A Pore Segment in DEG/ENaC $\text{Na}^{+}$ Channels*

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DEG/ENaC $\text{Na}^{+}$ channels have diverse functions, including $\text{Na}^{+}$ absorption, neurotransmission, and sensory transduction. The ability of these channels to discriminate between different ions is critical for their normal function. Several findings suggest that DEG/ENaC channels have a pore structure similar to $K^+$ channels. To test this hypothesis, we examined the accessibility of native and introduced cysteines in the putative P loop of ENaC. We identified residues that span a barrier that excludes amiloride as well as anionic and large methanethiosulfonate reagents from the pore. This segment contains a structural element (S/G)CS involved in selectivity of ENaC. The results are not consistent with predictions from the $K^+$ channel pore, suggesting that DEG/ENaC $\text{Na}^{+}$ channels have a novel pore structure.

Members of the DEG/ENaC family of $\text{Na}^{+}$ channels have diverse functions, but each moves $\text{Na}^{+}$ across the cell membrane. In the epithelial $\text{Na}^{+}$ channel (ENaC), constitutive channel activity results in $\text{Na}^{+}$ absorption across epithelia including the kidney, lung, and colon (1, 2). Mutations in ENaC disrupt $\text{Na}^{+}$ homeostasis to cause hypertension (3–5) or a salt-wasting disorder (3). In other members of the DEG/ENaC family, the channel is opened by chemical and possibly mechanical stimuli (6–10). This gated movement of $\text{Na}^{+}$ may be involved in sensory transduction and neurotransmission by depolarizing the cell.

Ion permeation is controlled by amino acids that form the channel pore. DEG/ENaC channels are homo- or heteromultimers, and ENaC is formed by three subunits, $\alpha$, $\beta$, and $\gamma$ (11, 12). Each subunit has two membrane-spanning segments, a large extracellular domain, and cytoplasmic N and C termini (13–15). Two different models have been proposed for the stoichiometry of ENaC and other DEG/ENaC channels, consisting of either nine subunits ($\alpha_3$, $\beta_3$, and $\gamma_3$) (16, 17) or four subunits ($\alpha_2$, $\beta_1$, and $\gamma_1$) (18–20). An important property of ENaC is its ability to discriminate between $\text{Na}^{+}$ and $K^+$; ENaC is impermeable to $K^+$ (1, 2). In addition, ENaC is slightly more permeable to $\text{Li}^{+}$ than to $\text{Na}^{+}$, and it is impermeable to anions. However, little is known about the structure of the ion conduction pathway of DEG/ENaC channels or the mechanism of selectivity.

The structure of another cation channel, the KcsA $K^+$ channel, was recently determined (21). Like DEG/ENaC subunits, KcsA has two membrane-spanning domains. The outer pore is a P loop formed by amino acids that precede the second transmembrane segment (M2) and that contains the GYG selectivity filter. Sequence similarity and experimental evidence suggest that this pore structure is shared by Shaker and other six membrane-spanning $K^+$ channels, and it may also be conserved in other tetrameric voltage-gated and cyclic nucleotide-gated channels (21). Several findings suggest that DEG/ENaC channels may also contain a P loop and have a pore structure similar to KcsA. First, genetic analysis suggested that DEG/ENaC channels and $K^+$ channels may have evolved from a common ancestral channel (22). Second, DEG/ENaC channels contain a hydrophobic domain that precedes M2; this may be similar to the $K^+$ channel hydrophobic P loop. Third, consistent with a P loop, protease sensitivity suggests that this hydrophobic domain may extend into the plasma membrane (14). Finally, tetraethylammonium (TEA),† which interacts with the P loop to block $K^+$ channels, also blocks the pore of a neuronal DEG/ENaC channel (BNC1) (23).

We therefore tested the hypothesis that the pore of DEG/ENaC $\text{Na}^{+}$ channels is similar in structure to the pore of $K^+$ channels. We used methanethiosulfonate (MTS) reagents to examine the accessibility of native and introduced cysteines in the putative P loop of ENaC, and we compared our results to predictions based on the structure of KcsA. A similar strategy has been successfully used in the structural analysis of a variety of transporters and ion channels including $K^+$ channels (24). In addition, we asked whether mutations in this domain altered the selectivity of ENaC. Because the three ENaC subunits have a very similar amino acid sequence, it is likely that they share a similar structure. Thus, we focused our studies on one subunit ($\gamma$) and then extended key findings to the other two subunits.

**Experimental Procedures**

DNA Constructs—cDNAs encoding human $\alpha$, $\beta$, and $\gamma$ENaC in pcDNA3 or pcDNA3 vectors were constructed as described previously (12). Site-directed mutations were generated by oligonucleotide-based mutagenesis using the Muta-Gen (Bio-Rad) or Quick-change (Stratagene) kits, and the accuracy of the mutation was confirmed by DNA sequencing.

Expression and Electrophysiology—We expressed ENaC in Xenopus oocytes by nuclear injection of cDNAs encoding each subunit (0.2–0.4 ng each). Following incubation of the oocytes for 16–24 h in modified Barth’s solution at 18 °C, we measured whole-cell current by two-electrode voltage clamp. Oocytes were bathed in NaCl Ringer (116 mM NaCl, 2 mM KCl, 0.4 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM Hepes; pH 7.4, with NaOH) or a solution in which NaCl was replaced by LiCl or KCl. Oocytes were voltage-clamped at a potential of –60 mV, and current-voltage relationships were obtained by stepping to potentials between –140 and +40 mV (20 mV steps) for 300 ms. Amiloride-sensitive current was determined by subtracting current obtained at –60 mV with a maximal dose of amiloride (or benzamil) in the bathing solution.

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† The abbreviations used are: TEA, tetraethylammonium; MTS, methanethiosulfonate; MTSEA, (2-aminoethyl)methanethiosulfonate hydrobromide; MTSET, (2-trimethylammonium)ethyl methanethiosulfonate bromide; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; MT-4-fluorescein, 2[(5-fluoresceinyl)aminocarbonyl]ethyl methanethiosulfonate.
from base-line current without amiloride. We used concentrations of amiloride and benzamil that completely blocked the channels. Mean amiloride-sensitive Na⁺ current for wild-type ENaC was 1.87 ± 0.22 μA (n = 34). Permeability ratios were calculated from changes in reversal potential with Na⁺, Li⁺, and K⁺ as the predominant cation in the extracellular bathing solution (25).

Response to MTS Reagents—We determined the sensitivity of wild-type or mutant ENaC to (2-aminoethyl)methanethiosulfonate hydrobromide (MTSET, 1 mM), [2-(trimethylammonium)ethyl]methanethiosulfonate bromide (MTSEA, 1 mM), 2-trimethylammoniumethyl[methanethiosulfonate (MTSEE, 1 mM), MTSET (1 mM), and MTSES (10 mM) were present in the extracellular bathing solution as indicated by the bars, and current is indicated by the dash. B, percent decrease in amiloride-sensitive Na⁺ current (I_amil.) (mean ± S.E.) in response to MTSEA (1 mM) in cells expressing the indicated combinations of ENaC subunits (n = 4–15). C, percent decrease in I_amil. in cells expressing α and βENaC with the indicated γ subunits. Asterisks indicate p < 0.05 versus wild-type (n = 6–15). F, percent decrease in I_amil. after treatment of wild-type ENaC for 100 s with 1 mM MTSEA (EA), 1 mM MTSET (ET), or 10 mM MTSES (ES), as indicated (n = 6–15). The indicated groups were pretreated with 1 mM MTSET or 10 mM MTSES prior to MTSEA. H, percent decrease in I_amil. when wild-type ENaC was treated with 1 mM MTSEA in the absence or presence of 100 μM amiloride, as indicated (n = 5). Inhibition in the presence of amiloride was not different than zero (p = 0.08).

RESULTS

Sensitivity of ENaC to MTS Reagents—ENaC contains three native cysteines in M2: Cys⁵⁴¹ in Cys⁵⁴⁵ in the γ subunit and Cys⁵³⁶ in β (see Fig. 5A below). To determine whether these cysteines were accessible to the extracellular bathing solution, we examined the effect of extracellular MTSEA on Na⁺ current. This compound modifies cysteines by covalently attaching a positively charged (S)-ethyl ammonium group. In Xenopus oocytes expressing α, β, and γENaC, addition of MTSEA to the extracellular bathing solution irreversibly decreased Na⁺ current by 60% (Fig. 1, A and B). The residual current was blocked by amiloride, a blocker of DEG/ENaC channels. This result suggests that MTSEA covalently modified one or more cysteine(s) in wild-type ENaC, resulting in irreversible inhibition of the channel.

To identify the subunit or subunits modified by MTSEA, we expressed the α subunit alone or in combination with β or γENaC. Although maximal Na⁺ current requires coexpression of all three subunits, expression of αENaC alone or coexpression of α with either β or γENaC produce functional Na⁺ channels (11, 12). When the α subunit was expressed alone or with βENaC, MTSEA produced only a small decrease in Na⁺ current (Fig. 1B). Thus, modification of βCys⁵³⁶ or other cysteines in β or γENaC was not responsible for inhibition of the wild-type channel. In contrast, when we coexpressed α and γ, MTSEA decreased Na⁺ current to the same extent as with expression of all three subunits, indicating that the γ subunit was the major target for MTSEA.

Mutation of both M2 cysteines in γ (γC⁵⁴¹A/C⁵⁴⁵A) significantly diminished inhibition by MTSEA (by 45%) (Fig. 1C). Mutation of Cys⁵⁴¹ (γC⁵⁴¹A) reproduced the effect of γC⁵⁴¹A/C⁵⁴⁵A, but mutation of Cys⁵⁴⁵ (γC⁵³⁶A) did not (Fig. 1C). These data suggest that Cys⁵⁴¹ was accessible to modification and was the main residue responsible for inhibition of ENaC by MTSEA. Other cysteines in ENaC may also be modified by MTSEA, since mutation of Cys⁵⁴¹ did not completely abolish inhibition, but such cysteines lie outside the M2 and pre-M2 segment.

In contrast to MTSEA, a larger cationic reagent, MTSET, did not inhibit ENaC (Fig. 1, D and F). Subsequent treatment with MTSEA (after MTSET) decreased Na⁺ current (Fig. 1, D and F), suggesting that Cys⁵⁴¹ had not been modified by MTSET and was therefore inaccessible to this reagent. An anionic reagent, MTSES, also did not decrease Na⁺ current and did not prevent MTSEA from subsequently inhibiting the channel (Fig. 1, E and F). Because MTSES is 10-fold less reactive than MTSEA to thiols in aqueous solution, we used a 10-fold higher concentration (10 mM). Thus, access to Cys⁵⁴¹ is constrained by the size and charge of the MTS reagent, consistent with loca-
Amiloride inhibits ENaC by directly or indirectly occluding the pore. Therefore, if Cys541 is located in the pore, amiloride might protect this residue from modification by MTSEA. To test this, we blocked ENaC with a maximal concentration of amiloride (100 μM) and then added MTSEA to the bathing solution in the continued presence of amiloride (Fig. 1G). Following washout of both ENaC and amiloride, whole-cell current was similar to current prior to treatment; MTSEA decreased Na+ current by only 8.6% (not statistically different than zero) when amiloride blocked the channel (Fig. 1, G and H). Subsequent treatment with MTSEA (without amiloride) irreversibly inhibited ENaC to the same extent (60%) as channels not previously treated with amiloride and MTSEA (Fig. 1G). Thus, amiloride prevented access to the cysteine or cysteines responsible for most of the inhibition by MTSEA. Because Cys541 makes an important contribution to this inhibition, these results suggest that amiloride prevented modification of Cys541 and, therefore, that this residue is located in the channel pore. Moreover, these results suggest that Cys541 lies deeper within the pore than the site of amiloride block. Although MTSEA is membrane-permeable in its unprotonated form (26), the ability of extracellular amiloride to protect Cys541 argues that MTSEA must enter the pore from the extracellular side to modify this residue.

**Effect of MTSET on Cysteine Substitutions in γENaC**—To examine the accessibility of other residues in the M2 and pre-M2 segments, we replaced amino acids in γENaC (540 to 519) individually with cysteine. Following expression of each mutant γ subunit with wild-type α and β, we examined the effect of extracellular MTSET, which did not inhibit wild-type ENaC (Fig. 2, A and B). Based on the response to MTSET, we identified three functional segments between amino acids 519 and 540.

In the most C-terminal segment, cysteines introduced at position 536 (γG536C) or 533 (γG533C) allowed MTSET to irreversibly decrease Na+ current (90 and 35%, respectively) (Fig. 2, A and B), indicating that these cysteines were accessible to modification by MTSET. When cysteines were placed at other positions from 531 to 540, MTSET did not alter Na+ current (Fig. 2B). Either these residues were not accessible to extracellular MTSET or modification did not alter Na+ current. In the α and β subunits, introduction of cysteine at the position equivalent to γGly536 (α536C and β532C) each resulted in inhibition by MTSET (76 and 67%, respectively).

In a second segment (526–530), cysteine modification stimulated ENaC. When we placed cysteine at position 530 (γS520C), 528 (γG528C), or 526 (γG526C), extracellular MTSET produced a large increase in Na+ current (Fig. 2, A and B). In each case, stimulation resulted from covalent modification of the introduced cysteine, since the increase was irreversible and the current was completely blocked by amiloride. MTSET also stimulated current when cysteines were placed in the α and β subunits within this domain (α524C, 77%; β520C, 328%).

MTSET inhibited ENaC when cysteines were placed at two positions in a third segment further toward the N terminus. When we mutated Ser523 and Ser519 individually to cysteine, MTSET produced a rapid and irreversible decrease in Na+ current (39 and 34%, respectively) (Fig. 2, A and B). Therefore, these residues were also accessible to modification by MTSET.

**Protection by Amiloride and Benzamil**—To localize the reactive cysteines relative to the site of amiloride block, we asked whether amiloride (or the amiloride analog benzamil) would prevent modification. Channels were treated with MTSET while they were blocked by a maximal dose of amiloride or benzamil (Fig. 3A). Following washout of both reagents, current was compared with base-line current before treatment. Fig. 3B plots the MTSET-induced change in Na+ current in the absence or presence of amiloride/benzamil. When the channel contained cysteine at position 536, current returned close to the original base line following washout (Fig. 3A). Thus, benzamil prevented modification of this residue (Fig. 3B), similar to our finding that amiloride protected wild-type ENaC from modification by MTSEA. Conversely, when cysteine was placed at position 533, MTSET inhibited the channel to the same extent in the absence or presence of amiloride (Fig. 3, A and B). Thus, amiloride did not prevent modification of residue 533. MTSET also modified cysteines at positions 530, 528, and 526 when the channel was blocked by amiloride (Fig. 3, A and B), resulting in increased Na+ current. Similarly, MTSET modified cysteines at positions 523 and 519 in the presence of amiloride, decreasing Na+ current (Fig. 3, A and B). These results localize Gly536 in the pore internal to the site of amiloride/benzamil block, whereas the reactive residues from 533 to 519 lie external to this site.

**Accessibility of Introduced Cysteines Is Limited by Charge and Size**—To define further the relative positions of the reactive amino acids, we took advantage of the ability of the ENaC
much larger side chain, MTS-4-fluorescein. This reagent does
reduce cysteines to an MTS reagent that covalently attaches a

533, 530, 523, and 519 lie external to this selectivity barrier. We tested the accessibility of the intro-

536 was inaccessible to MTSES or if

versus

0.05

whether Cys 536 was inaccessible to MTSES or if

permeating ENaC. Cys541 and Gly536 lie internal and residues

Cys533. Cysteines at positions 530, 523, and 519 were also

by MTSET (Fig. 3

4-fluorescein (200

G536C), as indicated (Fig.

Asterisks indicate p < 0.05 versus no amiloride/benzamil (B) and no pretreatment (C) (n = 4–7).

pore to exclude anions. MTSES is similar in size to MTSET, but

inaccessible to MTSES. Similarly, MTSES did not alter Na

current when ENaC contained cysteine at position 536, in contrast to MTSET which decreased Na

current 90%. To determine whether Cys536 was inaccessible to MTSES or if modification by MTSES did not alter Na

current, we asked whether this residue could be modified by MTSET following treatment with MTSES. MTSET decreased Na

current to the same extent as in cells not pretreated with MTSET (Fig. 3C), indicating that the cysteine at position 536 was inaccessible to MTSES. In contrast to Cys536, pretreatment of cysteine at position 533 with MTSES prevented inhibition of the channel by MTSET (Fig. 3C). This indicates that MTSES modified Cys533. Cysteines at positions 530, 523, and 519 were also accessible to MTSES, as indicated by the lack of response to MTSET following pretreatment by MTSET (Fig. 3C). These results suggest that the segment from 536 to 533 spans a selectivity barrier that excludes anions, although it is unclear if the same mechanism prevents small anions (e.g. Cl

) from permeating ENaC. Cys541 and Gly536 lie internal and residues 533, 530, 523, and 519 lie external to this selectivity barrier.

We also took advantage of the ability of the pore to discrimi-

nate based on size. We tested the accessibility of the intro-

duced cysteines to an MTS reagent that covalently attaches a

much larger side chain, MTS-4-fluorescein. This reagent does not alter Na

current through wild-type ENaC. Similar to our studies with MTSES above, we asked whether MTSET would alter Na

current following pretreatment with MTS-4-fluorescein. MTS-4-fluorescein did not prevent inhibition of channels containing Cys536 (Fig. 3C), indicating that this cysteine was not accessible to this reagent. Conversely, cysteines at positions 533, 530, 523, and 519 were accessible to modification by MTSET-4-fluorescein; pretreatment with MTS-4-fluorescein prevented modification by MTSET (Fig. 3C). Thus, the segment from 536 to 533 also spans a size barrier; Cys541 and Gly536 are internal and residues 533, 530, 523, and 519 are external to this size barrier.

Mutations in M2 Alter Channel Selectivity—A defining char-

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the shift in reversal potential when $\text{Li}^+$ replaced $\text{Na}^+$ in the bathing solution (Fig. 4, A and B). This mutation also had no effect on $\text{K}^+$ permeability (Fig. 4, A and B). Interestingly, the amino acid at the equivalent position in $\alpha$ and $\beta$ ENaC, and in all other members of the DEG/ENaC family, is glycine (Fig. 5A). However, this amino acid difference is functionally conservative; mutation of Ser 540 in $\gamma$ to glycine produced permeability properties identical to wild-type ENaC (not shown).

In contrast to these effects on $\text{Na}^+/\text{Li}^+$ selectivity, mutation of another neighboring amino acid, Ser$^{542}$, increased the permeability of the channel to $\text{K}^+$ but did not alter the $\text{Na}^+/\text{Li}^+$ permeability ratio (Fig. 4, A and B). Although this mutant channel was still more permeable to $\text{Na}^+$ than $\text{K}^+$ ($P_{\text{K}^+}/P_{\text{Na}^+} = 0.15 \pm 0.02$), the inward $\text{K}^+$ current observed was in stark contrast to the relative $\text{K}^+$ impermeability of wild-type ENaC (Fig. 4A). All members of the DEG/ENaC family studied to date are selective for $\text{Na}^+$ over $\text{K}^+$, and Ser$^{542}$ is conserved in each of these channels. Thus, this conserved serine might be an important determinant of $\text{Na}^+/\text{K}^+$ selectivity.

**DISCUSSION**

To test the hypothesis that DEG/ENaC subunits and $\text{K}^+$ channels have a similar pore structure, we aligned their sequences based on hydrophobicity plots of the M2 and pre-M2 segments (Fig. 5A). Despite only limited sequence similarity between ENaC and $\text{K}^+$ channels, hydrophobicity plots for these channels are remarkably similar in this region. An identical alignment was previously proposed (22). We then compared the accessibility of cysteines in $\gamma$ ENaC to predictions made from the alignment and known structure of KcsA. Fig. 5B shows two (of four) subunits of KcsA. We superimposed the predicted positions of reactive cysteines in $\gamma$ ENaC based on the sequence alignment in A. C, working model of outer part of ENaC pore, showing contribution of one $\gamma$ subunit. The role of M1 in formation of the pore was not addressed in these studies. A–C, reactive cysteines are colored according to their response to MTSET (or MTSEA for 541); blue (533, 536, and 541) and red (519 and 523) residues were inhibited, and yellow residues (526, 528, and 530) were stimulated.

If ENaC and $\text{K}^+$ channels have a similar pore structure, the KcsA structure predicts that Cys 541 would lie outside the pore (Fig. 5B). However, four findings suggest not only that Cys$^{541}$ is located in the pore but in a relatively narrow region of the pore.

First, Cys$^{541}$ was covalently modified by a small MTS reagent (MTSEA), but not by larger (MTSET, MTS-4-fluorescein) or anionic (MTSES) reagents. Second, the rate of modification by MTSEA was slow requiring more than 100 s, consistent with a relatively inaccessible location. Third, the data suggest that amiloride prevented modification of Cys$^{541}$, indicating that this residue lies internal to the site of amiloride block. Fourth, mutation of neighboring residues altered channel selectivity, suggesting that Cys$^{541}$ is located in a segment ((S/G)CS) that may contribute to the selectivity filter of ENaC.

In $\text{K}^+$ channels, the residue predicted to be equivalent to
Gly\textsuperscript{536} (Tyr\textsuperscript{532} in KcsA) is the binding site for external TEA (Fig. 5, A and B) (21). This is interesting since mutation of Gly\textsuperscript{536} decreased the affinity of ENaC for amiloride (27) and benzamil.\textsuperscript{5} The predicted equivalent to Gly\textsuperscript{533} (Gly\textsuperscript{79} in KcsA) is part of the selectivity filter of the K\textsuperscript{+} channel (Fig. 5, A and B) (21). However, mutation of Gly\textsuperscript{533} and adjacent amino acids did not disrupt the selectivity of ENaC. Based on the structure of KcsA, we would predict that Gly\textsuperscript{536} would lie internal to Gly\textsuperscript{536} (Fig. 5B). Both residues were accessible to MTSET. However, in contrast to the prediction, Gly\textsuperscript{533} was also accessible to anionic (MTSES) and large (MTS-4-fluorescein) reagents, whereas Gly\textsuperscript{536} was not. In addition benzamil protected Gly\textsuperscript{536} from modification but did not protect Gly\textsuperscript{533}. Thus, Gly\textsuperscript{536} lies deeper within the pore than Gly\textsuperscript{533}, opposite to the orientation predicted from alignment with KcsA.

The alignment predicts that residues in the stimulatory segment of γENaC (residues 526–530) are located internal to the selectivity filter. Asn\textsuperscript{530} lines up with Val\textsuperscript{76} in KcsA which lies at the internal end of the selectivity filter (Fig. 5A), and Leu\textsuperscript{528} in γENaC is the predicted equivalent to the residue that forms the binding site for internal TEA in K\textsuperscript{+} channels (Thr\textsuperscript{241} in KcsA, Fig. 5, A and B) (28). Thus, if the pore of ENaC is similar to K\textsuperscript{+} channels, residues 528 and 530 would be internal to Gly\textsuperscript{536} and not accessible to modification from the extracellular side (Fig. 5B). However, external MTSET did modify these residues. In addition, several findings suggest that these residues lie in a relatively exposed position external to Gly\textsuperscript{536}.

First, modification by MTSET increased Na\textsuperscript{+} current. If these residues were located deep within the pore, we would expect that addition of a large cationic side chain (by MTSET modification) would impede the flow of Na\textsuperscript{+} through the pore, decreasing current. Second, MTSES and MTS-4-fluorescein also modified a residue in this domain, suggesting that it lies external to the selectivity and size barrier that restricts access to Gly\textsuperscript{536}. Third, amiloride did not protect these residues from modification, suggesting that they lie external to the site of amiloride block. Finally, in another DEG/ENaC family member, BNC1, mutation of Gly\textsuperscript{530} to phenylalanine in this domain created a binding site for external TEA (23).

Therefore, the data are not consistent with the hypothesis that ENaC and K\textsuperscript{+} channels have a similar pore structure and suggest that the pore of ENaC has a novel structure. Fig. 5C shows a working model. The results suggest that Cys\textsuperscript{541} and the (S/G)CS motif lie in a relatively narrow part of the pore and may contribute to the selectivity filter, analogous to the GYG motif in K\textsuperscript{+} channels. However, in ENaC this sequence is not predicted to be located in a pore loop but near the extracellular end of M2. The equivalent segment in the α and β subunits may also contribute to selectivity; mutation of the residues in αENaC equivalent to γCys\textsuperscript{541} and γSer\textsuperscript{542} produced a small change in the Na\textsuperscript{+}/Li\textsuperscript{+} selectivity (29) and in the Na\textsuperscript{+}/K\textsuperscript{+} selectivity (30), respectively. Based on this altered Na\textsuperscript{+}/K\textsuperscript{+} selectivity, Kellenberger and co-workers (30) independently proposed a model in which αSer\textsuperscript{542} contributes to the cation selectivity of ENaC. The (S/G)CS motif might participate in selectivity by forming a cation-binding site. It is interesting that serine and glycine at position 540 were equivalent, suggesting that a specific side chain was not required for normal selectivity. Perhaps the cation-binding site is not formed by the side chain but by the main chain carbonyl oxygens, as has been proposed for K\textsuperscript{+} channels (21). Alternatively, mutations of the (S/G)CS motif might indirectly alter selectivity by causing a structural change. This seems less likely since the mutations that we tested outside of this motif did not alter selectivity. However, since we only tested the effect of cysteine substitutions, we cannot exclude the possibility that mutation to other amino acids might alter selectivity.

The data suggest that Gly\textsuperscript{536} is located in a wider and more superficial part of the pore than Cys\textsuperscript{541} (Fig. 5C); a cysteine introduced at position 536 was modified not only by MTSEA but also by the larger reagent MTSET. Our results suggest that the equivalent residues in α and β make a similar contribution to the pore and are consistent with previous reports regarding the accessibility of these residues to Zn\textsuperscript{2+} (27) and (for mouse αENaC) MTSEA (19). The contribution of this residue to the pore is also conserved in another DEG/ENaC family member; in BNC1, MTSEA inhibited the channel when a cysteine was introduced at Gly\textsuperscript{437} (equivalent to γGly\textsuperscript{536}) (31).

Gly\textsuperscript{533} lies external to Gly\textsuperscript{536}, as discussed above. Our results localize the site of amiloride and benzamil block to this short segment of amino acids between 533 and 536 (Fig. 5C). In addition, these amino acids form a barrier that can exclude MTS reagents based on their charge and size. The mechanism of amiloride block is not clear. Amiloride block is voltage-dependent, sensing 15–30% of the electrical field (32–34). In addition, as discussed above, mutation of Gly\textsuperscript{536} decreased the affinity of ENaC for amiloride. Thus, the simplest interpretation is that amiloride directly blocks the pore by binding to a site between Gly\textsuperscript{533} and Gly\textsuperscript{536} (as illustrated in Fig. 5C). However, it has been reported that mutation of residues at a site in the extracellular domain also disrupted block by amiloride, raising the possibility that amiloride might block the channel indirectly through an allosteric mechanism (35). If this is the case, then the site between Gly\textsuperscript{533} and Gly\textsuperscript{536} might not be a binding site for amiloride, but rather the site of a conformational change that occludes the pore in response to the binding of amiloride to a distant site. Although this mechanism seems less likely, it remains consistent with the location of Gly\textsuperscript{533} and Gly\textsuperscript{536} in the channel pore.

Our results suggest that residues 526, 528, and 530 also lie external to Gly\textsuperscript{536}. Although their position relative to Gly\textsuperscript{533} is less clear, the simplest interpretation is that they lie external to this residue (Fig. 5C) since modification increased Na\textsuperscript{+} current, rather than blocking the channel. In the α and β subunits, modification of cysteines placed in this domain also increased Na\textsuperscript{+} current, suggesting that they occupy relatively equivalent positions in the channel. Interestingly, these residues are located at the equivalent position to “DEG” mutations in other DEG/ENaC family members; mutation of a specific residue (e.g., Gly\textsuperscript{530} in BNC1) to an amino acid with a large side chain causes neuronal swelling and/or channel activation (6, 9, 10, 36, 37). Although the mechanism of increased current is unknown, our recent results suggest that in BNC1, the large side chain disrupts channel gating by interfering with closing of the channel (37). In ENaC, modification of cysteines introduced in this DEG domain may have an analogous effect by increasing the size of the cysteine side chain. It is interesting that in γENaC stimulation was not specific to the single DEG residue but could be produced by modification of cysteines at three different positions within this functional segment.

Ser\textsuperscript{523} and Ser\textsuperscript{519} appear to lie external to Gly\textsuperscript{536} based on the inability of amiloride to protect these residues from modification and on the accessibility of these residues to MTSES and MTS-4-fluorescein. Their location relative to the stimulatory segment (residues 526–530) is unclear. The hydrophobicity plot suggests that the pre-M2 segment may form a loop, which is consistent with the results of previous protease sensitivity experiments (14). Therefore, in our model we tentatively placed these amino acids in the pore internal to residues.

\textsuperscript{2}P. M. Snyder, D. R. Olson, and D. B. Bucher, unpublished observations.
526–530 (Fig. 5C). Such a position is consistent with our finding that modification by MTSET decreased Na⁺ current, possibly by obstructing the flow of Na⁺ through the pore. This overall architecture is also consistent with an αβ barrel model for the outer pore of ENaC proposed by Guy and Durell (38) using theoretical considerations. However, it is also possible that Ser⁵²³ and Ser⁵¹⁹ lie external to residues 526–530, with modification inhibiting the channel through an indirect conformational change. The model that we propose assumes that the MTS reagents can only access cysteines via an aqueous pathway, an assumption that is consistent with previous reports (24). However, if MTS reagents modified cysteines through other non-aqueous pathways, the data could be consistent with other pore models.

Our studies focused on the γ subunit. However, studies on key residues in the α and β subunits suggest that the three subunits share a similar structure in the pore domain and that each subunit makes a similar contribution to the pore of ENaC. In previous studies, investigators swapped the M2 segments between αENaC and MEC-4, a related protein from Caenorhabditis elegans (29, 39). Interestingly, this produced functional proteins, suggesting that M2 from one family member could substitute for M2 of another. In addition, several observations from BNC1 fit nicely with the model proposed for the pore of ENaC. Thus, it seems likely that members of the DEG/ENaC family share a common pore architecture.

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