows representative current traces for wild type ENaC and one of the mutants, γD₁₆₆A. This mutation reduced ENaC stimulation by acidic pH. In Fig. 4B, we plotted the total change in ENaC current between pH 9.0 and pH 5.25 for each of the 15 mutants (relative to wild type ENaC). We found that seven of the mutations (γD₁₆₆A, γQ₁₆₅A, γD₁₆₆A, γE₂₉₂A, γD₃₁₅A, γH₄₃₉A, and γE₄₅₅A) decreased activation of ENaC current by acidic pH (Fig. 4B). In contrast,
the other mutations had no effect or slightly increased the pH response (Fig. 4B).

As no single mutation abolished the response to pH, the data indicate that multiple residues in the extracellular domain of human γENaC are required. To test this idea further, we generated a human γENaC construct in which the seven residues that reduced the response to acidic pH were mutated simultaneously (γ_{D164A,Q165A,D166A,E292A,D335A,H439A,E455A} labeled γ_{7Ala}). Cells expressing human αβγ_{7Ala}ENaC had a greater reduction in their response to acidic pH than any single mutation alone (Fig. 4C and D), consistent with a role for multiple residues. However, the response to acidic pH was not abolished, indicating that additional ENaC residues also contribute. Interestingly, ENaC inhibition by alkaline pH (pH 7.4–9.0) was identical to wild type ENaC (Fig. 4D). Thus, different residues are responsible for ENaC regulation by acidic and alkaline pH.
pH Regulation of ENaC

In previous work, we found that extracellular Cl⁻ inhibits ENaC. To test the specificity of these mutations for pH, we measured the inhibition of ENaC activity by extracellular Cl⁻. A reduction in extracellular Cl⁻ increased the γ₇Ala mutant current to an extent similar to wild type ENaC (Fig. 4, E and F). This demonstrates that the decrease in the response to acidic pH seen with the γ₇Ala mutations did not result from a nonspecific change in channel activity.

Residues Equivalent to γ₄₄₄ in α- and βENaC Also Modulate the pH Response.—Because the γ₇Ala mutations did not abolish ENaC regulation by acidic pH, we searched for additional contributing residues. We turned our attention to α- and βENaC; our finding that human γENaC alone was not sufficient to explain the species difference in pH regulation supported a role for one or more additional subunit(s). Of the seven human γENaC residues that contributed to pH regulation, one was conserved in αENaC (α₁₂₃₅₋γ₁₃₃₃), and two were conserved in βENaC (β₂₂₈₁₋γ₂₂₉₂ and β₃₄₄₆₋γ₃₄₅₆, Fig. 5A). To test their role in pH regulation, we mutated each residue individually to Ala. Two of the mutations (α₁₂₃₅₋γ₁₃₃₃ and β₂₂₈₁₋γ₂₂₉₂) did not reduce the effect of pH on ENaC current (Fig. 5C). In contrast, β₃₄₄₆₋γ₃₄₅₆ had a large effect, decreasing the acidic pH response by 75% (Fig. 5, B and C). Fig. 5E shows the pH dose-response relationship for β₃₄₄₆₋γ₃₄₅₆; this mutation selectively reduced ENaC stimulation by acidic pH but had no effect on inhibition by alkaline pH. In αENaC, the corresponding residue carries a positive charge (α₄₄₄). In contrast to results with β- and γENaC, mutation of α₄₄₄ to Ala increased ENaC stimulation by acidic pH (Fig. 5, D and E).

Together, the data suggest that the negative charge of β₃₄₄₆ and equivalent positions in α- and γENaC are important for protons to modulate ENaC activity. To investigate this possibility further, we replaced β₃₄₄₆ with Cys. This mutation reduced ENaC activation by acidic pH to the same degree as the Ala mutation at this position (Fig. 5F). Modification of the Cys with MTSES, to restore the negative charge, partially rescued pH regulation of ENaC (Fig. 5F). Mutating β₃₄₄₆ to Lys elicited a pH dose response nearly identical to β₃₄₄₆A or β₃₄₄₆C (data not shown). These findings indicate that the negative charge at this position is important for ENaC activation by acidic pH.

We performed a similar series of experiments at the α₄₄₄ position. We mutated α₄₄₄ to Cys. This mutation increased the response to acidic pH to a degree similar to the Ala mutation (Fig. 5G). When we introduced a negative charge by modifying the Cys mutant with MTSES, the response to acidic pH increased even further (Fig. 5G). These data indicate that a negative charge at this position in α, β-, or γENaC allows ENaC to be activated by protons.

Mutation of Titratable Residues in β- and γENaC Specifically Eliminates the Response to Acidic pH.—In Fig. 6, A and B, we co-expressed β₁₄₄₆ with γ₀Ala (and wild type α). Acidic pH failed to stimulate this mutant channel, whereas inhibition by alkaline pH was intact. Cells expressing α₁₄₄₆γ₀Ala also showed increased amiloride-sensitive current at pH 7.4 (in 116 mM NaCl Ringer’s), suggesting that Ala mutations mimic ENaC stimulation by the protonated state of the wild type acidic residues (Fig. 6C). However, ENaC inhibition by extracellular Cl⁻ was not significantly reduced (Fig. 6D), demonstrating that the mutations are specific to activation by extracellular pH. These results identify eight residues in the extracellular domains of β- and γENaC that are required for regulation by acidic pH but are not involved in regulation by alkaline pH or Cl⁻.

Ala Mutations Disrupt Na⁺ Self-inhibition.—In previous work, we found that protons stimulate ENaC in part by reducing Na⁺ self-inhibition, a process by which extracellular Na⁺ decreases ENaC activity. Here we asked whether protons modulate Na⁺ self-inhibition through the eight titratable residues we identified in β- and γENaC. We varied the extracellular pH (7.4 or 5.5) and measured Na⁺ self-inhibition by shifting the extracellular Na⁺ concentration from 1 to 116 mM. This produced a large peak inward current (Iₒ) that decreased to a lower steady-state current (Iₛ, Fig. 7A); the fraction of current inhibited by Na⁺ (Na⁺ self-inhibition) is plotted in Fig. 7C. For wild type ENaC, Na⁺ inhibited channel activity to a greater extent at pH 7.4 than at pH 5.5 (Fig. 7, A and C), consistent with our previous work (14). Mutation of the eight residues (β₁₄₄₆A and γ₀Ala) dramatically reduced Na⁺ self-inhibition at pH 7.4 and nearly abolished the effect of acidic pH on Na⁺ self-inhibition (Fig. 7, B and C). Mutation of the γENaC subunit alone (γ₀Ala) had an intermediate effect (Fig. 7C). Intriguingly, Na⁺ self-inhibition for wild type ENaC and the mutants converged at pH 5.5 (Fig. 7C), suggesting that neutralization of the acidic residues by protonation or mutagenesis have equivalent effects on Na⁺ self-inhibition.

DISCUSSION

In this work, we identified eight residues that participate in the regulation of ENaC activity by extracellular acidification. Mutation of seven residues in γENaC (Asp-164, Gln-165, Asp-166, Glu-292, Asp-335, His-439, and Glu-455) and one residue in βENaC (Glu-446) each reduced ENaC stimulation by acidic pH, whereas simultaneous mutation of all eight abolished stimulation. Although ENaC is a constitutively active channel, a wide variety of extracellular stimuli can modulate its activity.
FIGURE 3. 15 titratable residues present in the extracellular domain of human but not rat γENaC. Partial sequence alignment of the extracellular domains of human and rat γENaC is shown. Residues that differ between the two subunits are indicated with an open box. Acidic residues and histidines (Asp, Glu, His) that differ between the two subunits are indicated with a gray box.

FIGURE 4. Mutation of titratable residues in the extracellular domain of γENaC decreases the response to acidic pH. A, C, and E, representative traces of current versus time recorded at −60 mV from Xenopus oocytes expressing human αβγENaC (A and C, black, scale bar = 1 μA, 5 s), or human αβ- and γD166AENaC (A, gray, scale bar = 2 μA, 6.5 s), or human αβ- and γD164A,Q165A,D166A,E292A,D335A,H439A,E455AENaC (E, gray, scale bar = 1 μA, 5 s). Currents were recorded under conditions of high Na⁺ (116 mM) and low extracellular Cl⁻ (5 mM) over a range of pH from 9.0 to 5.25 (only pH 5.25 is shown). ENaC-dependent inward Na⁺ current was determined by application of amiloride at the end of the experiment. B, total pH response for each acidic residue identified in Fig. 3 (calculated as the difference in ENaC activity between pH 9.0 and 5.25) relative to wild type human αβγENaC (mean ± S.E., error bars, n = 3–8, *p < .05). D, dose–response relationship showing the change in ENaC activity versus extracellular pH relative to pH 7.4 for cells expressing wild type (black) and human αβγD166AENaC (gray, mean ± S.E., error bars are hidden by data symbols, n = 11). E, representative trace of Cl⁻ inhibition recorded at −60 mV from oocytes expressing wild type (black) or αβγD166AENaC (gray). F, Cl⁻ inhibition relative to wild type ENaC (mean ± S.E., n = 11).
including $H^+$, $Cl^-$, $Na^+$, $Cu^{2+}$, $Zn^{2+}$, $Ni^{2+}$, shear stress, and proteases (8, 11, 14, 17–20). In this regard, it is clear that the extracellular domain functions as a sensor to detect changes in the composition and quantity of the highly varied extracellular milieu in which ENaC resides.

The residues we identified could function as proton sensors through titration of their side chains. Alternatively, they could function downstream of proton binding by transducing conformational changes that underlie channel gating. Several lines of evidence support the first model in which these residues are directly titrated by protons. First, all of the residues have side chains that are titrated in the acidic pH range (Glu, Asp, His), with the exception of Gln-165. Although the role of Gln-165 in the acidic pH response may seem contrary to this idea, its location may alter the $pK_a$ or accessibility of two surrounding acidic residues (Asp-164 and Asp-166). Second, the charge of the residues was critical. Neutralization of the charge, by mutation to Ala, mimicked the effect of acidic pH by increasing ENaC current. Conversely, restoration of the negative charge (by MTSES modification of $E_{446C}$) restored ENaC regulation by acidic pH. Likewise, introduction of a negative charge at the equivalent position in $\alpha_2$ENaC (via MTSES modification of $K_{477C}$) enhanced ENaC activation by acidic pH. Finally, mutation of these eight residues abolished ENaC regulation by acidic pH, but did not alter its regulation by alkaline pH or $Cl^-$.

**FIGURE 5.** Residues equivalent to $E_{455}$ in $\alpha_1$ and $\beta_1$ENaC also modulate the pH response. A, seven residues in $\gamma_2$ENaC that decrease the response to acidic pH and the equivalent residues in $\alpha_1$ and $\beta_1$ENaC. B and D, representative traces of current versus time recorded at $-60$ mV from Xenopus oocytes expressing human $\alpha_1\beta_1\gamma_2$ENaC (B and D, black, scale bar = 1 $\mu$A, 5 s), $\alpha_2\beta_2\gamma_2$ENaC (B, gray, scale bar = 2 $\mu$A, 5 s), or $\alpha_2\beta_2\gamma_2$ENaC (D, gray, scale bar = 2 $\mu$A, 5 s). Currents were recorded under conditions of high Na$^+$ (116 mM) and low extracellular Cl$^-$ (5 mM) over a range of pH from 9.0 to 5.25 (only pH 5.25 is shown). ENaC-dependent inward Na$^+$ current was determined by application of amiloride at the end of the experiment. C, total change in ENaC activity between pH 7.4 and 5.25 relative to wild type (mean ± S.E. (error bars), n = 7–10, *, p < .05). E–G, dose-response relationships showing the change in ENaC activity versus extracellular pH (relative to pH 7.4) for cells expressing wild type ENaC (E–G, black), $\alpha_{K477A}$ (E, dark gray) or $\beta_{E446C}$ mutations (E, light gray, mean ± S.E., n = 6–9, error bars hidden by data symbols); $\beta_{E446A}$ after modification with MTSES (F, dark gray), $\beta_{E446A}$ (F, dark gray triangles), and $\beta_{E446C}$ (F, black squares), mean ± S.E., n = 3–9, some error bars hidden by data symbols); $\alpha_{K477A}$ (G, gray triangles), $\alpha_{K477C}$ (G, black squares), and $\alpha_{K477C}$ after modification with MTSES (G, dark gray, mean ± S.E., n = 3–9, some error bars hidden by data symbols).
This finding also indicates that different residues mediate ENaC regulation by acidic pH and by alkaline pH. Thus we favor a model in which the eight identified residues are titrated by protons but we cannot exclude other potential mechanisms.

Although ENaC subunits share a high degree of sequence similarity, there is growing evidence to suggest that the functional roles of each subunit are strikingly different. Several examples exist. In earlier work, we demonstrated that the three ENaC subunits play differing roles in regulation by extracellular pH relative to pH 7.4 for cells expressing wild type or ENaC mutations (gray, mean ± S.E., n = 6, error bars hidden by data symbols), C, amiloride-sensitive current relative to wild type ENaC (mean ± S.E., n = 6, *p < .05). D, Cl− inhibition relative to wild type ENaC (mean ± S.E., n = 6).

We took advantage of species differences in ENaC regulation to identify residues required for regulation by pH; rat (and mouse) ENaC is much less sensitive to pH than human ENaC. We do not yet know why pH regulation of ENaC varies among species. 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 and mice lacked selective pressure to develop or retain the pH response because of a less varied diet.

ENaC is exposed to extreme changes in pH. Our data suggest that the ENaC extracellular domain functions as a sensor to detect these pH changes and to respond with appropriate changes in ENaC activity, and hence, in Na+ absorption. With hypervolemia, the inhibitory effect of acidic pH has dual effects on ENaC activity. In addition to stimulatory effect investigated here, acidic pH also reduces ENaC activity by enhancing Cl− inhibition (11). This inhibitory effect occurs through αE446A and γE455A which contribute to Cl− binding sites in the extracellular domain of ENaC. Protonation of these residues is thought to enhance Cl− binding through electrostatic effects. These opposing effects of pH may function to fine-tune renal Na+ absorption. With hypovolemia, delivery of NaCl to the collecting duct is low and the urine is acidic. Under these low Cl− conditions, the stimulatory effect of protons predominates, maximizing Na+ absorption. Conversely, with hypervolemia, the inhibitory effect of protons is enhanced by rising Cl− concentrations. This serves to reduce Na+ absorption. By modulating renal sodium absorption, changes in extracellular pH and ion concentrations may collaborate to maintain volume homeostasis and to regulate blood pressure.
pH Regulation of ENaC

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