Na\textsuperscript{+} Inhibits the Epithelial Na\textsuperscript{+} Channel by Binding to a Site in an Extracellular Acidic Cleft*

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Background: External Na\textsuperscript{+} inhibits ENaC.
Results: Mutations centered about a key aspartate in an acidic cleft weakened Na\textsuperscript{+} inhibition and altered inhibitor selectivity.
Conclusion: The acidic cleft hosts an inhibitory Na\textsuperscript{+} binding site.
Significance: The acidic cleft harbors a key Na\textsuperscript{+} binding site for ENaC and perhaps sites for ligands that regulate other members of the ENaC/degenerin family.

The epithelial Na\textsuperscript{+} channel (ENaC) has a key role in the regulation of extracellular fluid volume and blood pressure. ENaC belongs to a family of ion channels that sense the external environment. These channels have large extracellular regions that are thought to interact with environmental cues, such as Na\textsuperscript{+}, Cl\textsuperscript{−}, protons, proteases, and shear stress, which modulate gating behavior. We sought to determine the molecular mechanism by which ENaC senses high external Na\textsuperscript{+} concentrations, resulting in an inhibition of channel activity. Both our structural model of an ENaC α subunit and the resolved structure of an acid-sensing ion channel (ASIC1) have conserved acidic pockets in the periphery of the extracellular region of the channel. We hypothesized that these acidic pockets host inhibitory allosteric Na\textsuperscript{+} binding sites. Through site-directed mutagenesis targeting the acidic pocket, we modified the inhibitory response to external Na\textsuperscript{+}. Mutations at selected sites altered the cation inhibitory preference to favor Li\textsuperscript{+} or K\textsuperscript{+} rather than Na\textsuperscript{+}. Channel activity was reduced in response to restraining movement within this region by cross-linking structures across the acidic pocket. Our results suggest that residues within the acidic pocket form an allosteric effector binding site for Na\textsuperscript{+}. Our study supports the hypothesis that an acidic cleft is a key ligand binding locus for ENaC and perhaps other members of the ENaC/degenerin family.

The epithelial Na\textsuperscript{+} channel (ENaC) is a highly Na\textsuperscript{+}-selective heterotrimeric channel whose activity is modulated by a variety of extracellular factors (1, 2). ENaC-mediated Na\textsuperscript{+} conductance through the apical membrane is rate-limiting for transepithelial Na\textsuperscript{+} absorption in many Na\textsuperscript{+}-transporting epithelia (2). In the distal nephron, this transporter has a crucial role in extracellular fluid volume regulation and is under the control of the volume-regulatory hormones aldosterone and vasopressin (2, 3). The role of ENaC in transepithelial Na\textsuperscript{+} transport is broad because the channel is expressed in many transporting epithelia, including the distal nephron, lung airway and alveoli, distal colon, sweat glands, lingual epithelia, and inner ear (4–6). ENaC subunits are also expressed in vascular smooth muscle and endothelial cells, where they appear to modulate vascular tone (7).

Sensitivity to extracellular factors is a characteristic of members of the ENaC/degenerin family. For ENaC, a high extracellular [Na\textsuperscript{+}] inhibits the channel through at least two mechanisms. First, external Na\textsuperscript{+} rapidly inhibits ENaC by directly binding to extracellular allosteric effector sites within the channel. This allosteric inhibition, referred to as Na\textsuperscript{+} self-inhibition, reflects a low-affinity (tens of micromolar), pH-sensitive, non-voltage-sensitive reduction in ENaC open probability (8–13). This inhibitory effect is also cation-selective because both Na\textsuperscript{+} and Li\textsuperscript{+} inhibit the channel, whereas K\textsuperscript{+} has a modest inhibitory effect at best, and protons activate the channel (9, 10). Second, an elevated intracellular Na\textsuperscript{+} concentration inhibits the channel over a slow time course (14, 15).

The extracellular domains of members of the ENaC gene family likely host allosteric effector sites that sense factors in the extracellular environment. For example, acid-sensing ion channels (ASICs) are proton-activated. The resolved structure of ASIC1 features an acidic pocket in each subunit that was proposed to contain proton binding sites competent to trigger gating (16). A major challenge in elucidating ENaC gene family function has been the ability to distinguish ligand binding from downstream transduction steps. The crystal structure of ASIC1 suggests several putative proton binding sites, and mutations at select sites affect acid activation of ASIC1 (17–19). However, one cannot readily distinguish a proton binding site from sites involved in transmitting conformational changes to the channel gate. In this work, we used inhibitor specificity to tackle this challenge.

ENaCs are activated by proteases, which cleave the α and γ subunits at specific sites, releasing imbedded inhibitory tracts (1, 20–23). On the basis of ASIC1 homology and our work that identified the binding site of a peptide derived from the α subunit inhibitory tract, we developed a structural model of the ENaC α subunit (24, 25). We found that the α subunit inhibi-


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tory tract lies adjacent to an acidic region that is homologous to the ASIC1 acidic cleft. We hypothesized that specific residues within the acidic cleft of the \(\alpha\) subunit form a Na\(^+\) binding site required for the Na\(^+\) self-inhibition response.

Na\(^+\) self-inhibition is an allosteric response. Channel modifications that affect the apparent strength of Na\(^+\) self-inhibition could alter channel behavior at different steps in this regulatory process. Experimental manipulations that affect Na\(^+\) self-inhibition could alter the Na\(^+\) binding site, transduction steps subsequent to Na\(^+\) binding, or the stability of the open state relative to the closed state of the channel independent of Na\(^+\) binding. The fact that ENaC is both regulated by external Na\(^+\) and conducts Na\(^+\) complicates our ability to differentiate the Na\(^+\) binding step from other steps in the Na\(^+\) self-inhibition mechanism. To define sites within the channel periphery that participate in Na\(^+\) binding, we studied properties that directly reflect this event. Accordingly, we measured the effect of mutations on the effector or cation specificity of the Na\(^+\) self-inhibition response. Select mutations within the acidic cleft weakened Na\(^+\) self-inhibition and resulted in channels that were more effectively inhibited by Li\(^+\) or K\(^+\) than by Na\(^+\), in contrast to the wild-type channel phenotype, where Na\(^+\) is the most potent inhibitor. We identified a single titratable residue whose mutation abrogated Na\(^+\) self-inhibition as well as channel activation by acidic pH. Through cross-linking, we demonstrate that constraining movements of adjacent structures within the acidic cleft reduced channel activity. Our data show that the acidic cleft of the \(\alpha\) subunit of ENaC hosts an allosteric effector binding site for Na\(^+\) and provide strong support for the concept that interactions of external factors with the extracellular region of ENaC have an important role in modulating channel activity.

**EXPERIMENTAL PROCEDURES**

**ENaC Expression and Mutagenesis**—Mouse ENaC subunits were mutated and expressed in *Xenopus* oocytes as described previously (26). All mutations were confirmed by direct sequencing.

**Current Measurement**—Electrophysiological measurements were performed using a GeneClamp 500B voltage clamp amplifier (Axon Instruments, Foster City, CA) and pClamp 10.2 software (Axon Instruments). Oocytes were placed in a 20-\(\mu\)l recording chamber (AutoMate Scientific, Berkeley, CA) with perfusion (3–5 ml/min) controlled by an eight-channel pinch valve system (AutoMate Scientific). For experiments that did not examine the cation specificity of ENaC inhibition, we used a high Na\(^+\) bath solution (110 mM NaCl, 2 mM KCl, 2 mM CaCl\(_2\), and 10 mM HEPES (pH 7.4)). We replaced HEPES with MES for lower pH versions of the high-Na\(^+\) bath solution. To make a low-Na\(^+\) bath solution, we replaced NaCl with 109 mM NMDG and 1 mM NaCl. For experiments that examined the cation specificity of ENaC inhibition, we used buffers that included K\(^+\) channel blockers as described previously (9) (100 mM NMDG-Cl, 0.82 mM MgCl\(_2\), 0.41 mM CaCl\(_2\), 10 mM NMDG-HEPES, 5 mM BaCl\(_2\), and 10 mM TEA-Cl for the NMDG\(^+\) solution and with 100 mM NaCl, 100 mM LiCl, and 100 mM KCl replacing NMDG-Cl for the Na\(^+\), Li\(^+\), and K\(^+\) solutions, respectively). Amiloride was dissolved in dimethyl sulfoxide at 100 mM and diluted in bath solution to 10 \(\mu\)M for fixed-voltage experiments or 100 \(\mu\)M for voltage ramp experiments.

**Current-Voltage Curve Analysis**—The comparison of ENaC inhibition by extracellular Na\(^+\), Li\(^+\), and K\(^+\) is complicated by the fact that ENaC is permeable to these cations by a 1.6:1: <0.01 (Li\(^+\):Na\(^+\):K\(^+\)) ratio (27). To measure ENaCi inhibition by these ions, we determined the relative Na\(^+\) permeability (\(P_{Na}\)) in the presence of each ion from the amiloride-sensitive steady-state current-voltage (I-V) curves by fitting each curve to the Goldman-Hodgkin-Katz model as described previously (9) with some modifications. When Na\(^+\) was the only permeant ion present, we fit I-V curves to equation 1,

\[
I = \frac{P_{Na}}{RT} \left[ \frac{[Na]}{[Na]_o} \right] \exp \left( \frac{EF}{RT} \right) \left[ 1 - \exp \left( \frac{-EF}{RT} \right) \right] \tag{1}
\]

where \(E_F\) is the membrane potential, Faraday constant, \([Na]_o\) and \([Na]_i\) are the membrane potential, and extracellular \([Li]_o\) and \([Na]_i\) are the membrane potential, Faraday constant, absolute temperature, intracellular \([Na]_i\), and extracellular \([Na]_o\), respectively. When the bath solution was switched to Li\(^+\), we fit I-V curves to equation 2,

\[
I = \frac{P_{Li}}{RT} \left[ \frac{[Li]}{[Na]_o} \right] \exp \left( \frac{EF}{RT} \right) \left[ 1 - \exp \left( \frac{-EF}{RT} \right) \right] \tag{2}
\]

where \([Li]_o\) is extracellular \([Li]_o\) and \(P_{Li}/P_{Na}\) was assumed to equal 1.6 on the basis of the ion selectivity of the pore (9, 27). We assumed that the mutations examined here, far from the pore, did not affect the value of \(P_{Li}/P_{Na}\). We also assumed that \([Na]_o\) remained constant throughout the experiment and that intracellular \([Li]_i\) was zero. To minimize changes in \([Na]_o\) during the experiment, we clamped oocytes at –20 mV between voltage ramps. We collected five amiloride-sensitive I-V curves for each oocyte, using bath solutions with different cations (NMDG\(^+\), Na\(^+\), Li\(^+\), K\(^+\), and NMDG\(^+\) again) for 1 min, followed by the same solution supplemented with 100 \(\mu\)M amiloride for 20 s. We fit the I-V curves from each oocyte simultaneously using a global fitting routine and Igor Pro 6 (Wavemetrics, Oswego, OR). This allowed us to use data under all conditions to determine a global value for \([Na]_o\), while independently determining local values for \(P_{Na}\) under each condition. We assigned values for the global parameters \(F\), \(R\), and \(T\) and the local parameters \([Na]_o\), or \([Li]_i\), \(P_{Li}/P_{Na}\), \(E\) was the independent variable. Each fit produced five \(P_{Na}\) values, one for each condition. We normalized each \(P_{Na}\) value to a corrected \(P_{Na}\) determined with bath NMDG\(^+\) at the beginning and end of the experiment to account for channel rundown, which we assumed was linear over the course of the experiment.

**Statistical Analyses**—Values presented are mean ± S.E. We performed multiple group comparisons using analysis of variance followed by Tukey post hoc test. We adjusted the significance threshold from \(p = 0.05\) for multiple comparisons using the Bonferroni correction. We performed pairwise compari-
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In the absence of extracellular acidification, mutations of acidic residues in the acidic cleft of ENaC subunits affects Na⁺ self-inhibition. The acidic cleft is a region at the juncture of the finger, β-ball, and thumb domains (Fig. 1C), which has the appearance of an acidic cleft (24). A homologous region in ASIC1 has been proposed to play a role in proton sensing (16). We hypothesized that residues within the acidic cleft of ENaC subunits coordinate Na⁺, the first step in the Na⁺ self-inhibition response.

To examine whether mutations of residues in the acidic cleft weakened Na⁺ self-inhibition, we performed a Trp mutagenesis screen of acidic cleft Asp and Glu residues (Fig. 1C, red spheres) and measured the effect of mutation on Na⁺ self-inhibition. Wild-type or mutant ENaCs were expressed in *Xenopus laevis* oocytes, and ENaC currents were measured by two-electrode voltage clamp. Because ENaC Na⁺ self-inhibition manifests slowly relative to bath solution exchange, acutely raising the bath [Na⁺] from a low concentration (where the channel open probability (Pₒ) is high) results in a peak inward current that subsequently declines (8, 11), reflecting Na⁺ binding events at extracellular allosteric site(s) that are transduced to reduce channel Pₒ (i.e., Na⁺ self-inhibition) (Fig. 1B) (12, 13, 29).

Of the acidic sites tested, we found that the Trp mutation at αAsp-176, αGlu-362, and αAsp-365 reduced Na⁺ self-inhibition (Fig. 1E, red columns). The latter two lie at the beginning of a loop connecting the sixth and seventh β strands (Fig. 1C; β6-β7 loop), whereas αAsp-176 on the α1 helix interacts with the β6-β7 loop (Fig. 2A).

We hypothesized that β6-β7 loop residues participate in Na⁺ binding. We performed Trp scanning mutagenesis of the residues lining the β6-β7 loop and determined the effects of individual mutations on Na⁺ self-inhibition (Fig. 1F). In our model, the β6-β7 loop extends from the β-ball toward helix α1 of the finger domain, with αLeu-369 at the apex of the loop interacting with αAsp-176 from helix α1 of the finger domain (Figs. 1C and 2A). We found that a Trp mutation at multiple residues flanking αLeu-369 strongly reduced Na⁺ self-inhibition, whereas αLeu369W enhanced Na⁺ self-inhibition.

**RESULTS**

Protons Activate Mouse ENaC—Collier and Snyder (10) and Collier et al. (28) reported previously that human ENaC is activated by extracellular acidification with an apparent pH50 of activation of 6.5. They found that rat ENaC was also activated by acid but at a lower pH (28). Because acidification was associated with reduced Na⁺ self-inhibition, they suggested that extracellular acidification activated ENaC by relieving Na⁺ self-inhibition. We examined the effect of extracellular acidification on mouse ENaC expressed in *Xenopus* oocytes by two-electrode voltage clamp (Fig. 1A). Although modest acidification (to pH 6.5) did not alter channel activity (see below), lowering the extracellular pH from 7.4 to 4.7 stimulated currents by 44% (Fig. 1A, red columns), which has the appearance of an acidic cleft (24). A homologous region in ASIC1 has been proposed to play a role in proton sensing (16). We hypothesized that residues within the acidic cleft of ENaC subunits coordinate Na⁺, the first step in the Na⁺ self-inhibition response.

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If Na\(^+\) binding to residues in the β6-β7 loop results in a decrease in channel activity, then the conformation of the β6-β7 loop must be linked to the conformation of the channel gate. It follows that constraining movement of the loop should alter channel activity. We used a cross-linking approach to test this hypothesis. Our model suggested that αLeu-369 at the apex of the β6-β7 loop interacts with αAsp-176 from helix α1 of the finger domain (Fig. 2A). We introduced Cys at αLeu-369 in the β6-β7 loop and, simultaneously, to αAsp-176 in helix α1 of the finger domain. Because αL369C and αD176C are predicted to be in close proximity, we examined whether these introduced Cys residues formed a natural disulfide bond, linking the β6-β7 loop with the α1 helix. DTT addition activated ENaC-bearing Cys residues at both sites (Fig. 2, B and C). This activation required the presence of both introduced Cys residues because DTT did not affect the wild type or channels with a single Cys substitution. The DTT-treated double mutant had modestly reduced Na\(^+\) self-inhibition compared with DTT-treated wild-type channels (22.8 ± 0.5% versus 38 ± 2%, n = 4, p < 0.05), suggesting that the introduced cysteine residues, per se, modestly altered channel function. These data suggest that conformational changes that involve the β6-β7 loop affect channel activity.

**Residues in the Acidic Cleft Determine the Cation Specificity of Inhibition**—To identify residues involved in the Na\(^+\) binding step, we measured the effect of specific mutations on the ligand specificity of Na\(^+\) self-inhibition. As noted above, mutations that alter the Na\(^+\) self-inhibition response could reflect changes in the Na\(^+\) binding step, transduction steps subsequent to Na\(^+\) binding, or the relative stability of the open state. To determine which of these possibilities is responsible, we performed experiment A (Fig. 3). Oocytes expressing wild-type ENaC and various mutants were treated for 1 min with each of Na\(^+\), Li\(^+\), K\(^+\), and NMDG\(^+\). Measurements of channel currents were made 20 s after perfusion with each solution. The resulting I-V curves were fit with equation 1 to determine the Na\(^+\) permeability (P\(_{\text{Na}}\)) and the Na\(^+\) conductance (G\(_{\text{Na}}\)).

**FIGURE 2.** Cross-linking the β6-β7 loop to the α1 helix reduces channel activity. A, Leu-369 on the β6-β7 loop and Asp-176 on helix α1 are adjacent in our model of the α subunit. B, oocytes expressing wild-type ENaC, either of the α subunit single mutants (αD176C or αL369C) or the αD176C/αL369C double mutant, were exposed to 10 mM DTT and then amiloride (10 μM) while currents were measured at a holding potential of −100 mV. Representative recordings are shown. Currents were normalized to the current immediately prior to DTT addition. Average currents prior to DTT were −1.7 ± 0.4 μA (wild type), −1.5 ± 0.3 μA (αD176C), −1.9 ± 0.4 μA (αL369C), and −1.1 ± 0.2 μA (αD176C/αL369C). C, amiloride-sensitive currents following DTT addition normalized to currents prior to DTT (n = 7–10). *, p < 0.01.

**FIGURE 3.** Mutations in the acidic cleft alter the cation selectivity of ENaC inhibition. A, ENaC-expressing oocytes were perfused for 1 min with each solution, after which currents were determined at varying holding potentials (−140 to 60 mV in 20-mV steps, 0.5 s/step). Amiloride (amil)-supplemented solutions were then perfused for 20 s, followed by measurement of currents at the different holding potentials. B, resultant I-V curves were fit with equation 1 or 2 to derive P\(_{\text{Na}}\) values in each solution (red, initial NMDG\(^+\); green, Na\(^+\); blue, Li\(^+\); black, K\(^+\); orange, final NMDG\(^+\)). Measurements in NMDG\(^+\) were taken at the beginning and end of each experiment to estimate channel rundown (−2%–7%/min), which was used to adjust P\(_{\text{Na}}\) values for channel rundown. C, correlation between Na\(^+\) self-inhibition measured under a constant voltage clamp (Fig. 1B) and the ratio of Na\(^+\) permeabilities in Na\(^+\) and NMDG\(^+\). D, ratio of Na\(^+\) permeabilities measured in different bath solutions: Li\(^+\) versus Na\(^+\) (top panel) and K\(^+\) versus Na\(^+\) (bottom panel); *, p < 0.003 versus wild type (n = 40 for the wild type and 5–12 for mutants).
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and/or closed states. We reasoned that the ability to discriminate Na<sup>+</sup> and Li<sup>+</sup> from other cations reflects ligand binding.

To compare the inhibition of ENaC by Na<sup>+</sup>, Li<sup>+</sup>, and K<sup>+</sup>, we examined steady-state Na<sup>+</sup> permeability in the presence of different extracellular cations using a method adapted from Bize and Horisberger (9). Amiloride sensitive I-V curves were obtained at steady state in oocytes sequentially perfused with buffers containing NMDG<sup>+</sup>, Na<sup>+</sup>, Li<sup>+</sup>, K<sup>+</sup>, and then NMDG<sup>+</sup> again as the predominant cation (Figs. 3A and B). We then fit the I-V curves to equations derived from the Goldman-Hodgkin-Katz model of ion flux to determine the relative permeability of ENaC to Na<sup>+</sup> (P<sub>Na</sub>) under each condition (Fig. 3B and equations 1 and 2, see “Experimental Procedures”). Reductions in P<sub>Na</sub> in Na<sup>+</sup>, Li<sup>+</sup>, or K<sup>+</sup> relative to P<sub>Na</sub> in NMDG<sup>+</sup> reflect ENaC inhibition by these effectors. We assumed that the Li<sup>+</sup>/Na<sup>+</sup> permeability ratio (P<sub>Li</sub>/P<sub>Na</sub>) i.e. the pore selectivity is 1.6 on the basis of published reports (27, 30) and is not affected by these mutations far from the pore. We assumed that the K<sup>+</sup>/Na<sup>+</sup> permeability is 0. We also assumed that intracellular [Na<sup>+</sup>] ([Na]) remains steady through the course of the experiment. With these assumptions, fitting each curve to the Goldman-Hodgkin-Katz model depends on two variables, P<sub>Na</sub> and [Na], which have little interdependency for curve fitting purposes (Fig. 3B). We corrected for channel rundown using P<sub>Na</sub> measurements in NMDG<sup>+</sup> at the beginning and end of the experiment and assuming a linear rundown.

The extent of Na<sup>+</sup> inhibition measured by this method correlated with Na<sup>+</sup> self-inhibition measured by rapidly switching solutions from NMDG<sup>+</sup> to Na<sup>+</sup> (Fig. 1B). Na<sup>+</sup> self-inhibition is given by the ratio of P<sub>Na</sub> with extracellular Na<sup>+</sup> to P<sub>Na</sub> with extracellular NMDG<sup>+</sup> (P<sub>Na</sub>(Na)/P<sub>Na</sub>(NMDG)). We plotted the results for the wild type, αD176W, and the β6-β7 loop Trp mutants (Fig. 3C). The fitted line gave a Pearson’s correlation coefficient of −0.75 (<i>p</i> < 0.005). Notably, the fitted line is shifted from its theoretical position (the diagonal from (0, 100) to (1, 0)) toward the origin. This may reflect, in part, an under-estimation of the peak current (e.g. Fig. 1B), which depends upon the efficiency of solution exchange and the kinetics of channel inhibition.

Wild-type mouse ENaC showed a slightly different effector specificity for channel inhibition (Na<sup>+</sup> > Li<sup>+</sup> > K<sup>+</sup> Fig. 3D) than what has been reported for human ENaC (Na<sup>+</sup> ≈ Li<sup>+</sup> > K<sup>+</sup>) (9). Trp substitutions at sites toward the beginning or end of the β6-β7 loop changed this order. For αQ363W, αD365W, and αV373W, Li<sup>+</sup> was a more potent inhibitor than Na<sup>+</sup>, whereas K<sup>+</sup> and Na<sup>+</sup> had similar inhibitory effects. The mutants αI367W, αS371W, and αT374W exhibited weakened Na<sup>+</sup> inhibition relative to K<sup>+</sup>, whereas αL370W exhibited weakened Li<sup>+</sup> inhibition relative to Na<sup>+</sup>. Trp mutation at other sites neighboring αGln-363 and αVal-373 showed trends toward changing inhibitor specificity but were not significantly different from the wild type.

To corroborate these results, we measured effector specificity using a second method that adapted a fixed-voltage protocol for measuring Na<sup>+</sup> self-inhibition (Fig. 1B). First we examined Na<sup>+</sup> inhibition by rapidly switching from an NMDG<sup>+</sup> solution to a Na<sup>+</sup> solution while voltage-clamping the oocyte at −100 mV (Fig. 4). As described above, this leads to a rapid inward peak current (<i>I</i><sub>P</sub>) that declines to a steady-state level (<i>I</i><sub>SS</sub>). The fall from <i>I</i><sub>P</sub> to <i>I</i><sub>SS</sub> reflects Na<sup>+</sup> self-inhibition. Because ENaC also conducts Li<sup>+</sup>, we measured Li<sup>+</sup> inhibition in a similar manner by rapidly switching from an NMDG<sup>+</sup> solution to a Li<sup>+</sup> solution. Because ENaC does not appreciably conduct K<sup>+</sup>, we measured K<sup>+</sup> inhibition indirectly by examining the effect of K<sup>+</sup> pretreatment on Na<sup>+</sup> inhibition. Instead of initially bathing the oocyte in an NMDG<sup>+</sup> solution, where ENaC <i>P</i><sub>Na</sub> is high (12), we used a K<sup>+</sup> solution. If channel inhibition by K<sup>+</sup> is much weaker than by Na<sup>+</sup>, switching from NMDG<sup>+</sup> or K<sup>+</sup> to Na<sup>+</sup> should produce similar Na<sup>+</sup> self-inhibition responses. This is what we observed for wild-type channels (Fig. 4). If channel inhibition by K<sup>+</sup> and Na<sup>+</sup> are similar, then pretreatment with K<sup>+</sup> should attenuate the inhibitory response to Na<sup>+</sup>, as we observed with the αV373W and αD365W mutants (Fig. 4). If K<sup>+</sup> inhibits an ENaC mutant more effectively than Na<sup>+</sup>, then switching from a K<sup>+</sup> to Na<sup>+</sup> bath should induce a rise in current that represents Na<sup>+</sup> relief of K<sup>+</sup> inhibition. This was seen with the αQ363W mutant (Fig. 4). Using this fixed-voltage protocol, we also found that Na<sup>+</sup> was a more effective inhibitor than Li<sup>+</sup> of wild-type mouse ENaC. αN364W exhibited a pattern similar to the wild type. For αV373W and αD365W, this pattern was reversed. For αQ363W, Li<sup>+</sup> and Na<sup>+</sup> were similarly effective inhibitors. For the wild type and αN364W, K<sup>+</sup> pretreatment did not alter Na<sup>+</sup> self-inhibition when compared with NMDG<sup>+</sup> pretreatment.
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**FIGURE 5.** Specific conserved sites on structures near αD365 weaken Na\(^+\) inhibition. **A**, structures near the β6-β7 loop in an ENaC α trimer model, including the β2-α1 loop of the α subunit and the beginning of helix α6 of the neighboring β subunit. **B** and **C**, sequence alignments of the β2-α1 loop (**B**) and the β10-α6 loop (**C**), with sites of interest indicated by arrows. **D**, 

\[ P_{Na}(X) / P_{Na}(NMHG) \]

**E**, representative experiment examining the effector specificity of inhibition of αN161A compared with the wild type (p < 0.05, Student’s t test). K\(^+\) pretreatment did not affect Na\(^+\) inhibition of either the wild type or αN161A compared with NMHG pretreatment (p = not significant, paired Student’s t test). In contrast to the wild type, Li\(^+\) inhibition was greater than Na\(^+\) inhibition for αN161A channels (p < 0.05, paired Student’s t test).

**Sites Adjacent to the β6-β7 Loop Affect Inhibitor Specificity**—Mutations in the β6-β7 loop affect the cation selectivity of channel inhibition. Our model of a hypothetical ENaC α trimer suggested that adjacent sites could coordinate a bound ion in concert with sites in the β6-β7 loop (Fig. 5A). αAsn-161 is at the end of a highly conserved sequence in the β2-α1 loop connecting the β ball and the finger domain in the α subunit and lies in proximity to the β6-β7 loop (Fig. 5B). The first Ser within a WPSXXS motif in the knuckle domain of a neighboring subunit lies across the plane of the α subunit β6-β7 loop (Fig. 5C). According to recent reports, the neighboring knuckle domain is part of the β subunit (31, 32). Notably, a polymorphism of the Trp residue in the WPSXXS motif of the human α subunit (αW493R) was associated with a loss of Na\(^+\) self-inhibition and an increased channel P\(_{Na}\) (33). We mutated αAsn-161 to Ala and βSer-462 to Cys and assessed the resultant inhibitor specificity. We compared P\(_{Na}\) values derived from steady state I-V curves using different perfusion buffers as described above. Both αN161A and βS462C weakened Na\(^+\) inhibition but did not affect Li\(^+\) or K\(^+\) inhibition (Fig. 5D). We corroborated these results for αN161A using the fixed-voltage protocol for measuring self-inhibition induced by Na\(^+\) or Li\(^+\) (Fig. 5E), which showed a pattern of inhibition similar to αD365W and αV373W and different from wild-type ENaC (Fig. 4).

**Protonation of αAsp-365 Activates ENaC**—Acidification of the extracellular solution increased mouse ENaC currents (Fig. 1A). We hypothesized that protonation of a Na\(^+\)-coordinating residue weakens Na\(^+\) binding, diminishing Na\(^+\) self-inhibition and activating the channel. To identify titratable residues responsible for this activation, we performed titrations of wild-type channels and three mutants: αD176W, αE362W, and αD365W (Fig. 6B). We performed each experiment as described for Fig. 1A and measured the acute amiloride-sensitive change in current upon acidification from pH 7.4. Although each mutant blunts Na\(^+\) self-inhibition (Fig. 1), we observed that only αD365W reversed the effect of acidification on ENaC currents, causing currents to modestly fall instead of rise (Figs. 1A and 6, A and B). Channels bearing αD176W or αE362W responded to acidification similarly to wild-type. For all groups tested, effects at pH 5.5 were intermediate to effects at pH 4.7. In contrast to human ENaC (10), effects at pH 6.5 were minor for all groups tested.

We also examined the effect of acidification on the Na\(^+\) self-inhibition response. At pH 4.7, the wild type and the three Trp mutants exhibited low Na\(^+\) self-inhibition responses (Fig. 6C and D). These data, taken together, suggest that reducing the pH to 4.7 or altering the Asp group at αAsp-365 removes an important coordinating group from one of the putative Na\(^+\)
**Na\(^+\) Inhibits ENaC by Binding in an Acidic Cleft**

![Diagram](image-url)

**FIGURE 6. αAsp-365 mutants lack acid-dependent channel activation.** A, acidification inhibits αD365W currents. B, effects of pH titration on the wild type and select mutants of acidic residues in the acidic cleft. Each experiment was performed as in Figs. 1A and 6A while varying the final pH. The acute amiloride-sensitive change in current upon acidification to pH 6.5, 5.5, or 4.7 was quantified. For experiments in which currents increased as a result of acidification, we used the maximal increase in current, which occurred at 10 ± 1 s after initiating solution exchange (Fig. 1A). For experiments in which the current decreased as a result of acidification, there was no transient nadir because both the fast and slow phases of the change affected the current in the same direction (A).

For quantification of the acute effect of changing pH on current, we used the current value at 10 s after initiating solution exchange. *p < 0.01 versus the wild type at pH 5.5; #, p < 0.005 versus the wild type at pH 4.7. For all groups, n = 5–8. C, acidification weakens Na\(^+\) self-inhibition. Shown are representative current recordings of oocytes expressing the wild type or α subunit mutants and voltage-clamped at −100 mV. Solutions were exchanged rapidly as indicated. D, Na\(^+\) self-inhibition was quantified at pH 4.7 as for Fig. 1E. No significant differences were detected (n = 5–8). E, experiments were performed as described for Fig. 3. P\(_{\text{Na}}\) values where Na\(^+\), Li\(^+\), or K\(^+\) were the principal cation were normalized to P\(_{\text{Na}}\) determined in NMDG\(^+\). *p < 0.005 versus the wild type (n = 40 for the wild type and 5–8 for mutants).

binding sites of ENaC. To further address this hypothesis, we examined the response of the channel to acidification after introducing conservative Glu or Asn substitutions at αAsp-365 or after reversing the charge by Lys substitution. All three αAsp-365 mutants (Glu, Asn, and Lys) prevented channel activation by acidification, echoing our result with αD365W (Fig. 6B). We also examined the effect of these mutations on Na\(^+\), Li\(^+\), or K\(^+\) inhibition at steady state using the voltage ramp method (Fig. 3). We found that each mutant specifically weakened Na\(^+\) inhibition (Fig. 6E). Our data suggest that the specific moiety at position α365, rather than charge per se, is needed to confer “full” sensitivity to external Na\(^+\).

**DISCUSSION**

ENaCs are members of a family of ion channels that respond to factors in the extracellular environment. One of the external factors that regulate ENaC is Na\(^+\). There are multiple lines of evidence supporting the notion that Na\(^+\) binding to one or more effector sites within the extracellular domains results in the inhibition of channel activity in an allosteric manner (1, 12, 13, 29, 34). Given that allosteric transitions are required for this process, it is not surprising that many substitutions throughout the extracellular regions of ENaC subunits altered the Na\(^+\) self-inhibition response. For example, we and others have reported mutations that weaken Na\(^+\) self-inhibition in the palm domain, thumb domain, β ball-thum domain interface, knuckle domain, finger domain, and subunit interfaces and among the Cys involved in disulfide bridges (13, 25, 28, 31, 33, 35–39). Furthermore, it is not surprising that mutations in transmembrane helical residues that interfere with pore closure and increase channel P\(_{\text{Na}}\) (e.g. βS518K) significantly weaken Na\(^+\) self-inhibition (38, 40). Likewise, other maneuvers that increase channel P\(_{\text{Na}}\), e.g. Zn\(^{2+}\) addition (41) or Cl\(^-\) removal (42), weaken apparent Na\(^+\) self-inhibition. In summary, on the basis of previous studies, it has been difficult to discern whether specific mutations that change Na\(^+\) self-inhibition do so by altering Na\(^+\) binding, downstream transduction steps, or the baseline stability of the open or closed states of the channel.

We postulated that Na\(^+\) binds to a site defined, in part, by acidic residues, consistent with Na\(^+\)-bound protein structures (43, 44) and the predominance of oxygen atoms coordinating Na\(^+\) in model small molecules (45). We also reasoned that the preference of the channel for Na\(^+\) over other cations as an inhibitory effector originates from the binding site(s) rather than from subsequent transduction steps. On these premises, we tested the ability of α subunit acidic cleft mutants to both affect Na\(^+\) self-inhibition and alter the relative inhibitory efficacy of Na\(^+\), Li\(^+\), and K\(^+\). Mutation of αAsp-365 or neighboring residues in our α subunit model weakened inhibition by external Na\(^+\) and altered the cation selectivity of channel inhibition. Even conservative αAsp-365 mutations (Glu or Asn) had this effect. αAsp-365 substitutions also prevented channel activation by acidification regardless of whether a conservative or non-conservative substitution was used. These results support the notion that αAsp-365 has a role in Na\(^+\) binding and the subsequent reduction in channel P\(_{\text{Na}}\) and that acidification activates ENaC by protonating αAsp-365 and disrupting Na\(^+\) binding at an effector site that includes αAsp-365.

Our results also suggest that there are multiple effector sites for Na\(^+\) self-inhibition. All mutants tested at or near αAsp-365 retained at least a small Na\(^+\) self-inhibition response. Acidifi-
cation had no effect on the Na⁺ self-inhibition response of αD365W, but reduced the response of the wild type and other mutants to similarly low but measurable values (compare Figs. 1E and 6D). We propose that there are additional Na⁺ effector sites. Because αD365W has a small but measurable Na⁺ self-inhibition response, at least one of the additional effector sites does not depend on residues titratable in the pH 7.4–4.7 range. Accordingly, conservative mutations of the β and γ sites that correspond to αAsp-365 (βD302N and γE322Q) exhibited Na⁺ self-inhibition similar to the wild type (βD302N, 33 ± 4%; γE322Q, 27 ± 3%; wild type, 32 ± 2%; n = 5–6; p = not significant).

Other members of the ENaC/Deg family have an Asp or Glu residue at the position equivalent to mouse αAsp-365 (e.g. Glu-229 in chicken ASIC1, Fig. 1D). This residue lies at the beginning of the β6-β7 loop, which connects the β-ball and palm domains (1, 16). Electrostatic calculations for the ASIC1 Glu (equivalent to αAsp-365) predicted a pKₐ of 7.4, a prerequisite for proton sensing in the physiological range (17). However, mutating this residue in ASIC1 to Gln had little effect on the pH sensitivity of channel activation (17, 18). The δ subunit of ENaC replaces the α subunit in the channel complex expressed in some tissues of several species, including humans (but not rodents). Interestingly, human δβγ channels are poorly inhibited by external Na⁺ (46). The δ subunit has a Pro residue at the equivalent site to αAsp-365. However, mutating δPro-314 to Asp did not sensitize human δβγ ENaC to external Na⁺ (data not shown). This observation suggests that there are residues in addition to αAsp-365 that are needed to form a Na⁺ binding site in the α subunit.

The β6-β7 loop of ASIC1 interacts with the finger and thumb domains and is six residues longer than its ENaC subunit counterparts (Fig. 1D). In our α subunit model, the shorter β6-β7 loop approaches the α1 helix in the finger domain but does not abut thumb domain residues (Fig. 1C) (24). Our results demonstrating that αD176C in the α1 helix and αL369C in the β6-β7 loop form a disulfide bond suggest that these structures are indeed in close proximity and that stabilizing interactions between the α1 helix and the β6-β7 loop is inhibitory. The ASIC1 β6-β7 loop includes Asp-238 and Glu-239 (cASIC1 numbering, Fig. 1D) that pair with thumb domain residues Asp-350 and Asp-346, respectively. These thumb domain residues have been proposed to sense protons (16). Krauson et al. (19) showed that mutating Asp-346 in mouse ASIC1a results in a biphasic pH activation curve, suggesting that this mutation specifically weakened one of multiple proton sensing sites. ASIC1 residues Asp-238 and Glu-239 and ENaC residues α371–373 are located in a similar region of the β6-β7 loop (Fig. 1D), where α subunit Trp substitutions weaken Na⁺ self-inhibition and alter the cation selectivity of this process (Figs. 1, 3, and 4). Baconguis and Gouaux (47) provided additional evidence for the role of the β6-β7 loop in modulating channel gating in response to external factors. They found that psalmotoxin, an ASIC1 inhibitor, extends an Arg-rich hairpin into the acidic pocket and forms hydrogen bonds with the β6-β7 loop backbone (47). These previous observations and our current findings, taken together, suggest that the β6-β7 loop has evolved within members of the ENaC/degenerin family members to facilitate the sensing of specific factors.

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