The epithelial Na\textsuperscript{+} channel (ENaC) is typically formed by three homologous subunits (α, β, and γ) that possess a characteristic large extracellular loop (ECL) containing 16 conserved cysteine (Cys) residues. We investigated the functional role of these Cys residues in Na\textsuperscript{+} self-inhibition, an allosteric inhibition of ENaC activity by extracellular Na\textsuperscript{+}. All 16 Cys residues within α and γ ECLs and selected β ECL Cys residues were individually mutated to alanine or serine residues. The Na\textsuperscript{+} self-inhibition response of wild type and mutant channels expressed in Xenopus oocytes was determined by whole cell voltage clamp. Individual mutation of eight α Cys residues (Cys-1, -4, -5, -6, -7, -10, -13, or -16), one β Cys (Cys-7), and nine γ Cys (Cys-3, -4, -6, -7, -10, -11, -12, -13, or -16) residues significantly reduced the magnitude of Na\textsuperscript{+} self-inhibition. Na\textsuperscript{+} self-inhibition was eliminated by simultaneous mutations of either the last three α ECL Cys residues (Cys-14, -15, and -16) or Cys-7 within both α and γ ECLs. By analyzing the Na\textsuperscript{+} self-inhibition responses and the effects of a methanethiosulfonate reagent on channel currents in single and double Cys mutants, we identified five Cys pairs within the αECL (αCys-1/αCys-6, αCys-4/αCys-5, αCys-7/αCys-16, αCys-10/αCys-13, and αCys-11/αCys-12) and one pair within the γECL (γCys-7/γCys-16) that likely form intrasubunit disulfide bonds. We conclude that approximately half of the ECL Cys residues in the α and γ ENaC subunits are required to establish the tertiary structure that ensures a proper Na\textsuperscript{+} self-inhibition response, likely by formation of multiple intrasubunit disulfide bonds.

Sodium transport across apical membranes via epithelial Na\textsuperscript{+} channels (ENaCs)\textsuperscript{2} is the rate-limiting step in Na\textsuperscript{+} reabsorption in the distal nephron, lung, and other Na\textsuperscript{+}-transporting epithelia. ENaC activity is dynamically regulated to maintain extracellular fluid volume homeostasis (1). Na\textsuperscript{+} self-inhibition represents an intrinsic regulation of Na\textsuperscript{+} channels specifically by extracellular Na\textsuperscript{+} (2–5). Recent reports have shown that Na\textsuperscript{+} self-inhibition reflects a reduction in the open probability of ENaC (5, 6). Na\textsuperscript{+} self-inhibition differs in many ways from feedback inhibition, a relatively slow down-regulation of ENaC function by intracellular Na\textsuperscript{+} (7). The structural requirements and to a larger degree the details of the mechanism of Na\textsuperscript{+} self-inhibition are not known, although the amino-terminal portion of the extracellular domain has been implicated in this regulatory phenomenon (5, 6, 8, 9).

Typically, a functional ENaC complex is formed by three homologous subunits, α, β, and γ, although the subunit stoichiometry is still in dispute (10–13). These pore-forming subunits have the same membrane topology as other two-transmembrane domain channels such as inward rectifier K\textsuperscript{+} channels and purinergic P\textsubscript{2}X receptors. A distinguishing feature of ENaC subunits among these two-transmembrane domain channels is a large extracellular loop (ECL) consisting of about 450 residues of the total 600–700 amino acids. Recent studies have suggested a number of functional roles for the ECL, including channel trafficking, amiloride binding, regulation of gating, binding to transition metals, protease cleavage, and Na\textsuperscript{+} self-inhibition (5, 6, 14–18). We previously identified a histidine residue in the γECL (γHis-239) as a required structural element for Na\textsuperscript{+} self-inhibition of mouse ENaC (mENaC), because substitutions at this site eliminated the inhibitory response (5). However, it is unlikely that γHis-239 is the sole participant in the allosteric inhibition by Na\textsuperscript{+}, given its steep temperature dependence that implicates a large scale conformational change associated with the inhibition (4, 19).

Each ECL contains 16 conserved Cys residues that can be grouped into two cysteine-rich domains (CRD): CRD-I, including cysteines 1–6, and CRD-II, including cysteines 7–16 (see Fig. 1). Theoretically, these Cys residues could form intrasubunit or intersubunit disulfide bonds that normally facilitate proper folding in the endoplasmic reticulum and maintain structural integrity (20). Firsov et al. (14) investigated the roles of the ECL Cys residues by a systematic mutation of all 16 Cys residues in the rat αECL, as well as selected mutations of Cys residues in the βECL and γECL. The authors concluded that the Cys-1 and Cys-6 residues of CRD-I in all three subunits and the Cys-11 and Cys-12 of CRD-II in α and β (but not in γ) ECLs are required for efficient expression of ENaC at the plasma membrane. However, the authors also reported that individual mutation of six of ten CRD-II Cys residues significantly increased Na\textsuperscript{+} currents without a proportional change in the number of channels expressed at the cell surface (14), suggesting that these Cys mutations increase the single channel activity. It has been known for many years that specific extracellular
Sulphydryl reagents activate ENaC currents by interfering with Na⁺ self-inhibition (4, 21). In addition, we recently demonstrated that thiophilic Zn²⁺ activates mouse αβγENaC by eliminating Na⁺ self-inhibition (22). In the context of these findings, we hypothesized that certain Cys residues in ENaC might be required for its Na⁺ self-inhibition response. We tested this hypothesis by mutating ECL Cys residues and comparing the Na⁺ self-inhibition response in oocytes expressing wild type (WT) or mutant ENaCs. Our results suggest that multiple Cys residues of both CRD-I and CRD-II in the α and γ subunits play significant roles in Na⁺ self-inhibition.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis and in Vitro Transcription—** Mouse α, β, and γ ENaC cDNAs in pBluescript SK- vector (Stratagene, La Jolla, CA) were used as templates to generate point mutations using a PCR-based method as previously described (23). Each of the αECL Cys residues was mutated to a Ser or Ala, and γECL Cys residues were systematically mutated to Ala. Target mutations were confirmed by direct sequencing. For clarity, the Cys residues within the ECLs are identified in the text (Cys-1 to Cys-16) or figures (C-1 to C-16) with their sequential numbers. The complementary RNAs (cRNAs) for WT and mutant ENaC subunits were synthesized with T3 RNA polymerase (Ambion, Inc.), purified with an RNA purification kit (Ambion, Inc.), and quantitated by spectrophotometry and density analyses of the RNA band in a denaturing agarose gel.

**ENaC Expression and Two-electrode Voltage Clamp—** ENaC expression in *Xenopus* oocytes and current measurements by two-electrode voltage clamp were performed as previously reported (23). Deolficated oocytes were injected with 1 ng of cRNA for each mENaC subunit (α, β, and γ) per oocyte and 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 gentamycin sulfate, pH 7.4). All of the experiments were performed 20–50 h following cRNA injections at room temperature (20–24 °C). The oocytes were placed in an oocyte recording chamber from Warner Instruments (Hamden, CT) and perfused with constant flow rate of 12–15 ml/min.

**Procedures for Observing Na⁺ Self-inhibition—** To examine Na⁺ self-inhibition, 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) was replaced rapidly by a high Na⁺ bath solution (NaCl-110; containing 110 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 10 mM HEPES, pH 7.4), while the oocytes were continuously clamped to −60 or −100 mV as indicated. Bath solution exchange was performed with a six-channel Teflon valve perfusion system from Warner Instruments. At the end of the experiment, 10 μM amiloride was added to the bath to determine the amiloride-insensitive component of the whole cell current. Currents remaining in the presence of 10 μM amiloride were generally less than 200 nA. The results from oocytes that showed unusually large amiloride-insensitive currents (> 5% of total currents) were discarded to minimize current contamination from endogenous channels and membrane leak. Given the well known variability of the *Xenopus* oocyte expression system and our initial observation that the Na⁺ self-inhibition responses of WT αβγmENaCs varied moderately among different batches of oocytes, despite a very small variation in the response within the same batch of oocytes, the responses of WT channels were always examined with mutants in the same batch of oocytes in an alternating manner.

The first 40 s of current decay was fit with an exponential equation by Clampfit 9.0 (Axon Instruments Inc.). The peak current (*I*ₚₑᵃᵏ) was the measured maximal inward current immediately after bath solution exchange from low Na⁺ to high Na⁺ concentration. The steady state current (*I*ₛₛ) represented the measured current at 40 s after *I*ₚₑᵃᵏ. The current ratio of *I*ₛₛ/*I*ₚₑᵃᵏ was calculated from amiloride-sensitive *I*ₛₛ and *I*ₚₑᵃᵏ obtained by subtracting amiloride-insensitive currents from *I*ₛₛ and *I*ₚₑᵃᵏ.

**FIGURE 1. A model of the α ENaC subunit.** A structural model of αENaC was generated based on prior reports and our findings in this study. The transmembrane domains (M1 and M2) are shown as cylinders, whereas the cytoplasmic amino terminus, carboxyl terminus, and ECL are displayed as curved lines. The conserved 16 Cys residues within the ECL are indicated by circles with serial numbering. Small loops were introduced where two nearby Cys residues are separated by more than three residues. The distances between the circles approximate the number of residues that separate the two neighboring Cys residues. The slash identifies two furin cleavage sites. Furin-dependent proteolysis increases ENaC activity by suppressing Na⁺ self-inhibition (6, 9, 18). The pentagon between Cys-3 and Cys-4 illustrates δHis²⁸², a residue homologous to γHis³⁹ whose mutation leads to loss of Na⁺ self-inhibition (5). The gray circles identify Cys residues whose mutations significantly reduced the Na⁺ self-inhibition response, and the circle with a thick circumference identifies αCys-11, whose substitution enhanced Na⁺ self-inhibition. Proposed disulfide bonds based on this study are shown as gray lines (14). The dashed line identifies a potential disulfide bond between αCys-8 and αCys-15 that was not demonstrated in this study. The dashed oval circle shows that the area around αCys-1/αCys-6 may be inaccessible to solvent based on a lack of modification by MTSET in the single Cys mutants.
ENaC Cys and Na⁺ Self-inhibition

Procedure for Observing the Effects of MTSET—The oocytes were clamped at −60 mV and perfused with the bath solution NaCl-110. After 1 min recording of the control current, the bath solution was replaced by one containing 1 mM 2-(trimethylammonium) ethyl methanethiosulfonate bromide (MTSET; Toronto Research Chemicals Inc.) for 2 or 3 min. The bath was then switched back to NaCl-110 to wash out MTSET for 1 min. Amiloride at 10 μM was added to the bath to determine amiloride-insensitive current. Because MTSET has a short life time in aqueous solution with pH 7.0 at 20 °C (11 min) (24), the reagent was dissolved in the NaCl-110 no earlier than 1 min prior to use. The oocytes with unstable currents were not used for these experiments.

Statistical Analysis—The data are presented as the means ± S.E. Significance comparisons between groups were performed with Student’s t tests. A p value of less than 0.01 was considered statistically different, because responses within the same group showed very little variation, which could increase the probability of a false positive result.

RESULTS

The 16 conserved ECL Cys residues that were mutated in this study are clustered in two cysteine-rich domains, CRD-I and CRD-II, which are separated by a stretch of less well conserved residues (Fig. 1). We examined the hypothesis that specific disulfide bonds formed by these extracellular Cys residues are required to generate or maintain the structures that determine the response of ENaC to extracellular Na⁺.

The response of Na⁺ self-inhibition was examined by clamping oocytes at −60 mV, whereas bath [Na⁺] was rapidly increased from 1 to 110 mM, as previously reported (5). WT channels were always examined and compared with mutants in the same batch of oocytes. The time constants (τ) for the inhibition in WT oocytes were in the range of 6.0 ± 0.2 and 8.1 ± 0.4 s, whereas the Iₚ/Iₚₐₚeak values varied in the range of 0.49 ± 0.02 and 0.66 ± 0.02 in 18 different batches of oocytes. The variability among different batches of oocytes was likely due, in part, to variability in the extent of proteolytic processing of ENaC subunits that regulate the Na⁺ self-inhibition response (6, 18, 25).

Alterations of Na⁺ Self-inhibition by Mutations of αECL Cys Residues—All of the mENaCs with mutant α subunits expressed amiloride-sensitive Na⁺ currents sufficient to examine Na⁺ self-inhibition. The oocytes expressing ENaCs with eight different α mutants showed a Na⁺-dependent current decay that was significantly smaller in magnitude (greater Iₚ/Iₚₐₚeak) than for cells expressing WT ENaC, indicating a reduced Na⁺ self-inhibition response (Figs. 2 and 3). These mutations were located in both CRD-I (αCys-1, -4, -5, and -6) and CRD-II (αCys-7, -10, -13, and -16) of the ECL (Fig. 1). An increased Iₚ/Iₚₐₚeak was typically accompanied by an increased time constant, except for ENaCs with mutants in the same batch of oocytes. The variability among different batches of oocytes was likely due, in part, to variability in the extent of proteolytic processing of ENaC subunits that regulate the Na⁺ self-inhibition response (6, 18, 25).

Alterations of ENaC Na⁺ Self-inhibition by Mutations of γECL Cys Residues—All of the mENaCs with mutant γ subunits were functional following cRNA injections in oocytes. Nine γ mutants showed a significantly reduced and slowed Na⁺ self-inhibition response (Figs. 4 and 5). Mutation of γCys-7 produced the greatest effect. Interestingly, six ENaCs with a mutant γ subunit, including γCys-4 (C266A), γCys-6 (C289A), γCys-7 (C378A), γCys-10 (C413A), γCys-13 (C440A), and γCys-16 (C463A) showed qualitatively similar changes to ENaCs with the corresponding mutations in α (Figs. 3 and 5).
ENaC Cys and Na⁺ Self-inhibition

However, unlike the α mutants, mutation of γCys-3 (C220S) and γCys-12 (C429A) blunted Na⁺ self-inhibition, whereas mutation of γCys-1 (C100A) and γCys-5 (C273A) did not change the response. In addition, mutation of γCys-11 (C415A) reduced the Na⁺ self-inhibition response, an effect opposite to that observed with mutation of αCys-11.

Effects of Mutations of βECL Cys Residues on Na⁺ Self-inhibition—Although all three ENaC subunits contribute to pore formation, mutations at corresponding sites of different subunits often result in nonidentical changes, suggestive of asymmetric roles for the subunits (26, 27). A previous study suggested that γENaC and αENaC subunits play larger roles in the Na⁺ self-inhibition response than does βENaC (5). We examined Na⁺ self-inhibition of selected β mutants including βCys-3, -4, -7, and -16, whose corresponding mutations in α and/or γ significantly reduced the Na⁺ self-inhibition response. As shown in Fig. 6, only mutation of βCys-7 (βC359A) moderately reduced the magnitude and speed of Na⁺ self-inhibition. Our results indicate that ECL Cys-7 in all three subunits (αCys-421, βCys-359, and γCys-378) is particularly sensitive to point mutations with regard to the Na⁺ self-inhibition response, suggestive of a critical role for these Cys residues.

Probing Disulfide Bond-forming Cys Pairs by Functional Additivity—One possible reason for the reduced Na⁺ self-inhibition response in the ENaCs with selected Cys mutations is a disruption of disulfide bonds that are required to stabilize the ECL structure pertinent to the mechanism of the Na⁺ inhibition. Firsov et al. (14) suggested that αCys-1 and αCys-6 in CRD-I as well as αCys-11 and αCys-12 in CRD-II form disulfide bonds, based on a comparison of changes in ENaC currents in oocytes expressing subunits with single or double Cys mutations. Using a similar approach, we tested whether those Cys residues whose substitutions significantly suppressed Na⁺ self-inhibition represented disulfide-linked residues by construction of double Cys mutants in the α or γ subunits. We reasoned that if a pair of Cys residues forms a disulfide bond that has a role in the Na⁺ self-inhibition response, mutation of either one or both Cys residues should result in a similar, but not additive change in the Na⁺ self-inhibition response. On the other hand, if two Cys residues do not form a disulfide bond, the change in Na⁺ self-inhibition response should be largely additive, resulting from their independent influence on Na⁺ self-inhibition. Therefore, we analyzed the relative changes of $I_{\text{ss}}/I_{\text{peak}}$. 

![Figure 3](image1.png)

**FIGURE 3.** Effects of mutations of individual αECL Cys residues on Na⁺ self-inhibition. Wild type β and γ ENaC subunits were co-expressed with either WT or a mutant α subunit. The Na⁺ self-inhibition responses in oocytes expressing WT or mutant channels were examined at a clamping voltage of −60 mV. The time constants and $I_{\text{ss}}/I_{\text{peak}}$ values were obtained as described under “Experimental Procedures.” The data were collected in different batches of oocytes. The WT (αβγ) values represent 108 observations pooled from 17 batches of oocytes, whereas mutant data are from 6–9 oocytes. Student’s t tests were performed in the same batch of oocytes to compare the responses of Na⁺ self-inhibition of WT and an individual mutant. The values that are significantly different from that of WT (p < 0.01) are shown as black bars.

![Figure 4](image2.png)

**FIGURE 4.** Na⁺ self-inhibition response of γ subunit mutants. The oocytes were clamped at −60 mV except for αβγC289A (γC-6), and whole cell currents were continuously recorded while bath [Na⁺] was rapidly increased from 1 mM (open bar) to 110 mM (gray bar). The oocytes injected with cRNAs for αβγC289A were clamped at −100 mV, because small currents were observed at −60 mV (< 500 nA). The units of current and time for the mutants are the same as for WT and are omitted for clarity. The traces represent at least five independent observations. The names in parentheses indicate the positions of the mutated Cys residues.
ENaC Cys and Na⁺ Self-inhibition

FIGURE 5. Effects of mutations of individual γECL Cys residues on Na⁺ self-inhibition. Wild type α and β ENaC subunits were co-expressed with either WT or a mutant γ subunit. The Na⁺ self-inhibition responses in oocytes expressing WT and mutant αβγ mENaCs were examined at a clamping voltage of −60 mV. The data were collected in different batches of oocytes. The WT (αβγ) values represent 108 observations pooled from 17 batches of oocytes, whereas data for mutants are from 6–11 oocytes. Student's t tests were performed in the same batch of oocytes to compare the responses of Na⁺ self-inhibition of WT and a mutant. Black bars indicate that the values are significantly different from that of WT (p < 0.01).

FIGURE 6. Effects of mutations of individual Cys residues of βECL on Na⁺ self-inhibition. Wild type α and γ ENaC subunits were co-expressed with either WT or a mutant β subunit. The responses of Na⁺ self-inhibition in oocytes expressing WT or mutant channels were examined at a clamping voltage of −60 mV. The data were collected in different batches of oocytes. The WT (αβγ) values represent 26 observations pooled from four batches of oocytes, whereas data for mutants are from 6–11 oocytes. Student's t tests were performed in the same batch of oocytes to compare the responses of Na⁺ self-inhibition of WT and a mutant. Black bars indicate that the values are significantly different from that of WT (p < 0.01).

(ΔI/p, peak: mutant I/p, peak minus WT I/p, peak) in mENaCs with single or double Cys mutations. As shown in Fig. 7A, the double mutation of αCys-1 and αCys-6 (αC-1/αC-6) resulted in a value of ΔI/p, peak (black bar) that was no more than those caused by either αCys-1 or αCys-6 single mutation but was much less than the value for an additive effect (αC-1 + αC-6, gray bar) that was predicted assuming independence of the two Cys residues (i.e. no disulfide bond). Because we observed a similar reduction in ENaC Na⁺ self-inhibition with either the αCys-1 or αCys-6 single mutation, which was not additive in the double mutant, these data are consistent with the presence of a disulfide bond between αCys-1 and αCys-6, in accordance with previous findings (14). Similarly, the Na⁺ self-inhibition response of mENaCs with double mutations at αCys-4/αCys-5, αCys-7/αCys-16, and αCys-10/αCys-13, showed nonadditive effects compared with ENaC with the corresponding single mutations (Fig. 7B–D), consistent with the presence of disulfide bonds between these three pairs of Cys residues. In contrast, mENaCs with the αCys-4/αCys-16, αCys-5/αCys-16, and αCys-10/αCys-16 double Cys mutations displayed additive effects (Fig. 7E–G), inconsistent with disulfide bonds between αCys-4 and αCys-16, αCys-5 and αCys-16, and αCys-13 and αCys-16, respectively. Our observation that ENaC with the αCys-11 mutation enhanced Na⁺ inhibition, whereas αCys-12 mutation did not change the response is not consistent with a disulfide bond between the two Cys residues. However, ENaCs with the double mutation αCys-11/αCys-12 behaved like channels with αCys-12 single mutation (Fig. 7H), suggesting interactions between these two Cys residues. Similar analyses of ENaCs with γCys-7/γCys-16 and γCys-4/γCys-16 double Cys mutations revealed an additive effect for ENaC with γCys-4/γCys-16 and nonadditive effect for ENaC with γCys-7/γCys-16. These data are consistent with a disulfide...
Although an additive effect was not clearly observed in the Na⁺ self-inhibition in oocytes co-expressing a mutant α subunit containing either single or double Cys mutations and WT α and β subunits were compared as described in the legend to Fig. 7. The bars have the same meaning as in Fig. 7. The numbers of observations are indicated in parentheses. An asterisk indicates p < 0.01 between single Cys mutant and double Cys mutant.

It is unknown whether the functional ENaC complex formed by heterologous αβγ subunits contains intersubunit disulfide bonds. The observed changes in Na⁺ self-inhibition resulting from substitution of ECL Cys residues could also result from a disruption of intersubunit as well as intrasubunit disulfide bonds, given the observation of significant reduction in Na⁺ self-inhibition by multiple α and γ mutations. As an initial test for the existence of intersubunit disulfide bonds, we examined the Na⁺ self-inhibition response of ENaCs expressing two different mutant subunits containing homologous Cys mutations of Cys-4, Cys-7, Cys-13, or Cys-16 and a WT subunit. ENaCs with homologous Cys mutations in α and γ (Fig. 9, A–D), α and β (C-7; Fig. 9B), or β and γ (C-7; Fig. 9B) showed clear additive effects in their suppression of self-inhibition with exception of αCys-7/γCys-7. The results do not support formation of intersubunit disulfide bonds between these Cys residue pairs and are in line with formation of intrasubunit bridges suggested above.

Mutations of both αCys-7 and γCys-7 eliminated the Na⁺ self-inhibition response (I_{ss}/I_{peak} = 0.99 ± 0.00, n = 8). The ΔI_{ss}/I_{peak} from the double mutations was significantly greater than that of either single mutation (p < 0.01). However, it did not reach the predicted change in Na⁺ self-inhibition, assuming an additive effect of these two mutations. This was due to an apparent “ceiling effect” that prevented the ΔI_{ss}/I_{peak} from going beyond a value of 0.41 (1 - I_{ss}/I_{peak} of WT = 0.59 ± 0.02, n = 6), the maximal change in Na⁺ self-inhibition if this response was eliminated (dashed line in Fig. 9B).

Although an additive effect was not clearly observed in αCys-7/γCys-7 mutant, the greater ΔI_{ss}/I_{peak} in the double mutant was not consistent with a disulfide bond between these two Cys residues.

Probing Disulfide Bond-forming Cys Pairs by Cys Modification—A second approach was used to identify disulfide bond forming Cys pairs. The basic strategy was that breaking a disulfide bond by mutating one of the paired Cys residues would allow the free Cys residue to be covalently modified by a methanethiosulfonate reagent. We chose MTSET for these studies, based on previous work showing that the activity of WT ENaC was not affected by externally applied MTSET (28, 29). This reagent is positively charged at neutral pH and is membrane-impermeant. The latter ensures that external MTSET will only react with extracellular Cys residue to form a mixed disulfide bond. A lack of membrane permeation is important; intracellular methanethiosulfonate reagents inhibit rat αβγ ENaC (30).

Fig. 10A illustrates the potential results that may occur in response to MTSET modification of Cys residues within the ECL of mutant ENaCs. MTSET will yield a positive result if three conditions are met: (i) there is a free extracellular Cys residue, (ii) that residue is readily accessible to solvent (thus to MTSET), and (iii) a change of channel activity occurs following Cys modification. MTSET will not alter the activity of a mutant channel with a free extracellular Cys if this residue is (i) inaccessible to solvent or (ii) the Cys modification produces no change in channel activity.

When a pair of bridged Cys residues is mutated, MTSET should not affect the double Cys mutant, similar to WT ENaC. On the other hand, mutation of a pair of nonbridged Cys residues would break two separate disulfide bonds and thus provide two free Cys residues whose modification by MTSET should alter whole cell Na⁺ currents if at least one of free Cys residues is modified by MTSET with a change in channel activity.

We observed two distinct responses of WT ENaCs to 1 mM MTSET, although a consistent response was seen within an individual batch of oocytes. We frequently observed no abrupt change in Na⁺ current when MTSET was added or washed out.
ENaC Cys and Na\(^+\) Self-inhibition

**A**

| Free -SH | Accessible | Change | Result |
|----------|------------|--------|--------|
| -        | +          | +      | N      |
| -        | +          | -      | P      |
| +        | -          | +      | N      |
| +        | -          | -      | P      |

(black trace in Fig. 10B). The Na\(^+\) current exhibited a slow and linear decline over time, consistent with channel run-down that is often observed when oocytes expressing ENaCs are continuously clamped at a negative potential in the presence of high [Na\(^+\)] in the bath (31, 32). We also observed a rapid but modest and transient increase in Na\(^+\)/H\(^+\) current in response to MTSET (gray trace in Fig. 10B). MTSET washout resulted in a rapid decrease in the current with a similar magnitude to the increase, indicating that this response did not reflect covalent modification of the channel by MTSET. Slow channel run-down was also noted. These data suggest that there are no free, solvent-accessible, and functionally important extracellular Cys residues in WT ENaC.

We focused on ECL Cys residues that were suggested to form disulfide bonds, based on analyses of Na\(^+\)/H\(^+\) self-inhibition. Among the ten Cys mutants examined, four (\(\alpha\)Cys-4, \(\alpha\)Cys-5, \(\alpha\)Cys-10, and \(\alpha\)Cys-11) were irreversibly inhibited, and one (\(\alpha\)Cys-16) was irreversibly stimulated by MTSET, indicating that these five Cys residues likely participate in formation of disulfide bonds in WT ENaC. Channels with an individual \(\alpha\)Cys-1 or \(\alpha\)Cys-6 mutation or a double \(\alpha\)Cys-1/\(\alpha\)Cys-6 mutation were not affected by MTSET (Fig. 10C).

Although the \(\alpha\)Cys-7 mutant was not affected by MTSET (Fig. 10D), the reversed increase and decrease in current was observed during application and washout of MTSET, respectively, and normalized amiloride-sensitive currents obtained after MTSET washout were reported. Mutant channels are identified by serial numbering. For C–H, the traces/bars for the first, second, and double mutants are shown in black, gray, and light gray, respectively. An asterisk indicates that the \(I_{MTSET}/I_{MTSET}^0\) value in a single mutant was significantly different from that of the double mutant (\(p < 0.01\)).

**FIGURE 10.** Responses of mutant ENaCs to extracellular MTSET. A, illustrations of potential scenarios and results regarding MTSET modification of Cys residues within the ECL. A portion of the channel is illustrated as a loop with a disulfide bridge in WT. Three distinct types of single mutants, SM1, SM2, and SM3, as well as two distinct types of double mutants, DM1 and DM2, are distinguished by (i) their availability of a free -SH group, (ii) the solvent accessibility of the -SH group, and (iii) the effect of MTSET modification (see "Results"). N and P indicate negative (no change in current) and positive result (change in current), respectively. In B–H, a representative experiment and the averaged data are shown. The oocytes were clamped at \(-60\) mV and perfused with bath solutions with or without 1 mM MTSET. Following 1 min recording of the control current (50 s shown), MTSET was applied to the bath for 2 min (except 3 min for D) as indicated by a black bar above the traces. MTSET was then washed out for 1 min. The relative currents in a recording were obtained by normalizing all of the current values to the current immediately prior to MTSET application. The bars in the charts \((I_{MTSET}/I_{MTSET}^0, \text{mean} \pm \text{S.E., } n = 3–7)\) represent currents (amiloride-sensitive) measured at 2 min following MTSET addition that were normalized to currents (amiloride-sensitive) prior to application of the reagent, with the exception of B (gray bar for WT2) and G. In the latter, a reversible increase and decrease in current was observed during application and washout of MTSET, respectively, and normalized amiloride-sensitive currents obtained after MTSET washout were reported. Mutant channels are identified by serial numbering. For C–H, the traces/bars for the first, second, and double mutants are shown in black, gray, and light gray, respectively. An asterisk indicates that the \(I_{MTSET}/I_{MTSET}^0\) value in a single mutant was significantly different from that of the double mutant (\(p < 0.01\)).
not affected by MTSET, the αCys-16 mutant was irreversibly stimulated by 20% (Fig. 10E). The loss of stimulation with the αCys-7/αCys-16 double mutant suggests that αCys-7 was modified by the MTSET in the αCys-16 mutant and that αCys-7 and αCys-16 form a disulfide bond. Results observed for another pair, αCys-10/αCys-13, were similar to αCys-7/αCys-16. MTSET inhibited αCys-10 mutant without affecting either the αCys-13 mutant or the αCys-10/αCys-13 double mutant (Fig. 10F), suggesting that these Cys residues form a disulfide bond.

For the αCys-11/αCys-12 pair, the αCys-11 mutant was strongly inhibited by MTSET, whereas the αCys-12 mutant was modestly and slowly inhibited by MTSET (following a brief modest activation (Fig. 10G)). The response of αCys-11/αCys-12 mutant was similar to WT, consistent with a disulfide bond between αCys-11 and αCys-12. We also examined the effect of MTSET on an αCys-13/αCys-16 double mutant. This pair of residues is not predicted to form a disulfide bond. The double mutant was irreversibly stimulated by MTSET, a response that was similar to that of the individual αCys-16 mutant (Fig. 10H). These results suggest that αCys-13 and αCys-16 do not form a disulfide bond.

Effect of Reducing Reagent on Na⁺ Self-inhibition—If certain disulfide bonds in the ECLs are required for Na⁺ self-inhibition of ENaCs, this response is also likely to be sensitive to reducing reagents. We examined the effects of 10 mM dithiothreitol (DTT) applied extracellularly on ENaC currents and the Na⁺ self-inhibition response. As shown in Fig. 11 (A and B), addition of 10 mM DTT to a high [Na⁺] bath solution produced a small and transient stimulation of the current (maximal effect less than 20%). The currents were typically increased to a maximal level within ~20 s and then tended to decrease afterward to control levels observed prior to DTT treatment within 2 min. Under continuous clamping, the currents continued to decline slowly, consistent with the reported run-down of ENaC activity (32). The small magnitude together with the quick onset and reversal of the stimulation by DTT made it difficult to judge whether the effect was due to reduction of disulfide bonds of the channels. The Na⁺ self-inhibition response in the presence of 10 mM DTT was not suppressed but rather appeared moderately faster and stronger as evidenced by the reduced time constant and $I_{\text{rest}}/I_{\text{peak}}$ (Fig. 11, C and D), which in part might be attributed to the run-down.

**DISCUSSION**

We observed that mutations of specific Cys residues at multiple sites within the α and γ subunit ECLs significantly blunted the Na⁺ self-inhibition response. In addition, the inhibition was eliminated by simultaneous mutations of two or more Cys residues at specific sites within the ECLs. These observations suggest that certain ECL Cys residues are required for the appropriate regulation of ENaC activity by extracellular Na⁺.

Therefore, the characteristic extracellular loops of ENaC subunits not only contain Cys residues essential for efficient trafficking of the channel complex to plasma membrane (14) but also harbor specific Cys residues required for the physiological regulation of the functional channels residing in the membrane. Our findings in this study are in line with recent studies implicating multiple functional roles of the ECLs, such as modulation of channel gating, metal binding, protease regulation, and response to reactive species (5, 6, 8, 9, 16–18, 22, 25, 33, 34).

A rapid inhibition of ENaC activity by a high extracellular [Na⁺] is thought to “buffer” the effect of external Na⁺ on the rate of Na⁺ influx and thus participate in the physiological regulation of Na⁺ absorption (2). A reduction or elimination of such autoregulation could lead to an increase in ENaC current and hence Na⁺ hyperabsorption. The latter can increase blood pressure via fluid volume expansion and impair lung mucus clearance because of depletion of airway surface liquid (1, 35). Therefore, these Cys mutations that suppress Na⁺ self-inhibition represent another possible group of gain-of-function mutations for ENaC, in addition to the well characterized Lidde’s and degenerin mutations (1, 7, 26).

Firsov et al. (14) identified Cys-1 and Cys-6 of α, β, and γ ECLs as well as Cys-11 and Cys-12 of α and β ECLs as essential residues for proper routing of the assembled ENaCs to plasma membrane. We observed that point mutations of αCys-1, -4, -5, -6, -7, -10, -13, or -16; βCys-7; or γCys-3, -4, -6, -7, -10, -11, -12, -13, or -16 significantly reduced the Na⁺ self-inhibition response (Figs. 2–6), suggesting that the regulation of ENaC activity by extracellular Na⁺ depends on the presence of multiple ECL Cys residues. These Cys residues are scattered along the entire ECL, implicating both CRD-I (Cys-1 through Cys-6) and CRD-II (Cys-7 through Cys-16) in the mechanism of Na⁺ self-inhibition. These observations agree with a large confor-
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mational change associated with the inhibition suggested by other investigators (4, 19).

Because the whole cell current is the product of the channel number, open probability, unitary conductance, and driving force, a suppression of Na⁺ self-inhibition may or may not increase the current, depending on whether channel number is also altered by a mutation. For example, substitution of αCys-7, -10, -13, or -16 significantly increased the whole cell current in the report by Firsov et al. (14) and reduced the magnitude of Na⁺ self-inhibition in this study. It is likely that the reduced response of Na⁺ self-inhibition is primarily responsible for the increased currents, because Firsov et al. (14) did not observe a change in surface expression with these mutants. The results from these two studies indicate that these mutations qualify as gain-of-function mutations. However, substitutions of αCys-1 or αCys-6 resulted in two distinct phenotypes, a suppression of Na⁺ inhibition (higher open probability), which is expected to increase channel current, and a reduction in surface channel number leading to a decrease in channel current (14). The whole cell current reduction observed with mutation of either Cys residue (14) likely reflects the net change from these two opposing effects with the impaired trafficking being dominant. These results are consistent with the notion that this pair of Cys residues (αCys-1 and αCys-6) is required for both Na⁺ self-inhibition and trafficking of ENaCs. Mutation of αCys-11 led to an enhanced Na⁺ self-inhibition (lower open probability) and reduced surface channel density, both of which contribute to the reduced whole cell current (14). These observations clearly identify the αCys-11 mutation as a loss-of-function mutation.

Our observation that Na⁺ self-inhibition was eliminated by the triple mutation of αCys-14/Cys-15/Cys-16 was a surprise, because no change in Na⁺ self-inhibition was observed with either individual αCys-14 or αCys-15 mutant. It is possible that structural alterations induced by αCys-14 or αCys-15 mutations are compensated by the αCys-7/αCys-16 disulfide bond and that simultaneous substitution of all three Cys residues would eliminate up to three disulfide bonds that stabilize the proper tertiary structure of CRD-II (Fig. 1). The three Cys residues form an interesting CXXXCXXX sequence (where X is any residue). An essential CXXX motif in the Sco family of proteins that are involved in the assembly of the dinuclear CuA site in cytochrome c oxidase either binds a copper ion or undergoes redox chemistry (36, 37).

Mutations of nine of the total 16 Cys residues within both α and γ ECLs significantly altered the Na⁺ self-inhibition response. These results, together with our previous report implicating a His residue in both α and γ ECLs (αHis-282/γHis-239) in the mechanism of Na⁺ self-inhibition (5), support the notion that the inhibition of ENaC by external Na⁺ requires at least the α and γ subunits. Although the βECL Cys residues were not systematically mutated in this study, the minimal change in the Na⁺ self-inhibition response of four β subunit mutants suggests that βECL Cys residues have a less important role in Na⁺ self-inhibition, compared with their counterparts in the α and γ ECLs. Based on this and previous studies, we speculate that Na⁺ self-inhibition encompasses an interaction between α and γ ECLs at one or more interfaces that remain to be determined. Recent studies on the regulation of ENaC gating have promoted the idea that the epithelial Na⁺ channel is a ligand-gated channel, like other members in the ENaC/degenerin superfamily (22, 38). In ligand-gated channels such as Cys-loop receptors, domain-domain interactions at subunit interfaces constitute the major elements in the channel gating (39).

Firsov et al. (14) proposed two pairs of disulfide bonds within the α ECL (αCys-1/αCys-6 and αCys-11/αCys-12) (14). Our results from the analyses of the Na⁺ self-inhibition responses of Cys mutants using an “additivity” approach (Fig. 7), similar to the approach used by Firsov et al. (14), suggest that the following four Cys pairs form disulfide bonds within αECL: αCys-1/αCys-6, αCys-4/αCys-5, αCys-7/αCys-16, and αCys-10/αCys-13. The last three pairs were confirmed by our analyses of the effects of MTSET on single and double Cys mutants. The MTSET approach also suggests a bond between αCys-11 and αCys-12 (Fig. 10). Collectively, all nine Cys residues within αECL whose mutations significantly altered the Na⁺ self-inhibition response (Fig. 3) are likely involved in five disulfide bonds. Based on our results and previous reports, we propose a structural model for αECL (Fig. 1). There may be additional disulfide bonds within αECL that we have not identified. We speculate that αCys-8 and αCys-15 may form another disulfide bond (Fig. 1), because their mutations increased the ratio of whole cell current over surface channel density (14). These four potential disulfide bonds (αCys-7/αCys-16, αCys-10/αCys-13, αCys-11/αCys-12, and αCys-8/αCys-15) would render the α CRD-II into a ladder-like tertiary structure (Fig. 1) reminiscent of the amino-terminal domain of insulin-like growth factor-binding protein 4 (40).

It should be noted that both methods used in our probe on the Cys pairs forming disulfide bonds have certain limitations. The identification of putative Cys pairs by analyses of Na⁺ self-inhibition of ENaC mutants is based on specific assumptions. These include the assumption that breaking a disulfide bond by the introduction of Cys mutants constitutes the dominant source of the functional change. This assumption may not be correct for all Cys pairs. For example, mutation of both bridged Cys residues may have additional consequences to breaking a disulfide bond, leading to an additive effect. Alternatively, multiple disulfide bonds might contribute in a collaborative manner to the stabilization of the structure of a functional domain.

For the MTSET method, a lack of response to MTSET does not discern whether there is a free Cys residue in a mutant channel (Fig. 10A), as is the case for the αCys-1 and αCys-6 mutants. The lack of an effect of MTSET in the αCys-1 and αCys-6 single mutants may reflect inaccessibility to solvent of the free Cys residue (Fig. 1). Interestingly, the first and sixth Cys residues in the ECL of P₂X₁ receptor reportedly form a disulfide bond but were not labeled by MTSEA-biotin when either Cys was mutated (41). Another caveat is the possibility of forming an alternative disulfide bond between two free Cys residues, when two Cys residues involved in separate bridges are mutated. This would leave no free Cys residue and thus result in a negative result. We believe that this event is unlikely, given the required proximity of two Cys residues to be oxidized to form a bridge (≤7 Å between the two α-carbons) (42).
Our results suggest that γCys-7 and γCys-16 may form a disulfide bond (Fig. 8). Although we have not investigated whether other γ subunit Cys residues are disulfide bond-forming Cys pairs within γECL, it is likely that there exist multiple disulfide bonds within γECL that contribute to the mechanism of Na⁺ self-inhibition. We also probed the possibility of formation of intrasubunit disulfide bonds between a limited numbers of selected residues within the three ENaC subunits. Our results do not support such bonds between these Cys pairs: αCys-4/γCys-4, αCys-7/βCys-7, αCys-7/γCys-7, βCys-7/γCys-7, αCys-13/γCys-13, and αCys-16/γCys-16 (Fig. 9). Because our analyses were limited to the above pairs involving homologous residues, the potential formation of intrasubunit disulfide bridges cannot be excluded. We favor the notion of an absence of intrasubunit disulfide bridges within the ECLs of ENaC subunits, as has been suggested for P₂X receptors (41, 43). The notion is consistent with our analyses on α Cys pairs. If all nine α Cys residues involved in Na⁺ self-inhibition indeed form intrasubunit disulfide bonds, as we have suggested, there would be no bridge between any of the nine α Cys residues and a Cys residue from either the β or γ subunit.

The exact role of the Cys residues in the mechanism of Na⁺ self-inhibition is unclear at this moment. Because external Na⁺-dependent reduction of open probability is associated with a presumably large conformational change (4, 19), it is possible that some Cys residues form disulfide bonds that are required to establish or maintain the proper structure of the putative “Na⁺ receptor” and/or “transducer” coupling the receptor to the channel gate. The lack of a suppressing effect on Na⁺ self-inhibition by the reducing reagent DTT suggests that the structure of functional channels that are correctly folded and assembled is not significantly altered by breaking disulfide bonds. Taken together, these observations suggest that certain Cys residues of ECLs have a critical role in establishing channel structure during biogenesis that is required for the channel response to extracellular Na⁺ but not essential in maintaining the structure once the channel complex resides at the plasma membrane. Like any protein, functional ENaC complexes are likely stabilized by multiple forces such as hydrogen bonds, hydrophobic interactions, and salt bridges in addition to disulfide bonds and therefore might not be dramatically affected by breaking disulfide bonds by DTT. Alternatively, the disulfide bonds important for maintaining the proper conformation for self-inhibition are not accessible to DTT, because native disulfide bonds are often found to be inaccessible to redox agents (44, 45). Interestingly, activity of the ATP-gated P₂X receptors, belonging to two-transmembrane domain channels and containing five pairs of disulfide bond forming Cys residues in their extracellular loops, was found to be insensitive to DTT (41, 43).

In summary, we have identified multiple Cys residues within extracellular loops of α and γ ENaC subunits where mutations result in a significant reduction in the response of Na⁺ self-inhibition. We propose that specific Cys residues form intrasubunit disulfide bonds that are required for the proper folding of the channel into its native conformation that is required for the Na⁺ self-inhibition response.

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