Base of the Thumb Domain Modulates Epithelial Sodium Channel Gating*

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The activity of the epithelial sodium channel (ENaC) is modulated by multiple external factors, including proteases, cations, anions and shear stress. The resolved crystal structure of acid-sensing ion channel 1 (ASIC1), a structurally related ion channel, and mutagenesis studies suggest that the large extracellular region is involved in recognizing external signals that regulate channel gating. The thumb domain in the extracellular region of ASIC1 has a cylinder-like structure with a loop at its base that is in proximity to the tract connecting the extracellular region to the transmembrane domains. This loop has been proposed to have a role in transmitting proton-induced conformational changes within the extracellular region to the gate. We examined whether loops at the base of the thumb domains within ENaC subunits have a similar role in transmitting conformational changes induced by external Na⁺ and shear stress. Mutations at selected sites within this loop in each of the subunits altered channel responses to both external Na⁺ and shear stress. The most robust changes were observed at the site adjacent to a conserved Tyr residue. In the context of channels that have a low open probability due to retention of an inhibitory tract, mutations in the loop activated channels in a subunit-specific manner. Our data suggest that this loop has a role in modulating channel gating in response to external stimuli, and are consistent with the hypothesis that external signals trigger movements within the extracellular regions of ENaC subunits that are transmitted to the channel gate.

Epithelial Na⁺ channels (ENaCs) are expressed in the distal segments of the nephron, airway and alveolae, and distal colon, where they mediate Na⁺ flux across apical membranes of epithelial cells. These channels have important roles in the regulation of airway surface liquid volume and mucociliary clearance, and in the regulation of extracellular fluid volume and blood pressure. ENaCs are composed of three homologous subunits, termed α, β, and γ, which have two hydrophobic transmembrane domains linked by large extracellular regions that include conserved Cys-rich domains.

Members of the ENaC/degenerin family are regulated by extracellular factors. For ENaC, these factors include proteases, protons, metals (including Na⁺), Cl⁻, and mechanical forces such as shear stress (1–6). ENaCs share about 20% identity at the amino acid level with acid-sensing ion channels (ASICs), members of the ENaC/degenerin family that are activated by extracellular acidification. The resolved structure of the extracellular and transmembrane portions of ASIC1 has provided clues into mechanisms by which external factors could modulate channel gating (7, 8). The extracellular domain of ASIC1 resembles an outstretched hand containing a ball, and has defined subdomains termed wrist, finger, thumb, palm, β-ball, and knuckle. Proton binding was proposed to lead conformational changes within specific extracellular domains, including the thumb domain (7).

A loop that links the 9th β sheet in the palm domain with the 4th α helix in the thumb domain (β9-α4 loop) interfaces with residues connecting the extracellular region to the transmembrane domains, where the channel gate resides (Fig. 1) (7, 9). Within this loop, a Trp residue has been proposed to couple movements within the extracellular region elicited by acidification to the transmembrane domains (7, 10). A mutation of this residue (Trp-288 in chicken ASIC1) disrupted proton-dependent gating of ASIC1a (10). A conserved Tyr residue is present at this site in the three ENaC subunits. Channels with a mutation of this Tyr residue in all three subunits exhibited whole cell Na⁺ currents in Xenopus oocytes that were similar in magnitude to wild-type channels.

In contrast to ASICs, ENaCs are active under basal conditions. However, their activity can be modulated by external factors, including external Na⁺ and laminar shear stress (LSS) (6, 11–16). We report that the responses of ENaC to changes in the external [Na⁺] and to LSS are altered by mutations within the β9-α4 loop. Our results support the hypothesis that external factors, including Na⁺ and shear stress, induce movements within the periphery of the extracellular regions of ENaC that, in part via the β9-α4 loop at the base of the thumb domain, influence channel gating.
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Site-directed Mutagenesis and in Vitro Transcription—Mouse wild-type or mutant α, β, and γ ENaC cDNAs in pBlue-script SK-vector (Stratagene, La Jolla, CA) were used as templates to generate point mutations using a PCR-based method as previously described (17) or with the QuikChange II XL site-directed mutagenesis kit (Stratagene). Targeted point mutations were confirmed by direct sequencing. The cRNAs for wild type and mutant ENaC subunits were synthesized with T3 mMessage mMachine (Ambion, Inc.) and quantified by spectrophotometry. The appropriate size of the cRNA was confirmed by agarose gel electrophoresis.

ENaC Expression and Two Electrode Voltage Clamp—Oocytes were harvested from Xenopus laevis using a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee. ENaCs were expressed in stage V-VI Xenopus oocytes by injecting 1 to 2 ng of cRNA per subunit of mouse α, β, and γ ENaC. The injected oocytes were incubated at 18 °C in modified Barth’s saline (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 15 mM HEPES, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 10 μg/ml sodium penicillin, 10 μg/ml streptomycin sulfate, 100 μg/ml gentamicin sulfate, pH 7.4). All experiments were performed at room temperature 20–48 h following cRNA injection.

Procedures for Examining Na⁺ Self-inhibition—Oocytes were placed in the recording chamber (Warner Instruments, Hamden, CT) and perfused with a flow rate of 12–18 ml/min. In the first 60 s, oocytes were perfused with a high Na⁺ bath solution (NaCl-110, containing 110 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 10 mM HEPES, pH 7.4), and then with a low Na⁺ bath solution (NaCl-1, containing 1 mM NaCl, 109 mM N-methyl-d-glucamine, 2 mM KCl, 2 mM CaCl₂, 10 mM HEPES, pH 7.4) for another 60 s. To initiate Na⁺ self-inhibition, the low Na⁺ bath solution was rapidly replaced by the high Na⁺ bath solution while the oocytes were clamped at −60 mV. At the end of each experiment, 10 μM amiloride in the NaCl-110 solution was added to the bath to determine the amiloride-insensitive component of the whole cell Na⁺ current. Bath solutions were exchanged with a Teflon valve perfusion system (Automate Scientific, Inc.). Currents remaining in the presence of 10 μM amiloride were typically less than 200 nA. To determine the time constant for Na⁺ self-inhibition (τ), the first 40 s of current decay following the switch to NaCl-110 was fit with an exponential equation by Clampfit 10.2 (Molecular Devices). Two current measurements were used to assess Na⁺ self-inhibition. The peak current (Ipeak) was measured as the maximal inward current immediately after the switch from the low to high Na⁺ concentration bath. The steady state current (I∞) was measured 40 s after Ipeak. The ratio of the amiloride-sensitive I∞ to Ipeak currents was used to assess the extent of the Na⁺ self-inhibition response.

Procedures for Examining LSS Response—Oocytes were maintained in a recording chamber (20 mm diameter) and perfused with the high [Na⁺] bath solution (NaCl-110) at a rate of 3.5 ml/min. LSS was applied by perfusion with bath solution through a vertical pipette (1.8 mm internal diameter) placed near the surface of the oocyte. The flow rate was adjusted to 1.6 ml/min, corresponding to 0.14 dynes/cm² of shear stress as previously described (6). To determine the kinetics of ENaC activation by shear stress, oocytes were clamped at a holding potential of −60 mV while whole cell currents were continuously recorded. The recording chamber was perfused with NaCl-110 for the first 25 s. LSS was then initiated by perfusion through the vertical pipette for 80 s. At the end of experiment, the ENaC-mediated component of the whole cell Na⁺ current was determined by perfusing with NaCl-110 supplemented with 5 μM benzamil. Whole cell Na⁺ currents were measured just prior to and following stimulation by LSS, and after bath perfusion with 5 μM benzamil. The benzamil-sensitive current measured during application of shear stress (ILSS) was normalized to the benzamil-sensitive basal current (Ibasal). To assess the relative magnitude of the flow response, Ibasal/I∞ values for each mutant were normalized to that of wild-type channels from the same batch. The time constant for channel activation (τ) was determined by fitting the first 40 s of current following the initiation of vertical perfusion with an exponential equation using Clampfit 10.2 (Molecular Devices).

Accessibility of a Cys-reactive Reagent—1 mM MTSET (2-(trimethylammonium)ethyl methanethiosulfonate bromide, Toronto Research Chemicals) was freshly prepared in NaCl-110 and delivered to oocytes by perfusion at the flow rate of 3.5 ml/min for 4 min while holding the membrane potential at 0 mV. The chamber was then perfused with NaCl-110 for 3 min to wash out excess MTSET. Subsequently, the holding potential was switched to −60 mV, and LSS was initiated by vertical perfusion as described above.

Statistical Analysis—Data were expressed as the mean ± S.E. unless otherwise indicated. Experiments were repeated with a minimum of two batches of oocytes obtained from different frogs. Given the variability in the response of channels to external Na⁺ (Na⁺ self-inhibition) and shear stress between different batches of oocytes, the response of mutant ENaCs was always compared with the response of wild-type channels within the same batch of oocytes. Electrophysiological data were analyzed with Clampfit 10.2 (Molecular Devices) and Origin 8.1 (OriginLab). Statistical comparisons were obtained
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RESULTS

ENaC gating is regulated by a variety of external factors including Na⁺, shear stress, and proteases. Increases in the external [Na⁺] reduce channel open probability (P₀), a process referred to as Na⁺ self-inhibition (11, 18, 19). Increases in LSS enhance channel activity, reflecting an increase in ENaC Po (6, 14, 20). It is likely that the extracellular regions of ENaC subunits have a key role in the channel response to external stimuli, including LSS and Na⁺. Mutations at defined sites in the extracellular regions have profound effects on Na⁺ self-inhibition (5, 11, 13, 19, 21). These results suggest that the binding of Na⁺ at external site(s) induces movements that are transmitted to the channel gate within the pore.

Mutations of Residues in the β9-α4 Loop Alter the Na⁺ Self-inhibition Response—The moderate sequence identity between the extracellular regions of ENaC subunits and ASIC1 (~25% identity excluding the finger domain) and the presence of conserved Cys residues that form defined disulfide bonds suggest that main structural features of the extracellular regions of ENaC subunits and ASIC1 are conserved (7, 12). We examined whether mutations in the putative β9-α4 loop of the three ENaC subunits were associated with changes in Na⁺ self-inhibition. As noted above, a Trp residue in β9-α4 loop of ASIC1 (Trp-288 in chicken ASIC1) has been proposed to couple H⁺-induced movements within the extracellular region to the transmembrane domain. A Tyr residue is at the homologous position in each of the three ENaC subunits (αY418, βY356, and γY375; see Fig. 2). These residues were mutated to Ala, Cys, or Trp, and the regulation of channels bearing a mutant subunit by external Na⁺ was examined. The Na⁺ self-inhibition response was determined in Xenopus oocytes expressing wild-type or mutant channels by clamping the membrane potential at −60 mV, and recording whole cell Na⁺ currents while rapidly changing the bath solution from a low (1 mM) to a high (110 mM) [Na⁺]. Following the increase in the bath [Na⁺], the whole cell current rapidly rises to a peak value, which then falls to a plateau current (see Fig. 3 and Table 1). The ratio of the steady state current to the maximal inward current (Iₜₕ/Iₚₑᵃᵏ) and the time constant of current reduction (𝜏) were used as measures of Na⁺ self-inhibition. The wild type channels exhibited an average Iₜₕ/Iₚₑᵃᵏ value of 0.63 ± 0.01 (n = 130; Table 1). The average time constant for Na⁺ self-inhibition for wild-type channels was 8.0 ± 0.2 s. Substitutions of αY418 with Ala, Cys, or Trp, or βY356 with Trp significantly increased Iₜₕ/Iₚₑᵃᵏ, reflecting a reduction in the Na⁺ self-inhibition response. No significant changes were observed in the time constant of the Na⁺ self-inhibition response. Channels with a Trp substitution of all 3 Tyr residues (i.e. αY418W/βY356W/γY375W) had a Na⁺ self-inhibition response that was similar to wild type.

If the β9-α4 loop has a role in transmitting structural changes from the channel periphery to the gate, substitutions of

from one-way ANOVA followed by Bonferroni test. A p value of less than 0.05 was considered statistically different.

FIGURE 2. Sequence alignment of mouse αENaC, βENaC, γENaC, and chicken ASIC1. Alignment of residues flanking the conserved Tyr in the β9-α4 loop at the base of the thumb domains of the α, β, and γ subunits. A Trp residue is present at the analogous position of ASIC1. The conserved Tyr residues are highlighted in bold. Other residues flanking the Tyr that were studied are indicated by the boxes.

FIGURE 3. Na⁺ self-inhibition is repressed by selected mutations of the Tyr residue in the β9-α4 loop. A, representative recordings for Na⁺ self-inhibition responses of wild type and selected mutant channels are shown. Oocytes were clamped at −60 mV and whole cell currents were continuously recorded while bath [Na⁺] was rapidly increased from 1 mM (open bar) to 110 mM (closed bar). Experiments were performed with 8 to 14 oocytes expressing mutated channels. B, amiloride-sensitive Iₚₑᵃᵏ/Iₚₑᵃᵏ ratios of mutant and wild-type channels are shown. The peak current (Iₚₑᵃᵏ) was measured as the maximal inward current immediately after bath exchange. The steady state current (Iₜₕ) was measured 40 s after Iₚₑᵃᵏ. The introduced mutations, Ala, Cys, or Trp, at the conserved Tyr residues are noted. 3W refers αY418W/βY356W/γY375W channels. C, time constants of current decay (𝜏) are shown. These were determined by fitting, with an exponential equation, the first 40 s of current decay following the maximal inward current. Dashed lines (β and C) show the averaged wild type values representing 130 observations, which are intended for reference and not for statistical comparisons. The responses of mutant ENaCs were always examined with wild-type channels within the same batch of oocytes to account for variability of Na⁺ self-inhibition responses within different batches of oocytes. Statistically significant differences between oocytes expressing wild-type channels and mutant channels were labeled with asterisks (*, p < 0.05, determined by one-way ANOVA followed by Bonferroni test (see “Experimental Procedures”).

| αENaC (413) | LEYNVTGCPE | βENaC (351) | PKGYPSPCTM | γENaC (370) | KLSEYQCTM | cASIC1 (283) | YLPFGDGCKA |
residues at sites adjacent to the conserved Tyr residue in β9-α4 loop might also alter the Na⁺ self-inhibition response. This is what we observed (Figs. 4 and 5). Substitutions of α414 with Ala or Trp, and substitutions of G415, G416, G419, and D420 with Trp significantly increased I_{ss}/I_{peak} (Figs. 4 and 5). Significant increases in the time constant of the Na⁺ self-inhibition response were observed with the α416W and α419W mutants. Several mutants exhibited an enhanced Na⁺ self-inhibition response (reduced I_{ss}/I_{peak} and τ), including αG419A and αG419C.

The loss of Na⁺ self-inhibition was most pronounced with the αG419W mutant. We examined whether substitutions at the analogous sites in the β and γ subunits (βS357 and γS376) affected the Na⁺ self-inhibition response (Fig. 5). Interestingly, we observed an enhanced Na⁺ self-inhibition (reduced I_{ss}/I_{peak}) with the βS357W and γS376W mutants. A notably increased rate of Na⁺ self-inhibition (lower τ) was observed with γS376W. Reduced Na⁺ self-inhibition (higher I_{ss}/I_{peak} and τ) was observed with βS357C. Channels with Trp substitutions of the 3 residues (αG419W/βS357W/γS376W) had a Na⁺ self-inhibition response that was modestly enhanced (lower I_{ss}/I_{peak}) relative to wild type.

**Mutations of Residues in the β9-α4 Loop Alter the Channel Response to Shear Stress**—In addition to responding to changes in the external [Na⁺], ENaCs respond to LSS with an increase in activity that reflects an increase in channel Po (6, 14, 20). If mechanical forces such as shear stress induces conformational changes in the periphery of the extracellular domains that are transmitted to the channel gate, mutations within the β9-α4 loop of ENaC subunits should alter the channel response to shear stress. This is what we have observed. Oocytes expressing wild type or mutant channels were subjected to LSS, as described under “Experimental Procedures.” We determined both the magnitude of the increase in Na⁺ current, as well as the time constant for the increase in Na⁺ current (τ) in response to LSS by applying fluid via a vertical pipette placed adjacent to the oocyte. Whole cell Na⁺ currents mediated by wild-type channels increased by 2.16 ± 0.06-fold with a τ of 8.4 ± 0.2 s, when subjected to vertical flow at a rate of 1.6 ml/min (Fig. 6 and Table 2 (n = 125)). The response to LSS was significantly enhanced in channels with substitutions of the conserved Tyr residue in the β9-α4 loop of the α subunit (αY418C and αY418W). Substitutions of the conserved Tyr residue in the β and γ subunits (βY356 and γY375) did not alter channel response to LSS. Channels with substitutions of all three Tyr residues (αY418W/βY356W/γY375W) showed a sig-

| Channel        | I_{ss}/I_{peak} | τ | Oocytes |
|----------------|-----------------|---|---------|
| WT             | 0.6 ± 0.1       | 3.0 ± 0.2 | 1.0    |
| αG414A         | 0.7 ± 0.2       | 3.2 ± 0.3 | 1.1    |
| αG415F         | 0.8 ± 0.3       | 3.5 ± 0.4 | 1.2    |
| αG416F         | 0.9 ± 0.4       | 3.8 ± 0.5 | 1.3    |
| αG419F         | 1.0 ± 0.5       | 4.0 ± 0.6 | 1.4    |

* p < 0.05 versus WT channels from the same batch.
The enhanced flow response was most pronounced with the αG419W mutant. We examined whether substitutions at the analogous sites in the β and γ subunits (βS357 and γS376) affected the shear stress response (Fig. 8). BS357C exhibited a significant increase in Na⁺ current in response to LSS when compared with wild type. In contrast, the rate of the increase in current in response to shear stress was significantly reduced with γS376W mutant. Interestingly, channels with Trp substitution of the three residues (αG419W/βS357W/γS376W) responded to shear stress with an increase in current that was similar to wild type, although the time constant for the increase in current was modestly increased.

The introduction of Cys residues at sites in the β9-α4 loop allows us to examine whether Cys-reactive reagents, such as MTSET, alters the channel’s response to LSS. Channels with Cys substitutions at αY418 and αG419 exhibited similar responses to LSS prior to and following treatment with MTSET. Similar results were observed with Cys mutants at the analogous sites in the β and γ subunits (βY356, γY375, and γS376) with the exception of βS357, where the flow response was significantly reduced following MTSET treatment (Fig. 9). These results suggest that the Cys side chain is accessible to MTSET and that modification of the introduced Cys blunts the
Experiments were performed with 13 to 18 oocytes expressing mutated responses to LSS of wild type and selected mutant channels are shown. The ILSS/Ibasal values of mutants were not altered by MTSET (Fig. 9).

Amiloride-sensitive currents of wild type and mutant channels to shear stress are shown prior to (control) and following treatment with MTSET. The ILSS/Ibasal values of the mutants were normalized to the ILSS/Ibasal of wild-type ENaC from the same batch. The introduced Ala, Cys, or Trp mutations at the different residues are noted. 3W refers αG419W/βS357W/γS376W channels. C, time constants of current decay (τ) are shown. Dashed lines (B and C) show the averaged wild-type values representing 125 observations, which are intended for reference and not for statistical comparisons. Significant differences between oocytes expressing wild-type channels and mutant channels were labeled with asterisks (*, p < 0.05).

### FIGURE 8. Shear stress response is enhanced by mutations of αG419 or its corresponding residues in the β and γ subunits. A, representative recordings for responses to LSS of wild type and selected mutant channels are shown. Experiments were performed with 10 to 15 oocytes expressing mutated channels. Relative whole cell Na⁺ current responses (ILSS/Ibasal) of mutant and wild-type channels to LSS are shown. The ILSS/Ibasal values of the mutants were normalized to the ILSS/Ibasal of wild-type ENaC from the same batch. The introduced Ala, Cys, or Trp mutations at the different residues are noted. 3W refers αG419W/βS357W/γS376W channels. C, time constants of current decay (τ) are shown. Dashed lines (B and C) show the averaged wild-type values representing 125 observations, which are intended for reference and not for statistical comparisons. Significant differences between oocytes expressing wild-type channels and mutant channels were labeled with asterisks (*, p < 0.05).

### TABLE 2

| Channel  | RFR (s) | τ (s) | Oocytes |
|----------|---------|-------|---------|
| WT (αβγ) | 1.00 ± 0.02 | 8.4 ± 0.2 | 125 |
| αL41Wβγ | 1.20 ± 0.09 | 8.5 ± 0.7 | 17 |
| αG415Wβγ | 1.22 ± 0.10 | 8.3 ± 1.0 | 14 |
| αG416Wβγ | 1.40 ± 0.13 | 6.9 ± 0.8 | 10 |
| αN417Wβγ | 1.30 ± 0.11 | 7.2 ± 0.9 | 18 |
| αY418Aβγ | 1.30 ± 0.13 | 9.9 ± 1.2 | 12 |
| αY418Cβγ | 1.41 ± 0.16 | 11.0 ± 1.1 | 11 |
| αY418Wβγ | 1.30 ± 0.07 | 8.3 ± 0.6 | 23 |
| αG419Aβγ | 1.07 ± 0.10 | 10.8 ± 0.7 | 11 |
| αG419Cβγ | 1.12 ± 0.11 | 9.3 ± 0.4 | 11 |
| αG419Wβγ | 1.54 ± 0.15 | 7.8 ± 0.5 | 15 |
| αD420Wγ | 1.25 ± 0.18 | 9.1 ± 1.0 | 16 |
| αY356Aγ | 1.20 ± 0.10 | 9.4 ± 0.6 | 13 |
| αY356Cγ | 1.18 ± 0.10 | 10.3 ± 0.8 | 11 |
| αY356Wγ | 1.29 ± 0.14 | 12.9 ± 1.4 | 9 |
| αβY375A | 1.07 ± 0.06 | 11.5 ± 0.8 | 13 |
| αβY375C | 1.16 ± 0.09 | 10.6 ± 0.7 | 13 |
| αβY375W | 1.21 ± 0.07 | 10.2 ± 0.8 | 14 |
| αβS357Cγ | 1.56 ± 0.14 | 10.1 ± 0.9 | 14 |
| αβS357Wγ | 1.05 ± 0.08 | 11.4 ± 1.1 | 10 |
| αβS376C | 0.93 ± 0.06 | 10.9 ± 1.0 | 13 |
| αβS376W | 0.85 ± 0.04 | 14.5 ± 1.8 | 12 |
| αY418WY356W-Y375W | 1.54 ± 0.11 | 9.5 ± 0.6 | 16 |
| αG419WβS357W-γS376W | 0.91 ± 0.08 | 10.3 ± 0.6 | 15 |

### FIGURE 9. MTSET inhibits the shear stress response of βS357C channels. A, representative recordings for responses to LSS of wild type and selected mutant channels prior to (control) and following treatment with MTSET are shown. Experiments were performed with 10–11 oocytes expressing mutated channels and 30 oocytes expressing wild-type channels. Relative whole cell Na⁺ current responses (ILSS/Ibasal) of mutant and wild-type channels to shear stress are shown prior to (open bars) and following (closed bars) treatment with MTSET. The ILSS/Ibasal values of the mutants were normalized to the ILSS/Ibasal of wild-type ENaC from the same batch prior to MTSET treatment. Significant changes in the RFR after treatment with 1 mM MTSET were labeled with asterisks (*, p < 0.05). C, basal amiloride-sensitive currents (μA) prior to (open bars) and following (closed bars) treatment with MTSET. In the presence of external Na⁺ and exhibit a markedly enhanced Na⁺ self-inhibition response when compared with wild type channels (Fig. 10 and Ref. 18). We have proposed that movements within the periphery of the α subunit in the presence of external Na⁺ that enhance Po are prevented by the
inhibitory tract (9). We examined whether αR205A, R231A channels with selected mutations in the β9-α4 loop relieve channel inhibition by external Na⁺. αR205A, R231A channels with a αY418C mutation showed a marked loss in the Na⁺ self-inhibition response, when compared with αR205A, R231A channels.

In addition to retaining the α subunit inhibitory tract, αR205A, R231A channels also retain the γ subunit inhibitory tract (2, 23). We observed that αR205A, R231A channels with a γY375C mutation also exhibited a significant reduction in the Na⁺ self-inhibition response, when compared with αR205A, R231A channels. In contrast, αR205A, R231A channels with a

DISCUSSION

The ENaC/degenerin family is composed of ion channels that are regulated by external factors. Limited information is available regarding the sites where external factors interact with these channels, and the subsequent structural transitions that lead to changes in channel gating. In this regard, sites within the more peripheral regions of ASIC subunits have been identified as putative proton binding sites. The resolved structure of ASIC1 revealed a potential mechanism by which movements within the periphery induced by H⁺ binding could be translated to movements within the transmembrane domains and gate, via the base of the thumb (i.e. the β9-α4 loop) (7).

The inhibition of ENaC by external Na⁺ and its activation by shear stress reflect changes in channel Po (11, 18, 19). If these factors (i.e. external Na⁺ and shear stress) induce structural changes within the channel periphery, and if the β9-α4 loop has a role in transmitting these structural changes to the gate, mutations within this loop should alter the channel response to external Na⁺ and shear stress in a similar manner (see Fig. 11). This is what we observed. For the mutants analyzed, we observed a significant correlation between changes in Na⁺ self-inhibition and the channel response to shear stress (Fig. 11). Mutants that exhibited a reduced Na⁺ self-inhibition response (higher Iss/Ipeak reflecting an increase in channel Po in the presence of external Na⁺ compared with wild type) also had an enhanced response to shear stress (reflecting an increased channel Po in the presence of shear stress).

Trp-288 within the β9-α4 loop of ASIC1 was suggested to have a key role in transmitting structural changes in response to H⁺ binding to the gate, based on its proximity to the external portions of the transmembrane regions within the resolved ASIC1 structure (7). Recent mutagenesis studies provided evidence in support of the functional role of Trp-288 in H⁺-dependent gating of ASIC1 (10). A Tyr residue is present at the base of the thumb domain of the three mouse ENaC subunits at the homologous position of ASIC1 Trp-288 (Fig. 2). We found

**FIGURE 10. Na⁺ self-inhibition response of channels with mutations of the α subunit furin cleavage sites is repressed by Cys mutations of either αY418 or γY375.** A, representative recordings of the Na⁺ self-inhibition responses of wild type channels, a furin mutant (αR205A, R231A (α/R/A)) channels, and channels with the α furin mutant and a Cys mutation of the conserved Tyr in the 9-4 loop at the base of the thumb domain. Experiments were performed with 13–23 oocytes expressing mutated channels and 24 oocytes expressing wild-type channels. B, amiloride-sensitive Iss to Ipeak ratios of wild type and mutant channels are shown. The introduced mutations at the different residues are noted. C, time constants of current decay (τ) are shown. Significant differences between oocytes expressing wild-type channels and mutant channels were labeled with an asterisk (*, p < 0.05). Significant differences between oocytes expressing the α furin mutant channel (αR205A, R231A (α/R/A)) and the other mutant channels were labeled with a double asterisk (**, p < 0.005).
that channels with mutations of these conserved Tyr residues in the $\alpha$ and $\beta$ subunits ($\alpha$Y418 and $\beta$Y356) exhibited an altered response to external Na$^+$ and/or LSS. Furthermore, channels with mutations in residues flanking the conserved Tyr in the $\alpha$ subunit ($\alpha$L414 to $\alpha$D420) also exhibited an altered response to external Na$^+$ and/or shear stress, when compared with wild-type channels. The most robust changes in the response to external Na$^+$ and shear stress were noted with $\alpha$G419 mutants. Channels with substitutions at the residues homologous to $\alpha$G419 in the $\beta$ and $\gamma$ subunits ($\beta$S357 and $\gamma$S376) also exhibited significant changes in their response to external Na$^+$ and shear stress, when compared with wild-type channels. Taken together, these results suggest that the base of the thumb domain of ENaC has a role in modifying channel gating properties in response to Na$^+$ and LSS. We cannot exclude the possibility that some mutations we examined indirectly altered the channel response to external Na$^+$ or shear stress. For example, selected mutants could directly or indirectly alter Na$^+$ binding.

We further examined whether selected mutations in the $\beta$-$\alpha$-$\delta$ loop would affect the properties of channels that have an intrinsically low Po due to the retention of inhibitory tracts in the $\alpha$ and $\gamma$ subunits. Interestingly, mutations in the $\beta$-$\alpha$-$\delta$ loop affected the response to external Na$^+$ in a subunit specific manner. Channels with a Cys substitution of the conserved Tyr residue in $\alpha$ subunit ($\alpha$Y418C) or $\gamma$ subunit ($\gamma$Y375C) blunted the inhibition by external Na$^+$ (Fig. 10). A Cys mutation of the corresponding Tyr residue in $\beta$ subunit (BY356C) had no effect. We recently proposed that the $\alpha$ subunit inhibitory tract interacts with residues in both the finger and thumb domains, limiting the relative movements of the domains and reducing channel Po in the presence of external Na$^+$ (9). Our results are consistent with the notion that limiting relative movements of finger and thumb domains may limit transitions in the pore to an open state (and/or enhance transitions to a closed state), and that the base of the thumb has a role in transmitting structural information from the channel periphery to the pore in a subunit-specific manner.

There are 16 conserved Cys residues in the extracellular regions of the three ENaC subunits. One of these conserved Cys residues ($\alpha$C421, $\beta$C359, and $\gamma$C378) is immediately distal to the tract of residues ($\alpha$L414-$\alpha$D420) that we examined at the base of the thumb by scanning mutagenesis. The resolved ASIC1 structure, and our previous work suggest that this Cys residue forms a disulfide bond with the most distal of the conserved extracellular Cys residues (7, 12). We previously reported that substitutions of $\alpha$C421, $\beta$C359, and $\gamma$C378 resulted in channels with a reduced Na$^+$ self-inhibition response (12).

Most mutations in the $\beta$-$\alpha$-$\delta$ loop of ENaC subunits that enhanced the Na$^+$ self-inhibition response were associated with lower baseline amiloride-sensitive inward Na$^+$ currents when compared with wild-type channels ($\alpha$G419A, $\alpha$G419C, $\gamma$S376W, and $\alpha$G419W/$\gamma$S375W $\gamma$S376W; see Table 1). Whereas channels with a reduced Na$^+$ self-inhibition response would be predicted to exhibit a higher Po, our inability to detect a significant difference in basal amiloride-sensitive Na$^+$ currents may reflect the overall variability in levels of current expression among oocytes (Table 1). These results are consistent with those reported by Li et al. (10), where channels with mutations in the conserved Tyr residue in the $\beta$-$\alpha$-$\delta$ loop of all three ENaC subunits expressed amiloride-sensitive inward currents that were similar in magnitude to wild-type channels.

We also observed subunit specificity in the response of channels bearing mutations in the $\beta$-$\alpha$-$\delta$ loops. Substitutions at analogous sites in the three subunits did not result in equivalent changes in the response to external Na$^+$ and to shear stress. For example, Na$^+$ self-inhibition was repressed by a Trp mutation of $\alpha$G419 (i.e. increased $I_{\text{Na}}/I_{\text{peak}}$), but was enhanced by Trp mutations at the corresponding residue in the $\gamma$ subunit (Fig. 5). A Cys mutation at position 419 in the $\alpha$ subunit enhanced Na$^+$ self-inhibition, but a mutation in the $\beta$ subunit repressed Na$^+$ self-inhibition. We have previously reported subunit specificity in the response of channels to external Na$^+$ and in the activation of channels by proteases (11, 19, 24).

It was interesting to note that substitutions in the thumb region were primarily associated with increases in $I_{\text{Na}}/I_{\text{peak}}$, or in the response to shear stress, likely reflecting increases in channel Po in response to external factors. However, a few mutants exhibited a decrease in $I_{\text{Na}}/I_{\text{peak}}$ or in the shear stress response, reflecting a decrease in channel Po.

There are three Gly residues in the tract flanking the residue Tyr-418 in the $\alpha$ subunit. Gly residues would be expected to provide flexibility within the $\beta$-$\alpha$-$\delta$ loop, and it is notable that substitutions with Trp residues at these sites reduced the channels response to external Na$^+$, and at two sites increased the channels response to shear stress.

In summary, substitutions at select sites within the $\beta$-$\alpha$-$\delta$ loop at the base of the thumb domain were associated with changes in the response to external Na$^+$ and LSS. Our results support the hypothesis that external factors, such Na$^+$ and shear stress, are sensed within the periphery of the extracellular domains and affect ENaC gating, in part, though conformational changes that are transmitted to the pore.

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