On the Interaction between Amiloride and Its Putative α-Subunit Epithelial Na\(^+\) Channel Binding Site*

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The epithelial Na\(^+\) channel (ENaC) belongs to the structurally conserved ENaC/Degenerin superfamily. These channels are blocked by amiloride and its analogues. Several amino acid residues have been implicated in amiloride binding. Primary among these are \(\alpha\)Ser-583, \(\beta\)Gly-525, and \(\gamma\)Gly-542, which are present at a homologous site within the three subunits of ENaC. Mutations of the \(\beta\) and \(\gamma\) glycines greatly weakened amiloride block, but, surprisingly, mutation of the serine of the \(\alpha\) subunit resulted in moderate (<5-fold) weakening of amiloride \(K_i\). We investigated the role of \(\alpha\)Ser-583 in amiloride binding by systematically mutating \(\alpha\)Ser-583 and analyzing the mutant channels with two-electrode voltage clamp. We observed that most mutations had moderate effects on amiloride block, whereas those introducing rings showed dramatic effects on amiloride block. In addition, mutations introducing a \(\beta\)-methyl group at this site altered the electric field of ENaC, affecting both amiloride binding and the voltage dependence of channel gating. We also found that the His mutation, in addition to greatly weakening amiloride binding, appends a voltage-sensitive gate within the pore of ENaC at low pH. Because diverse residues at \(\alpha\)Ser-583, such as Asn, Gln, Ser, Gly, Thr, and Ala, have similar amiloride binding affinities, our results suggest that the wild type Ser side chain is not important for amiloride binding. However, given that some \(\alpha\)Ser-583 mutations affect the electrical properties of the channel whereas those introducing rings greatly weaken amiloride block, we conclude that amiloride binds at or near this site and that \(\alpha\)Ser-583 may have a role in ion permeation through ENaC.

The epithelial sodium channel (ENaC)\(^1\) is the primary target of the potassium-sparing diuretic, amiloride. ENaC is expressed in the apical membrane of sodium-absorptive epithelia such as the distal nephron, lung airway and alveoli, and descending colon (1). As such, ENaC plays a critical role in maintaining Na\(^+\) homeostasis, controlling blood pressure and airway fluid volume. ENaC is a member of the ENaC/Degenerin superfamily, all of which conduct Na\(^+\), are inhibited by amiloride, and have some structural features in common. They each have two hydrophobic membrane-spanning domains (M1 and M2) with intracellular N and C termini and a large extracellular loop containing two or three cysteine-rich domains (2). To date, \(\alpha\), \(\beta\), \(\gamma\), and \(\delta\) ENaC subunits, which share 30–40% sequence identity, have been identified in mammals. ENaC is largely found in the kidney, lung, and colon as comprised of \(\alpha\), \(\beta\), and \(\gamma\) subunits, although the subunit stoichiometry of functional channels remains controversial. Several groups have proposed a tetrameric \(\alpha\)\(\beta\)\(\gamma\) quaternary structure, although a nonameric \(\alpha\)\(\beta\)\(\gamma\)\(\delta\) arrangement has also been proposed (3–6). The \(\delta\) subunit may substitute for \(\alpha\) in some tissues.

Understanding the mode of amiloride binding and the mechanism by which it inhibits ENaC has been the objective of numerous studies (2). Apparent amiloride binding affinity was found to be attenuated by mutations at several specific sites. Mutations at \(\alpha\)Ser-583, \(\beta\)Gly-525, and \(\gamma\)Gly-542 (\(\gamma\)Gly-537 using rat ENaC numbering), which are located in a region preceding the second membrane-spanning domain, altered amiloride \(K_i\) (7–9). Other sites were identified that localized to the extracellular loop of the \(\alpha\) subunit (10–13). Two general mechanisms, ionic block and allostery, have been proposed for the inhibition of ENaC currents by amiloride (14). Support of the ionic block model was grounded on the findings that amiloride block was voltage sensitive and competitive with Na\(^+\) in certain species and that amiloride may bind to the extracellular mouth of the pore (7–9, 15, 16). Support of the allostery model derived from evidence that amiloride may interact with non-pore-associated regions within ENaC, is non-competitive with Na\(^+\) in certain species, and some amiloride analogues stimulate Na\(^+\) current (10–13, 17–19).

In this study, we sought to understand the role, if any, of the \(\alpha\)Ser-583 side chain in binding amiloride. Previously, mutations at \(\alpha\)Ser-583 have been found to cause only modest changes in apparent amiloride binding affinity, in stark contrast to all mutations made at the analogous positions in the \(\beta\) and \(\gamma\) subunits (\(\beta\)Gly-525 and \(\gamma\)Gly-542) where any mutation of the Gly residue greatly weakened amiloride block (8), suggesting that the backbone assumes \(\phi\) and \(\psi\) angles uniquely accessible to Gly at this position. In the only resolved structure of a protein with bound amiloride, that of urokinase-type plasminogen activator, several Ser and Gly residues were involved in the coordination of amiloride through either the backbone carbonyl or the Ser hydroxyl (20). Here, we have generated a series of point mutations at \(\alpha\)Ser-583 to explore the role of this residue in amiloride block. We found that residues containing rings have large effects on amiloride block, whereas substitutions that do not introduce rings have moderate or no effect on amiloride block. Interestingly, the presence of a side chain capable of forming H-bonds is not required for high affinity.
block. Furthermore, certain mutations altered the electric field of the conducting pore, a change that altered the gating behavior of ENaC. In addition, one of the mutations at this site generated a simple voltage-sensitive gate. These findings support a pore block model for amiloride inhibition of ENaC and give insight to the orientation of αSer-583 in the channel pore.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—All ENaC subunits cloned in this study are mouse ENaC subunits whose cDNAs were inserted into pbLuescript SK (Stratagene, La Jolla, CA). (21). Point mutations were generated by using a method as previously described (22).

**ENaC Expression**—Stage V and VI oocytes free of follicle cell layers were injected with 1–4 ng of cRNA for each mouse ENaC subunit/oocyte and incubated at 18 °C for 24–72 h in modified Barth’s saline (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 15 mM HEPES, 0.3 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 10 μg/ml sodium penicillin, 10 μg/ml streptomycin sulfate, 100 μg/ml gentamicin sulfate, pH 7.4). Where indicated, cRNAs encoding β and γ subunits truncated at Arg-564 and Arg-583, respectively, were injected instead of cRNAs encoding wild type β and γ subunits to increase functional expression (23).

**Two-electrode Voltage Clamp**—Two-electrode voltage clamp was performed using a DigiData 1320A interface and a GeneClamp 500B voltage clamp amplifier (Axon Instruments, Foster City, CA). Data acquisition and analysis were performed using pClamp 8.2 (Axon Instruments) on a 1.5-GHz Pentium 4 PC (Gateway 2000 Inc., North Sioux City, S.D.). Pipettes were pulled from borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL) with a Micropipette Puller (Sutter Instrument Co., Novato, CA) and had resistances of 0.5–5 megaohms when filled with 3M KCl and inserted into the bath solution. Oocytes were maintained in a recording chamber (Automatic Scientific, San Francisco, CA) with 20 μl of bath solution and continuously perfused with bath solution at a flow rate of 4–5 μl/min. The bath solution contained 110 mM NaCl, 2 mM KCl, 2 mM CaCl2, 10 mM HEPES, pH 7.4. All experiments were performed at ambient temperatures (20–24 °C).

For experiments measuring apparent drug affinity, inhibitors were diluted from 0.5 mM stock solutions in Me2SO into the bath solution delivered to the oocyte by perfusion. Prior to measurement at each indicated inhibitor concentration, oocytes were perfused for 10 s at that concentration. Amiloride Ki was determined by nonlinear curve fitting (Igor Pro 4.0.9.1, Osage, OR) using Equation 1.

\[
\frac{I}{I_N} = \frac{K_i}{K_i + [Inh]} + C
\]

where I is the Na+ current, IN is the Na+ current measured in the absence of inhibitor, [Inh] is the inhibitor concentration, and C is the inhibitor-insensitive component of the current.

Concentration-dependencies were determined by clamping oocytes in a series of voltage steps (500 ms) from −140 to 60 mV in 20-mV increments, whereas whole cell currents were measured 400 ms after initiation of each voltage step. Oocytes were clamped at 0 mV for 50 ms between voltage steps. A tail protocol was also employed. Na+ currents were measured from oocytes clamped at VTR for 2 s, followed by Vtest for 2 s. VTR was 20 mV, and Vtest varied from −140 to 60 mV in 40-mV increments unless otherwise indicated. IT and IN were measured 50 and 1950 ms, respectively, after application of Vtest.

**Patch Clamp**—Vitelline membranes of oocytes were removed manually following incubation of the oocytes at room temperature in modified Barth’s saline supplemented with 200 μM sucrose. Oocytes were then transferred to a recording chamber with bath solution and allowed to recover for 10 min before clamping. The bath and pipette solutions contained 110 mM NaCl, 2 mM KCl, 2 mM CaCl2, 10 mM HEPES, pH 7.4. Glass pipettes with tip resistances of 5–20 megaohms were used. For single channel conductance measurements, single channel currents were recorded in excised inside-out configuration using an Axopatch 200B amplifier (Axon Instruments) and a DigiData 1322A interface (Axon Instruments) connected to a Pentium 4 PC (Gateway). Single channel currents were acquired using pClamp 9.0 (Axon Instruments) at 20 kHz, filtered at 1000 Hz by a 4-pole low pass Bessel filter built in the amplifier, and stored on the hard disk. Single channel currents were further filtered at 100 Hz with a Gaussian filter for display and analysis. Transitions with duration <20 ms due to amplifier noise were not included for analysis. For NPo measurements, single channel currents were recorded in cell-attached mode using a PC ONE patch clamp amplifier (Dagan Corp., Minneapolis, MN), DigiData 1322A interface, and Clampfit 8.1 software (Axon Instruments). Single channel currents were further filtered at 100 Hz with a Gaussian filter for display and analysis. NPo estimates were made using recordings of duration ≥5 min using Clampfit 9.2 software (Axon Instruments).

**Statistical Analysis**—Data are presented as mean ± S.E. unless otherwise indicated. Statistical significance was analyzed by Student’s t-test. Curve fittings were performed with Clampfit 9.0 (Axon Instruments) and Igor Pro 4.0.9.1 (WaveMetrics, Oswego, OR).

**RESULTS**

**Effect of αSer-583 Mutations on Amiloride Block**—To study the role that αSer-583 may play in amiloride binding, we constructed a series of point mutations at this site. We selected both conservative (e.g. Ala, Cys, Thr) and non-conservative (e.g. Gln, Phe, His, Leu) substitutions for Ser to determine what binding specificity amiloride has toward αSer-583. cRNAs encoding wild type (wt) or mutant α subunits were mixed with those encoding wild type β and γ subunits and injected into Xenopus laevis oocytes. With the His, Phe, and Tyr α Ser-583 mutants, it was necessary to co-inject cRNAs encoding truncated β and γ subunits (denoted βγ and γγ respectively) in order to increase functional expression (23). These channels were benzamil sensitive (see below) and sodium selective (data not shown). Two mutants, αSS583K and αSS83D, did not perform functional expression 72 h after co-injection with βγ and γγ.

Expressing oocytes were perfused with increasing concentrations of amiloride while currents were measured at a holding potential of −100 mV. Currents were normalized to the current measured in the absence of drug, and resultant dose-response curves were fit with Equation 1 (Fig. 1, see “Experimental Procedures”). The order of apparent amiloride affinity for these mutants was Leu >Asn, Gln, Ser (wt) >Gly, Cys >Ile, Thr >Ala >Val >His >Phe >Tyr with wt-ENaC having a Ki of 130 ± 40 μM (Table I). Seven of the mutations had modest effects on apparent amiloride affinity, causing changes that were 16-fold or less. αSS83V had a greater effect, weakening apparent affinity by 35-fold. Notably, αSS83N and αSS83S did not significantly alter amiloride Ki, whereas αSS83L increased apparent affinity by 4-fold. In contrast, all three ring residues had strong effects: αSS83H weakened amiloride Ki by 230-fold, whereas both αSS83F and αSS83Y weakened amiloride Ki by more than three orders of magnitude.

Parallel experiments with amiloride, we also measured the apparent affinity of these mutants for benzamil, an amiloride derivative with a benzyl substitution off of the guanidinium moiety. Our results with benzamil are similar to those we found with amiloride (Fig. 1). The order of apparent benzamil affinity for these mutants was Leu >Asn, Gln, Ser (wt) >Gly, Cys >Ile, Thr >Ala >Val >His >Phe >Tyr with wt-ENaC having a Ki of 5.1 ± 0.2 μM (Table I). Five of the mutations had modest effects on apparent benzamil affinity, causing less than an 8-fold change. The Cys, Ile, Ala, and Val mutations had stronger effects, weakening benzamil Ki by 24-, 30-, 55-, and 120-fold, respectively. Dramatically, however, and paralleling our amiloride results, all three mutations containing ring structures weakened apparent benzamil binding by ~four orders of magnitude or more. Interestingly, αSS83G and wt-ENaC had similar apparent affinities for benzamil, which contrasts with αSS83Gβγ having a weaker apparent affinity for amiloride as compared with wt-ENaC. This is because αSS83Gβγ has a 75-fold greater apparent affinity for benzamil than amiloride, the largest such difference among all mutants.

**Effect of αSer-583 Mutations on Electrical Depth of Amiloride Binding**—To further study the role of αSer-583 in the interaction between ENaC and amiloride, we examined the voltage dependence of amiloride block of the mutant channels. We measured amiloride Ki at 20-mV increments between −140 and −20 mV for each of the mutants studied here (Fig. 2). The

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Fits to Equation 1 are shown for wt with a solid line and for each mutant with a dashed line. Fit results are in Table I. 

| @Ser-583 | Amiloride $K_i^{100\text{ mV}}$ | Benzamil $K_i^{100\text{ mV}}$ |
|-----------|-----------------|-----------------|
| Ser (wt)  | $0.13 \pm 0.04$ | $0.19 \pm 0.03$ | $0.0051 \pm 0.0002$ |
| Ala       | $2.1 \pm 0.3^*$  | $1.8 \pm 0.02$  | $0.28 \pm 0.06^*$  |
| Cys       | $0.6 \pm 0.06^*$ | $0.43 \pm 0.03^*$ | $0.12 \pm 0.03^*$  |
| Phe       | $1.70 \pm 10^6$ | $0.24 \pm 0.02$  | $54 \pm 2^*$      |
| Gly       | $0.6 \pm 0.2^*$  | $0.14 \pm 0.03$  | $0.0083 \pm 0.002$ |
| His       | $5 \pm 30^6$     | $0.35 \pm 0.03^*$ | $46 \pm 9^*$      |
| Ile       | $1.56 \pm 0.03^*$| $0.70 \pm 0.03^*$| $0.16 \pm 0.04^*$ |
| Leu       | $0.031 \pm 0.002^*$| $0.11 \pm 0.02^*$| $0.0047 \pm 0.0002$|
| Asn       | $0.076 \pm 0.007$| $0.15 \pm 0.03$  | $0.011 \pm 0.001$ |
| Gln       | $0.088 \pm 0.002$| $0.22 \pm 0.03$  | $0.026 \pm 0.005^*$|
| Thr       | $6 \pm 1.6^*$    | $0.54 \pm 0.03^*$| $0.04 \pm 0.001^*$|
| Val       | $5 \pm 4.5 \pm 0.9^*$| $0.71 \pm 0.03^*$| $0.6 \pm 0.1^*$    |
| Tyr       | $8 \pm >300$    | ND              | $171 \pm 8^*$     |

* $p < 0.05$ versus $\text{wt}$ by Student's $t$-test.

The voltage dependence of amiloride inhibition was then characterized by fitting the data in Fig. 2 to Woodhull's ionic block model, which provides an estimate for the electrical depth of binding of the amiloride binding site within the channel (24, 25). This model is derived from the application of Eyring rate theory to a simple scheme where the ionic blocker only accesses its binding site in the channel from the external side of the membrane, as shown in Equation 2.

$$K_i(V) = K_i^\circ \exp\left(\frac{-2FV}{RT}\right)$$  \hspace{1cm} (Eq. 2)

Here, $K_i(V)$ is the amiloride $K_i$ at a given electric potential, $K_i^\circ$ is the amiloride $K_i$ in the absence of an electric field, $\delta$ is the fraction of the membrane potential acting on the site, and $z$, $F$, $V$, $R$, and $T$ are the valence of the blocking ion, the Faraday, the electrical potential across the membrane, the gas constant, and the absolute temperature, respectively. Five mutants, namely $\alpha S83A$, $\alpha S83F$, $\alpha S83G$, $\alpha S83N$, and $\alpha S83Q$, had $\delta$ values that were not significantly different from that of wt-ENaC (Table I). Five other mutants, namely $\alpha S83H$, $\alpha S83C$, $\alpha S83T$, $\alpha S83I$, and $\alpha S83V$, had $\delta$ values that were significantly higher than that of wt-ENaC. Interestingly, the three mutations that had the highest $\delta$ values, causing an average 3.4-fold increase versus wt-ENaC, were those with amino acid side chains that introduced a $\beta$-methyl group at $\alpha$-83 (i.e. Thr, Ile, and Val).

**Effect of @Ser-583 Mutations on Current Rectification—** Shown in Fig. 3 are the current-voltage (I-V) relationships for wt-ENaC and selected mutant ENaC channels in the absence of amiloride, measured 400 ms after application of the indicated voltages. As has been previously reported, wt-ENaC expressed in oocytes with a high internal [Na $^+$] exhibits slight inward rectification and therefore has a near linear I-V relationship (26). Five of the @Ser-583 mutations, namely Leu, Gly, Asn, Gln, and Ala,
behaved similarly to wt-ENaC (data not shown), whereas the remaining seven exhibited different I-V relationships.

In the cases of αS583F and αS583Y, we observed outward rectification. The current-voltage relationship was simulated by characterizing the population of channels as having two distinct activity states with the voltage-dependent transition between the two states governed by a voltage-dependent Boltzmann distribution. This model is described by Equations 3 and 4

\[
P(V) = \frac{1}{1 + e^{-V - V_{1/2}}}
\]

(Eq. 3)

\[
I = A\cdot(V - V_{1/2})\cdot[P(V) + r\cdot[1 - P(V)]]
\]

(Eq. 4)

where \(P(V)\) is the probability of the channel being in the high activity state at a given voltage, \(V_{1/2}\) is the midpoint voltage of the transition, \(s\) is the slope of the S-shaped function at the inflection point, \(V_{Na}\) is the reversal potential for Na\(^+\), \(r\) is the ratio between the high and low channel activity states, and \(A\) is a scaling factor so that \(I\) is \(-1\) at \(-100\) mV. Fits of this model to the data for αS583FβγT and αS583YβγT channels are shown in Fig. 3. Whole cell conductance was 1.6- and 2.7-fold higher at depolarizing versus hyperpolarizing clamping voltages for oocytes expressing αS583FβγT and αS583YβγT channels, respectively (Table II). For both mutants, \(V_{1/2}\) was similar to the reverse potential, suggesting that rectification is closely associated with the direction of the ion flux. Indeed, equivalent fits were obtained with one less variable by setting \(V_{1/2}\) equal to \(V_{Na}\) (fit not shown).

In the case of oocytes expressing αS583Cβγ, αS583Iβγ, αS583Tβγ, or αS583Vβγ channels, we observed greater inward rectification relative to wt-ENaC. All four mutant channels were satisfactorily simulated by the model described above. The degree of rectification among three of these mutants was similar, having an average 2.8 ± 0.2-fold enhanced whole cell conductance at highly hyperpolarizing versus depolarizing potentials, with the rectification of αS583C being less pronounced with only a 1.5-fold change in activity. The \(V_{1/2}\) values were also similar among the four, having an average value of \(-83 \pm 28\) mV. Interestingly, three of these mutations are the only naturally occurring amino acids that have a methyl group on the β-carbon (see “Discussion”).

**Effect of αSer-583 Mutations on Single Channel Properties**

Given that the time between current measurements during the I-V protocol was less than 1 s, we hypothesized that the rectification induced by these mutations was due to either a voltage-dependent conductance (\(g\)) or open probability (\(P_o\)) effect and not due to changes in the number of channels in the membrane. To test this hypothesis, we performed single channel recordings of the αS583Tβγ channel (Fig. 4). In excised inside-out patches, in which both the bath and the pipette contained identical solutions with 110 mM Li\(^+\), we observed that the I-V relationship was linear from \(-120\) to 60 mV (Fig. 4A). We found that the αS583T mutation reduced the single channel conductance with Li\(^+\) as the charge carrier, with \(g\) equal to 3.2 ± 0.1 pS, compared with wt-ENaC, which has a reported \(g\) value of 8.1 pS (27). These data suggest that the inward rectification of αS583Tβγ is the result of a voltage-sensitive \(P_o\) effect rather than a voltage-dependent \(g\) effect.
To measure this effect directly, we measured the $N\cdot P_o$ of $\alpha$SS83T$\beta y$ in cell-attached patches, with 110 mM Li$^+$ in the pipette solution (Fig. 4B). We observed that $N\cdot P_o$ increased with hyperpolarization, such that from −40 to −80 mV the value of $N\cdot P_o$ almost doubles. Assuming that $N$ remains constant for each patch during the time of the experiment, these data demonstrate that the inward rectification of $\alpha$SS83T$\beta y$ is the result of a voltage-dependent $P_o$ effect.

We were unable to perform single channel experiments with the outwardly rectifying mutants $\alpha$SS83F and $\alpha$SS83Y, perhaps because of the low conductance evidenced by the necessity of coexpressing these mutants with $\beta y$ and $\gamma T$ subunits to achieve functional expression. In lieu of single channel data, we performed a tail current protocol using two-electrode voltage clamp (Fig. 5). In these experiments, the membrane potential is held at a given voltage ($V_{\text{hold}}$, 20 mV) for a time sufficient to achieve steady state, after which the membrane potential is changed to a test value ($V_{\text{test}}$). This is then repeated for a series of test potentials (Fig. 5, A and B). I-V relationships measured immediately upon change to $V_{\text{test}}$ (current defined as $I_{\text{tail}}$) that

Table II

| Parameters | $V_{\text{1/2}}$ | $V_{\text{Na}}$ | $r$ |
|------------|-----------------|-----------------|-----|
| aSS83 (wt) | 11 | $-66 \pm 7$ | $3.3 \pm 0.2$ | 1.08 $\pm 0.01$ |
| Thr | 11 | $-88 \pm 19$ | $1.1 \pm 1.4$ | 2.7 $\pm 1$ |
| Cys | 12 | $-58 \pm 4$ | $5.1 \pm 0.7$ | 1.5 $\pm 0.1$ |
| Ile | 22 | $-65 \pm 7$ | $-5 \pm 2$ | 2.0 $\pm 0.5$ |
| Val | 10 | $-120 \pm 60$ | $-7 \pm 4$ | 3 $\pm 3$ |
| Phe | 28 | $-11 \pm 18$ | $-2.7 \pm 0.8$ | 1.6 $\pm 0.2$ |
| Tyr | 24 | $5 \pm 8$ | $-2.5 \pm 1.2$ | 2.7 $\pm 0.1$ |

Table II - Fitted values for I-V plots of selected aSer-583 mutants

Parameters are as defined for Equations 3, 4. Parameter values are best fit ± S.D. from Fig. 3 using Equation 4.

Fig. 4. Single channel recordings of $\alpha$SS83T$\beta y$. A, Li$^+$ currents were measured from excised inside-out patch recordings carried out at the indicated membrane potentials. Pipette and bath solutions were identical and contained 110 mM Li$^+$ as described under “Experimental Procedures.” Representative recordings at indicated voltages are shown from an individual patch. Each data point is the average of 3–7 recordings ± S.E. Straight line fit of the data gave a single channel conductance of 3.2 ± 0.1 pS. B, Li$^+$ currents were measured from cell-attached patch recordings carried out at the indicated membrane potentials. The pipette contained 110 mM Li$^+$ . Representative recordings at −40 and −80 mV from an individual patch are shown. $N\cdot P_o$ was estimated using Clampfit 9.2. Each point represents the $N\cdot P_o$ estimated from a single patch at the indicated membrane potential. Lines connect $N\cdot P_o$ estimates from the same patch at different voltages.
Crate constants are in Table III. was fit for each channel with a single exponential equation. Derived Each data point represents the mean of at least six measurements panel A using mutations caused outward rectification at voltage potentials and outward rectification at depolarizing potentials (Fig. 6A, pH 7.4). Given an average pK of 6.2 for His and the ability of the electric field to influence equilibria involving charged species, we hypothesized that the outward rectification that we observed at depolarizing potentials parallels the results above for aS583Fβγγ and aS583Yβγγ and represents the current when His is not protonated. Furthermore, at hyperpolarizing membrane potentials His protonation becomes more favorable, and protonation would likely affect the I-V relationship.

To test this hypothesis, we measured the current-voltage relationship of aS583Hβγγ under various pH conditions (Fig. 6A). At pH 8.5, aS583Hβγγ behaved more like aS583Fβγγ and aS583Yβγγ, exhibiting less inward rectification at negative potentials than at pH 7.4. At pH 4.6, outward rectification is largely absent, whereas at highly negative potentials voltage-dependent inhibition was observed. Data at pH 5.7 and 6.5 represent states intermediate to those at pH 4.6 and 7.4. The model described by Equations 3 and 4 was inadequate to describe the behavior of this channel. We also note that current levels for aS583Hβγγ under acidic or neutral conditions were similar, whereas currents at pH 8.5 were higher (Fig. 6B). Control experiments with wt-ENaC showed little or no effect of pH on current levels.

To model these data, we applied Woodhull’s ionic blockage model as described above, except that K terms were replaced with K’ terms describing proton dissociation (24, 25). Because there are at least two α subunits/channel, the probabilities that either (Poff), one (P1H), or both (P2H) His residues are protonated at a given potential are given by Equations 7–9.

\[
P_{\text{off}}^{-1} = \frac{1}{1 + [H^+]K_a(V)} \tag{7}
\]

\[
P_{1H} = \frac{2[H^+]K_a(V)}{[1 + [H^+]K_a(V)]} \tag{8}
\]

\[
P_{2H} = \frac{[H^+]K_a(V)^2}{1 + [H^+]K_a(V)} \tag{9}
\]

It then becomes possible to apply Equations 3 and 4 to the unprotonated channels, ascribe no current to the doubly protonated channels, and describe the singly protonated channels as either blocked or having a characteristic activity (Scheme I and Equation 10, see below). However, such a model was inadequate to simulate the data (Fig. 6A, dashed line). Thus, it was necessary to also introduce a voltage-dependent change in channel activity to the protonated channels, perhaps reflecting a conformational change causing blockage. This model is described by Scheme II and Equation 10.

Effect of pH on aS583H—aS583Hβγγ channels exhibited both inward rectification at highly hyperpolarizing clamp potentials and outward rectification at depolarizing potentials (Fig. 6A, pH 7.4). Given an average pK of 6.2 for His and the ability of the electric field to influence equilibria involving charged species, we hypothesized that the outward rectification that we observed at depolarizing potentials parallels the results above for aS583Fβγγ and aS583Yβγγ and represents the current when His is not protonated. Furthermore, at hyperpolarizing membrane potentials His protonation becomes more favorable, and protonation would likely affect the I-V relationship.

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Effect of pH on aS583H—αS583Hβγγ channels exhibited both inward rectification at highly hyperpolarizing clamp

| A | B | C |
|---|---|---|
| S (wt) | T | C |
| I | V | F |
| Y | Y | Y |

Fig. 5. Voltage-dependent apparent P change of selected mutants. A, representative recordings using the tail protocol for wt-ENaC and each aS583 mutant are shown. Vertical and horizontal scale bars represent 2 μA and 500 ms, respectively. B, current-voltage relationships at I (○) and I (●) are shown for wt-ENaC and each mutant. Each data point represents the mean of at least six measurements ± S.E. The time course of current increase in panel A at V = −100 mV was fit for each channel with a single exponential equation. Derived rate constants are in Table III. C, apparent P (○) was calculated using \( P = \frac{I_{\text{off}}}{I_{\text{off}} + I_{\text{on}}} \times 100\% \). Each data point represents the mean of at least six measurements ± S.E. Data for each channel were fit using Equation 6 with \( x \) values reported in Table III. For comparison, \( P \) was also calculated using aS583T single channel data (○, see Fig. 4) using \( P = \frac{(N-P_n^\alpha mV - N-P_n^{\alpha-40 mV})}{N-P_n^{\alpha-40 mV}} \times 100\% \).
V_{1/2} in the PV_{1/2} term is equal to V_{Na} (see above).

Equation 10 describes a model in which voltage-dependent block is possible only when both His are protonated, the best fit of which is shown as a solid line in Fig. 6A. If the value of PV_{1/2} is set to 0, Equation 10 also describes Scheme I. All of the data sets in Fig. 6A were simultaneously fit with K_a, δ, V_{50}, s_1, s_2, r_1, and r_2 as global parameters, and V_{Na} as a local parameter; all parameters were well determined. The best fit gave a pK_a for His of 5.9 ± 0.1, close to its canonical value of 6.2. For electrical depth of the site, δ, the best fit value was 0.30 ± 0.05, within the margin of error of δ measured for amiloride binding of 0.35 ± 0.03 for this mutant (see Table I). Voltage-induced block of channels containing two protonated His occurred at highly hyperpolarizing potentials, with V_{1/2} equal to −182 ± 1 mV. For the conductivity ratio of His^{out}:His^{in}, given by r_1, the value was 4.0 ± 0.5, somewhat higher than that for either αS563Fβγγ or αS563Yβγγ based on their I-V relationships (Table II). Interestingly, the channel activities of His^{out} and His^{H^+} were similar, with r_1/r_2 having a value of 1.30 ± 0.08. The fit results thus indicate that His^{out} and His^{H^+} have similar channel activities, whereas the channel activity of His^{in} is reduced by 3–4-fold by comparison. That His^{H^+} has greater channel activity than His^{in} suggests that, in addition to causing channel block at hyperpolarizing potentials, His protonation also alters channel activity in the absence of voltage-dependent block.

With the same number of fitting parameters, it is also possible to describe a model in which voltage-dependent block occurs when at least one His is protonated. However, fits to such a model were worse than the model requiring two protonated His for channel block, giving almost triple the χ^2 value.
that the processes in prior to the increase in current develops in the presence of $\text{H}1\text{I}1002$ P mutants, the increases in current after application of observed with the other mutants. For wt-ENaC and the other $\text{H}9251$ direct interaction between the side chain of $\text{H}9251$ sought to determine the nature of interaction, if any, between $\text{H}9251$ gating properties of $\text{H}9251$ differently. First, low pH increased the voltage-dependent $\text{H}9251$ 11005/H11002 1 in the absence of block at $\text{H}9251$ 3 quaternary structure, indicating that the data do not fit not shown). Additionally, a fit equivalent to that achieved using Equation 10 was obtained with similar parameter values assuming three His/channel, in accordance with a nonameric $\alpha_3\beta_3\gamma_3$ quaternary structure, indicating that the data do not allow for discrimination between previously proposed channel subunit stoichiometries (3–6).

To determine which single channel properties are responsible for the various aspects of the IV relationships of $\alpha\text{SS}383\text{H}$, we applied the tail protocol at pH 7.4 and 4.6 to this mutant (Fig. 6C). At pH 7.4, $\alpha\text{SS}383\text{H}$ behaves like $\alpha\text{SS}383\text{Y}$, outwardly rectifying at $I_{\text{tail}}$ with a slightly voltage-dependent $P_o$ $(z_g = 0.03 \pm 0.01)$. At pH 4.6, $\alpha\text{SS}383\text{H}$ behaves quite differently. First, low pH increased the voltage-dependent gating properties of $\alpha\text{SS}383\text{H}$ $(z_g = -0.20 \pm 0.02)$. Second, at $I_{\text{tail}}$, proton-dependent block at hyperpolarizing potentials appears instantly, but it is largely relieved over time. However, the kinetics of $\alpha\text{SS}383\text{H}$ block relief, which feature a lag phase, are different from the kinetics of apparent $P_o$ increase observed with the other mutants. For wt-ENaC and the other mutants, the increases in current after application of $V_{\text{test}}$ were well described by a first order exponential equation, giving rate constant values of 1.4–1.6 s$^{-1}$ (Fig. 5, Table III). Assuming gating is a first order process, the relationship $k_{\text{rel}} = k_1 + k_{-1}$ gives channel open and close times on the order of seconds, consistent with ENaC gating observed both here (Fig. 4) and previously (28). At pH 7.4, $\alpha\text{SS}383\text{H}$ behaves similarly, giving a rate constant of 1.4 s$^{-1}$ (Fig. 6C). However, at pH 4.6 this rate slows to 0.6 s$^{-1}$ in the absence of block at $-100$ mV, perhaps because of His protonation. In addition, a lag phase prior to the increase in current develops in the presence of voltage-induced block at $-180$ mV. These results suggest that the processes in $\alpha\text{SS}383\text{H}$ that lead to rapid voltage-dependent block and slow apparent $P_o$ increase (or block relief) are distinct yet may influence each other to affect both gating kinetics and $P_o$ voltage dependence. However, these data are insufficient to resolve the contributions of each component that influence current; therefore, conclusions regarding interactions between these processes remain tenuous.

**Discussion**

Based on earlier work by our group and others (4, 8, 9, 29), we previously proposed that amiloride binds to ENaC such that its pyrazine ring is close to the site defined by $\alpha\text{Ser}-583$, $\beta\text{Gly}-525$, and $\gamma\text{Gly}-542$, and its charged guanidinium moiety is close to the outer mouth of the selectivity filter (29). In this work, we sought to determine the nature of interaction, if any, between $\alpha\text{Ser}-583$ and amiloride. Our results clearly indicate that a direct interaction between the side chain of $\alpha\text{Ser}-583$ and amiloride, if any, is not an important determinant of amiloride binding. This characteristic of amiloride block is manifest in the effects of mutations at this position on both amiloride and benzamil block. For serine, three conservative mutations are available: threonine, which adds a $\beta$-methyl group; alanine, which eliminates the hydroxyl group; and cysteine, which substitutes the hydroxyl group with a sulfhydryl group. If the hydroxyl group of $\alpha\text{Ser}-583$ had a contributory interaction with amiloride, apparent binding affinity should be most affected by Ala, followed by Cys, and least affected by Thr. For amiloride, this is not the case. Although Ala does have the largest effect among these three (16-fold increase in $K_i$), Thr has a similar effect (12-fold), and Cys has a smaller effect (5-fold). In contrast, for benzamil this appears to be the case. Ala weakens benzamil $K_i$ 55-fold, whereas Cys and Thr weaken benzamil $K_i$ 11- and 8-fold, respectively. However, further inspection dispels any notion of a specific, contributory H-bonding interaction between any $\alpha\text{SS}383$ side chains and either drug. For both drugs, the four $\alpha\text{SS}383$ mutations with 5-fold or smaller effects are Gly, Leu, Asn, and Gln, which have neither functional groups nor overall volume in common.

There are two aspects among the mutations we examined that have common effects on the apparent binding affinity of both drugs. The first is a ring structure, present on Phe, Tyr, and His, whose presence had the most dramatic effects on weakening apparent drug binding affinity. Notably, the apparent affinity of ring-introducing mutants for amiloride decreased with residue volume so that, in order of apparent binding affinity, His > Phe > Tyr. The exception to this trend is that the His and Phe mutants have similar $K_i$ values for benzamil. It is also interesting that these three mutations weakened benzamil $K_i$ to a greater degree than amiloride $K_i$ vis-à-vis wt-ENaC, because benzamil has 40% more mass than amiloride. There was no such correlation between $K_i$ and residue volume for those mutants without ring side chains at $\alpha\text{SS}383$. The introduced rings have large volumes with few degrees of freedom with which to maneuver and exhibit large effects on apparent drug binding affinity, suggesting that these residues protrude into the pore lumen and at least partially occupy the putative amiloride binding site (Fig. 7A). Other similarly large residues (e.g. Leu and Gln) that have more degrees of freedom do not weaken amiloride binding, suggesting that these residues are able to fold away from the amiloride binding site. If this site is also within the conduction pathway, and the mutants with rings are able to adopt multiple conformations, the presence and direction of ion flux may influence which conformation is preferred. Such a structure can account for the large loss in apparent binding affinity, the greater effects on benzamil block, and the dependence of channel activity on the direction of ion flux, which is supported by $V_{\text{Na}} > V_{\text{Na}}$ for rectification observed with these three mutants (Figs. 3, 6, Table II).

The second interesting finding is the effect of a $\beta$-methyl group, present on Thr, Ile, and Val. Two of these can be directly compared with structurally related amino acids, namely Thr to Ser, and Ile to Leu. In both cases, and for both drugs, the effect of the $\beta$-methyl group is to weaken $K_i$, $-10$-fold for Thr versus Ser and $-50$-fold for Ile versus Leu. Valine has no such closely related analogue but can be compared with the structurally, more distantly related residues Ala and Leu. Compared with both of these residues, the effect of Val mutation is to weaken apparent drug binding affinity, 2-fold versus Ala and $-140$-fold versus Leu.

In addition to weakening the apparent affinity of the channel for amiloride, the presence of a $\beta$-methyl group also changes the electrical properties of the channel, an effect shared with

**Table III**

Voltage dependence of selected $\alpha\text{Ser}-583$ mutants

$k$ refers to the rate constant for changes in apparent $P_o$, $z_g$ refers to the gating charge. Values of $k$ are derived from Figs. 5A, 6C by fitting the current at $-100$ mV between $I_{\text{tail}}$ and $I_{0}$ to a first-order exponential equation and are mean $\pm$ S.E. Values of $z_g$ are derived from Figs. 5C, 6C using Equation 6. Parameter values are best fit $\pm$ S.D., * $p < 0.05$ versus wt by Student’s $t$-test.

| $\alpha\text{SS}383$ | $n$ | $k$ | $z_g$ |
|----------------------|-----|-----|-------|
| Ser (wt)             | 6   | 1.41 ± 0.04 | $-0.04 \pm 0.02$ |
| Thr                  | 8   | 1.38 ± 0.01 | $-0.15 \pm 0.02^*$ |
| Cys                  | 6   | 1.53 ± 0.02 | $-0.11 \pm 0.01^*$ |
| Ile                  | 6   | 1.38 ± 0.01 | $-0.14 \pm 0.02^*$ |
| Val                  | 6   | 2.2 ± 0.45  | $-0.12 \pm 0.02^*$ |
| Phe                  | 6   | 1.58 ± 0.02 | $-0.11 \pm 0.04^*$ |
| Tyr                  | 6   | 1.42 ± 0.03 | $-0.05 \pm 0.02$ |
| His, pH 7.4          | 8   | 1.41 ± 0.02 | $-0.03 \pm 0.01$ |
| His, pH 4.6          | 8   | 0.60 ± 0.02 | $-0.20 \pm 0.02^*$ |
the Cys mutant. One change was to increase the value of \( \delta \) for amiloride binding. Palmer's laboratory (30) previously reported that divalent blockers exhibit a voltage dependence twice that of monovalent blockers, suggesting that the value of \( \delta \) represents the electrical depth of the blocker binding site. Schild et al. (8) observed that Ca\(^{2+}\) block of \( \alpha\text{S580D} \) by also had a voltage dependence approximately twice that of amiloride. In contrast, monovalent and divalent blockers of inwardly rectifying K\(^+\) channels have similar voltage dependences, which has led to the proposal that \( \delta \) arises from the displacement of conducting ions from the pore by these K\(^+\) channel blockers (31–33). The other electrical property change was to increase the voltage dependence of \( P_o \), causing greater inward rectification for these mutants. Given both of these changes and given that apparent drug affinity is only moderately weakened, we find it unlikely that the presence of a \( \beta\)-methyl group at \( \alpha583 \) grossly changes the binding position of either drug or alters net charge movement through physical space during gating. More likely, the presence of a \( \beta\)-methyl group or Cys at \( \alpha583 \) alters the electric field. Such an alteration of the electric field would account for both the increased gating charge (\( z_p \)) and the increased fraction of the field that amiloride senses (\( \delta \)) in these mutants by increasing the electrical distance associated with both physical events. In fact, the effect of a \( \beta\)-methyl group at \( \alpha583 \) on the values of \( z_p \) and \( \delta \) for amiloride binding were parallel, causing an average 3.4-fold increase in both. The effects of the Cys mutation on both \( z_p \) and \( \delta \) were also parallel, causing an average 2.5-fold increase in both. To account for these changes, we propose that these \( \beta\)-methyl groups protrude into the conducting pore of the channel (Fig. 7A). Likewise, based on the properties of the Cys mutant being more similar to the Ile, Val, and Thr mutants than to the wt-Ser, we propose that the Cys at \( \alpha583 \) adopts a conformation similar to the \( \beta\)-methyl groups and different from the wt-Ser and thus also protrudes into the conduction pore of the channel. The proposed orientation for the side chain of \( \alpha\text{S583C} \) is consistent with previous work suggesting that the sulfhydryl group of this mutant is solvent accessible, because it is sensitive to sulfhydryl-reactive reagents (4, 8, 34, 35). This model assumes that the protein backbone is largely unaltered by these mutations and therefore the positions of the \( \alpha \) and \( \beta \) carbons at \( \alpha583 \) are relatively fixed.

With the \( \alpha\text{S583H} \) channel, we have generated a proton-dependent, voltage-gated channel. This mutant has three distinct features. First, at high pH, this channel behaves similarly to \( \alpha\text{S583F} \) and \( \alpha\text{S583Y} \) outwardly rectifying with a \( V_{1/2} \) equivalent to the reverse potential for Na\(^+\). Second, at low pH, voltage-dependent inhibition occurs at highly hyperpolarizing potentials. Third, under both pH conditions, there is a slow increase in \( P_o \) with hyperpolarization, albeit to a greater extent under acidic conditions. These three phenomena may be attributed to distinct events within the channel. At high pH, the ring is uncharged and presumably partially protrudes into the pore lumen. Because the ring is uncharged, it is unlikely to be affected by the electric field. However, analogous to the Phe and Tyr rings, its conformation may be affected by the direction of ion flux, such that outward flux favors a conformation that allows for higher flux. At low pH, the ring is protonated and charged and is affected by the electric field. As merely the presence of one or two protonated His is insufficient to account for the block, the positively charged rings are likely drawn inward with hyperpolarization, blocking the channel (Fig. 7B).

Both of these events, which we propose involve ring movement, occur on a fast time scale (Fig. 6C). In addition to these events, there is an increase in \( P_o \) with hyperpolarization, which occurs on a slower time scale. We have measured this rate to be \( 0.6–1.4 \text{ s}^{-1} \), which is consistent with the re-equilibration of the \( P_o \) upon change of the membrane potential. Block relief occurs on a time scale similar to that of \( P_o \) equilibration; however, its kinetics are more complicated, having an initial lag phase (Fig. 6C, pH 4.6, dotted line). The lag phase suggests that there may be interactions between the gate and the conformation of the introduced His. Thus, block occurs on a faster time scale than apparent block relief, which takes place in the context of voltage-dependent gating. On the basis of their dissimilar kinetic time scales, the gate of ENaC can be distinguished from His conformational changes, which in effect function as a gate. This suggests that the \( \alpha\text{S583H} \) mutation appended a proton-dependent voltage-sensitive gate, whose \( P_o \) decreases with hyperpolarization, within the conducting pore. This gate is functionally distinct from the normal gate of ENaC, whose \( P_o \) slightly increases with hyperpolarization (36–39). In addition, the block caused by these positively charged His residues may mimic the block caused by amiloride.

The data presented here indicate that \( \alpha583 \) side chain shape is the primary factor that determines the effect of \( \alpha\text{Ser-583} \) mutation on amiloride binding affinity. Amiloride does not seem to have a specific interaction with the \( \alpha\text{Ser-583} \) side chain, and amiloride affinity is most affected by intrusions of \( \alpha583 \) side chains into the amiloride binding pocket. This model of amiloride binding is also consistent with data that have been previously published. Schild et al. (8) showed that mutations at either \( \beta\)-Gly-525 or \( \gamma\)-Gly-537, sites in rat \( \beta \) and \( \gamma \) analogous to Gly, that were responsible for the loss in apparent amiloride affinity. Kellenberger et al. (9) showed that the amiloride binding effects of mutations at the \( \alpha \) and \( \beta \) binding sites were largely accounted for by an increase in
the microscopic $k_{\text{off}}$ rate, indicating a disruption of bound complex. Whereas it is now clear that the wild type side chains at this site in the $\alpha$, $\beta$, and $\gamma$ subunits do not have strong contribu-
tory interactions with amiloride, and yet amiloride binds at or near this site, it is not clear what mechanism is responsible for the effects of mutations at this site on amiloride binding. Poss-
sibilities include altering access to nearby functional groups, either from the backbone or nearby side chains, or simply causing unfavorable steric interactions, or combinations of both.

Our data are also consistent with previous models that amiloride binds ENaC in its conducting pore and is thus a pore blocker (40, 41). Earlier experiments showed that amiloride binding is sensitive to the electric field and that $\text{Na}^+$ is competitive with amiloride binding (16, 28, 30, 40, 42, 43). Here we have shown that specific mutations alter the electric depth of amiloride binding. Our data also provide no evidence for cooperative amiloride binding for either wild type or mutant chan-

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