Epithelial Sodium Channel Pore Region

STRUCTURE AND ROLE IN GATING

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Epithelial sodium channels (ENaCs) have a crucial role in the regulation of extracellular fluid volume and blood pressure. To study the structure of the pore region of ENaC, the susceptibility of introduced cysteine residues to sulfhydryl-reactive methanethiosulfonate derivatives (2-aminoethyl)-methanethiosulfonate hydrobromide (MTSEA) and (2-(trimethylammonium)ethyl)methanethiosulfonate bromide (MTSET)) and to Cd2+ was determined. Selected mutants within the amino-terminal portion (αVal568–αTrp592) of the pore region responded to MTSEA, MTSET, or Cd2+ with stimulation or inhibition of whole cell Na+ current. The reactive residues were not contiguous but were separated by 2–3 residues where substituted cysteine residues did not respond to the reagents and line one face of an α-helix. The activation of αSS80Cβγ mENaC by MTSET was associated with a large increase in channel open probability. Within the carboxyl-terminal portion (αSer588–αSer592) of the pore region, only one mutation (αSS583C) conferred a rapid, nearly complete block by MTSEA, MTSET, and Cd2+, whereas several other mutant channels were partially blocked by MTSEA or Cd2+ but not by MTSET. Our data suggest that the outer pore of ENaC is formed by an α-helix, followed by an extended region that forms a selectivity filter. Furthermore, our data suggest that the pore region participates in ENaC gating.

Epithelial sodium channels (ENaCs) are composed of three homologous subunits, termed α-, β-, and γENaC (1, 2). These subunits assemble to form a hetero-oligomeric, Na+-selective ion channel with a subunit stoichiometry of 2α:1β:1γ (3, 4), although an alternative subunit stoichiometry has been proposed (5, 6). All three Na+ channel subunits have cytoplasmic amino and carboxyl termini, two transmembrane domains (termed M1 and M2), and a large ectodomain (7–9). Previous studies have shown that selected point mutations within the pore region preceding M2 of each subunit altered functional properties of the channel, including cation selectivity, single channel conductance, and sensitivity to the blocker amiloride (4, 10–14). Specific mutations of residues in a conserved three-residue tract, (G/S)XS (where X is Ser, Gly, or Cys), within the pore region of the three ENaC subunits, rendered channels K+-permeable. Snyder et al. (15) examined the accessibility of a sulfhydryl-reactive methane thiosulfonate (MTS) derivative to substituted cysteine residues within the pore region of human γENaC, and they proposed a structural model of the channel pore similar to that proposed by Kellenberger et al. (11) but distinct from the resolved structure of the KcsA K+ channel pore (16).

We previously reported that selected cysteine substitutions within the carboxyl-terminal domain of the pore region of mouse αENaC (αSer588–αSer592) altered the cation selectivity and amiloride sensitivity of the channel and proposed that this region forms the selectivity filter of the channel (14). In the current study, we systematically examined accessibility of sulfhydryl reagents to αβγmENaCs with engineered cysteine within the 24-residue pore region of the α-subunit. Channels with selected cysteine mutations within the carboxyl-terminal portion of the pore region responded to the external application of MTS derivatives with an inhibition of amiloride-sensitive Na+ currents. In contrast, we observed a significant increase in amiloride-sensitive Na+ currents following the external application of MTS derivatives or Cd2+ when cysteine residues were introduced at selected sites within the amino-terminal portion of the pore region of αmENaC. The pattern of distribution of cysteine mutations that led to MTS-induced activation of Na+ currents suggests that this region has an α-helical structure. In addition, the activation of αSS580Cβγ by an MTS reagent was associated with a dramatic increase in channel open probability. We propose that the ENaC pore region forms part of the outer pore vestibule with an α-helix followed by an extended region. ENaC may have limited structural similarities with the KcsA K+ channel (16, 17). In addition, our results suggest that the pore region has a role in ENaC gating.

EXPERIMENTAL PROCEDURES

Reagents—All chemicals were from Sigma unless stated otherwise.

Cysteine-scanning Mutagenesis—Site-directed mutagenesis was performed on mouse αENaC (18) with a sequential polymerase chain reaction method using Pfu DNA polymerase (Stratagene, La Jolla, CA). Amino acids αVal568–αSer592 of ααmENaC were replaced individually with a cysteine residue, and target mutations were confirmed by automated DNA sequencing, as described previously (14).

Functional Expression of the Mutant mENaCs in Xenopus Oocytes—Complementary RNAs (cRNAs) for wild type and mutant α-, wild type β-, and γmENaC were synthesized with T7 RNA polymerase (Ambion Inc., Austin, TX). Stage V–VI Xenopus oocyte was injected with 2–4 ng of cRNA for each subunit in 50 nl of H2O. Injected oocytes were main-

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tained at 18 °C in modified Barth’s saline (MBS, 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 15 mM HEPES, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 10 μg/ml sodium penicillin, 10 μg/ml streptomycin sulfate, 100 μg/ml gentamicin sulfate, pH 7.2).

**Two-electrode Voltage Clamp—**Two-electrode voltage clamp was performed 20–72 h after injection at room temperature (22–25 °C) as described previously (14). Data acquisition and analysis were performed using pClamp 6.03 software (Axon Instruments) on a Pentium PC. Oocytes were maintained in a recording chamber with 1 ml of bath solution containing (in mM) 100 sodium gluconate, 2 KCl, 1.8 CaCl₂, 5 BaCl₂, 10 HEPES, pH 7.2, and continuously perfused at the flow rate of 4–5 ml/min. Pipettes filled with 3 × KCl had resistances of 5–5 MΩ. Typically, oocytes were clamped to a series of voltage steps from −140 to +40 mV in 20-mV increments for 450 ms every 2 s, and the whole cell currents were measured at 400 ms. Amiloride-sensitive Na⁺ currents were defined as the absence of Na⁺ currents in the absence and presence of 100 μM amiloride in the bath solution.

The susceptibility of mutant channel with engineered sulfhydryl groups to sulfhydryl reagents was examined with the sulfhydryl reagents (α-methyl) methanethiosulfonate hydrobromide (MTSEA), [(2-trimethylammonium) ethyl] methanethiosulfonate bromide (MTSET), sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES), and [(2-(trimethylammonium) ethyl] methanethiosulfonate bromide (MT-SET), which is a sulfur-mimetic ion. MTSES solution giving a 1 mM final concentration of MTSET, and currents were recorded at 1–3 min following perfusion of the oocytes with a solution containing 2 mM MTSES to the bath solution (Figs. 2 A and 4 A). Within the carboxyl-terminal portion of the pore region of αENaC, only 1 of 8 mutants examined (αS583Cβγ) responded to both MTSET (I/I₀ = 0.03 ± 0.01, n = 4) and MTSET (I/I₀ = 0.31 ± 0.09, n = 4) with a large inhibition of the amiloride-sensitive inward Na⁺ current (Fig. 2 A and Fig. 3, B and C). Several mutants (αS588C, αV590C, and αL591C) responded to MTSEA with an inhibition of the amiloride-sensitive inward Na⁺ current that was significantly greater than WT. These residues are located in close proximity to and on either side of a three-residue tract (αSer⁵⁸⁶αSer⁵⁸⁷αSer⁵⁸⁸), that has a critical role in restricting K⁺ permeation through the channel (11, 12, 14, 15). One mutant (αS588C) that responded to MTSEA with a partial inhibition of whole cell Na⁺ current was located within this three-residue tract. The MTSEA- and MTSET-induced inhibition of whole cell Na⁺ currents remained after these reagents were removed from the bath solution (Fig. 3 C).

In contrast, channels with cysteine substitutions at multiple sites within the amino-terminal portion of the pore region of αENaC responded to MTSEA (i.e. αV572C, αS576C, αS580C, αS581C, and αW582C) or MTSET (i.e. αV572C, αS576C, αN577C, and αS580C) with a significant increase in amiloride-sensitive inward Na⁺ currents (Fig. 2 A). Only one mutant (αS573Cβγ) responded to MTSET with an inhibition of whole cell Na⁺ current (I/I₀ = 0.64 ± 0.03, n = 5). The residues within the amino-terminal portion of the pore region where cysteine substitutions responded to MTSEA reagents with a large change in whole cell Na⁺ current line one face of an α-helix, with the exception of αW582C (Fig. 2 B), suggesting that this region is α-helical in structure.

These increases in whole cell currents occurred rapidly (within a minute) after application of MTSEA and were not reversible after MTSEA or MTSET were removed from the bath solution (Fig. 3 C).

**RESULTS**

**Responses of Mutant mENaCs to External MTS Derivatives—**ENaC pore regions are highly conserved among members of the ENaC/degenerin family (Fig. 1 A). Secondary structure predictions of the pore region suggest that the amino-terminal portion may exist as either α-helix or β-sheet, whereas the carboxy-terminal portion appears to be more irregular in structure. The center portion is predicted to be a turn region (Fig. 1 B). To probe the pore region structure, all residues within the OMENaC pore region (αVal⁶⁶⁹Ser⁶⁷⁶) were systematically mutated to cysteine and coexpressed with WT β- and γENaC subunits in Xenopus oocytes. We previously observed that all mutants with cysteine substitutions within the pore region of αENaC retained channel activity, although low levels of expressed currents (<200 nA) were observed with two mutants (αG587Cβγ and αS589Cβγ) (14).

Wild type αβγENaC responded to 2.5 mM MTSEA with a partial inhibition of whole cell Na⁺ currents (I/I₀ = 0.75 ± 0.05, n = 17; Fig. 2 A). This reduction in current is similar to that reported by other investigators (5, 15). The partial inhibition of wild type ENaC by MTSEA is likely due to covalent modification of Cys⁴⁴⁷ in γENaC that aligns to Ser⁶⁸⁶ in αENaC, as proposed by Snyder et al. (15). Mutation of γCys⁴⁴⁷ to serine largely eliminated the MTSEA-induced partial inhibition of Na⁺ currents, whereas mutation of an adjacent cysteine (γCys⁵⁵₁) had no effect on the partial inhibition of Na⁺ currents by MTSEA (data not show). Amiloride-sensitive whole cell Na⁺ currents in oocytes expressing wild type αβγENaC were not significantly altered following addition of 1 mM MTSET or 5 mM MTSE₃ to the bath solution (Figs. 2 A and 4 A).

We observed distinct effects of MTS reagents on ENaCs with cysteine substitutions within the amino-terminal (αVal⁶⁶⁹αTrp⁶⁷⁰) and carboxy-terminal (αSer⁵⁸⁶αSer⁵⁸⁷αSer⁵⁸⁸) domains of the pore region of the mouse α-subunit (Fig. 2 A). Within the carboxyl-terminal portion of the pore region of αENaC, only 1 of 8 mutants examined (αS583Cβγ) responded to both MTSET (I/I₀ = 0.04 ± 0.01, n = 4) and MTSET (I/I₀ = 0.31 ± 0.09, n = 4) with a large inhibition of the amiloride-sensitive inward Na⁺ current (Fig. 2 A and Fig. 3, B and C). Several mutants (αS588C, αV590C, and αL591C) responded to MTSEA with an inhibition of the amiloride-sensitive inward Na⁺ current that was significantly greater than WT. These residues are located in close proximity to and on either side of a three-residue tract (αGly⁵⁸⁶αSer⁵⁸⁷αSer⁵⁸⁸), that has a critical role in restricting K⁺ permeation through the channel (11, 12, 14, 15). One mutant (αS588C) that responded to MTSEA with a partial inhibition of whole cell Na⁺ current was located within this three-residue tract. The MTSEA- and MTSET-induced inhibition of whole cell Na⁺ currents remained after these reagents were removed from the bath solution (Fig. 3 C).
sulfhydryl reagents are marked with *. Residue Gly 579 is not marked.

Data presented as mean ± S.E. from 4 to 8 oocytes except for wild type response to MTSEA (17 oocytes). Filled bars indicate statistical significance (p < 0.05, mutant versus WT). ND indicates not determined due to low level of expressed currents with αS580Cβγ and αS580Cβγ. B, helical wheel analysis of mENaC pore residues Thr570–Ser583. Residues with substitution in mENaC pore residues Thr570–Ser583. Residues demonstrated that the introduction of residues with large side chains at the site analogous to αSer536 (i.e. γG536C) was modified by MTSET but not by MTSES.

Responses of Mutant mENaCs to External Cd²⁺—Group IIB divalent cations such as Cd²⁺ and Zn²⁺ are able to bind free sulphydryls with high affinity and therefore have been used as biophysical probes to study the pore structure of ion channels (19–22). Cd²⁺ was used in this study as its crystal radius (0.92 Å) (21) is nearly same as that of Na⁺ (0.95 Å) (23). We examined whether extracellular Cd²⁺ (5 mM) altered whole cell Na⁺ currents in oocytes expressing either WT or mutant mENaCs. A modest increase in amiloride-sensitive whole cell Na⁺ current was observed in response to Cd²⁺ in oocytes expressing WT αpymENaC. A similar response to extracellular Cd²⁺ was observed with 16 of the 22 mENaC mutants examined. Several mutations within the αmENaC pore region responded to Cd²⁺ with a significant increase (αN577C, αS580C, and αW582C) or a modest decrease (αG579C and αL584C) in whole cell Na⁺ current. Similar to MTSET, Cd²⁺ abolished whole cell amiloride-sensitive Na⁺ currents in oocytes expressing αS583Cβγ (I/I₀ = 0.03 ± 0.01, n = 4, Fig. 2A). The blocking effect of Cd²⁺ on αS583Cβγ was both fast and voltage-dependent, as evidenced by the minimal block of outward currents, compared with the large inhibition of inward currents (Fig. 3B, right panel). This is consistent with the observation of voltage-dependent block of rat αS583Cβγ ENaC by external Zn²⁺ (10).

Role of Pore Region in ENaC Gating—The introduction of cysteine residues at αVal572, αSer576, αAsp577, αSer580, αGly581, and αTrp586 led to channels that responded to MTSEA, MTSET, MTSES, or Cd²⁺ with an increase in whole cell Na⁺ current. These increases in whole cell currents occurred rapidly (within a minute) after external application of the MTS reagent or Cd²⁺ (Fig. 3C), suggesting that changes in single channel Na⁺ conductance or open probability occurred previously. Previous studies demonstrated that the introduction of residues with large side chains at the site analogous to αSer576 of mENaC led to a significant increase of currents in oocytes expressing ASIC2 (or BNC1), an ENaC-related H⁺-gated ion channel (24). Similar mutations of deg-1 or mec-4 (mechanosensitive proteins in Caenorhabditis elegans) led to neuronal degeneration (25, 26). We examined whether the introduction of a residue with a large side chain at or in proximity to αSer576 increased whole cell Na⁺ currents. Whole cell Na⁺ currents in oocytes expressing either αV572Fβγ or αS576Cβγ were significantly greater than that observed in oocytes expressing WT ENaC, although Na⁺ currents measured in oocytes expressing αV572Fβγ or αS576Fβγ were similar in magnitude to WT. In contrast, whole
Fig. 3. Representative two-electrode voltage clamp recordings showing the responses of mutant mENaCs to external sulfhydryl reagents. A, effect of external 1 mM MTSET on αS580Cβγ is shown. Whole cell Na⁺ current traces were obtained before (MTSET⁻) and after (MTSET⁺) application of the reagent, after washout of the reagent, and after perfusion with 0.1 mM amiloride. Current-voltage curves (IV) were obtained by plotting amiloride-sensitive Na⁺ currents against clamp voltages in the range of −100 to 60 mV. Filled circles represent basal currents, and open circles indicate the currents recorded after MTSET treatment. B, responses of αS583Cβγ to MTSEA (left) and MTSET (center) are displayed as IV curves before (filled circles) and after (open circles) external application of the reagents. Currents are amiloride-sensitive Na⁺ currents. The right panel shows representative voltage ramp recordings from an oocyte expressing αS583Cβγ. The oocyte was clamped at 6-s intervals with a linear ramp from −100 mV to 60 mV over 1 s. Top traces are the currents recorded before (0 s) and after (6 and 12 s) perfusion of a bath solution containing 5 mM Cd²⁺. Current trace in the presence of amiloride is close to zero current level. The zero current level is indicated by a dashed line. C, time courses of MTSET-induced changes in amiloride-sensitive Na⁺ currents from oocytes expressing WT mENaC (○), αS576Cβγ (△), αS580Cβγ (■), or αS583Cβγ (×). Amiloride-sensitive Na⁺ currents were measured at 5-s intervals at −100 mV and normalized to the current level immediately prior to delivery of a reagent (at time = 1 min). Normalized currents (I/I₀) are shown. Solid bar indicates the period (1–4 min) when the oocyte was bathed with 1 mM MTSET. MTSET was washed out over a 3-min period (4–7 min) with bath solution. Dashed bar indicates the period (7–8 min) when 100 μM amiloride was present in the bath solution.

Cell currents measured in oocytes expressing αS580Fβγ or αS580Cβγ were significantly less than that observed in oocytes expressing WT ENaC (Fig. 5). We performed single channel analyses of αS580Cβγ before and after treatment with MTSET, to test whether the modification of the introduced cysteine residue altered channel gating (Fig. 6). The single channel slope conductance for Na⁺ of αS580Cβγ was 3.7 ± 0.3 pS (n = 11), slightly less than the conductance of wild type mENaC (4.3 pS) (14). This is consistent with our previous observation that the single channel slope conductance for Li⁺ of αS580Cβγ was nearly identical to WT ENaC and that the Li⁺/Na⁺ current ratio for αS580Cβγ was 1.36-fold greater than that of WT (14). The open probability of αS580Cβγ was 0.07 ± 0.02 (n = 5), determined at potentials between −100 and −60 mV. The reduced single channel Na⁺ conductance and open probability were consistent with the reduced whole cell Na⁺ currents observed in oocytes expressing αS580Cβγ, when compared with oocytes expressing WT ENaC (Fig. 5). Following treatment with MTSET, αS580Cβγ exhibited a dramatic change in gating characteristics. When patches were made shortly (within minutes) following MTSET treatment, channels were primarily open but exhibited frequent transitions to the closed state (Fig. 6B). However, when patches were made minutes later following MTSET treatment, channels remained open, and very few brief closures were observed (Fig. 6, C and D). The open probability was not determined due to too few transitions between open and closed states despite long (>10 min) recordings; however, open probability was clearly >0.9 (Fig. 6, C and D). The single channel conductance of MTSET-modified αS580Cβγ was 2.3 ± 0.1 pS (n = 4), lower than that of unmodified channels (equaling a 38% decrease). In many recordings we only observed noise, comparable to open channel noise, with no clear transitions suggesting that the channel remained open over a recording period of 5–10 min (data not shown). These data indicate that MTSET converted αS580Cβγ to a lower conductance channel, but one that was nearly continuously open. These MTSET-induced changes in conductance and open probability are the likely mechanisms of the MTSET-induced increase in whole cell Na⁺ current observed with this mutant channel (Figs. 2A and 3A).

Similar single channel analyses were performed with Li⁺ as the conducting ion in the pipette, as well as in the bath solution. We observed that MTSET treatment of oocytes expressing αS580Cβγ reduced the unitary Li⁺ current from 0.7 to 0.3 pA.
at –80 mV and locked the channel in an open state as only brief closures were observed (Fig. 6F). The Li⁺ current reduction in response to MTSET was larger than Na⁺ current reduction following MTSET treatment. These data obtained from single channel analyses with Li⁺ as the conducting ion were consistent with the effect of MTSET on amiloride-sensitive whole cell Li⁺ currents measured in oocytes expressing αS580Cβγ. Unlike the effects of MTSET on whole cell αS580Cβγ Na⁺ currents, Li⁺ currents were not significantly increased by MTSET (I/Li = 0.93 ± 0.06, n = 4). It is likely that the MTSET-induced reduction in Li⁺ unitary current balances the MTSET-induced increase in αS580Cβγ open probability.

**DISCUSSION**

The substituted cysteine accessibility method has been used to probe pore structure of various ion channels (27). In this study, we used this approach to examine the pore structure of αENaC. Several distinct effects of MTS reagents and Cd²⁺ on αβγ mENaCs were observed with cysteine substitutions within the pore region of the α-subunit. MTSEA, MTSET, and Cd²⁺ inhibited whole cell Na⁺ currents in oocytes expressing αS580Cβγ, as previously reported (4, 10). Surprisingly, this is the only mutant that displayed significant block by these three reagents. The lack of an inhibitory effect of MTS reagents on engineered cysteines near αSer⁵⁸³ indicates that this residue is located within a restricted site. Interestingly, another MTS reagent (MTSES) with a negative charge did not inhibit αS583Cβγ. Furthermore, MTSES pretreatment of oocytes expressing this mutant channel failed to prevent the subsequent inhibition by MTSEA (Fig. 4D), indicating that MTSES did not efficiently modify αS853Cβγ. Schall et al. (10) proposed that αSer⁵⁸³ is located in the electrical field of the ENaC pore, as Zn²⁺-induced inhibition of αS583Cβγ was voltage-dependent. We also observed that Cd²⁺-induced block of αS853Cβγ mENaC was voltage-dependent (Fig. 3B). These results suggest that a negative potential within the vicinity of αSer⁵⁸³ hinders the access of MTSES. This notion is reminiscent of the proposal that the pore helix of KcsA K⁺ channels generates a negative potential at its carboxyl terminus that contributes to

**FIG. 4.** Effects of external MTSES (5 mM) on wild-type and mutant mENaCs. A, external application of the negatively charged MTSES increased Na⁺ currents of αS576Cβγ and αS580Cβγ mENaCs without affecting the currents of wild type and αS583Cβγ mENaCs. Bars represent amiloride-sensitive Na⁺ currents (mean ± S.E., from 4 to 8 oocytes) in the presence of 5 mM MTSES normalized to the current level in the absence of MTSES (I/0). Solid bars indicates values are significantly different from wild type (p < 0.01). B, pretreatment with 5 mM MTSES significantly reduced MTSEA-induced increases in whole cell Na⁺ currents in oocytes expressing αS576Cβγ or αS580Cβγ, and the MTSEA induced current inhibition of αS853Cβγ. Open bars indicate ratios of currents after and before application of 2.5 mM MTSEA (I/0) measured in oocytes pretreated with 5 mM MTSES. * indicates a statistically significant difference (p < 0.001, mutant versus WT). # indicates a statistically significant difference (p < 0.05, MTSES-pretreated oocyte versus none-pretreated oocyte).

**FIG. 5.** Expressed amiloride-sensitive Na⁺ currents (mean ± S.E., n = 6–11 oocytes) of wild type and selected mENaC mutants were obtained from paired measurements. Filled bars indicate statistically significant differences in whole cell amiloride-sensitive Na⁺ currents (p < 0.05, mutant versus WT). Each oocyte was injected with 1 ng of cRNA for each subunit and clamped in an alternate order 20–30 h after injection.

**FIG. 6.** External MTSET markedly increased the open probability of αS850Cβγ mENaC. Single channel recordings were performed in the cell-attached configuration at −80 or −100 mV (negative value of pipette potential) with 110 mM NaCl solution (A–D) or 110 mM LiCl solution (E and F) in both the pipette and bath. Scales are displayed next to the recordings on the right and closed states are marked with short bars. Traces in A and E are single channel recording traces of the mutant channel prior to MTSET treatment. The channel is characterized with short open time and long close time that result in a low open probability. Trace in B shows a representative recording obtained shortly (within minutes) after MTSET treatment. It displays increased open probability due to increased open time and reduced close time as well as decreased single channel conductance. The estimated Na⁺ conductance was 2.3 ± 0.1 pS (n = 4) from MTSET-treated cells and 3.7 ± 0.3 pS (n = 11) from untreated cells. Recordings in C and D were obtained more than 5 min after MTSET treatment. The channel stays open most of the time with very short close states. F shows a Li⁺ current trace following MTSET treatment. The channel was primarily open but exhibited a few transitions to the closed state that can be observed when the trace is displayed in expanded time scale (low trace). The unitary Li⁺ current was decreased (0.3 pA) when compared with untreated channels (0.7 pA) at −80 mV.
the stabilization of cations in the pore cavity (28).

At sites amino-terminal to αSer583, only αSS573Cβγ and αG579Cβγ responded to MTSET or Cd\(^{2+}\) with a partial inhibition of the whole cell Na\(^{+}\) current. Several mutant channels with cysteine mutations within the amino-terminal portion of the pore region (i.e. amino-terminal to αSer583) responded to sulfhydryl reagents with significant increases in whole cell current. These residues (αVal572, αSer576, αAsn577, αSer580, αGln581, and αTrp582) line one face of an α-helix, with the exception of αW582C (Fig. 2B), consistent with an α-helical structure as suggested by secondary structure predictions (Fig. 1B). These stimulatory effects do not appear to rely on the positive charges carried by these reagents, as the negatively charged MTSES also stimulated αSS576Cβγ and αSS80Cβγ (Fig. 4A). Snyder et al. (15) also observed MTSET-induced activation of Na\(^{+}\) currents with cysteine mutations in this region of human γENaC, although only three mutants responded with a gain of function in response to MTSET, and the location of these residues was not suggestive of an α-helical structure. These differences in responses of α- and γ-subunit mutants to MTS reagents may reflect, in part, the presence of two α-subunits and only one γ-subunit within each channel protein. Another possibility is that the three ENaC subunits may not be arranged symmetrically along pore axis. This latter notion is supported by the observations that mutations at homologous sites in different subunits led to different changes in channel selectivity, amiloride sensitivity, and divergent cation sensitivity (10, 12, 15). Although KcsA core pore is formed by four identical subunits in a symmetrical manner (16), studies on voltage-gated Na\(^{+}\) channels suggested that the four-pore segments from Domains I–IV are arranged asymmetrically (19, 20).

ENaCs with cysteine substitutions at sites carboxy-terminal to αSer583 either did not respond to MTS reagents and Cd\(^{2+}\) or, alternatively, were partially inhibited (i.e. αL584Cβγ, αS588Cβγ, αV589Cβγ, and αL591Cβγ). MTSET did not inhibit channels with introduced cysteines carboxy-terminal to αSer583, a region encompassing the proposed selectivity filter. Given several reports indicating αGly587 and αSer589 have an important role in conferring cation selectivity and restricting K\(^{+}\) permeation (11, 12, 14), residues αSer587–αSer589 and adjacent residues are likely facing the conducting pore. A simple explanation for a lack of an inhibitory effect of MTSET on channels with cysteine substitutions in this region is that these sites are not accessible to the reagent.

**Structure of the ENaC Pore Region**—The effects of MTS reagents and Cd\(^{2+}\) were most prominent when cysteine residues were placed at sites amino-terminal to αSer583. Only modest changes in whole cell currents were observed in response to MTSEA or Cd\(^{2+}\) when cysteine residues were placed carboxy-terminal to αSer583. As these accessible residues (defined by a large response to MTS reagents or Cd\(^{2+}\)) preceded αLeu584, our results support a model of the pore region of the channel that has been proposed by both Kellenberger et al. (11) and Snyder et al. (15). These groups suggested that pore is formed by residues that enter the membrane spanning region from an extracellular site and transitions to an α-helical second membrane spanning domain. As the pore enters the membrane, it gradually narrows to form a site that restricts K\(^{+}\), analogous to a funnel that narrows to its spout. This model is based, in part, on the observation that the substitution of cysteine residues in the β- or γ-subunits at a position analogous to αSer583 (βG525C and γG542C) resulted in a large increase in the K\(^{+}\) values of amiloride. Schuld and co-workers (10, 11) proposed that amiloride interacts directly with these residues and that αSer583 must be external to αSer589. A model of the outer vestibule of the pore, incorporating an α-helical structure that transitions to a narrow selectivity filter, is illustrated in Fig. 7B. This model is consistent with that proposed by Palmer (29) 10 years ago.

Schild et al. (10) previously demonstrated that ENaCs with acidic residues at position αSer580 (or at the analogous positions βGly525 and γGly534) were inhibited by extracellular Ca\(^{2+}\) in a voltage-dependent manner, suggesting that these residues (i.e. amino-terminal to αSer583, βGly525, and γGly534, respectively) were accessible to extracellular Ca\(^{2+}\). These data support the proposed pore structure of Kellenberger et al. (11) and Snyder et al. (15), whereby the interaction of Ca\(^{2+}\) with αSS80D would block the pore of the channel (see Fig. 7B). However, we observed that oocytes expressing αSS80Cβγ mENaC responded to MTSEA, MTSET, and Cd\(^{2+}\) with a large increase in amiloride-sensitive currents. If the αSS80C side chain extends into the pore lumen, its modification by MTS reagents or Cd\(^{2+}\) would be expected to inhibit the channel and not activate it. Furthermore, channels with substituted cysteine residues at selected sites near αSer580 (i.e. αVal572, αSer576, αAsn577, αGln581, or αTrp582) also responded to MTSEA, MTSET, or Cd\(^{2+}\) with an activation of amiloride-sensitive currents. If this proposed pore structure is correct, it is reasonable to predict that channels with cysteine residues substituted at multiple sites amino-terminal to the selectivity filter would respond to MTSEA, MTSET, or Cd\(^{2+}\) with a large inhibition of whole cell currents. However, of the mutants we have examined, only αSS83Cβγ responded to these reagents with a large inhibition of Na\(^{+}\) current. Our results suggest that periodic residues within the amino-terminal portion of the mENaC pore region are accessible to sulfhydryl reagents externally applied but do not directly face the conducting pore.

The outer pore of the KcsA K\(^{+}\) channel is formed by an α-helix that enters the membrane followed by an extended region directed toward the extracellular space (16) (Fig. 7A). Is it necessary to propose a pore structure for ENaC that basically differs from other highly selective cation channels? Previous studies have suggested that the pore regions of the voltage-gated Na\(^{+}\) and Ca\(^{2+}\) channels are similar in structure to voltage-gated K\(^{+}\) channels, although they clearly differ in mechanisms for achieving cation selectivity (30). Our data suggest that pore region residues amino-terminal to αGln581 form an α-helix, and our previous results (14) suggested that residues extending from αSer580–αSer589 form an extended selectivity filter. This secondary structure is similar to that of the KcsA K\(^{+}\) channel. Furthermore, within the pore regions of K\(^{+}\) channels (Shaker and inward rectifier Kir2.1) and omENaC, the pattern of accessibility to substituted cysteine residues to cysteine-reactive reagents is strikingly similar (Fig. 7C) (31–34).

Our previous observation that mutation of the GSS tract within the selectivity filter of the α-subunit of mENaC (αGly587–αSer589) to the K\(^{+}\) channel selectivity filter signature sequence GYG rendered the mutant channel K\(^{+}\)-selective (14) is also consistent with the notion that K\(^{+}\) channels and ENaC may have similar pore structures.

Fig. 7A illustrates a model of the ENaC α-subunit pore region using the structure of the KcsA K\(^{+}\) channel, aligning the GYG tract within KcsA with the GSS tract within αENaC. Residues αVal572–αSer580 form an α-helix; αLeu584–αSer589 form an extended selectivity filter; and αGln581–αSer583 are located at the turn region where the α-helix transitions into the selectivity filter. Residues where substituted cysteines responded to MTS reagents or Cd\(^{2+}\) line one face of the helix, with the exception of αTrp582 (Fig. 2B). Our model places αSer589 at a location external to the GSS track, consistent with the previous observation that the mutant αSS92Iβγ rat ENaC
was blocked by external Ca\(^{2+}\) in a voltage-dependent manner (13). This model (Fig. 7A) is not consistent with amiloride interacting directly with \(b\)G525C or \(g\)G542C, residues proposed to interact directly with amiloride (10). These residues would be within the internal portion of the selectivity filter. However, these mutations (i.e. \(b\)G525C or \(g\)G542C) might change the structure of the pore and indirectly alter the \(K_i\) values of amiloride, as suggested by Schild et al. (10).

The introduction of cysteine residues carboxy-terminal to the GYG tract of \(K_1\) channels conferred sensitivity to MTS reagents (Fig. 7, A and C). If \(ENaC\) and \(K_1\) channels share a similar pore structure, the introduction of cysteine residues carboxy-terminal to \(\alpha\)Ser599 would be expected to confer sensitivity to MTS reagents. However, we only observed a modest block of Na\(^{+}\) currents in response to MTSEA when cysteine residues were present at \(\alpha\)Val590 or \(\alpha\)Leu591, and no changes in whole cell Na\(^{+}\) currents were observed in response to MTSET or Cd\(^{2+}\). These results suggest that \(K_1\) channels and \(ENaC\) differ in their pore structure, although the nature of these differences has yet to be defined. Based on our data and previous studies, we propose that the amino-terminal portion of the \(\alpha\)ENaC pore region forms an \(\alpha\)-helix, and the carboxy-terminal

![Fig. 7. Structural models of the \(\alpha\)ENaC pore region. A, structure of KcsA pore region (right) and a model for \(\alpha\)mENaC pore region (left) are presented in stick model with ribbon rendering (9 shin green lines) using the modeling software HyperChem 5.1 (Hypercube Inc., Gainesville, FL). For KcsA K\(^{+}\) channel, structure of residues P41-Y60 was generated from coordinates obtained from the Protein Data Bank (code: 1BL8) and the numbering of the residues is shown in C of this figure. The structural model for ENaC pore region was generated by mutating KcsA pore region residues to Val\(^{590}\)–Ser\(^{592}\) of \(\alpha\)mENaC according to the alignments shown in C. Residue \(\alpha\)Phe\(^{566}\) was omitted and energy minimization was not performed. Element colors are as followings: cyan for carbon, blue for nitrogen, and red for oxygen. Residues corresponding to Val\(^{572}\), Ser\(^{573}\), Ser\(^{576}\), Asp\(^{577}\), Gln\(^{581}\), Trp\(^{582}\), and Ser\(^{583}\) of \(\alpha\)mENaC are displayed in violet color. Substituted cysteines at these sites were accessible to sulfhydryl reagents from extracellular side judged by significant changes in amiloride-sensitive Na\(^{+}\) currents in response to these reagents. KcsA residues homologous to the residues in voltage-gated and inward rectifier \(K_1\) channels that are accessible to external sulfhydryl reagents are also shown in \textit{violet color}. B, an alternative model for the \(\alpha\)mENaC pore region was generated by rotating residues Ser\(^{583}\)–Ser\(^{592}\) (corresponding to Ser\(^{583}\)–Ser\(^{592}\) of \(\alpha\)mENaC) from the model A (left panel) by 180° along the X axis. Externally accessible residues in \(\alpha\)mENaC are also highlighted in \textit{violet color} as in A. This model includes all 24 residues (Val\(^{569}\)–Ser\(^{592}\)) of \(\alpha\)mENaC pore region. The key residues retaining \(K_1/Na_1\) selectivity (Gly\(^{587}\) and Ser\(^{589}\), corresponding to Gly\(^{587}\) and Ser\(^{590}\) of \(\alpha\)mENaC) are located in the narrowest region of the pore. C, sequence alignments and comparison of accessibility to external sulfhydryl reagents between \(K_1\) channels and ENaC. The alignments of the pore region residues of KcsA, Shaker \(K_1\) channel, inward rectifier \(K_1\) channel Kir 2.1 and \(\alpha\)mENaC were performed by aligning the Gly\(^{587}\)–Ser\(^{588}\)–Ser\(^{589}\) of \(\alpha\)mENaC to the \(K_1\) channel GYG track. A gap was introduced in \(K_1\) channels to enhance overall alignments. The plus sign (+) indicates sites where cysteine substitutions within the pore region result in channels that were inhibited (or activated) by MTS reagents, Ag\(^{+}\), or Cd\(^{2+}\), and the minus sign (–) shows residues not accessible to the reagents. The plus/minus sign (±) indicates sites where cysteine substitutions result in channels that were modestly inhibited by MTSEA or Cd\(^{2+}\). The X indicates the sites not tested for sulfhydryl reagent accessibility.
portion forms a selectivity filter, like that of K⁺ channels. Furthermore, we propose that the transition from selectivity filter to α-helical second membrane spanning domain within ENaC has a structure that differs from K⁺ channels.

**Role of Pore Region in ENaC Gating**—Our observation that mutant mENaCs with cysteine substitutions within the amino-terminal portion of the pore region (preceding αSer⁵⁸⁶) responded to MTSEA, MTSET, or Cd²⁺ with an increase in whole cell Na⁺ current led us to examine whether this region has a role in ENaC gating. In agreement with our observation that MTS reagents activated whole cell Na⁺ currents in oocytes expressing αSS80Cβγ, MTSET induced a large increase in open probability of αSS80Cβγ, indicating that this residue is within a domain that controls ENaC gating. Our data suggest αSer⁵⁷⁶ and αSer⁵⁸⁰ are two crucial residues in this gating domain, as all three MTS reagents dramatically stimulated whole cell Na⁺ currents in oocytes expressing these mutant channels. This proposed role of αSer⁵⁷⁶ in channel gating is in agreement with previous observations suggesting a similar role of the residue at the analogous position in degenerins and in H⁻-gated Na⁺ channels. The introduction of bulky residues at a site analogous to αS576C within the *C. elegans* proteins deg-1 and mec-4 lead to neurodegeneration that is proposed to occur as a result of an unregulated activation of a putative mechanosensitive cation channel (35, 36). Similarly, selected mutations at an analogous site within this pore region of related H⁻-gated Na⁺ channels, termed BNC (or ASIC2), resulted in sustained channel activation that was independent of acidification (24). Based on these observations, we propose that the ENaC pore region participates in channel gating.

Aside from increasing open probability, external application of MTSET reduced both Na⁺ and Li⁺ unitary currents of αSS80Cβγ. The observed changes in whole cell amiloride-sensitive Na⁺ currents of αSS80Cβγ in response to MTSET reflects opposing effects on open probability (increased) and unitary current (decreased). The mechanism of MTSET-induced reduction in unitary current is unknown. This may reflect changes in the conformation of the open channel due to covalent binding of MTSET to αS880C; alternatively, the positively charged MTSET may partially block the pore.

Waldmann et al. (13) reported that αSS89I and αSS89F (analogous to αSer⁵⁸⁶ of mENaC) reduced rat ENaC open probability from 0.89 to 0.09 and 0.04, respectively, and increased Na⁺ unitary conductance from 4.6 to 10 pS without changing Li⁺ conductance. This study provided evidence that αSer⁵⁸⁸ located within the selectivity filter of ENaC, has a role in ENaC gating. We have also observed that αSS88Cβγ mENaC has very low open probability resulting from short open and long close times (data not shown). Fyfe et al. (37) studied the functional properties of ENaCs with chimeric γ-β subunits. Their results also suggested that this region regulates ENaC gating. These observations that mutations within both amino-terminal and carboxyl-terminal portions of the pore region resulted in changes in channel gating suggest a collaborative role of these two regions in ENaC gating through intradomain interactions. We propose a working model for ENaC gating. In this model, the pore helix (amino-terminal portion of pore region) undergoes rotational movement. This movement could be in response to conformational changes at other sites within ENaC, such as the ectodomain, the M2 domains, or cytoplasmic domains (i.e. the amino terminus (38)). The rotation of the pore helix leads to changes in the diameter of the selectivity filter, which in turn allows ion translocation through the pore. A recent study of KcsA gating observed movement of reporter cysteines introduced at the carboxyl-terminal end of the pore helix in association with the channel gating (45). If the ENaC pore structure shares the fundamental design of the KcsA K⁺ channel pore, αSer⁵⁷⁶ and αSer⁵⁸⁰ are expected to be close to the carboxy-terminal end of the pore helix of ENaC. Substitution of large side chains at these two sites might hinder rotational movement of the amino-terminal and carboxyl-terminal portions of the pore region, and we propose that this occurs, in part, through hydrogen bonding. ENaC pore regions have a large number of serine residues (7 in omENaC, 2 in βmENaC, and 4 in ymENaC). Given the proposed subunit stoichiometry of 2α, 1β, and 1γ, a total of 20 serine residues are present within the pore regions of the channel. These serine residues, as well as other polar residues and backbone carbonyl oxygens, are capable of forming a network of hydrogen bonds. A sliding model of gating is also plausible. A relative sliding movement between αSer⁵⁷⁶_Ser⁵⁸⁰ and αSer⁵⁸⁸_Ser⁵⁸⁹ could lead to channel transitions between open and closed states.

Our model predicts a connection between ENaC gating and permeation and favors a dynamic selectivity filter. Gating and permeation have been generally considered as independent processes of ionic channels since 1952 (39). However, recent studies have challenged this concept (40, 41). The notion that pore regions function as ion channel gates has been proposed for cyclic nucleotide-gated channel (42). Recent studies support a dynamic selectivity filter challenging another long term notion of a rigid selectivity filter (43).

Alternatively, if the ENaC pore is similar to that proposed by Kellenberger et al. (11) and Snyder et al. (15) (Fig. 7B), gating might also involve rotational movement of the α-helical region preceding the selectivity filter. This gating mechanism was proposed by Adams et al. (24) in their studies of activation of the H⁻-gated Na⁺ channel BNC (or ASIC2).

We observed that several αENaC mutants in the pore helix, including αV572Fβγ and αSS76Cβγ, expressed whole cell currents in oocytes that were significantly greater than that observed with WT ENaC, consistent with the notion that mutations within this region affect channel gating. We anticipate that selected mutations within this domain of human ENaC are likely to be found in the clinical setting of salt-sensitive hypertension due to enhanced ENaC-mediated Na⁺ transport in the distal nephron. In this context, Melander et al. (44) reported a mutation within human ENaC (γN530K), a position analogous to mouse αAsn⁵⁷⁷, in a patient with hypertension and diabetes, although a causal relationship was not established. In summary, our data suggest that the amino-terminal pore region of αENaC is α-helical in structure and that this region is involved in channel gating.

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Epithelial Sodium Channel Pore Region: STRUCTURE AND ROLE IN GATING
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