Side Chain Orientation of Residues Lining the Selectivity Filter of Epithelial Na\textsuperscript{+} Channels*

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Epithelial Na\textsuperscript{+} channels (ENaCs) selectively conduct Na\textsuperscript{+} and Li\textsuperscript{+} but exclude K\textsuperscript{+}. A three-residue tract ((G/S)XS) present within all three subunits has been identified as a key structure forming a putative selectivity filter. We investigated the side chain orientation of residues within this tract by analyzing accessibility of the introduced sulfhydryl groups to thiophilic Cd\textsuperscript{2+}. Xenopus oocytes were used to express wild-type or mutant mouse αβ\textsubscript{ENaC}. The blocking effect of external Cd\textsuperscript{2+} was examined by comparing amiloride-sensitive Na\textsuperscript{+} currents measured by two-electrode voltage clamp in the absence and presence of Cd\textsuperscript{2+} in the bath solution. The currents in mutant channels containing a single Cys substitution at the first or third position within the (G/S)XS tract (αG587C, αS589C, βG529C, βS531C, γS546C, and γS548C) were blocked by Cd\textsuperscript{2+} with varying inhibitory constants (0.06–13 mM), whereas the currents in control channels were largely insensitive to Cd\textsuperscript{2+} at concentrations up to 10 mM. The Cd\textsuperscript{2+} blocking effects were fast, with time constants in the range of seconds, and were only partially reversible. The blocked currents were restored by 10 mM dithiothreitol. Mutant channels containing alanine or serine substitutions at these sites within the α subunit were only poorly and reversibly blocked by 10 mM Cd\textsuperscript{2+}. These results indicate that the introduced sulfhydryl groups face the conduction pore and suggest that serine hydroxyl groups within the selectivity filter in wild-type ENaCs face the conduction pore and may contribute to cation selectivity by participating in coordination of permeating cations.

Epithelial Na\textsuperscript{+} channels (ENaCs)\textsuperscript{1} mediate Na\textsuperscript{+} transport across apical membranes of high resistance epithelia and participate in body fluid homeostasis, control of blood pressure, and airway fluid balance (1). Four subunits have been cloned from mammalian tissues and are termed αENaC, βENaC, γENaC, and δENaC. Na\textsuperscript{+}-transporting epithelial cells typically express α, β, and γ subunits. ENaC subunits belong to the ENaC/DEG superfamily with degenerins (DEG and MEC) from Caenorhabditis elegans mechanosensitive neurons, acid-sensing ion channels, and peptide-gated Na\textsuperscript{+} channels (2). ENaC/DEG members have limited sequence homology and share a membrane topology similar to that of other two-transmembrane domain channels such as inward rectifier K\textsuperscript{+} channels and P2X receptors (3). A tetrameric structure has been reported for αβENaC (two α subunit, one β subunit, and one γ subunit), α subunit-only channels, and FMRF amide-gated Na\textsuperscript{+} channel (4–7). However, a channel composition of more than four subunits has also been suggested (8, 9).

Studies on cloned and native channels have shown that typical ENaCs are highly selective for the small alkali cations Li\textsuperscript{+} and Na\textsuperscript{+} (10). Cloned αβγENaC has a Li\textsuperscript{+}/Na\textsuperscript{+} selectivity of 1.5–2.0 and is essentially impermeable to K\textsuperscript{+}, with a K\textsuperscript{+}/Na\textsuperscript{+} selectivity of <0.01 (11–13). Using site-directed mutagenesis, we and others have identified key residues that dictate ENaC cation selectivity (11–15). A three-residue tract ((G/S)XS) within a region (pre-M2 or pore region) preceding the second transmembrane domain (referred to as M2) of α, β, and γ subunits is considered to be the key element forming a putative selectivity filter based on complete or partial loss of selectivity among Na\textsuperscript{+}, Li\textsuperscript{+}, and K\textsuperscript{+} in mutant channels containing point mutations at the first or third position within this tract (11–15). The contributions of the three subunits to ion selectivity are not equal, suggesting that the selectivity filter has an asymmetric structure that is distinct from the nearly perfect symmetry of K\textsuperscript{+} channel selectivity filters (15). In addition to the three-residue tract, mutations of nearby residues or of selected residues in M2 or post-M2 regions also alter cation selectivity (13, 15–17). Based on results from previous studies, the general consensus is that the ENaC pore is formed by the pre-M2 and M2 domains of all three subunits; however, the orientation of these two segments (pre-M2 and M2) within the three-dimensional ENaC structure is unknown, and different models have been proposed (11–13, 15, 18).

Crystal structures of bacterial K\textsuperscript{+} channels have provided insight regarding mechanisms by which K\textsuperscript{+} channels achieve a K\textsuperscript{+}-selective phenotype (19, 20). Four K\textsuperscript{+}-binding sites within the 12-Å-long selectivity filter are formed by 16 carbonyl oxygen atoms from the signature sequence TVGY and four hydroxyl oxygen atoms from the threonine residues (20). In contrast, the ion-binding sites within the selectivity filter of voltage-gated Na\textsuperscript{+} and Ca\textsuperscript{2+} channels are thought to come from side chains of key residues (DEKA for the Na\textsuperscript{+} channels and EEEE for the Ca\textsuperscript{2+} channels) (21). It is not clear whether backbone oxygens, side chain oxygens, or both coordinate permeating Na\textsuperscript{+} and Li\textsuperscript{+} ions in ENaCs. Kellenberger et al. (22) recently proposed that the four carbonyl oxygen atoms from four serine residues at the third position within the (G/S)XS tracts (two from αENaC and one from βENaC and γENaC each) provide a Na\textsuperscript{+}-binding site and that the side chains point...
to the intersubunit spaces and do not interact with cations passing through the selectivity filter. The proposed side chain orientation of the Ser residues at the third position was largely based on a positive correlation between increases in $K^+$ permeability and the volumes of several substituted residues at $\alpha$Ser<sup>589</sup>. Our previous study suggested that the hydroxyl group of $\alpha$Ser<sup>589</sup> might contribute to Na<sup>+</sup> binding and cation selectivity since only a Thr substitution at $\alpha$Ser<sup>589</sup> retained the ability to discriminate between Na<sup>+</sup> and K<sup>+</sup> (13). As the Van der Waals volume of the Thr residue (93 Å<sup>3</sup>) is 27% greater than that of the Ser residue (73 Å<sup>3</sup>) (23), our results did not support the model proposed by Kellenberger et al. (22) and raised the possibility that the hydroxyl side chain at position 589 of the a subunit might interact with ions traversing the selectivity filter. To probe the side chain orientation of filtering residues, we examined the accessibility of engineered sulphydryl groups at the first and third positions in mouse $\alpha$ENaC, $\beta$ENaC, and $\gamma$ENaC to external Cd<sup>2+</sup>. Our results indicate that sulphydryl groups from Cys residues introduced at the first and third positions of the selectivity filter tract ((G/S)X) interact with external Cd<sup>2+</sup> and suggest that the side chains of Ser residues within the selectivity filter, including $\alpha$Ser<sup>589</sup>, may point to the pore lumen and interact with cations within the filter.

**EXPERIMENTAL PROCEDURES**

*Site-directed Mutagenesis—Site-directed mutagenesis was performed on cDNAs for mouse $\alpha$ENaC, $\beta$ENaC, and $\gamma$ENaC cloned into pBluescript SK (Stratagene) as reported previously (13). Target mutations were confirmed by direct sequencing. The first and third residues within the three-residue tract ((G/S)X) of $\alpha$ENaC, $\beta$ENaC, and $\gamma$ENaC were individually substituted with Cys. To enhance functional expression in oocytes, we generated $\beta$ and $\gamma$ subunits with an introduced Liddle’s mutation ($\beta$R564X or $\gamma$R583X, denoted $\beta_T$ or $\gamma_T$, respectively) that truncates their intracellular C termini.*

*Channel Expression in Xenopus Oocytes—The cRNAs for wild-type and mutant ENaC subunits were synthesized with T7 RNA polymerase (Ambion Inc.). Oocytes harvested from *Xenopus laevis* were treated with collagenase to remove follicle layers. Stage V and VI oocytes were injected with cRNAs for wild-type or mutant mouse $\alpha$ENaC, $\beta$ENaC, and $\gamma$ENaC at 1–4 ng/subunit/oocyte. Injected oocytes were incubated at 18 °C in modified Barth’s saline (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO<sub>3</sub>, 15 mM HEPES, 0.3 mM CaCl<sub>2</sub>, 0.82 mM MgSO<sub>4</sub>, 10 μM sodium penicillin, 10 μM streptomycin sulfate, and 100 μg/ml gentamicin sulfate, pH 7.4).*

*Two-electrode Voltage Clamp—All experiments were performed 20–96 h after cRNA injections at room temperature (20–24 °C). Oocytes were placed in an oocyte recording chamber from Warner Instrument Corp. (Hamden, CT) and perfused with a bath solution containing 110 mM NaCl, 2 mM KCl, 2 mM CaCl<sub>2</sub>, and 10 mM HEPES, pH 7.4. Whole cell Na<sup>+</sup> currents were measured by two-electrode voltage clamp as described previously (13). To obtain a current-voltage relationship, oocytes were clamped from −140 to 60 mV in 20-mV increments for 0.5 s. For time course experiments, oocytes were continuously clamped at −100 mV. Amiloride-sensitive currents are defined as the difference between total currents and the currents with 100 μM amiloride in the bath solution.

*Cd<sup>2+</sup> Accessibility—The effects of Cd<sup>2+</sup> on mutant ENaC channels were examined by comparing currents before and after external application of CdCl<sub>2</sub>. Increasing concentrations of CdCl<sub>2</sub> (from 10<sup>−7</sup> to 10<sup>−2</sup> M) were used to determine dose-response relationships. As some mutations within the selectivity filter tract decrease amiloride sensitivity (15, 24), we considered statistically significant. Nonlinear least-square curve fittings were performed with OriginPro 7 (OriginLab Corp., Northampton, MA).*

**RESULTS**

We previously observed low levels of functional ENaC expression in *Xenopus oocytes* expressing αβγENaCs with a Cys substitution at the first or third residue within the (G/S)X tract of the putative selectivity filter, with whole cell Na<sup>+</sup> currents at −100 mV typically below 500 nA (13, 15). Taking advantage of the enhanced surface expression of channels with Liddle’s mutations, we investigated accessibility of external Cd<sup>2+</sup> to engineered sulphydryl groups within the (G/S)X tract. A sequence alignment of residues near the (G/S)X tracts in the ENaC/DEG family is shown in Fig. 1A. Our rationale was that Cys-substituted channels would be blocked by external Cd<sup>2+</sup> if introduced sulphydryl groups point to the filter lumen as illus-
A–F examined the reversibility of the block. As shown in Fig. 4, the addition of the reducing reagent DTT (10 mM) restored currents to near control levels. Currents restored by DTT were blocked by 0.1 mM amiloride, eliminating the possibility that DTT-induced currents were mediated by endogenous channels. Moreover, application and washout of 10 mM DTT did not produce noticeable effects on currents in oocytes expressing the same mutant channels without prior application of Cd\textsuperscript{2+}. These results suggest that the observed Cd\textsuperscript{2+} block of the mutant ENaCs containing Cys substitutions within the putative selectivity filter was due to Cd\textsuperscript{2+} interaction with thiol groups rather than with oxygens.

**Effects of External Cd\textsuperscript{2+} on Non-Cys Mutants**—To eliminate the possibility that the Cd\textsuperscript{2+} block of the Cys mutants was due to Cd\textsuperscript{2+} binding to endogenous Cys in the ENaC pore, we examined the effects of Cd\textsuperscript{2+} on channels containing an Ala substitution at αGly\textsuperscript{557} or αSer\textsuperscript{558} or a Ser substitution at αGly\textsuperscript{557}. External application of Cd\textsuperscript{2+} at concentrations up to 1 mM did not block the currents in oocytes expressing αG557A, αG587S, or αS589A (Fig. 5), where either the β or γ subunit had a Liddle’s mutation. 10 mM Cd\textsuperscript{2+} blocked <40% of the currents in these mutants, and the block was completely reversed by Cd\textsuperscript{2+} washout from the bath solution, in contrast to the poor reversibility of the Cd\textsuperscript{2+} inhibition of αG587Cβ\textgammaγ and αS589Cβ\textgammaγ (Fig. 4). The partial and low affinity block by 10 mM Cd\textsuperscript{2+} likely reflects binding of divalent Cd\textsuperscript{2+} to non-thiol ligands within the selectivity filter of the mutant channels and suggests that the observed Cd\textsuperscript{2+} block of the Cys mutant channels at submillimolar concentrations resulted from Cd\textsuperscript{2+} binding to the introduced sulfhydryl groups.

**Cd\textsuperscript{2+} Block and the Endogenous Cys Residue**—The γ subunit has a natural Cys within the (G/S)X tract (Cys\textsuperscript{457}). This residue is not conserved among ENaC/DEG family members (Fig. 1) and is not required for cation selectivity, as point mutations of this Cys do not alter L/I Na\textsuperscript{+} or K\textsuperscript{+} Na\textsuperscript{+} selectivity (12). To investigate whether γ-Cys\textsuperscript{1} was involved in the Cd\textsuperscript{2+} block of the Cys-substituted mutant channels, we expressed channels with Cys introduced into the (G/S)XS tract in the setting of γCys\textsuperscript{457}. Unfortunately, the additional mutation (i.e. γC547S) in the presence of a Liddle’s mutation decreased functional channel expression to levels that were too low to allow us to perform a typical Cd\textsuperscript{2+} dose-response experiment. As an alternative, we examined the blocking effects of 0.1 and 1 mM Cd\textsuperscript{2+} on αG587Cβ\textgammaγC547S and αS589Cβ\textgammaγC547S. We observed that 0.1 mM Cd\textsuperscript{2+} blocked about half of the current in oocytes expressing αG587Cβ\textgammaγC547S and that 1 mM Cd\textsuperscript{2+} blocked approximately half of the current in oocytes expressing αS589Cβ\textgammaγC547S. These results indicate that the γC547S mutation did not affect the Cd\textsuperscript{2+} block of the Cys mutants, sug-

### Table 1

| mENaC | No. of oocytes | \(I_{\text{na}}\) | \(K_i\) | Hill coefficient |
|-------|---------------|----------------|---------|-----------------|
| αG587Cβ\textgammaγ | 10 | 3.5 ± 0.6 | 0.06 ± 0.01 | 1.01 ± 0.04 |
| αS589Cβ\textgammaγ | 9 | 2.8 ± 0.4 | 1.31 ± 0.06 | 1.02 ± 0.04 |
| αG529Cβ\textgammaγ | 8 | 2.9 ± 0.8 | 1.35 ± 0.39 | 0.69 ± 0.07 |
| αS531Cβ\textgammaγ | 9 | 6.5 ± 1.3 | 13.5 ± 1.4 | 1.5 ± 0.0 |
| αβ\textgammaγS546C | 9 | 10.4 ± 1.2 | 0.94 ± 0.06 | 1.68 ± 0.04 |
| αβ\textgammaγS548C | 5 | 5.1 ± 1.3 | 8.44 ± 1.98 | 0.57 ± 0.08 |
| αS583Cβ\textgamma | 9 | 4.3 ± 1.4 | 0.05 ± 0.01 | 1.04 ± 0.05 |
| αG525Cβ\textgamma | 10 | 3.9 ± 1.4 | 0.35 ± 0.07 | 1.15 ± 0.11 |
| αγG542C | 10 | 2.1 ± 0.3 | 0.28 ± 0.01 | 1.92 ± 0.06 |
| αβ\textgammaγ | 9 | 20.4 ± 2.8 | ND | ND |
| αβ\textgammaγ | 8 | 26.3 ± 4.2 | ND | ND |
| αβ\textgammaγ | 12 | 13.5 ± 2.1 | ND | ND |

*a Fixed at 1 in curve fittings (otherwise unable to fit).*

**trated in Fig. 1B. Conversely, channels would not be blocked by Cd\textsuperscript{2+} if the introduced sulfhydryl groups face away from the pore lumen as shown in Fig. 1C.**

**External Cd\textsuperscript{2+} Blocks ENaCs with a Single Cysteine Substitution within the Putative Selectivity Filter**—Whole cell currents in oocytes expressing αβγmENaCs with a single Cys substitution and a Liddle’s mutation were in the range of 1–5 μA at −100 mV (Table I). Amiloride at 100 μM blocked the currents of the mutants containing a Cys substitution at the third Ser in the α, β, or γ subunit to <200 nA, a level typically observed in non-injected oocytes. However, amiloride failed to block the currents of channels containing a Cys mutation at the first position of the selectivity filter tract of the α or β subunit to the same level. This is consistent with previous reports that mutations at αGly\textsuperscript{557} and βGly\textsuperscript{558} decrease the amiloride sensitivity of the mutant channels (14, 15). The effects of external Cd\textsuperscript{2+} on ENaC currents were examined by analyzing the current changes in the presence of increasing concentrations of CdCl\textsubscript{2} (from 10⁻⁷ to 10⁻² M) in the bath solution. Representative recordings are shown in Fig. 2. All six mutant channels were blocked by external Cd\textsuperscript{2+} in a dose-dependent manner (Fig. 3). The estimated inhibitory constants (\(K_i\)) and Hill coefficients are listed in Table I. In contrast, control channels containing a Liddle’s mutation but with no Cys substitution were not blocked by Cd\textsuperscript{2+}. These results indicate that the introduced sulfhydryl groups at the selectivity filter-lining sites in all three subunits were accessible to external Cd\textsuperscript{2+}. It was apparent that mutant channels with a Cys substitution at the first position within the filter tract (αG587C, βG529C, and γS546C) showed higher sensitivity to Cd\textsuperscript{2+} than channels containing a Cys mutation at the third position (αS589C, βS531C, and γS548C) of the same subunit (\(p < 0.05\)). With Cys substitutions at the first or third position of the (G/S)XS tract, α mutants were more sensitive to Cd\textsuperscript{2+} than β and γ mutants (\(p < 0.05\)), whereas β and γ mutants showed similar Cd\textsuperscript{2+} sensitivity (\(p > 0.05\)).

**Cd\textsuperscript{2+} Block Is Reversed by the Reducing Reagent Dithiothreitol (DTT)**—As a group 12 metal, Cd\textsuperscript{2+} binds free sulfhydryl groups with high affinity in a “near-covalent” manner (25). To confirm that the Cd\textsuperscript{2+}-induced block of the Cys mutant ENaCs was due to Cd\textsuperscript{2+} binding to the introduced sulfhydryl group, we examined the reversibility of the block. As shown in Fig. 4 (A–F), currents only partially recovered following Cd\textsuperscript{2+} washout from the bath solution. We also tested the reversibility of the Cd\textsuperscript{2+} block with a single voltage (−100 mV) clamp protocol (Fig. 4, G and H). Consistent with the above results, Na\textsuperscript{+} currents in oocytes expressing αG587Cβ\textgammaγ or αS589Cβ\textgammaγ were only partially restored following washout of Cd\textsuperscript{2+} from the bath solution. The addition of the reducing reagent DTT (10 mM) restored currents to near control levels. Currents restored by DTT were blocked by 0.1 mM amiloride, eliminating the possibility that DTT-induced currents were mediated by endogenous channels. Moreover, application and washout of 10 mM DTT did not produce noticeable effects on currents in oocytes expressing the same mutant channels without prior application of Cd\textsuperscript{2+}. These results suggest that the observed Cd\textsuperscript{2+} block of the mutant ENaCs containing Cys substitutions within the putative selectivity filter was due to Cd\textsuperscript{2+} interaction with thiol groups rather than with oxygens.
suggesting that the observed Cd$^{2+}$ block was due to its interaction with the engineered sulfhydryl groups.

**Rates of Cd$^{2+}$ Block**—Rates of Cd$^{2+}$ block are often used to compare the relative Cd$^{2+}$ accessibility to engineered Cys residues. We measured the rate of 1 or 10 mM Cd$^{2+}$ block of Na$^{+}$ currents in oocytes expressing channels with a single Cys mutant in the (G/S)XS tract using a voltage clamp protocol as described under “Experimental Procedures.” Fig. 6 shows representative recordings from these experiments. Time constants ($\tau$) for Cd$^{2+}$-dependent inhibition of mutant channels and the calculated rates of Cd$^{2+}$ block are listed in Table II. In agreement with the relative affinities of the Cd$^{2+}$ block, Cd$^{2+}$ inhibi-
itory rates for mutant channels with a Cys substitution at the first position of the (G/S)XS tract were greater than those observed for channels with a Cys substitution at the third position (G587C versus G529C, p < 0.001; G587C versus S531C, p < 0.01; and G529C versus S546C, p < 0.001). Interestingly, the rate of Cd²⁺ block of the αG587C mutant was similar to that of βG529C mutants (p > 0.05) and greater than that of the γS546C mutant (p < 0.001), despite the order of

![Figure 3. Cd²⁺ dose-response curves.](http://www.jbc.org/)

The 

![Figure 4. Reversibility of the Cd²⁺ block following Cd²⁺ washout and following DTT treatment.](http://www.jbc.org/)

The
perfusion with 10 mM CdCl₂, following Cd²⁺ removal are shown as means ± S.E., and Hill coefficient for αG587CβγTγ was 0.76 ± 0.03 (n = 8). This decrease in amiloride sensitivity was much greater than has been observed with Cys substitutions at other sites within the pore region of αENaC, including αSer⁵⁸³, result in only small increases (~3.5-fold) in the amiloride Kᵢ for the Cd²⁺ block of αG587Cβγ (0.05 ± 0.01 mm) was close to that for αG587CβγTγ (0.06 ± 0.01 mm; p > 0.05). Channels with βG525C showed a 4-fold higher sensitivity to Cd²⁺ than βG529C mutants (p < 0.05), and γG542C channels were 3-fold more sensitive to Cd²⁺ than γS546C mutants (p < 0.001). The rates of Cd²⁺ block of these amiloride-binding site mutants were significantly higher than those of their corresponding filter mutants (αS583C versus αG587C, p < 0.001; βG525C versus βG529C, p < 0.01; and γG542C versus γS546C, p < 0.001) (Table II).

Effects of Mutations within the Selectivity Filter on the Amiloride Block of mENaC Currents—Amiloride blocks ENaCs with an inhibitory constant of ~100 nm. αSer⁵⁸³, βGly⁵⁴⁵, and γGly⁵₄⁴ have been identified as key residues within a putative amiloride-binding site based on changes in the amiloride Kᵢ that were observed with point mutations (24) and are four residues away from the (G/S)X tract. -1000-fold increases in the amiloride Kᵢ have been reported with a Cys substitution at either βGly⁵₄⁵ or γGly⁵₄⁴, although Cys substitutions at selected sites within the pore region of αmENaC, including αSer⁵⁸³, result in only small increases (~3.5-fold) in the amiloride Kᵢ (13). Mutations of the first residue within the (G/S)X tract also decrease amiloride sensitivity (14, 15). The αG587S mutant increases the amiloride Kᵢ by 6.8-fold, although αG587A slightly decreases the amiloride Kᵢ (14). In oocytes expressing αG587CβγTγ, we observed that the reduction in whole cell current with 1 mM Cd²⁺ was greater than that observed with 100 μM amiloride. These data suggested that the block of αG587CβγTγ by 100 μM amiloride was incomplete. We therefore examined the response of αG587CβγTγ and αS589CβγTγ to increasing concentrations of amiloride (Fig. 8). The estimated Kᵢ for αG587CβγTγ was 3.16 ± 0.24 μM (n = 8), and the Hill coefficient was 0.76 ± 0.03 (n = 8). This decrease in amiloride sensitivity was much greater than has been observed with Cys substitutions at other sites within the α pore region. In contrast, the Kᵢ and Hill coefficient for αS589CβγTγ were 41.8 ± 6.5 μM (n = 4) and 0.85 ± 0.01 (n = 4), respectively.

**DISCUSSION**

Our study addresses a key question regarding the structure of the ENaC selectivity filter and the mechanisms of cation selectivity. Do hydroxyl oxygen atoms of the Ser residues at the first and third positions within the (G/S)X tract point to the pore to coordinate permeant cations (Li⁺ or Na⁺)? Our results indicate that the engineered sulfhydryl groups within the putative selectivity filter are accessible to externally applied Cd²⁺ and suggest that hydroxyl groups from wild-type Ser residues within the putative selectivity filter are also accessible to cations traversing the pore. These results are consistent with the
notion that hydroxyl groups from the Ser residues within the putative selectivity filter contribute to cation binding and therefore to selectivity.

Our conclusions appear to conflict with the pore model proposed by Kellenberger et al. (22), in which Na\(^{+}\) within the selectivity filter is coordinated exclusively by four carbonyl oxygen atoms contributed from four Ser residues within the selectivity filter tract, whereas the Ser side chains point to intersubunit interfaces. This hypothesis was primarily based on the positive correlation between the relative changes in cation selectivity and the volumes of the residues introduced at αSer\(^{589}\). However, this positive correlation was observed for a
ENaC Selectivity Filter

The third serine residue within the selectivity filter tract is conserved among all ENaC/DEG family members, consistent with a crucial role within the filter. Indeed, αSer589 is highly sensitive to point mutations. Substitution of αSer589 with Gly, Ala, Cys, Asp, Asn, Glu, or His results in K⁺-permeable channels with a reduced discrimination between Na⁺ and K⁺ (11, 13, 22). Nonfunctional channels were observed when αSer589 was mutated to Val, Leu, Met, Glu, Lys, Arg, Phe, or Trp (22). The only substitution at αSer589 that retains K⁺ exclusion is Thr, which also bears a hydroxyl group (13). Although mutations of this Ser residue in γENaC have also been reported to result in K⁺-permeable channels (12), the Ser at the third position in βENaC does not appear to have a key role in conserving ENaC cation selectivity, as βS531A and βS531C are not K⁺-permeable (11, 15).

In contrast, the first residue within the three-residue tract of βENaC (Gly) contributes to K⁺/Na⁺ selectivity, as channels with mutation of γGly229 to Ser, Cys, or Asp are K⁺-permeable (14, 15). Although we reported that channels with mutations of the first residue within the (G/S)XS tract in αENaC (αG587C and αG587A) are K⁺-permeable (13), Kellenberger et al. (14) reported no change in the K⁺/Na⁺ current ratio in the αG587A mutant. In addition, mutations of the first residue in γENaC (Ser546 in mENaC) are not K⁺-permeable (12, 14). In summary, mutagenesis data suggest that the third Ser in both αENaC and γENaC and the first Gly in βENaC and perhaps also in αENaC are important in restricting K⁺ permeation.

The apparent requirement of a Ser residue at the third position in the selectivity filter of αENaC and γENaC is interesting. Ser is not present in the selectivity signature sequence (DEKA) of voltage-gated Na⁺ channels. However, Ser residues in specific Na⁺/substrate symporters have been found to have important roles in determining Na⁺ binding affinity (30). Wheat HKT1 is a Na⁺-coupled K⁺ transporter that is related to fungal Trk and bacterial TrhK H⁺ transporters. These HKT1-related transporters are predicted to have four membrane domain-pore region-membrane domain repeats, and all four pore loop-like regions contain a highly conserved Gly that is considered crucial for K⁺ selectivity, analogous to the first Gly in the K⁺ channel selectivity filter sequence GYG (31). Interestingly, Arabidopsis thaliana HKT1 functions as a selective Na⁺ transporter (32). It was recently demonstrated that the Na⁺-selective phenotype is due to a Ser residue that replaces a Gly residue within the first membrane domain-pore region-membrane domain (33). This is reminiscent of the weaver mouse mutation, where a Gly-to-Ser mutation (G156S) within the selectivity filter of the G protein-gated inward rectifier K⁺ channel GIRK2 results in an increase in GIRK2 Na⁺ permeability (34). These observations suggest that the presence of Ser residues within selectivity filters or Na⁺-binding sites favors interactions with Na⁺ rather than K⁺. The retained K⁺/Na⁺ selectivity of αS589Tβγ suggests that Na⁺ selectivity may be dependent on the presence of hydroxyl groups. In light of our current results regarding the external Cd²⁺ block of αS589Cβγ and αβγS546C, we propose that hydroxyl oxygens from selected Ser residues within the (G/S)XS tract participate in coordinating Na⁺ or Li⁺ during ion permeation.

The Cd²⁺ block of Cys mutant channels was fast, with time constants of <3.2 s (Table II), which is consistent with the direct interaction of Cd²⁺ with the ENaC pore. Both carboxyl oxygen and side chain sulfur atoms may provide Cd²⁺-binding sites and allow for the Cd²⁺ block of the channel currents. We believe that the Cd²⁺ block primarily results from Cd²⁺ interaction with sulphydryl groups rather than carboxyl oxygen

**FIG. 7.** External Cd²⁺ blocks mENaCs with a Cys substitution at the putative amiloride-binding site. Cd²⁺ dose-response curves are shown for wild-type, αS589Cβγ, αG525Cγ and αβγS542C mENaCs. The dashed line for the wild-type mENaC was without curve fitting. The solid lines were from the best fit of the dose-response data with the Hill equation. Values are means ± S.E. The averaged fitting parameters and the number of observations are listed in Table I.

**FIG. 8.** Mutation αG587C reduces amiloride affinity. The amiloride dose-response curves for αG587Cβγ and αS589Cβγ are shown. Increasing concentrations of amiloride were sequentially added to the bath solution. Currents measured at −100 mV were normalized to the basal currents prior to amiloride application to obtain relative currents. The lines are from the best fit of the data with the Hill equation.

limited number of mutants (αS589C, αS589D, αS589N, and αS589H). Other mutations at αSer589 either eliminate channel activity (αS589V, αS589E, αS589L, αS589M, αS589K, αS589F, αS589R, and αS589W) or result in selectivity changes that are not consistent with this correlation (e.g. αS589G, αS589A, and αS589Q). Specifically, substitution of αSer589 with either Gly or Ala reduces the residual volume, but increases the K⁺/Na⁺ current ratio. Furthermore, channels with a Thr substitution at αSer268 retain the ability to discriminate Na⁺ and K⁺ (13), although the volume of Thr is −27% greater than that of Ser (23).

Nevertheless, molecular sieving is clearly one of the determining factors governing ENaC selectivity (3, 11, 22, 26–29). However, it is still unclear whether size exclusion is sufficient for ENaCs to achieve ion selectivity. Other factors (such as a high field strength site within the channel pore that might preferentially bind smaller cations such as Li⁺ and Na⁺) might influence cation selectivity (26–28). We speculate that ENaC cation selectivity is achieved through multiple mechanisms, including size exclusion, electrostatic interactions between ions and amino acid residues lining the pore, preferential cation coordination number and geometry, and pore flexibility. We propose that a mixture of carbonyl and hydroxyl oxygen atoms within the ENaC narrow selectivity filter coordinates permeating Na⁺ or Li⁺ ions and that the selectivity filter has an asymmetric design.
atoms based on the following observations. First, minimal Cd$^{2+}$ sensitivity was observed with Ala substitutions of αGly$^{587}$ and αSer$^{589}$. In these cases, currents were restored when Cd$^{2+}$ was simply removed from the bath solution, consistent with weak and reversible binding to hard donors such as oxygens. Second, the Cd$^{2+}$ block of channels with Cys mutants in the (G/S)XS tract was only partially reversed following washout of Cd$^{2+}$ from the bath solution, but was nearly completely reversed upon the addition of 10 mM DTT. This is consistent with the predominantly covalent bond characteristics of Cd$^{2+}$ interaction with free sulfhydryl groups (25).

Channels with a Cys substitution at either the first or third position of the (G/S)XS tract in αENaC showed significantly higher Cd$^{2+}$ sensitivity than channels with the corresponding Cys substitution in βENaC or γENaC. The Cd$^{2+}$ sensitivity of channels with a Cys substitution in either βENaC or γENaC was similar. These differences may reflect the presence of more than one sulfhydryl group that could participate in Cd$^{2+}$ binding when Cys was introduced into αENaC, whereas only one sulfhydryl group would participate in Cd$^{2+}$ binding when Cys was introduced into either the β or γ subunit, assuming a tetrameric αβγγ architecture (4, 5). Alternatively, sulfhydryl groups introduced at homologous sites in different subunits might be located at different depths within the conduction pore, as we previously suggested (15).

For all three subunits, the Cd$^{2+}$ sensitivities of ENaCs with a Cys substitution at the first position of the (G/S)XS tract were at least 10-fold greater than those observed for channels with a Cys substitution at the third position. Furthermore, the rates of Cd$^{2+}$ block of channels with a Cys substitution at the first position were also more rapid than those observed with a Cys substitution at the third position. These differences likely reflect the relative depth of the introduced sulfhydryl groups within the channel pore and suggest that the first residue in the (G/S)XS tract is external to the third residue. The putative amiloride-binding site has been proposed to be external to the selectivity filter (24). Channels containing a Cys substitution at the amiloride-binding site (αS583Cβγ, αG525SCγ, and αβG542C) were blocked by external Cd$^{2+}$, indicating that the introduced sulfhydryl groups were accessible to Cd$^{2+}$. The result is consistent with previous observations that Zn$^{2+}$ and methanethiosulfonate reagents block these mutant channels (5, 12, 15, 18, 24). The greater Cd$^{2+}$ sensitivities and block rates of the amiloride-binding site mutants compared with those of the selectivity filter mutants are also consistent with the notion that the amiloride-binding site in the ENaC pore is external to the selectivity filter and with a gradually narrowing pore structure as proposed by Snyder et al. (12), Kellenberger et al. (14), and Palmer (29).

Based on our current observations and previous reports (11–15), we propose that the ENaC selectivity filter has an asymmetric structure that differs from the symmetric selectivity filter present in K$^+$ channels. Our working model for an Na$^+$ coordination shell within the selectivity filter is shown in Fig. 9B. In contrast to the model proposed by Kellenberger et al. (22), a Na$^+$-binding site at αSer$^{589}$-βSer$^{531}$-γSer$^{548}$ involves four carbonyl oxygens from the four Ser residues (assuming an αβγγ subunit stoichiometry) plus two hydroxyl oxygens from two αSer$^{589}$ residues. This model agrees with the following observations regarding ENaCs and Na$^+$ coordination geometry. (i) A sulfhydryl group introduced at αSer$^{589}$ is accessible to external Cd$^{2+}$, suggesting that the hydroxyl group of αSer$^{589}$ is accessible to permeating cations. (ii) K$^+$/Na$^+$ discrimination is retained only when αSer$^{589}$ is replaced with Thr (13), but not with other amino acids (Gly, Ala, Cys, Asp, Asn, Gln, and His) (11, 13, 22). These data suggest that the hydroxyl group of αSer$^{589}$ has a key role in conferring K$^+$/Na$^+$ selectivity. (iii) The side chain of βSer$^{531}$ is dispensable with regard to K$^+$/Na$^+$ selectivity (11, 15). Although channels with an Ala substitution...
of γSer工业化 retain K+/Na⁺ selectivity, αβγS548C is K⁺-permeable (11, 12). In addition, the low affinities and rates of Cd²⁺ block of the βS531C and γS548C mutants suggest a limited accessibility of the introduced sulfhydryl groups to Cd²⁺. (iv) The most common coordination number for Na⁺ in proteins and small molecules is six (35). As the roles of βSer and γSer in conferring ENaC cation selectivity have been addressed by analyzing a limited number of mutants, the possibility that their hydroxyl groups face the filter lumen and are involved in Na⁺ binding cannot be excluded. The major difference between our model and that of Kellenberger et al. (22) is the orientation of the hydroxyl groups of αSer. In our model, the αSer hydroxyl groups are positioned toward the conduction pore and participate in Na⁺ binding. According to the model of Kellenberger et al., these side chains point away from the pore lumen.

Mutagenesis studies suggest that there are both structural similarities and differences between ENaC and K⁺ channel pore regions. Two models of ENaC pore regions have been proposed based on mutation analyses. One model is based on the fold of the KcsA K⁺ channels (13). However, several groups have suggested that this model is unlikely, as it places the putative amiloride-binding site below the selectivity filter. Furthermore, substituted Cys residues at sites immediately following the (G/S)XS tracts are not modified by sulfhydryl-reactive reagents (12, 14, 15, 18). An alternative pore model with a gradually narrowing outer pore extending from the amiloride-binding site (consisting of αSer εβGly εγGly) to the selectivity filter is consistent with most mutagenesis studies (3). Our results in this study largely agree with this second model. We propose an updated model for the pore region structure (see Fig. 18 and Fig. 19). The pore region has two distinct structures that form the outer pore. The N-terminal portions are α helices that are tilted at an angle with respect to the membrane normal, and the C-terminal portions are non-helical. We previously proposed that αSer εβGly εγGly reside at the transition point between the helical and non-helical structures (18). Our model in Fig. 19E instead illustrates a transition at αSer εβGly εγGly. The diameter of the pore at αSer εβGly εγGly is proposed to be ~6 Å based on observations on the modifications of αS531Cβγ, αβγS548Cβγ, and αβγS548C by sulfhydryl reagents or thiophilic Zn²⁺ and Cd²⁺ (5, 12, 15, 18, 24). The diameter of the selectivity filter space at the level of αSer εβGly εγGly is proposed to have a dimension of ~2 Å, estimated from the averaged Na⁺–O distance of 2.42 Å in protein structures (35).

We also observed that αG587C significantly reduced the amiloride block. This change in amiloride affinity was greater than that observed for any other Cys substitution within the pore region of αENaC, including αS533Cβγ. As some mutations at the first position in a and β subunits reduce the amiloride block (14, 15), it is possible that part of the amiloride molecule may interact directly with residues in the selectivity filter. We propose that the positively charged guanidine moiety interacts with the first residue within the (G/S)XS tract of the selectivity filter and that the pyrazine ring interacts with a more external stretch of residues, including αSer εβGly εγGly (Fig. 19F). The first residue within the (G/S)XS tract may also provide a binding site for Na⁺, consistent with a previous report suggesting that Na⁺ and amiloride interact with ENaCs at a common site (36). Our model is also consistent with our previous observation that Cys substitutions at multiple sites neighboring αSer εβGly εγGly moderately reduce the amiloride sensitivity of mutant ENaCs (13, 15).

In summary, we observed that sulfhydryl groups introduced into the putative selectivity filter of ENaCs are exposed to the conduction pore. Our results are consistent with a pore structure in which some of the hydroxyl groups of filter-lining Ser residues (especially αSer εβGly εγGly) point to the filter lumen and participate in coordinating Na⁺ or Li⁺ in determining cation selectivity.

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