Intersubunit conformational changes mediate epithelial sodium channel gating

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The epithelial Na\(^+\) channel (ENaC) functions as a pathway for Na\(^+\) absorption in the kidney and lung, where it is crucial for Na\(^+\) homeostasis and blood pressure regulation. However, the basic mechanisms that control ENaC gating are poorly understood. Here we define a role in gating for residues forming interfaces between the extracellular domains of the three ENaC subunits. Using cysteine substitution combined with chemical cross-linking, we determined that residues located at equivalent positions in the three subunits (\(\alpha_{477}\), \(\beta_{446}\), and \(\gamma_{455}\)) form interfaces with residues in adjacent subunits (\(\beta_{230}\), \(\gamma_{287}\), and \(\alpha_{512}\), respectively). Cross-linking of these residues altered ENaC activity in a length-dependent manner; long cross-linkers increased ENaC current by increasing its open probability, whereas short cross-linkers reduced ENaC open probability. Cross-linking also disrupted ENaC gating responses to extracellular pH and Na\(^+\), signals which modulate ENaC activity during shifts in volume status. Introduction of charged side chains at the interfacing residues altered ENaC activity in a charge-dependent manner. Current increased when like charges were present at both interfacing residues, whereas opposing charges reduced current. Together, these data indicate that conformational changes at intersubunit interfaces participate in ENaC transitions between the open and closed states; movements that increase intersubunit distance favor the open state, whereas the closed state is favored when the distance is reduced. This provides a mechanism to modulate ENaC gating in response to changing extracellular conditions that threaten Na\(^+\) homeostasis.

INTRODUCTION

The epithelial Na\(^+\) channel (ENaC), a heterotrimer of \(\alpha\), \(\beta\), and \(\gamma\) subunits, functions as a pathway for Na\(^+\) absorption across epithelia in the kidney collecting duct and connecting tubule, lung, distal colon, and sweat duct (Schild, 2004; Snyder, 2005). In this role, the channel is critical for the maintenance of extracellular Na\(^+\) and blood pressure regulation. However, the basic mechanisms that control ENaC gating are poorly understood. Here we define a role in gating for residues forming interfaces between the extracellular domains of the three ENaC subunits. Using cysteine substitution combined with chemical cross-linking, we determined that residues located at equivalent positions in the three subunits (\(\alpha_{477}\), \(\beta_{446}\), and \(\gamma_{455}\)) form interfaces with residues in adjacent subunits (\(\beta_{230}\), \(\gamma_{287}\), and \(\alpha_{512}\), respectively). Cross-linking of these residues altered ENaC activity in a length-dependent manner; long cross-linkers increased ENaC current by increasing its open probability, whereas short cross-linkers reduced ENaC open probability. Cross-linking also disrupted ENaC gating responses to extracellular pH and Na\(^+\), signals which modulate ENaC activity during shifts in volume status. Introduction of charged side chains at the interfacing residues altered ENaC activity in a charge-dependent manner. Current increased when like charges were present at both interfacing residues, whereas opposing charges reduced current. Together, these data indicate that conformational changes at intersubunit interfaces participate in ENaC transitions between the open and closed states; movements that increase intersubunit distance favor the open state, whereas the closed state is favored when the distance is reduced. This provides a mechanism to modulate ENaC gating in response to changing extracellular conditions that threaten Na\(^+\) homeostasis.
Although changes in ENaC gating are critical for regulating epithelial Na⁺ transport, the basic mechanisms that control ENaC opening and closing are poorly understood. However, there are some recent data to guide us. Crystallization of a related DEG/ENaC ion channel (cASIC1a) in desensitized (Jasti et al., 2007; Gonzalez et al., 2009) and toxin-bound (Bacongins and Gouaux, 2012; Dawson et al., 2012; Bacongins et al., 2014) states has facilitated the generation of ENaC structural models, and mutagenesis has begun to identify residues necessary for regulation of ENaC gating. In the current work, we explore the mechanisms that underlie gating by taking advantage of extracellular domain residues located at an equivalent position in the three ENaC subunits. βENaC-E446 and γENaC-E455 function as putative proton sensors; mutation of these residues reduced ENaC activation by acidic pH (Collier et al., 2012). In contrast, the equivalent residue in αENaC (K477) carried a positive charge and its mutation enhanced acid activation of ENaC (Collier et al., 2012). Here we demonstrate that these residues are located at intersubunit interfaces, where they participate in conformational changes required for ENaC to transition between open and closed states.

**MATERIALS AND METHODS**

**DNA constructs**

cDNAs for human α-, β-, and γENaC in pMT3 were cloned as previously described (McDonald et al., 1994, 1995). Mutations were generated by site-directed mutagenesis (QuickChange II XL; Agilent Technologies) and sequenced in the University of Iowa DNA Core.

**Homology modeling and molecular graphics**

A model of αβγENaC based on the chicken ASIC1 crystal structure (PDB ID 4NYK) was generated using Phyre2 (Kelley and Sternberg, 2009). Structures of each subunit were aligned to the appropriate subunit of the ASIC1a homotrimer using UCSF Chimera. Molecular graphics and analyses were performed with the UCSF Chimera package (Resource for Biocomputing, Visualization, and Informatics, University of California, San Francisco, supported by NIGMS P41-GM103311; Petersen et al., 2004).

**Biochemical cross-linking**

HEK 293T cells cultured in Dulbecco’s modified Eagle’s medium (DMEM) were transfected with cDNAs encoding human α-, β-, and γENaC using Lipofectamine 2000 (Invitrogen). After transfection, 10 µM amiloride was added to the culture medium. To reduce spontaneous disulfide bonds, cells were incubated with 50 mM di-thiothreitol (DTT) for 15 min at 37°C in Ringer’s solution (135 mM NaCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 2.4 mM KH₂PO₄, and 10 mM HEPES, pH 7.4) with 10 µM amiloride. To cross-link cysteines, cells were treated with MTS reagents (100 µM for 30 s; MTS-2-MTS, 1,2-ethanediyl bis-MTS; MTS-4-MTS, 1,4-butanediyl bis-MTS; MTS-6-MTS, 1,6-hexanediyl bis-MTS; MTS-11-O3-MTS, 3,6,9,12-tetraoxatetradecane-1,14-diyl-bis-MTS; MTS-14-O4-MTS, 3,6,9,12-tetraoxatetradecane-1,14-diyl-bis-MTS; MTSES, sodium (2-sulfonatooethyl)MTS; MTSET, [2-(trimethylammonium)ethyl]MTS bromide) or reduced with DTT (30 mM for 30 s). ENaC current was determined by addition of 10 µM amiloride to the bathing solution. To quantitate Na⁺ self-inhibition, cells were bathed in low sodium (1 mM NaCl, 115 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH adjusted to 7.4 with NaOH). Cysteines were modified by treatment with MTS reagents (100 µM for 30 s; MTS-2-MTS, 1,2-ethanediyl bis-MTS; MTS-4-MTS, 1,4-butanediyl bis-MTS; MTS-6-MTS, 1,6-hexanediyl bis-MTS; MTS-11-O3-MTS, 3,6,9,12-tetraoxatetradecane-1,14-diyl-bis-MTS; MTS-14-O4-MTS, 3,6,9,12-tetraoxatetradecane-1,14-diyl-bis-MTS; MTSES, sodium (2-sulfonatooethyl)MTS; MTSET, [2-(trimethylammonium)ethyl]MTS bromide) or reduced with DTT (30 mM for 30 s). ENaC current was determined by addition of 10 µM amiloride to the bathing solution. To quantitate Na⁺ self-inhibition, cells were bathed in low sodium (1 mM NaCl, 115 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH adjusted with HCl) and then rapidly shifted to high sodium (116 mM NaCl). Na⁺ self-inhibition was quantitated as [(peak current – steady-state current)/peak current]. pH responses were measured in low Cl⁻ bath solution (58 mM Na₂SO₄, 58 mM N-mannitol, 2 mM KCl, 0.4 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH adjusted with NaOH).

**Single-channel studies in Xenopus oocytes**

ENaC was expressed in Xenopus oocytes by cytoplasmic injection of equal concentrations of α-, β-, and γENaC cRNA (1 µg/µl each) generated by in vitro transcription using mMessage mMachine T7 (Ambion). Injection volumes were varied between 30 and 20 nl to titrate ENaC expression to optimize the likelihood of recording from patches containing only one to three channels. Single-channel currents were recorded from devitellogized oocytes in the cell-attached patch-clamp configuration 1–3 d after injection. The pipette solution contained 110 mM LiCl, 2 mM KCl, 1.54 mM CaCl₂, and 10 mM HEPES, pH 7.4 with LiOH; or 1 mM LiCl, 109 mM NMDG, 2 mM KCl, 1.54 mM CaCl₂, and 10 mM HEPES, pH 7.4 with HCl. In all experiments, the bath solution contained 110 mM LiCl, 2 mM KCl, 1.54 mM CaCl₂, 10 mM HEPES, pH 7.4 with LiOH. Li⁺ was used in place of Na⁺ to increase the signal to noise ratio. Currents were amplified using an Axopatch 200B amplifier (Axon Instruments), acquired at 4 kHz and filtered with a 1 kHz lowpass filter.
RESULTS

Localization of intersubunit interfaces

We hypothesized that αK477, βE446, and γE455 participate in conformational changes that underlie ENaC gating. To pursue this hypothesis, we first examined their locations in the ENaC channel complex. Fig. 1 A shows a partial sequence alignment of the three homologous ENaC subunits and ASIC1a; αK477, βE446, and γE455 are located between two cysteines that are conserved throughout the DEG/ENaC family of ion channels. Fig. 1 B shows a homology model of ENaC based on the crystal structure of ASIC1a (PDB ID 4NYK; Gonzales et al., 2009). The structure of a single subunit resembles the anatomy of a left hand. The region above the transmembrane domain is the wrist. The cysteine-rich region of the channel is the thumb. The central β-ball region sits within the palm domain, and the extremities of the structure are referred to as the knuckle and finger domains (Jasti et al., 2007). Our previous work indicates that the three ENaC subunits assemble in an αωβ orientation (listed clockwise) when viewing the channel from the extracellular side (Collier and Snyder, 2011). αK477, βE446, and γE455 are located in a loop that connects the base of the thumb domain to the palm domain (Fig. 1 B). At this location, each residue is predicted to lie at an interface with its neighboring subunit. For example, βE446 is located at the interface with γENaC, close to γV85 (Fig. 1 B). Likewise, αK477 is predicted to lie at an interface close to βV85, and γE455 at an interface with αL120 (Fig. 1 B). These three residues lie within the palm domain β1–β2 linker.

To begin to validate this model, we tested whether the putative pH-sensing residues at these potential interfaces could be chemically cross-linked to their predicted neighbors. We initially focused on the interface between αK477 and βV85, replacing both residues with cysteine. After treatment with bifunctional cysteine-reactive compounds of differing lengths (MTS-x-MTS, where “x” indicates number of atoms in the linker backbone), we labeled and isolated ENaC at the cell surface by biotinylation, resolved ENaC on a nonreducing gel (to maintain subunit cross-linking), and then detected βENaC by immunoblot. With wild-type ENaC, we detected a band of ~90 kD, corresponding to monomeric βENaC (Fig. 2 A). Treatment with MTS-14-O4-MTS or MTS-2-MTS did not alter migration of βENaC. This indicates that native cysteines are not sufficient to cross-link ENaC subunits. In contrast, when we expressed αK477C/βV85CγENaC, both MTS-14-O4-ENaC and MTS-2-ENaC induced appearance of a higher molecular mass band, corresponding to the predicted size for a β-α dimer (Fig. 2 A). Thus, αK477C and βV85C are located in close enough proximity to be cross-linked. The higher mass band was not present in the absence of cross-linker (DMSO), indicating that cross-links did not form spontaneously. As a control for specificity, we coexpressed βV85C with γE455C (position equivalent...
to αK477C. We did not detect cross-linking between these residues after MTS-14-O4-MTS treatment, consistent with the prediction that these residues are not located at the same interface (Fig. 2 A).

We performed similar experiments at the other two predicted intersubunit interfaces. With expression of αβE446CγV87C, MTS-14-O4-MTS and MTS-2-MTS induced cross-linking to form β-γ dimers (Fig. 2 B). However, with αL120CβγE455C we detected a higher molecular mass band in the absence of cross-linking reagent (Fig. 2 C). This finding suggests that a spontaneous disulfide bond was formed between αL120C and γE455C. Consistent with this possibility, the dimer band was abolished by the reducing agent DTT (Fig. 2 C). Together, the biochemical cross-linking data support the model that putative pH-sensing residues are located at interfaces with residues in the β1-β2 linkers of adjacent ENaC subunits (Fig. 1 B).

Cross-linking alters ENaC activity
If conformational changes at the intersubunit interfaces contribute to ENaC gating, then cross-linking might alter ENaC activity by introducing constraints to movement. We began by testing the effects of a long cross-linker, MTS-14-O4-MTS. In Xenopus oocytes expressing αβγENaC, we quantitated ENaC current using the open channel blocker amiloride (Fig. 3 A). We then treated the cells with MTS-14-O4-MTS in the continued presence of amiloride (to minimize modification of endogenous pore cysteines [Snyder et al., 1999]) and compared current after washout of MTS-14-O4-MTS and amiloride with current before treatment (Fig. 3, A, B, D, and E, blue lines). In cells expressing wild-type ENaC, MTS-14-O4-MTS produced a small (16.9 ± 3.1%) decrease in current (Fig. 3, A and C), likely by reacting with endogenous pore cysteines (Snyder et al., 1999). In contrast, when we expressed αK477CβV85CγENaC, MTS-14-O4-MTS produced a large (421.4 ± 32.4%), irreversible increase in ENaC current (Fig. 3, B and C). This stimulation required cysteines at both positions; MTS-14-O4-MTS did not increase current when either cysteine was introduced individually (Fig. 3 C). The requirement for both cysteines indicates that simultaneous modification of both was required for ENaC stimulation, consistent with the formation of a cross-link between the residues. As an additional control for specificity, we introduced a cysteine at a neighboring position in βENaC (S84C). As part of a β sheet, its side chain is predicted to be in the opposite orientation as V85C. When we coexpressed βS84C with αK477C and γENaC, MTS-14-O4-MTS failed to stimulate ENaC current (Fig. 3 C).

We used the same strategy to examine the other two interfaces. At the interface between βE446C and γV87C,

![Figure 2. Cross-linking of intersubunit residues. (A–C) Representative immunoblots of βENaC (A and B) and γENaC (C) after isolation of the biotinylated cell surface fraction from HEK 293 cells expressing the indicated ENaC subunits. Cells were treated with DMSO (control), MTS-14-O4-MTS, MTS-2-MTS, or DTT, as indicated. Molecular mass is indicated in kilodaltons. Models illustrate the three ENaC subunits (viewed from the top) and locations of cross-linked residues.](image-url)
cross-linking with MTS-14-O4-MTS increased current (28.9 ± 6.3%; Fig. 3, D and F), albeit to a lesser extent than its effect at the interface between αK477 and βV85. At the interface between αL120C and γE455C (which formed a spontaneous disulfide bond), MTS-14-O4-MTS also increased ENaC current (136.1 ± 12.8%; Fig. 3, E and F).

Thus, a fraction of these channels likely lack spontaneous disulfide bonds, rendering them susceptible to cross-linking by MTS-14-O4-MTS. Together, the data lend additional support to a model in which αK477, βE446, and γE455 are located at intersubunit interfaces with βV85, γV87, and αL120, respectively. Moreover, at each interface, cross-linking with MTS-14-O4-MTS increased ENaC current.

**Length dependence of cross-linking**

To determine whether ENaC function is dependent on the distance between the residues at the intersubunit interface, we tested a range of different length cross-linkers. We focused on the interface between αK477 and βV85 because cross-linking at this location produced the largest effect on ENaC current. Fig. 4 shows the changes in ENaC current produced by cross-linkers ranging from 5.2 Å (MTS-2-MTS) to 20.8 Å (MTS-14-O4-MTS) in length (Loo and Clarke, 2001). When both αK477 and βV85 were replaced with cysteine, ENaC current was increased by MTS-14-O4-MTS and MTS-11-O3-MTS (Fig. 4). In contrast, shorter cross-linkers (MTS-6-MTS, MTS-4-MTS, and MTS-2-MTS) produced small decreases in ENaC current (Fig. 4). However, it is unclear whether they resulted from cross-linking of αK477 and βV85 because similar decreases were observed with wild-type ENaC or with single cysteine substitutions (with the exception of αK477C, which was stimulated by MTS-2-MTS). Thus, the effect of cross-linking reagents on ENaC current was length dependent; only long reagents were sufficient to increase ENaC current. This suggests that an increase in intersubunit distance favors the open state.

As an additional test of this paradigm, we examined the functional effect of the spontaneous disulfide bond that formed at the interface between αL120C and γE455C (Fig. 2 C). This bond constrains the interfacing residues in close proximity to one another. When we reduced this disulfide bond with DTT, there was a large increase in amiloride-sensitive current (Fig. 5, A and B). DTT produced a very small increase in current in cells expressing αβE446C γV87C ENaC, but had no effect on αK477C βV85C γENaC or wild-type ENaC (Fig. 5 B). Thus, in contrast to the effect of long cross-linkers, shortening the distance between αL120C and γE455C (by spontaneous disulfide bonding) inhibits ENaC activity. Together, the data support a model in which increased intersubunit distance favors
ENaC opening, whereas reduced distance favors the closed state. In the next section, we tested this model more directly at the single-channel level.

Cross-linking alters ENaC gating

We tested the effect of cross-linking on ENaC gating by recording single-channel currents (cell-attached patch clamp with Li⁺ as the permeant cation to increase the signal to noise ratio). Fig. 6 A shows a representative trace for α477Cβ85CγENaC that was not treated with cross-linker; the \( P_o \) was 0.40 ± 0.06 (Fig. 6, A and B; and Table 1), similar to wild-type ENaC (Snyder et al., 1995). Treatment with MTS-14-O4-MTS increased ENaC \( P_o \) to 0.60 ± 0.04 (Fig. 6, A and B; and Table 1). In contrast, it had no significant effect on single-channel conductance (8.06 ± 0.07 pS without modification \([ n = 8]\), 6.51 ± 0.90 pS with modification \([ n = 5]\), \( P = 0.103\)). Thus, cross-linking by MTS-14-O4-MTS increased current by altering channel gating. However, the increase in \( P_o \) was less than we expected, based on the fivefold increase in whole-cell current produced by MTS-14-O4-MTS. Quantitation of ENaC gating is complicated by a high degree of variability in \( P_o \), in part because of differences in the proteolytic cleavage state of individual ENaC channels (Hughey et al., 2003, 2004; Caldwell et al., 2004; Carattino et al., 2008; Kashlan et al., 2012); ENaC activity is enhanced by proteolytic removal of α- and γENaC extracellular domain segments. To reduce variability, we eliminated cleavage of γENaC by mutating its proteolytic cleavage sites, which converts channels to a low-activity state (Carattino et al., 2008). When coexpressed with αK477C and β85C, \( P_o \) was 0.03 ± 0.01 (Fig. 6, C and D; and Table 1). MTS-14-O4-MTS increased \( P_o \) fourfold to 0.12 ± 0.02 (Fig. 6, C and D; and Table 1), consistent with the increase in ENaC activity observed at the whole cell level.

We also tested the effect of a short cross-linker, MTS-2-MTS, on ENaC gating. We hypothesized that by reducing intersubunit distance, MTS-2-MTS would decrease ENaC \( P_o \). To facilitate detection of such a change, we exposed the outside of the channel to a low cation concentration (1 mM Li⁺ in pipette) to enhance ENaC activity (Bize and Horisberger, 2007) and measured outward current at a holding potential of 60 mV. Under these conditions, the \( P_o \) of unmodified channels was high (0.67 ± 0.13; Fig. 6, E and F; and Table 1), and treatment with MTS-2-MTS reduced \( P_o \) to 0.20 ± 0.08 (Fig. 6,

![Figure 5](image_url)

**Figure 5.** Spontaneous disulfide bond reduces ENaC current. (A) Representative current trace (voltage clamped to –60 mV) in a *Xenopus* oocyte expressing αL120CβγENaC. Amiloride-sensitive ENaC current was measured before and after treatment with 30 mM DTT (white bar). ENaC current was determined by block with 10 μM amiloride (Amil, black bars). (B) Percent change in ENaC current induced by DTT in oocytes expressing αL120CβγENaC without (top traces) or with (bottom traces) MTS-14-O4-MTS pretreatment. (B) Open probability determined from patches described in A \([ n = 5–8]\; *; \( P < 0.045\)). (D) Open probability determined from patches described in C \([ n = 5; *; \( P < 0.025\)).

![Figure 6](image_url)

**Figure 6.** Cross-linking alters ENaC open probability. (A–D) Representative single-channel current traces (cell-attached configuration) obtained at –100 mV (110 mM LiCl in bath and pipette) in oocytes expressing αK477CβV85CγENaC (A and B) or αK477Cβ85CγENaC (C and D) without (top traces) or with (bottom traces) MTS-14-O4-MTS pretreatment. (B) Open probability determined from patches described in A \([ n = 5–8]\; *; \( P < 0.045\)). (D) Open probability determined from patches described in C \([ n = 5; *; \( P < 0.025\)).

(E) Representative single-channel current traces (cell-attached configuration) obtained at 60 mV (110 mM LiCl in bath and 1 mM LiCl in pipette) in oocytes expressing αK477Cβ85CγENaC without (top trace) or with (bottom trace) MTS-2-MTS pretreatment. (F) Open probability determined from patches described in E \([ n = 4–7]\; *; \( P < 0.015\)). Mean ± SEM is shown.
Cross-linking disrupts ENaC regulation by extracellular pH and Na⁺

ENaC gating is modulated by extracellular signals. We tested whether intersubunit conformational changes underlie this regulation by cross-linking interfaces to constrain movements. We first examined ENaC regulation by extracellular pH, which requires acidic residues at two of the interfaces (E446 and E455; Collier et al., 2012). To keep these residues intact, we used the interface between K477C and V85C to introduce the cross-link. Before E and F; and Table 1). Thus, cross-linking with MTS-2-MTS altered ENaC gating by favoring the closed state.

Electrostatic effects at interfaces alter ENaC activity

The opposing effects of short and long cross-linkers on ENaC activity suggest that shortening the distance between interfacing residues favors channel closing, whereas lengthening of the distance favors channel opening. As an additional strategy to assess this model, we tested the effects of electrostatic interactions between the interfacing residues.

We first examined the interface between the positively charged residue K477 and V85. We replaced V85 with cysteine, coexpressed this subunit with wild-type α- and γENaC in Xenopus oocytes, and then acutely varied the charge at this position by covalent modification with MTSET and MTSES (which have no effects on wild-type ENaC [Snyder et al., 1999]). Like charges at the interfacing residues should increase the distance between them through electrostatic repulsion, whereas opposing (attractive) charges should reduce the distance. When we introduced a positively charged side chain, by exposure of oocytes to MTSET, ENaC current was irreversibly increased (Fig. 7, A and C). Thus, like charges increased ENaC activity. Conversely, introduction of a negative charge (MTSES) irreversibly decreased ENaC current (Fig. 7, B and C); opposing charges reduced ENaC activity.

We examined a second intersubunit interface between negatively charged E455 and L120 (which we replaced with cysteine). In this case, introduction of a positive charge (MTSET) irreversibly decreased ENaC current, whereas a negative charge (MTSES) irreversibly increased ENaC current (Fig. 7 D). Thus, at both interfaces, repulsive (like) charges increased ENaC activity, whereas attractive (opposite) charges reduced activity. These data support the concept that intersubunit conformational changes contribute to ENaC gating, with movement of the residues farther apart favoring channel opening and movement closer together favoring channel closing.

| Construct | n | N | t | NPO | PO |
|-----------|---|---|---|-----|----|
| αK477βV85γ | 8 | 1.9 ± 0.4 | 8.7 ± 0.6 | 0.74 ± 0.18 | 0.40 ± 0.06 |
| αK477βV85γ + MTS-14-O4-MTS | 5 | 1.6 ± 0.2 | 9.0 ± 0.5 | 0.98 ± 0.32 | 0.60 ± 0.09 |
| αK477βV85γUncleaved | 5 | 1 | 6.7 ± 0.9 | 0.03 ± 0.01 | 0.03 ± 0.01 |
| αK477βV85γUncleaved + MTS-14-O4-MTS | 5 | 1.6 ± 0.4 | 9.3 ± 0.5 | 0.22 ± 0.11 | 0.12 ± 0.03 |
| αK477βV85γLow Li⁺ | 4 | 1.5 ± 0.3 | 9.8 ± 0.1 | 0.92 ± 0.15 | 0.67 ± 0.13 |
| αK477βV85γLow Li⁺ + MTS-14-O4-MTS | 7 | 1.9 ± 0.4 | 8.0 ± 1.0 | 0.46 ± 0.18 | 0.20 ± 0.08 |

Values of ENaC single-channel properties shown in Fig. 7, including the number of patches studied (n), mean number of channels per patch (N), mean duration of the experiments in minutes (t), mean product of the number of channels per patch, and the estimated open probability (NP₀) and mean estimated channel open probability (PO). No analyzed patch contained more than three channels.

Figure 7. Electrostatic effects at interfaces alter ENaC activity. (A and B) Representative current traces in Xenopus oocytes (voltage clamped to −60 mV) expressing wild-type ENaC (blue) or βV85C ENaC (black) treated with MTSET (white bar, A) or MTSES (white bar, B). ENaC current was determined by block with 10 µM amiloride (Amil, black bars). (C and D) Percent change in ENaC current after treatment with MTSET (ET+) or MTSES (ES−) for cells expressing αβV85CγENaC (C) or αL120CβγENaC (D). Mean ± SEM is shown (n = 12; *, P < 0.0002).
cross-linking, acidic extracellular solution increased amiloride-sensitive current (Fig. 8, A and B), whereas alkaline pH reduced current (Fig. 8 B). After treatment with MTS-14-04-MTS, there was a large decrease in ENaC stimulation by acidic pH (Fig. 8, A and B; traces were scaled to facilitate comparison of pH responses) and a smaller decrease in inhibition by alkaline pH (Fig. 8 B). MTS-2-MTS had a similar effect (Fig. 8 B). Thus, cross-linking disrupted ENaC regulation by pH, implicating a critical role for intersubunit conformational changes.

ENaC activity is also regulated by extracellular Na⁺ through a negative feedback mechanism known as Na⁺ self-inhibition. To test whether intersubunit conformational changes are also required for this process, we asked if it would be disrupted by cross-linking. We began by examining the pairing of α₁L120C and γ₁E455C, which form a spontaneous disulfide bond. We incubated ENaC in low extracellular Na⁺ (1 mM; Fig. 9 A), which converts ENaC to a high Pₒ state (Chraïbi and Horisberger, 2002). We then rapidly shifted to a high-Na⁺ (116 mM) solution, generating a large peak in Na⁺ current (Fig. 9 A, red lines) that decreased over time to a lower steady-state level (Fig. 9 A, blue lines). This decrease in current reflects the degree to which ENaC is inhibited by extracellular Na⁺. When cells expressing α₁L120Cβ₁E455C-ENaC, DTT increased the fraction of current inhibited by Na⁺ by 35 ± 8% (Fig. 9, A and B). DTT had no effect on Na⁺ self-inhibition for wild-type ENaC or channels with cysteines at the other two interfaces (Fig. 9 B). These data suggest that spontaneous cross-linking of α₁L120C and γ₁E455C disrupts conformational changes required for ENaC Na⁺ self-inhibition.

We interrogated the interface between α₅K477C and β₅V85C using bifunctional cysteine-reactive reagents. In cells expressing α₅K477Cβ₅V85CγENaC, MTS-14-04-MTS reduced Na⁺ self-inhibition by 37.7 ± 3.3% (Fig. 9, C, D, and G). In contrast, MTS-14-04-MTS did not reduce Na⁺ self-inhibition for wild-type ENaC or for ENaC containing a single cysteine substitution (α₅K477C or β₅V85C; Fig. 9 G). Thus, cross-linking α₅K477 and β₅V85 with MTS-14-04-MTS reduced Na⁺ self-inhibition.

In Fig. 9 (E–G), we tested the effect of the shorter cross-linker MTS-2-MTS on Na⁺ self-inhibition. In cells expressing α₅K477Cβ₅V85CγENaC, MTS-2-MTS produced a large decrease in Na⁺ self-inhibition (74.6 ± 6.9%), much greater than the effects of MTS-2-MTS on wild-type ENaC or the single cysteine mutants (Fig. 9 G). This resulted from a decrease in the peak current elicited by addition of Na⁺ to the extracellular solution. Similarly, MTS-4-MTS, MTS-6-MTS, and MTS-11-O3-MTS also decreased Na⁺ self-inhibition (Fig. 9 G). Thus, reagents with a wide range of lengths were capable of cross-linking cysteines at the interface between α₅K477 and β₅V85. Although only the relatively long cross-linkers were sufficient to increase ENaC current, cross-linkers reduced Na⁺ self-inhibition independent of their length. By constraining movements at this interface, cross-linking likely disrupts ENaC transitions between the open and closed states.

**DISCUSSION**

This work elucidates a mechanism that mediates ENaC gating. We found that conformational changes at interfaces located between the three ENaC subunits participate in the transition between the open and closed states (illustrated in Fig. 10). Movements that increase the distance between interfacing residues favor channel opening, an effect mimicked by introduction of long cross-linkers or repulsive charges. Conversely, movements that shorten intersubunit distances favor ENaC closing, a state mimicked by short cross-linkers or attractive charges. Thus, the interfaces between ENaC subunits are important locations for the regulation of ENaC gating.

The data allow us to estimate the minimum distances between the interfacing residues. MTS-2-MTS cross-linked α₅K477C to β₅V85C and β₅E446C to γ₅V87C, indicating that the sulfur atoms within these residue pairs are located within 5 Å of one another. The distance between γ₅E455C and α₁L120C is ≤2 Å based on their ability to form a spontaneous disulfide bond. These reflect the intersubunit distances when the channel is closed. In the open state, the intersubunit distances increase. At the interface between α₅K477C and β₅V85C, only cross-linkers 16.9 Å or longer were sufficient to increase ENaC activity. The actual intersubunit distance in the open state may be approximately 14 Å.

**Figure 8.** Cross-linking disrupts pH regulation. (A) Representative current traces in *Xenopus* oocytes (voltage clamped to −60 mV) expressing α₅K477Cβ₅V85CγENaC before (black, −MTS-14) and after (blue, +MTS-14) treatment with MTS-14-04-MTS. Extracellular pH was shifted from 7.4 to 5.25, as indicated. The two traces were obtained from different cells and are scaled to facilitate comparisons. (B) Percent change in ENaC current (relative to current at pH 7.4) in response to changes in extracellular pH before (black) and after treatment with MTS-14-04-MTS (blue) or MTS-2-MTS (green). Mean ± SEM is shown (n = 3–6; some data symbols and error bars are hidden by other data symbols).
shorter than this because the cross-linkers are flexible (Zhou et al., 2008). However, they are likely to push apart the interfacing residues through the bulk of their long spacers. Importantly, our conclusions are supported by a second strategy using electrostatic repulsive and attractive effects to alter intersubunit distance.

The interfaces we identified play key roles in ENaC regulation by protons. Acidic residues at two of the three interfaces are required for protons to increase ENaC activity, likely by functioning as protonation sites (Collier et al., 2012). We speculate that protonation drives conformational changes that increase intersubunit distances through an electrostatic process, similar to the effects of long cross-linkers and repulsive charges. However, the ENaC homology model does not provide sufficient resolution to identify the potential charged interaction partners for these residues.

In addition to their role in pH regulation, interfaces between ENaC subunits contribute to modulation by other extracellular ions. For example, we found that constraining movements between interfacing residues (by cross-linking) disrupted ENaC inhibition by Na⁺. Both short and long cross-linkers reduced Na⁺ self-inhibition, presumably by preventing the channel from transitioning between open and closed conformations. Thus, conformational changes at the intersubunit interfaces are required for ENaC Na⁺ self-inhibition. In previous work, we identified two Cl⁻ binding sites at a different location between adjacent subunits. Binding of Cl⁻ to

Figure 9. Cross-linking decreases Na⁺ self-inhibition. (A) Representative current traces in *Xenopus* oocytes (voltage clamped to −60 mV) expressing αL120CβV85CENaC, before and after treatment with 30 mM DTT for 30 s. To quantitate Na⁺ self-inhibition, extracellular Na⁺ was transiently reduced to 1 mM, as indicated by the black bar, and then rapidly shifted back to 116 mM Na⁺. ENaC current was blocked by 10 µM amiloride (Amil, black bar). (B) Percent change in Na⁺ self-inhibition (SSI) induced by DTT in oocytes expressing αβγENaC (subunits are wild type or the indicated mutants). Mean ± SEM is shown (n = 6–7; *, P < 0.005). (C–F) Representative current traces in oocytes (voltage clamped to −60 mV) expressing αK477CβV85CENaC before (C and E) and after (D and F) treatment with MTS-14-O4-MTS or MTS-2-MTS. The extracellular solution contained 1 mM Na⁺ (black bar) or 116 mM Na⁺ (white bar). (G) Percent change in Na⁺ self-inhibition (SSI) for cells expressing αβγENaC (black), αK477Cβγ (green), αβV85Cγ (red), or αK477CβV85CγENaC (blue) induced by treatment with cross-linking reagents of different lengths (MTS-x-MTS, where “x” indicates the number of atoms in the linker backbone). Mean ± SEM is shown (n = 3–15; some error bars are hidden by data symbols).

Figure 10. ENaC gating model. Two state model of heterotrimetric ENaC as viewed from the top of the channel. Each subunit is represented as an irregular trapezoid. Approximate location of αK477 and βV85 are indicated on the model. Cross-linking with MTS-2-MTS favors the ENaC closed state and MTS-14-O4-MTS favors the open state. ENaC gating is regulated by extracellular protons (stimulatory) and Na⁺ (inhibitory); cross-linking disrupted this regulation by preventing transitions between the open and closed states.
one interface (α-β) inhibits ENaC activity by increasing Na’ self-inhibition, whereas Cl’ binding to the second interface (β-γ) inhibits ENaC in a Na’-independent manner (Collier and Snyder, 2011). External Cu2+ also inhibits ENaC at interfaces between the ENaC subunits (Chen et al., 2011). Thus, the interfaces harbor binding sites that allow ENaC to respond to a variety of extracellular modulators. In other channels, intersubunit interfaces have a similar function. For example, ligand-gated channels including the acetylcholine receptor and glycine receptor are activated through agonist binding to interfaces between adjacent subunits (Czajkowski et al., 1995; Czajkowski and Karlin, 1995; Todorovic et al., 2010). Activity of kainate subtype glutamate receptors is modulated by binding of Na’ and Cl’ to dimer interfaces in the extracellular domain (Chaudhry et al., 2009).

Our data indicate that movements at all three intersubunit interfaces contribute to ENaC gating. However, it is interesting that there is asymmetry between them. First, there are charge differences. Two of the sites contain negatively charged residues (βE446 and γE455) that function as putative proton sensors, whereas the third interface contains a positively charged residue (αK477). Second, a spontaneous disulfide bond formed between γE455C and αL120C but did not occur at the other two interfaces. This suggests differences in the minimum distances between residues at the three interfaces. Third, although cross-linking each interface with MTS-14-O4-MTS increased ENaC activity, there were quantitative differences; cross-linking βE446C to γV87C increased current much less than cross-linking the other two interfaces. It is not yet clear whether movements occur at all three interfaces in a concerted manner or whether movements at a single interface are sufficient to modulate ENaC gating.

It is also not yet clear how movements at the interfaces are transduced into changes in ENaC gating. Previous work implicated movements in the outer vestibule at the DEG residue, named because an equivalent mutation in a related channel (MEC-4) causes neurodegeneration in Caenorhabditis elegans (Driscol and Chalfie, 1991). Bulky or charged residues at this location lock ENaC in an open state, and accessibility to its side chain is state dependent, occurring selectively in the open state (Snyder et al., 2000). Similar movements at this location occur in ASICs (Adams et al., 1998; Tolino et al., 2011). Recently, chicken ASIC1a was crystallized in putative open states, bound to psalmotoxin 1 (PDB IDs 4FZ0 and 4FZ1; Baconguis and Gouaux, 2012) and to snake toxin (4NTW; Baconguis et al., 2014). Both toxins produce dimeric insets in the extracellular domain (location of αL120, βV85, and γV87 in ENaC), which were transduced into transmembrane domain movements thought to open the channel pore (Baconguis and Gouaux, 2012; Baconguis et al., 2014). Importantly, in the putative open (toxin bound) states, there was an increase in the distance between intersubunit interface residues (Met 364 and Val 81) equivalent to those we identified in ENaC. Although it is not known whether the structures of the toxin-bound ASIC1a reflect conformational changes that occur during physiological activation or whether gating-associated conformation changes are conserved between ENaC and ASIC1, the data support the concept that movements at the intersubunit interfaces contribute to gating of DEG/ENaC ion channels.

Other extracellular domain regions have also been implicated in ENaC gating. Proteolytic cleavage releases inhibitory segments from α- and γENaC, which increases ENaC activity. Elegant structural modeling of αENaC suggests that the inhibitory segment constrains gating motions at a location between the thumb and finger domains (Kashlan et al., 2011, 2012). Gating is also disrupted by mutations at the base of the thumb domain (Shi et al., 2011), in the finger domain (Shi et al., 2012a), and in the wrist domain (Shi et al., 2012b). Additional work will be necessary to determine whether these regions function coordinately with the intersubunit interfaces or whether they alter ENaC gating through independent mechanisms.

ENaC is a constitutively active channel, but a wide variety of extracellular stimuli can modulate its activity. In the collecting duct and connecting tubule of the kidney, ENaC is exposed to extreme changes in the concentrations of H+, Na’, and Cl’ during changes in volume status and diet. The ENaC extracellular domain functions as a sensor to detect changes in the composition of the highly variable extracellular milieu in which ENaC resides. We speculate that this rapid tuning of channel activity in response to changing extracellular conditions may allow ENaC to respond to a variety of challenges that threaten to disrupt Na’ transport and fluid homeostasis. Conformational changes at intersubunit interfaces play a critical role in this regulation and provide an attractive site to target pharmacological modulation of Na’ absorption.

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