

Permeability Properties of ENaC Selectivity Filter Mutants

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ABSTRACT The epithelial Na⁺ channel (ENaC), located in the apical membrane of tight epithelia, allows vectorial Na⁺ absorption. The amiloride-sensitive ENaC is highly selective for Na⁺ and Li⁺ ions. There is growing evidence that the short stretch of amino acid residues (preM2) preceding the putative second transmembrane domain M2 forms the outer channel pore with the amiloride binding site and the narrow ion-selective region of the pore. We have shown previously that mutations of the α S589 residue in the preM2 segment change the ion selectivity, making the channel permeant to K⁺ ions. To understand the molecular basis of this important change in ionic selectivity, we have substituted α S589 with amino acids of different sizes and physicochemical properties. Here, we show that the molecular cutoff of the channel pore for inorganic and organic cations increases with the size of the amino acid residue at position α 589, indicating that α S589 mutations enlarge the pore at the selectivity filter. Mutants with an increased permeability to large cations show a decrease in the ENaC unitary conductance of small cations such as Na⁺ and Li⁺. These findings demonstrate the critical role of the pore size at the α S589 residue for the selectivity properties of ENaC. Our data are consistent with the main chain carbonyl oxygens of the α S589 residues lining the channel pore at the selectivity filter with their side chain pointing away from the pore lumen. We propose that the α S589 side chain is oriented toward the subunit–subunit interface and that substitution of α S589 by larger residues increases the pore diameter by adding extra volume at the subunit–subunit interface.

KEY WORDS: ion channel • molecular sieving • pore • *Xenopus* oocyte • epithelial Na⁺ channel

INTRODUCTION

The epithelial Na⁺ channel (ENaC)* is expressed in the apical membrane of epithelial cells of the distal nephron, the colon, and the lung, where it mediates vectorial transepithelial Na⁺ absorption. The Na⁺ ions enter the cell through ENaC at the apical membrane and are transported out of the cell by the Na⁺/K⁺ ATPase at the basolateral membrane. In the distal nephron, aldosterone and vasopressin regulate ENaC activity, serving to maintain Na⁺ balance, extracellular volume, and blood pressure (Garty and Palmer, 1997).

ENaC belongs to the ENaC/degenerin (DEG) family of ion channels. Members of this family are present in nematodes, flies, snails, and mammals, where they are involved in Na⁺ transport, neurotransmission, mechanotransduction, and nociception (Mano and Driscoll, 1999). Members of the ENaC/DEG gene family are cation channels that share a common membrane topology

characterized by intracellular NH₂- and COOH termini, two transmembrane domains, and a large extracellular loop. ENaC differs from the other members of the ENaC/DEG family by its high selectivity for Na⁺ and Li⁺ over K⁺ ions and its high affinity for amiloride, a channel pore blocker. ENaC is a heterotetramer made of two α , one β , and one γ homologous subunits arranged around the central channel pore (Canessa et al., 1994; Firsov et al., 1998). All three subunits are involved in basic channel functions such as ionic selectivity, channel gating, and channel block by amiloride. The amiloride binding site has been localized in the short segment (preM2 segment) preceding the second transmembrane segment M2 of the ENaC subunits by mutations of either the α (S583), β (G525), or γ subunit (G537) that decrease channel affinity for amiloride by two to three orders of magnitude (Schild et al., 1997; Fig. 1). Mutations of α G587 and homologous residues in β and γ subunits (β G529 and γ S541; Fig. 1), four residues downstream of the amiloride binding site affect channel conductance for Na⁺ and Li⁺ ions by changing the channel affinity for these permeant ions. These findings suggest that at this site the pore is narrow and the ions interact tightly with the Gly and Ser residues lining the pore (Kellenberger et al., 1999b). Mutations of the conserved Ser residue α S589 and the homologous residue in γ ENaC result in channels that are permeable to cations larger than Na⁺ and Li⁺ such as K⁺, Rb⁺, and Cs⁺ (Kellenberger et al.,

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*Abbreviations used in this paper: DEG, degenerin; DMA, dimethylammonium; ENaC, epithelial Na⁺ channel; I_{Li}, amiloride-sensitive Li⁺ current; I_{Na}, amiloride-sensitive Na⁺ current; I_{NH₄}, amiloride-sensitive NH₄⁺ current; M2, second transmembrane domain; M₂Ab, anti-FLAG M₂ mouse mAb; pre-M2, segment NH₂-terminal of M2; MA, methylammonium; MTSEA, aminoethyl methanethiosulfate; TriMA, trimethylammonium; wt, wild type.

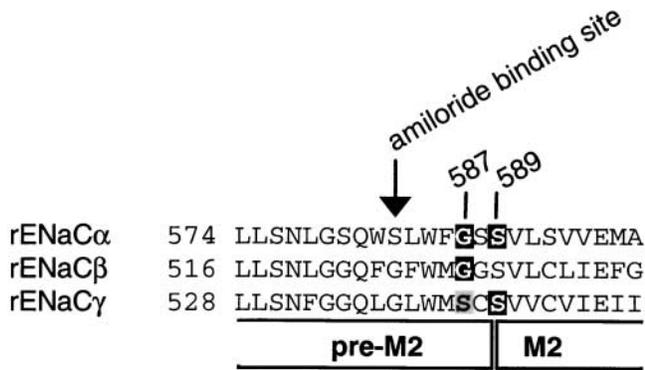
Site-directed Mutagenesis, Expression in Xenopus laevis Oocytes and Electrophysiological Analysis

FIGURE 1. Sequence alignment of the preM2/M2 segments of rat α , β , and γ ENaC. The number of the first residue shown is indicated. The putative start of M2 and the position of the amiloride binding site (α S583, β G525, and γ G537) are indicated. Mutations that change ionic selectivity are mutations of α G587 and α S589 as well as residues at the homologous positions in β and γ subunits. Residues shown in white on a dark background change Na^+/K^+ selectivity when mutated, and mutation of α G587 and the homologous residues in β and γ subunits change Li^+/Na^+ selectivity. Mutation of γ S541 (shown in black on gray background) changes Li^+/Na^+ , but not Na^+/K^+ selectivity.

1999a; Sheng et al., 1999; Snyder et al., 1999). The effects of mutations of residues 587–589 on ENaC conductance and ion selectivity identify this short stretch of amino acid residues in the preM2 segment as the narrow ion-selective region of the channel pore.

To gain further information on the role of the residue α S589 in maintaining the channel pore structure necessary for ion discrimination, we have substituted α S589 with amino acids of different volumes. We have analyzed channel selectivity properties of the α S589 mutants for inorganic and organic cations of varying diameter and shape. We found that the K^+ permeability as well as the molecular cutoff of the channel permeability for large cations increases with the size of the amino acid residue at position α S589. This is consistent with an increase of the pore diameter at the selectivity filter. The consequences of this enlargement of the pore are an increase in channel permeation to larger cations, and at the same time, a decrease in the unitary conductance of these mutant channels to small cations such as Na^+ and Li^+ . The observation that mutant channels with large side chains of residue α 589 form a wide pore indicates that the side chain of the residue at position 589 points away from the ion permeation pathway. We propose that it points toward the interface between the subunits lining the pore, and that substitutions of α S589 with large amino acid residues increase the diameter of the narrowest part of the channel pore by adding extra volume to the subunit–subunit interface. The largest amino acid substitutions cause structural changes that are incompatible with channel expression at the cell surface.

Site-directed mutagenesis was performed on rat ENaC cDNA as described previously (Schild et al., 1997). Complementary RNAs of each α , β , γ subunit were synthesized *in vitro*. Healthy stage V and VI *Xenopus* oocytes were pressure-injected with 100 nl of a solution containing equal amounts of $\alpha\beta\gamma$ ENaC subunits at a total concentration of 100 ng/ μl . For simplicity, mutants are named by the mutated subunit only, although all three subunits (α , β , and γ) always were coexpressed.

Electrophysiological measurements were taken at 16–48 h after injection. Macroscopic amiloride-sensitive currents, defined as the difference between ionic currents obtained in the presence and absence of 100 μM amiloride (Sigma-Aldrich) in the bath were recorded using the two-electrode voltage-clamp technique. All macroscopic currents shown are amiloride-sensitive currents as defined above. Currents were recorded with an amplifier (model TEV-200; Dagan Corp.) equipped with two bath electrodes. The standard bath solution contained 110 mM NaCl, 1.8 mM CaCl_2 , and 10 mM HEPES-NaOH, pH 7.35. For selectivity measurements, Na^+ was replaced with Li^+ , K^+ , Rb^+ , Cs^+ , or the organic cations at the same concentration. Pulses for current-voltage curves were applied, and data were acquired using a PC-based data acquisition system (Pulse; HEKA Electronic). Single-channel currents were measured in the outside-out configuration of the patch-clamp technique essentially as described previously (Kellenberger et al., 1999b). The bath solution in patch-clamp experiments was the standard bath solution described above, with Li^+ or Na^+ as the cation. The pipette solution contained 75 mM CsF, 17 mM NMDG-HCl, 10 mM EGTA, and 10 mM HEPES, pH 7.35. Pipettes were pulled from Borosilicate glass (World Precision Instruments). In patch-clamp experiments, currents were recorded with a List EPC-9 patch-clamp amplifier (HEKA Electronic) and filtered at 100 Hz for analysis. Aqueous stock solutions of MTS ethylammonium (MTSEA; Toronto Research Chemicals) were prepared just before the experiment, maintained on ice, and diluted into the bath solution immediately before use.

Binding Experiments

The FLAG reporter octapeptide that had been introduced in β and γ subunits is recognized by the anti-FLAG M_2 (M_2Ab) mouse mAb (Sigma-Aldrich). M_2Ab was iodinated as described by Firsov et al. (1996). Iodinated M_2Ab had a specific activity of 5–20 $\cdot 10^{17}$ cpm/mol, and were used up to 2 months after synthesis. On the day after mRNA injection, oocytes were transferred to a 2-ml Eppendorf tube containing modified Barth's saline ([in mM] 10 NaCl, 90 NMDG-HCl, 0.8 MgSO_4 , 0.4 CaCl_2 , and 5 HEPES, pH 7.2) supplemented with 10% heat-inactivated calf serum, and incubated for 30 min on ice. The binding was started upon addition of 12 nM $^{125}\text{I}\text{-M}_2\text{Ab}$ (final concentration) in a volume of 5–6 μl /oocyte. After 1 h of incubation on ice, the oocytes were washed eight times with 1 ml modified Barth's saline supplemented with 5% heat-inactivated calf serum, and then transferred individually into tubes for γ counting containing 250 μl of the same solution. The samples were counted, and nonspecific binding was determined from parallel assays of noninjected oocytes.

Immunoprecipitation

Oocytes were injected with FLAG-tagged and untagged subunits, as specified. After overnight incubation of the injected oocytes, microsomal membranes were obtained as described previously

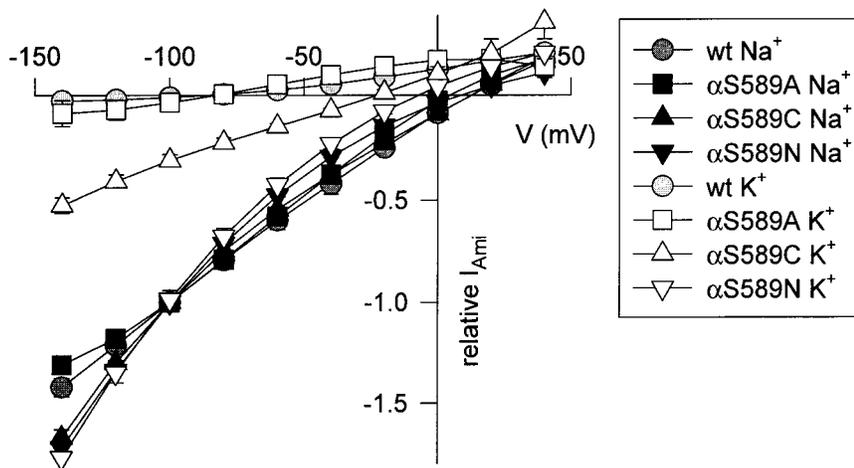


FIGURE 2. The K^+/Na^+ permeability ratio is increased by α S589 mutations. Current-voltage relationship in 120-mM Na^+ and 120-mM K^+ bath solution from oocytes expressing α S589A, α S589C, and α S589N measured with two-electrode voltage clamp. Currents were measured during 1-s voltage steps from a holding potential of -20 mV to test potentials of -140 to $+40$ mV in 20-mV increments. Currents measured in the presence of 10 μ M amiloride were subtracted from currents measured in the absence of amiloride. In each experiment, the amiloride-sensitive Na^+ and K^+ currents were normalized to the amiloride-sensitive Na^+ current at -100 mV. To allow Na^+ to enter the oocyte during the expression phase without downregulation of channel activity, mutant α subunits were

coexpressed with β subunits containing the Liddle mutation β Y618A together with wt γ ENaC and oocytes were kept in a solution containing 90 mM Na^+ during the expression phase (Kellenberger et al., 1998). The mutation β Y618A does not affect ion selectivity. $E_{rev,Na} - E_{rev,K}$ was 114 mV (wt), 96 mV (α S589A), 37 mV (α S589C), and 15 mV (α S589N), $n = 4-5$. I_{Na} at -100 mV was $30.0 \pm 3.6 \mu A$ (α S589A), $8.3 \pm 1.7 \mu A$ (α S589C), and $9.3 \pm 2.1 \mu A$ (α S589N).

(Geering et al., 1989) and solubilized in a Triton X-100 buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM HEPES, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml each of leupeptin, pepstatin A, and aprotinin).

Immunoprecipitations were performed in Triton X-100 buffer overnight at $4^\circ C$ with the anti-FLAG M2 antibody (M_2 Ab) and protein G-Sepharose. After SDS-PAGE, polypeptides were transferred for immunostaining onto nitrocellulose membranes (Schleicher & Schuell) and immunoblotted using anti-rat α ENaC antibody (May et al., 1997) as primary and goat anti-rabbit horseradish peroxidase (Amersham) as secondary antibody and visualized using Super Signal[®] West Dura Extended Duration Substrate (Pierce Chemical Co.).

RESULTS

Amino Acid Substitutions of Ser 589 in α ENaC

We have substituted the amino acid α S589 with residues of different sizes and physicochemical properties. The mutant α subunits were coexpressed with wild-type (wt) $\beta\gamma$ ENaC subunits in *Xenopus* oocytes. Ionic currents through wt and mutant channels were completely blocked by 100 μ M amiloride. The K^+ over Na^+ selectivity of the α S589 mutants was determined by the amiloride-sensitive current ratio (I_K/I_{Na}) obtained at -100 mV in the presence of external Na^+ (120 mM) and after Na^+ substitution by K^+ ions, as shown in Table I. Increasing the size of the substituting amino acid side chain at position α 589 increased the channel permeability to K^+ , as shown by the I_K/I_{Na} ratio. The α S589H substitution mutation that represents a 60% increase in the residue's volume renders the channel nonselective between K^+ and Na^+ . Changes in the K^+ over Na^+ selectivity were further evidenced from measurements of reversal potentials of the currents of α S589 mutants in the presence of extracellular Na^+ or

K^+ solution. Fig. 2 shows the current-voltage relationship of amiloride-sensitive currents in the presence of extracellular Na^+ and K^+ of ENaC with substitutions of α S589. I_{Na} and I_K were normalized in each experiment to I_{Na} at -100 mV. The current-voltage relationship with extracellular Na^+ solution is similar in wt and the α S589A, α S589C, and α S589N ENaC mutants. However, no inward K^+ current could be measured in ENaC wt even at potentials more negative than -100 mV. I_K was low in α S589A, intermediate in α S589C and almost equal to I_{Na} in α S589N. The K^+/Na^+ permeability ratio calculated from the difference of the reversal potential in extracellular K^+ and Na^+ solution, respectively, is 0.01 for wt, 0.02 for α S589A, 0.24 for α S589C, and 0.56 for α S589N (Hille, 1992). These permeability ratios are consistent with the I_K/I_{Na} current ratio values measured at -100 mV (Table I).

The α S589 mutations tend to decrease I_{Na} with increasing size of the substituting amino acid, and for substitutions with residues larger than His ($\sim 118 \text{ \AA}^3$), no amiloride-sensitive current could be detected (Table I). To determine whether the absence of I_{Na} observed with expression of the large α S589 substitution mutants is due to nonpermeant channels at the cell surface or to alterations in the biosynthesis of these mutants, we have compared the surface expression of selected α S589 mutant channels with ENaC wt. Wild-type and mutant ENaC channels were tagged with FLAG epitopes in the extracellular domain, and binding of the iodinated anti-FLAG antibody to intact oocytes was measured (Firsov et al., 1996). Expression of wt ENaC containing flagged β and γ subunits ($\alpha\beta^F\gamma^F$) increased surface binding of anti-FLAG mAb to 8.4 ± 1.9 -fold over noninjected oocytes. Coexpression of α S589 mu-

TABLE I
Current Expression and Ion Selectivity of ENaC Mutants

| Mutant | I_{Na} | I_K/I_{Na} | I_{NH4}/I_{Na} | g_{Na} | g_L | Van der Waals volume of amino acid |
|---------------------------------------|----------------|------------------|------------------|---------------|---------------|---------------------------------------|
| | μA | | | pS | pS | \AA^3 |
| $\alpha S589G$ | 9.4 ± 1.1 | 0.04 ± 0.00 | 0.02 ± 0.00 | 5.0 ± 0.4 | 7.8 ± 0.3 | 48 |
| $\alpha S589A^a$ | 41.3 ± 3.0 | 0.05 ± 0.00 | 0.03 ± 0.00 | 4.3 ± 0.2 | 5.9 ± 0.7 | 67 |
| $\alpha S589C^a$ | 5.2 ± 0.5 | 0.24 ± 0.02 | 0.23 ± 0.06 | $<1^b$ | 1.5 ± 0.1 | 86 |
| $\alpha S589D^a$ | 6.4 ± 0.8 | 0.30 ± 0.02 | 0.32 ± 0.02 | 1.7 ± 0.1 | 1.5 ± 0.2 | 91 |
| $\alpha S589N$ | 3.0 ± 0.4 | 0.70 ± 0.02 | 1.13 ± 0.10 | $<1^b$ | $<1^b$ | 96 |
| $\alpha S589V$ | 0.0 ± 0.0 | n.d. | n.d. | n.d. | n.d. | 105 |
| $\alpha S589E$ | 0.1 ± 0.0 | n.d. | n.d. | n.d. | n.d. | 109 |
| $\alpha S589Q$ | 0.9 ± 0.1 | 0.24 ± 0.02 | 0.30 ± 0.03 | $<1^b$ | 1.9 ± 0.5 | 114 |
| $\alpha S589H$ | 0.4 ± 0.1 | 1.02 ± 0.04 | 1.20 ± 0.00 | $<1^b$ | $<1^b$ | 118 |
| $\alpha S589L, M, K,$ F, R, W | 0.0 ± 0.0 | n.d. | n.d. | n.d. | n.d. | ≥ 124 |
| $\alpha L591C$ to $\alpha M610C^c$ | 15.8 ± 3.0 | 0.01 ± 0.00 | n.d. | n.d. | n.d. | |
| wt | 17.9 ± 1.9 | -0.01 ± 0.00 | 0.00 ± 0.00 | 5.0 ± 0.2 | 9.0 ± 0.3 | 73 |

Whole-cell amiloride-sensitive current ($n = 3-64$) and single-channel conductance ($n = 3-7$) of mutant α subunits coexpressed with wt β and γ ENaC. $n = 3-64$. n.d., not determined.

^aThese mutants have been partially characterized in our previous study (Kellenberger et al., 1999a).

^bSingle-channel conductance was clearly below 1 pS, and single-channel current amplitudes could not be resolved reliably.

^cMean of the Cys mutants of these 20 residues; the E598C mutant was not functional, but the E598A mutant was functional and used for this analysis.

tants with flagged β and γ subunits increased the mA binding 6.0 ± 1.6 -fold over noninjected oocytes for $\alpha S589A$ and 8.6 ± 4.5 -fold with $\alpha S589C$ ($n = 12-48$ oocytes). No significant binding was detected for the non-functional mutants $\alpha S589V$, $\alpha S589L$, and $\alpha S589W$ (e.g., the ratio was 1.1 ± 0.4 for $\alpha S589V$, 1.6 ± 0.6 for $\alpha S589L$, and 0.9 ± 0.3 for $\alpha S589W$, $n = 12-18$ oocytes), indicating the absence of channels at the cell surface.

The absence of cell-surface expression of $\alpha S589V$, $\alpha S589L$, and $\alpha S589W$ mutants is not due to an im-

paired translation of the mutant subunit. After solubilization from oocytes expressing wt $\alpha\beta\gamma$ or the mutant $\alpha S589$ subunits with wt $\beta\gamma$ ENaC, wt and mutant α subunits could be detected on Western blots using an anti-rat α ENaC antibody (May et al., 1997; Fig. 3 A), indicating that the mutant ENaC subunits are efficiently translated. The ability of the mutant α subunits to assemble with β subunits was tested by coimmunoprecipitation experiments in oocytes coexpressing the wt or mutant α subunits with β subunits that carry a FLAG epitope,

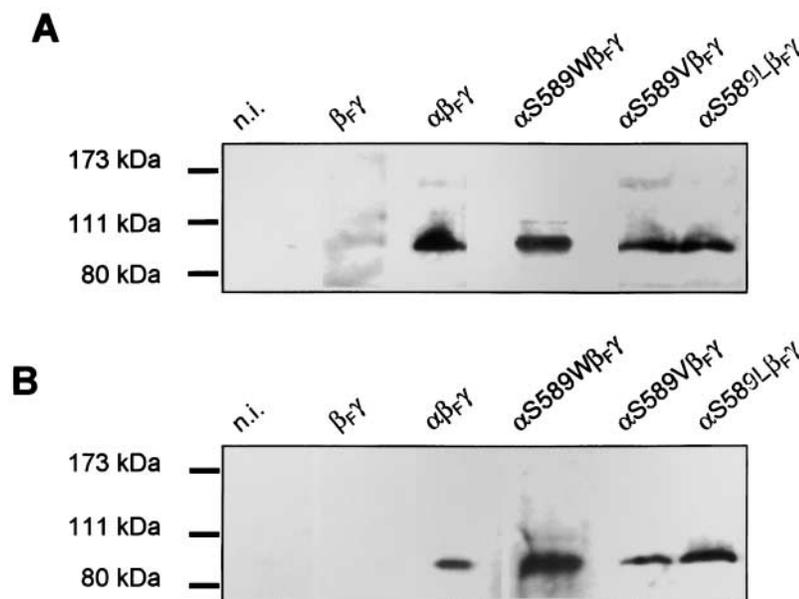


FIGURE 3. Analysis of translation of wt and mutant α subunits and of assembly with β subunits. Oocytes were not injected or injected with RNAs encoding ENaC carrying a FLAG epitope on the β subunit, $\alpha\beta_F\gamma$, $\alpha S589L\beta_F\gamma$, $\alpha S589V\beta_F\gamma$, $\alpha S589W\beta_F\gamma$, or $\beta_F\gamma$, as indicated. (A) Western blot immunostaining of solubilized wt and mutant ENaC subunits. Solubilized proteins were subjected to SDS-PAGE, and α ENaC was visualized on Western blots using an anti-rat α ENaC antibody (May et al., 1997). Molecular markers are indicated. (B) Western blot immunostaining for coimmunoprecipitation of wt and mutant ENaC under non-denaturing conditions. Non-denaturing coimmunoprecipitation was performed with an anti-FLAG antibody. Immunoprecipitates were subjected to SDS-PAGE and α ENaC was visualized on Western blots using an anti-rat α ENaC antibody. Specificity of the anti-rat α ENaC antibody is demonstrated by a control in which no α ENaC RNA was injected ($\beta_F\gamma$).

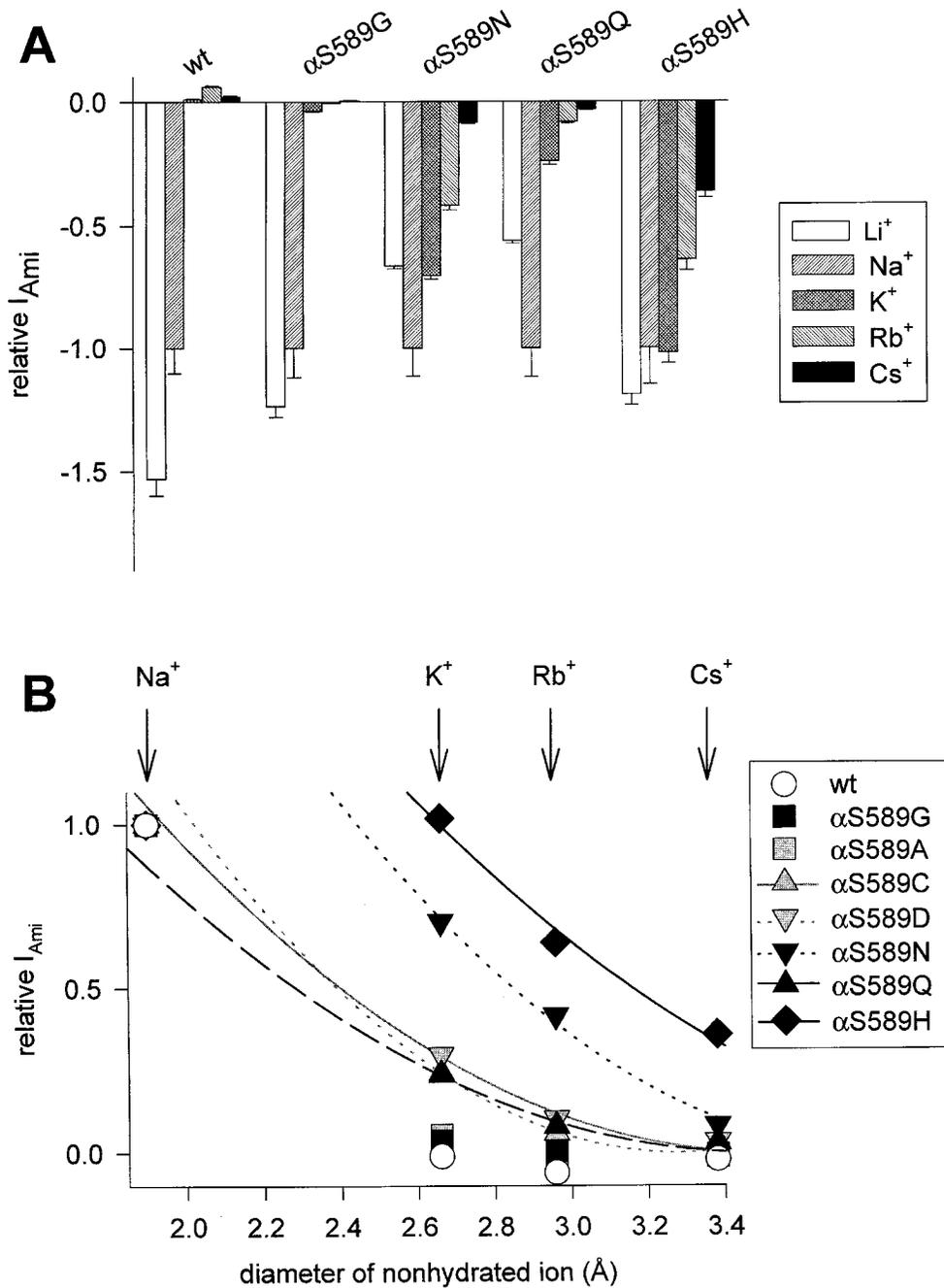


FIGURE 4. Selectivity of α S589 mutants to alkali metal cations. Two-electrode voltage-clamp recordings from *Xenopus* oocytes expressing ENaC wt or α S589 mutants. (A) Macroscopic amiloride-sensitive currents were measured at -100 mV in oocytes superfused with Li^+ , Na^+ , K^+ , Rb^+ , or Cs^+ external solutions (MATERIALS AND METHODS). Amiloride-sensitive currents are normalized to I_{Na} , and presented as mean \pm SEM ($n = 4-83$ per condition). Positive current values correspond to outward currents. (B) Relationship between the size of the ion and permeability. The normalized amiloride-sensitive currents are plotted as a function of the diameter of the nonhydrated ion. Fits to the equation $I_x/I_{Na} = f x [1 - (d_s/2)/(d_c/2)]^2$, where f is a scaling factor, d_s is the diameter of permeating spheres, and d_c is the diameter of the cylinder (the pore; Dwyer et al., 1980) are shown for the α S589 mutants that are permeable to at least K^+ and Rb^+ . Data and fits of the previously studied mutants α S589A, C, and D are shown in gray. The values of d_c obtained from the fit are 3.8 Å for α S589N, 3.5 Å for α S589Q, and 4.4 Å for α S589H.

i.e., $\alpha\beta_{F\gamma}$, α S589V $\beta_{F\gamma}$, α S589L $\beta_{F\gamma}$, α S589W $\beta_{F\gamma}$, and as a negative control, $\beta_{F\gamma}$ (Firsov et al., 1996). The ENaC channel complexes tagged with the FLAG epitope on β ENaC were immunoprecipitated with the anti-FLAG antibody under nondenaturing conditions, subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with an anti-rat α ENaC antibody. An α ENaC-specific band was detected with $\alpha\beta_{F\gamma}$ and α S589V/L/W $\beta_{F\gamma}$ but not $\beta_{F\gamma}$ (Fig. 3 B), indicating that the mutant subunits α S589V, α S589L, and α S589W retain their ability to assemble with β subunits. Thus, mutant α subunits with large amino acid substitutions at

position α 589 retain some ability to assemble with the β subunit but do not reach the cell surface.

Permeability of ENaC Mutants to Inorganic Cations

We have initially hypothesized that the increase in channel permeability to K^+ of the α S589 mutants was due to changes in the pore diameter (Kellenberger et al., 1999a). To obtain further evidence for such alterations in pore geometry, we measured the permeability properties of the different channel mutants to alkali and organic cations of different sizes and shapes. Fig. 4

T A B L E I I
Selectivity Sequences of Na⁺ and Larger Alkali Metal Cations and Organic Cations

| Mutant | Selectivity sequence | Cations not permeable ^a |
|--------------|--|---|
| wt | Na ⁺ | K ⁺ , Rb ⁺ , Cs ⁺ , NH ₄ ⁺ , MA, DMA, TriMA, guanidine |
| αS589C | Na ⁺ > K ⁺ = NH ₄ ⁺ > Rb ⁺ > guanidine | Cs ⁺ , MA, DMA, TriMA |
| αS589D | Na ⁺ > NH ₄ ⁺ = K ⁺ > Rb ⁺ > Cs ⁺ > guanidine | MA, DMA, TriMA |
| αS589N | NH ₄ ⁺ > Na ⁺ > K ⁺ > Rb ⁺ > Cs ⁺ > guanidine > MA | DMA, TriMA |
| αS589Q | Na ⁺ > NH ₄ ⁺ = K ⁺ > DMA = Rb ⁺ ≥ MA = guanidine = Cs ⁺ = TriMA | |
| αS589H | NH ₄ ⁺ ≥ Na ⁺ = K ⁺ > Rb ⁺ > guanidine > Cs ⁺ > MA ≥ DMA > TriMA | |
| βG529D, C, S | Na ⁺ > NH ₄ ⁺ ≥ K ⁺ > Rb ⁺ | MA ^b |

The selectivity sequences are based on data of experiments shown in Figs. 4 and 6, Table I, and Kellenberger et al. (1999a). “>” in the selectivity sequence means that the relative permeability between the two ions is significantly different ($P < 0.05$).

^a“Not permeable” means $I_x/I_{Na} < 0.02$.

^bPermeability of DMA, TriMA, and guanidine was not determined in these mutants.

A illustrates the amiloride-sensitive currents measured in the presence of different alkali metal cations at -100 mV, normalized to the values obtained with Na⁺ as the charge carrier (I_x/I_{Na}). When the main cation in the extracellular solution was K⁺, Rb⁺, or Cs⁺, wt ENaC showed small outward amiloride-sensitive currents at -100 mV (Fig. 4 A, upward bars) carried by intracellular Na⁺ ions. This indicates that ENaC wt is virtually impermeable to K⁺, Rb⁺, and Cs⁺. Among the mutants αS589G, αS589Q, αS589N, and αS589H, the ones with the highest permeability to K⁺ ions also show the highest conductance for Rb⁺ and Cs⁺. For all the mutants tested, the permeability sequence follows the size of the permeant ions, i.e., Na⁺ ≥ K⁺ > Rb⁺ > Cs⁺ (Table II). These results confirm the observation for amino acid substitutions of smaller sizes at position αS589, i.e., αS589A, αS589C, and αS589D (Kellenberger et al., 1999a) and further support the conclusion that αS589 mutations with larger side chain residues increase in pore diameter at the selectivity filter. For K⁺, Rb⁺, and Cs⁺ ions that are similar in size (Pauling diam, 2.7–3.4 Å), the permeability in αS589 mutants is inversely correlated with the size of the ion. This is demonstrated in Fig. 4 B, where the relationship between the relative permeability of the cation and its diameter is fitted by a simple equation that describes permeation based purely on molecular sieving (Dwyer et al., 1980; Kellenberger et al., 1999a; see Fig. 4 legend). However, the relative permeability of cations of very different sizes, as Na⁺ (1.9 Å) versus K⁺, Rb⁺, and Cs⁺, cannot be fitted by the above equation in most αS589 mutants (Fig. 4 B), indicating that mechanisms other than geometrical and frictional effects are critical for Na⁺ permeability. The fact that Na⁺ permeability is lower than expected for its diameter in the mutants αS589N and αS589H suggests that the selectivity filter becomes less favorable for permeation of Na⁺ ions when its diameter becomes

larger. Such an effect could also contribute to the lower macroscopic I_{Na} expressed by the αS589 mutants with large amino acid substitutions (Table I).

To test the possibility that the larger pore diameter makes it more difficult for small ions such as Na⁺ and Li⁺ to permeate the channel, we have performed single-channel recordings of different αS589 substitution mutants. Patch-clamp recordings of the functional αS589 mutants showed that the unitary conductance for Li⁺ and Na⁺ decreased with the increasing size of the substituent amino acid (Fig. 5 and Table I). For instance, the single-channel Li⁺ conductance was 9.0 pS for wt, 7.8 pS for αS589G, 1.5 pS for αS589C, and 1.9 pS for αS589Q, and <1 pS for the mutants αS589N and αS589H where single-channel openings could not be resolved. Thus, it appears that increasing the pore diameter at the selectivity filter with αS589 substitutions allows larger cations to permeate the channel to the prejudice of smaller ions such as Na⁺ or Li⁺.

Permeability of ENaC Mutants to Organic Cations

Organic cations such as ammonium and its methyl derivatives methyl-, dimethyl-, and trimethylammonium (MA, DMA, and TriMA) or guanidine have been used to estimate the minimum diameter of the pore of channels such as voltage-gated Na⁺, K⁺, and Ca²⁺ channels (Hille, 1992). These organic cations are not completely symmetrical like the alkali metal cations. To determine the permeabilities of organic cations in ENaC wt and the functional αS589 mutants, the amiloride-sensitive current carried by organic cations was measured at -100 mV and normalized to I_{Na} . Fig. 6 A shows the current-voltage relationship of the amiloride-sensitive current carried by Na⁺ or NH₄⁺ ions in oocytes expressing either ENaC wt or the αS589D mutant. Both, ENaC wt and the αS589 mutant show clear inward Na⁺ currents (Fig. 6 A, closed symbols). The αS589D Na⁺ current shows a

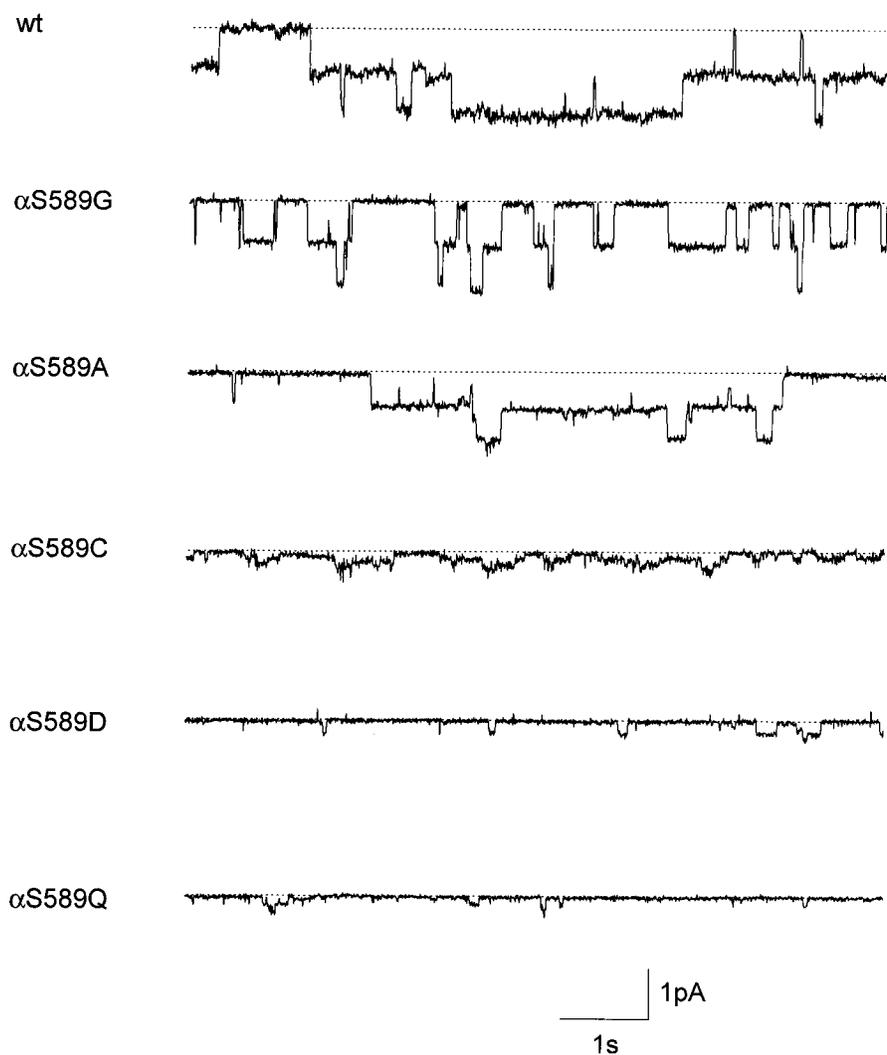


FIGURE 5. Single-channel records of ENaC wt and α 589 mutants. Traces are from outside-out patches from *Xenopus* oocytes at a holding potential of -100 mV in extracellular Li^+ solution. The dotted lines indicate the baseline when all ENaC channels in the patch are closed. The outside-out configuration was chosen to verify amiloride-sensitivity of channel activity.

strong inward rectification, which is due to the fact that the expression level of the mutant compared with the wt was low in this experiment and intracellular Na^+ concentration was much lower in the mutant compared with the wt (Kellenberger et al., 1998). For ENaC wt, no NH_4^+ inward current could be measured in oocytes even at very negative potentials (Fig. 6 A, open squares), whereas a substantial NH_4^+ inward current was evident in the mutant α S589D (Fig. 6 A, open circles). All α S589 mutants are permeant to NH_4^+ , as shown in Fig. 6 B. The permeability ratios of the α S589 mutants for K^+ or NH_4^+ ions are comparable, and as for K^+ ions, the channel permeability to NH_4^+ increases with the size of the residue at position α S589 (Table I). Regarding ammonium derivatives, the smaller substitution mutants (α S589G/A/C/D) are not permeable to any of the ammonium derivatives. α S589N is only permeant to the smallest derivative, MA, and α S589Q and α S589H show significant but low permeability to all three derivatives tested (MA, DMA,

and TriMA; Fig. 6 B). α S589C, D, N, Q, and H are permeable to guanidine.

The permeability data obtained with organic and inorganic cations from all functional α S589 mutants are summarized in Fig. 7. In this representation, the ions are aligned according to their increasing size from left to right (Fig. 7 legend) and the mutants are shown with increasing volume of the substituting amino acid from front to back. Fig. 7 illustrates two aspects of organic and inorganic cation permeability of α S589 mutants. First, substitution of α S589 by large amino acid residues increases the relative permeability of large inorganic and organic cations, i.e., increasing the size of the amino acid at position α 589 increases the pore diameter. Second, large cations have generally a lower permeability than small cations (Table II). NH_4^+ and guanidine carry higher currents than expected from their size. For the functional mutants with substituting residues larger than Ser, there is a significant correlation between the increase in volume of the amino acid

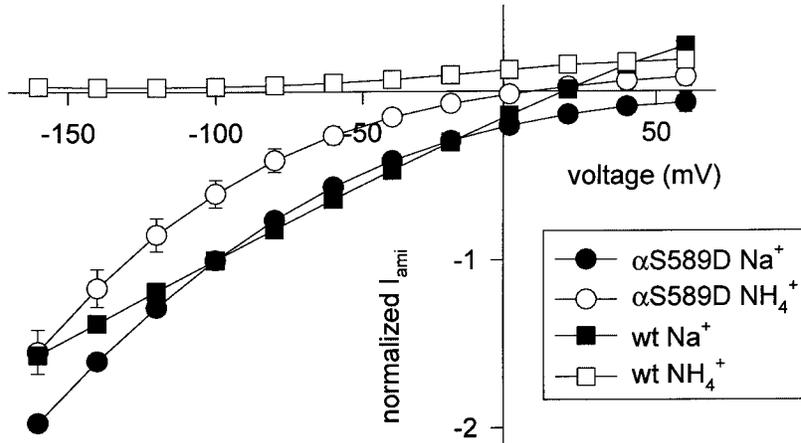
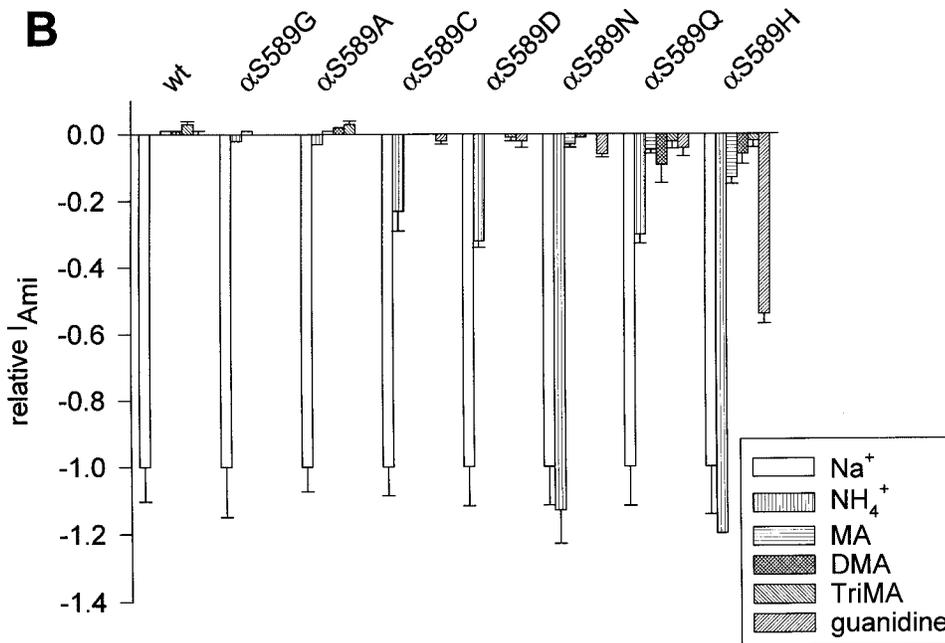
A**B**

FIGURE 6. Selectivity of α S589 mutants to organic cations. (A) I-V relationship of amiloride-sensitive Na^+ and NH_4^+ currents in *Xenopus* oocytes expressing either ENaC wt or the α S589D mutant. Currents were measured during 500-ms voltage-steps from a holding potential of -100 mV to test potentials of -160 to $+60$ mV in 20-mV increments. Currents measured in the presence of $5 \mu\text{M}$ amiloride were subtracted from currents measured in the absence of amiloride. Amiloride-sensitive currents were in each oocyte normalized to the amiloride-sensitive Na^+ current at -100 mV. (B) Macroscopic amiloride-sensitive currents were measured at -100 mV in oocytes superfused with Na^+ , NH_4^+ , MA, DMA, TriMA, or guanidine external solution (MATERIALS AND METHODS). Amiloride-sensitive currents are normalized to I_{Na} , and presented as mean \pm SEM ($n = 2-20$ per condition). Positive current values correspond to outward currents.

and the relative permeability to K^+ and NH_4^+ . Only for the α S589Q mutant, the K^+ and NH_4^+ permeability relative to Na^+ is lower than expected from its size. However, the α S589Q mutant still remains highly permeant to large cations such as K^+ and NH_4^+ , which is consistent with the general conclusion that substitution of α S589 by large amino acids increases the pore diameter at the selectivity filter. The particularity of the α S589Q mutant indicates that the size of the residue at position α 589 is not the only determinant of the pore diameter at the selectivity filter. Regarding the second observation addressed here, we found, in most mutants, a relative permeability for NH_4^+ and guanidine that was higher than expected from their size. NH_4^+ is somewhat flexible due to its ability to form hydrogen bonds (see DISCUSSION). Guanidine is of a similar size as

TriMA, but is a planar molecule in contrast to the more spherical shape of ammonium derivatives. α S589C, D, N, Q, and H are permeable to guanidine. The highest guanidine permeability ratio was obtained for α S589H ($I_{\text{guanidine}}/I_{\text{Na}} = 0.54 \pm 0.03$, $n = 10$; Fig. 6 B). The higher guanidine permeability relative to ammonium derivatives in the α S589C, D, N, and H mutants suggests that the pore at the selectivity filter of these mutants is not completely symmetrical but has the shape of a slotlike rectangle.

In addition to mutations of α S589, mutations of β G529 to Ser, Cys, and Asp (but not mutations to Ala and Arg) make ENaC permeable to K^+ . The $I_{\text{K}}/I_{\text{Na}}$ ratio was ~ 0.2 for β G529D and β G529S and 0.06 for β G529C (Kellenberger et al., 1999b). Mutations of β G529 that increase the $I_{\text{K}}/I_{\text{Na}}$ ratio also increase the $I_{\text{Rb}}/I_{\text{Na}}$ ratio,

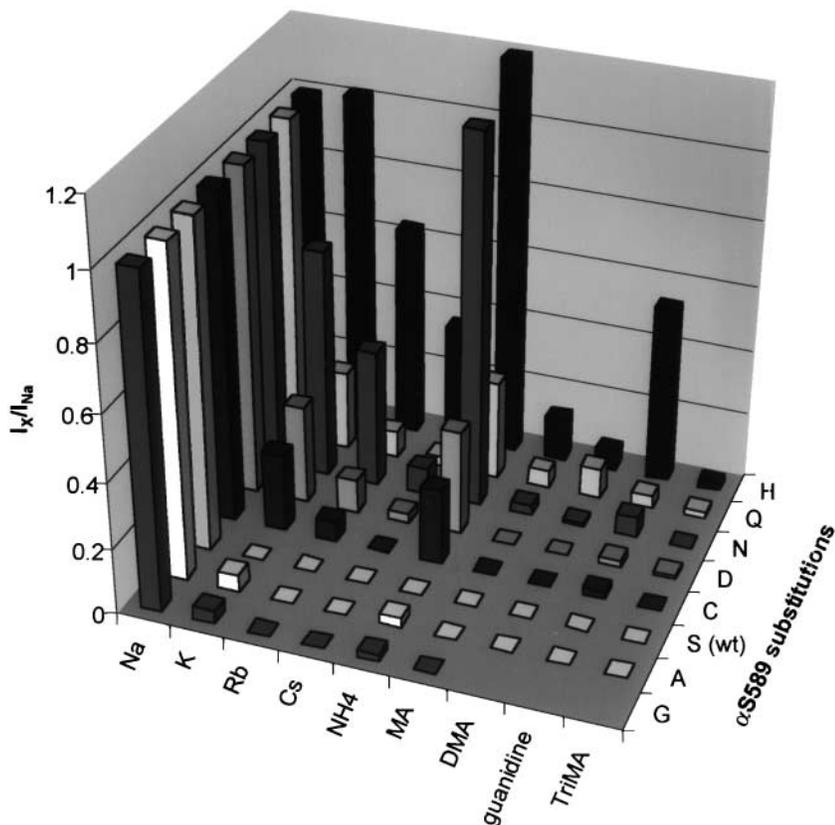


FIGURE 7. Graphical summary of the main results with inorganic and organic cations. Bar graph summarizing the amiloride-sensitive current ratios I_x/I_{Na} for all ions in all α S589 mutants tested. On the axis “ α S589 substitutions,” the different mutants are labeled by the single-letter code of the substituting amino acid. The ions are represented in the order of their size (diameter derived from Pauling radius for alkali metal cations and minimum diameter [Sun et al., 1997] for organic cations). The minimum diameter is as follows (in Å): 3.6 for NH_4^+ , 3.8 for MA, 4.6 for DMA, 6.0 for TriMA, and 5.8 for guanidine.

however, these mutant channels are not permeant to Cs^+ (Kellenberger et al., 1999b). The I_{NH_4}/I_{Na} ratio is 0.19 ± 0.09 , 0.46 ± 0.07 , and 0.17 ± 0.01 ($n = 5$) for β G529D, S, and D, respectively, and these three mutants are impermeable to MA (unpublished data). This is consistent with a mechanism similar to that of α S589 mutations in which these β G529 mutations change the ionic selectivity by enlarging the pore. The low permeability to large cations indicates that the β G529 mutations enlarge the pore by less than the α S589 mutations.

Coexpression of α S589N with β G529S and wt γ yielded a channel with reduced but still detectable current expression with an amiloride-sensitive Na^+ current at -100 mV of 0.1 ± 0.05 μA . The current ratio I_K/I_{Na} was 1.46 ± 0.23 ($n = 4$), showing a slightly higher selectivity to K^+ ions than the single substitution mutant α S589N. Thus, the effects of the α S589 and β G529 mutations on channel permeability to K^+ ions are additive, as expected for α and β subunits lining the channel pore and contributing to the selectivity filter.

Contributions of the Second Transmembrane Segment M2 and the Intracellular COOH Terminus to Ionic Selectivity

The increase in relative channel permeability to large cations in α S589 mutants indicates that α S589 is part of the selectivity filter. Adjacent residues not directly facing the pore lumen might also contribute to the ionic selec-

tivity of the channel by holding the selectivity filter in place and at the correct diameter. In addition it is possible that other amino acids along the ion permeation pathway may contribute to ion selectivity. We have replaced systematically all residues from L591 to M610 in the putative M2 segment of the α subunit with Cys residues to investigate the role of the second membrane-spanning segment M2 in ion selectivity. We have tested the accessibility of these engineered Cys residues to extracellularly applied sulfhydryl reagents. The hydrophilic sulfhydryl reagent MTSEA attaches covalently and selectively a charged group to Cys residues. If a substituted Cys residue lies on the water-accessible surface of the external pore, it should react with MTSEA, which would affect ionic current. The ratio of the I_{Na} after a 2-min extracellular application of 2.5 mM MTSEA relative to the initial I_{Na} was 0.77 ± 0.33 for ENaC wt and was not significantly different for the mutants (unpublished data). This suggests that the Cys residues in the M2 segment of α ENaC are not accessible to extracellular MTSEA, or that their modification does not change channel function, in contrast to residues in the preM2 segment of the ENaC subunits (Kosari et al., 1998; Snyder et al., 1999). When coexpressed with wt β and γ subunits, all mutants apart from α E598C produced functional channels. However, mutation α E598A was functional and was not different from wt with regard to ion selectivity and amiloride block. The mutants α L591C to

α M610C exhibit a I_K/I_{Na} in the range of 0.00–0.03 compared with 0.01 ± 0.00 of ENaC wt, indicating that the M2 segment of α ENaC does not contribute significantly to the ionic selectivity of the channel (Table I).

It was recently reported that truncation of the COOH terminus of rat α ENaC at residue R613 changed the Li^+ single-channel conductance and the Li^+/K^+ selectivity as determined from macroscopic currents (Jovov et al., 1999). In the context of this work, we have analyzed the properties of α R613X $\beta\gamma$ ENaC expressed in *Xenopus* oocytes. The unitary conductance, measured in outside-out patches was 4.7 ± 0.2 pS for Na^+ and 8.0 ± 0.4 pS for Li^+ ($n = 5$ patches each), directly compared with 4.6 ± 0.2 pS (Na^+) and 8.8 ± 0.4 pS (Li^+ ; $n = 2$ patches each) for ENaC wt. The α R613X $\beta\gamma$ mutant remained totally impermeant to K^+ ions, as shown by the macroscopic current ratio I_K/I_{Na} at -100 mV of zero as for ENaC wt. Thus, we have no indication that residues within the intracellular COOH terminus or M2 determine ion selectivity or unitary conductance. Therefore, we conclude that the selectivity filter is restricted to α G587– α S589 and the analogous residues in β and γ subunits.

Amiloride Sensitivity

Amiloride is a pore blocker and interacts with the permeating ions. We have addressed the possibility that in addition to changing ion selectivity α S589 mutations might also affect channel block by amiloride, since amiloride binds in the preM2 segment in the proximity of the selectivity filter (Fig. 1). We have analyzed the block by amiloride of currents generated by all α S589 mutants. In most mutants, amiloride block was not different from wt, except for α S589N, which marginally increased the IC_{50} and α S589H, which increased the IC_{50} for amiloride block by a factor of 16. With Na^+ as the main extracellular cation, the IC_{50} for amiloride block was 0.44 ± 0.05 and 2.80 ± 0.65 μ M for α S589N and α S589H, respectively, compared with 0.18 ± 0.08 μ M for ENaC wt ($n = 6$ –16). The important structural changes of the selectivity filter induced by the α S589H mutation that enlarges the channel pore allowing large cations to pass may extend to the nearby amiloride binding site and also impair channel block. We measured amiloride block of the NH_4^+ current for two mutants, α S589D and α S589N. The IC_{50} values for amiloride block of the NH_4^+ current were 0.10 ± 0.02 and 0.19 ± 0.06 μ M for α S589D and α S589N, respectively ($n = 6$ –7 each), indicating that amiloride blocks NH_4^+ currents with similar affinity as currents carried by Na^+ . The Cys substitution mutations in the M2 segment have no effect on amiloride block with an IC_{50} for the mutants α L591C to α M610C ranging from 0.06 to 0.33 μ M (unpublished data).

DISCUSSION

It was initially found that mutations of two residues, α S589 and β G529, change Na^+/K^+ selectivity and make ENaC permeable to K^+ and to a lesser extent to Rb^+ and Cs^+ (Kellenberger et al., 1999a,b). Subsequently, two additional mutations were shown to affect the channel K^+/Na^+ selectivity, α G587C at the position homologous to β G529 (Sheng et al., 1999) and γ S542C at the position homologous to α S589 (Snyder et al., 1999). The present work provides evidence that the changes in ionic selectivity caused by α S589 and β G529 mutations are due to an enlargement of the pore at the selectivity filter. Substitutions of α S589 by a variety of amino acids of different volume and subsequent analysis of the permeability of these mutant channels to different organic and inorganic cations of varying sizes show that the larger the substituting amino acid at position α S589, the easier it becomes for large organic and inorganic cations to permeate the channel. Enlargement of the pore at the selectivity filter not only allows large cations to permeate ENaC mutants, but also decreases channel permeability to small cations such as Na^+ or Li^+ ions. Thus, the pore size at the α S589 residue is critical for the selectivity properties of ENaC.

Ion Selectivity in wt and Mutant ENaC

Ionic selectivity of ENaC wt allows Na^+ and smaller cations such as Li^+ and H^+ to permeate the pore, whereas large cations such as K^+ , Rb^+ , and Cs^+ are excluded (Palmer, 1990). However, these monovalent nonpermeant cations block ENaC in a voltage-dependent manner, and as amiloride they are competitive blockers with regard to Na^+ ions. These observations indicate that K^+ , Rb^+ , and Cs^+ ions interact with Na^+ ions in the outer part of the channel pore down to the site where amiloride binds (i.e., α S583 and the corresponding residues β G525 and γ G537), but do not pass the selectivity filter. Thus, the outer pore of ENaC acts as a molecular sieve to discriminate among inorganic cations.

What are the consequences of enlarging the channel pore at the selectivity filter on the ion permeation properties of ENaC? Enlargement of the channel pore at the selectivity filter due to substitutions of α S589 by bulkier residues allows larger cations to pass through the selectivity filter (Kellenberger et al., 1999a). For all α S589 mutants, the permeability to K^+ , Rb^+ , and Cs^+ still depends on the relative size of the ion and of the pore as supported by the fit to the molecular sieving model (Fig. 4 B). However, enlargement of the pore is also associated with a decrease in permeability to small ions such as Na^+ and Li^+ . We observed that the permeability to Na^+ relative to K^+ ions of the α S589H and α S589N mutants is lower than expected for the size of Na^+ ions from the fit to the hydrodynamic model (Fig.

4 B). But more importantly, we demonstrated a reduction in the single-channel conductance for Li^+ and Na^+ ions in αS589 ENaC mutants that is inversely related to the increase in channel permeability to K^+ , Rb^+ , and Cs^+ ions. These two observations indicate that the higher the permeability of the αS589 mutant becomes to larger cations such as K^+ , Rb^+ , or Cs^+ , the more difficult it is for small ions such as Na^+ and Li^+ to permeate the channel. Thus, the geometry of the ENaC pore at the selectivity filter is critical for the relative permeability to Na^+ over K^+ and larger ions. A similar but more direct conclusion came from the solution of the 3-D structure of the K^+ channel KcsA. All K^+ channels show a selectivity sequence of $\text{K}^+ \approx \text{Rb}^+ > \text{Cs}^+$, whereas permeability for the smallest alkali metal cations Na^+ and Li^+ is immeasurably low. In the KcsA channel, the high selectivity for K^+ over smaller ions (Li^+ and Na^+) is based on the tight interaction of the permeating K^+ ion with the carbonyl oxygens of the backbone of the GYG sequence that lines the selectivity filter (Doyle et al., 1998). Permeant ions have to dehydrate to pass through the narrow channel pore. The ion-pore interaction is critical to compensate for the energetic cost of dehydration, and requires an optimal size ratio of the permeating ion and the selectivity filter. Na^+ ions are too small to tightly fit in the K^+ channel selectivity filter. As a consequence, the ion-pore interaction energy is too low to compensate for the cost of dehydration of Na^+ ions, making Na^+ permeation through K^+ channels almost impossible. Similarly, as the ENaC pore becomes larger in the αS589 mutants, accommodation of a Na^+ ion in the selectivity filter likely becomes energetically less favorable because of a lower interaction energy with the pore-lining residues, resulting in the observed decrease in unitary conductance.

Permeability to Organic Cations

NH_4^+ and its methyl derivatives, MA, DMA, and TriMA, all have the same basic geometry and differ only by the substitution of methyl groups for hydrogen atoms. Examples of channel proteins that select this series of cations entirely by molecular sieving are the nicotinic acetylcholine receptor (Adams et al., 1980; Dwyer et al., 1980) and voltage-gated Ca^{2+} channels in the absence of calcium (McCleskey and Almers, 1985). In these channels, the permeability to NH_4^+ -methyl derivatives decreases in a monotonic fashion with size or volume, as expected for pure molecular sieving. The analysis of the permeability of αS589 ENaC mutants to NH_4^+ and its methyl derivatives did not show a graded decrease in permeability with increasing size of the ion (Figs. 6 and 7 and Table II). All αS589 mutants except for αS589G and αS589A have a high permeability to NH_4^+ and all of these mutants are not, or only slightly, permeable to the methyl derivatives of ammonium. This suggests that

factors such as the shape of the ion and the interaction between the ion and the pore-lining residues codetermine ionic permeability in ENaC αS589 mutants. The magnitude of the current carried by NH_4^+ (I_{NH_4}) is in many αS589 mutants similar to I_{K} despite the fact that NH_4^+ is larger than K^+ , Rb^+ , and Cs^+ ions (diam = 3.6 Å; Sun et al., 1997). The high relative $\text{NH}_4^+/\text{Na}^+$ permeability ratio stresses the importance of interactions between the permeating ion and the pore for ion discrimination. NH_4^+ ions can readily permeate relatively small openings by formation of hydrogen bonds with pore-lining oxygen-containing groups thereby reducing the N-O distances (Hille, 1992). The effective radius of the NH_3^+ group when probed by an oxygen ligand (of the channel pore) was estimated to be 1.4 Å (Hille, 1992). The high permeability of NH_4^+ in αS589 mutants suggests that NH_4^+ indeed does form hydrogen bonds in the ENaC pore. Due to this flexibility, NH_4^+ can adapt to different pore conformations, resulting in a higher permeability in αS589N or αS589H for NH_4^+ compared with Na^+ or K^+ ions. NH_4^+ can, in principle, form hydrogen bonds either with carbonyl oxygens of the peptide backbone of the residue at position α589 or in the case of the mutants αS589D , αS589Q , and αS589H with the oxygen of the side chain. High permeability to NH_4^+ was not only observed in the substitution mutants containing oxygens in the α589 side chain, but also in mutants without oxygens in the side chain at positions α589 and βG529 ($\alpha\text{S589C/H}$ mutants as well as $\beta\text{G529C/S}$ mutants). To explain the high NH_4^+ permeability of the latter mutants, the carbonyl oxygens have to be involved in hydrogen bonds with NH_4^+ , supporting the hypothesis that, as for the KcsA channel, the carbonyl residues of αS589 line the channel pore at the selectivity filter.

An Estimate of the Size of the ENaC Pore

An estimate of the minimum diameter of the wt and mutant ENaC selectivity filter can be obtained from the largest permeant and the smallest nonpermeant ion. It is assumed that ions permeating highly selective channels are completely dehydrated or carry at most one water molecule with them in the narrowest part of the pore (Doyle et al., 1998), and therefore the size of the nonhydrated ion gives a good estimate of the pore at this site. Ionic (Pauling) radii are established from contact distances between touching, nonbonding atoms in crystals (Hille, 1992). The diameter of Na^+ and K^+ ions derived from their Pauling radius is 1.9 and 2.7 Å, respectively. Therefore, the minimum pore diameter of ENaC wt at its narrowest point is estimated to 1.9–2.7 Å. The pore diameters of αS589A and αS589G , which are permeant to K^+ but not to Rb^+ , is estimated to be between 2.7 and 3.0 Å, and that of αS589C (permeant to Rb^+ but not to Cs^+) is between 3.0 and 3.4 Å. The fits to the relation-

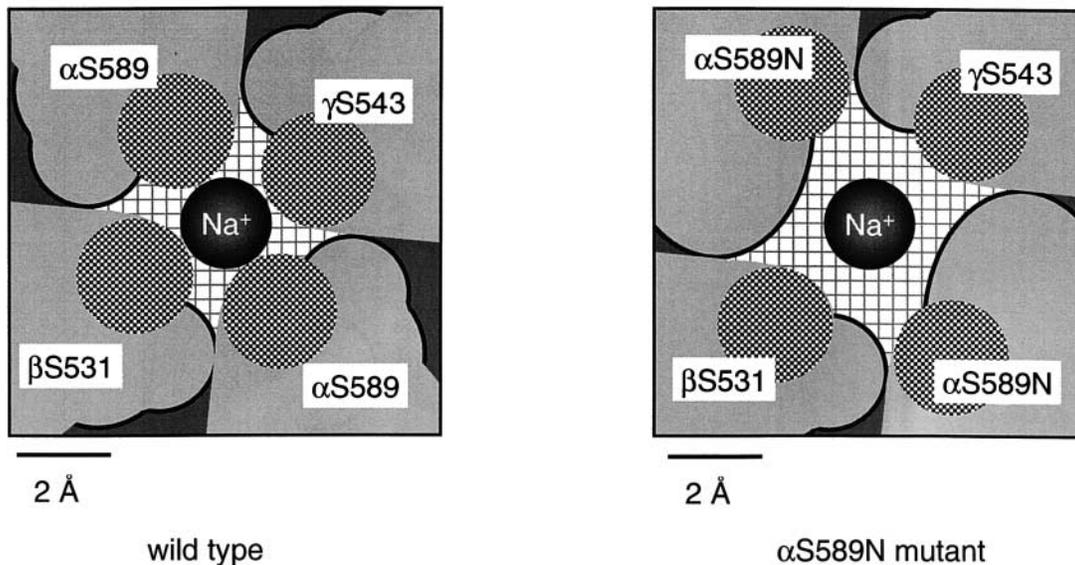


FIGURE 8. Model illustrating the mechanism by which mutations of α S589 may enlarge the channel pore at the selectivity filter. Residue α S589 and analogues in the β and γ subunit arranged around the channel pore are shown in a cross-section of the pore seen from the top for wt ENaC (left) and the mutant α S589N (right). Each residue is represented by its backbone part in gray, with the carbonyl oxygen shown as a hatched circle pointing toward the pore lumen and the side chain identified by the black edge. The side chains are located at the interface between the subunits. Increasing the size of the side chain of the two α S589 residues adds extra volume at the subunit-subunit interface and makes the pore wider.

ship between the alkali cation permeability and the ion diameter in α S589 mutants that are permeant to Cs^+ (Fig. 4 B) predict a pore radius in these mutants of the range of 3.5 (α S589D) to 4.4 Å (α S589H).

The organic cations that we have tested are not perfect spheres as the alkali metal cations. Judged from their minimum diameter, i.e., the smallest possible diameter of a circular pore through which the molecule would pass, all organic cations tested are larger than Cs^+ (Sun et al., 1997). The permeability pattern of these asymmetric cations (Figs. 6 and 7) allows predictions about the shape of the ENaC pore at the selectivity filter. The observation that the planar guanidine can permeate the α S589C mutant which is not permeable to Cs^+ (3.4 Å) and the fact that guanidine has a higher permeability than NH_4^+ derivatives in the α S589N, Q and H mutants indicate that the pore of mutant ENaC rather forms a slotlike rectangle. Voltage-gated Na^+ channels are permeable to NH_4^+ ($I_{\text{NH}_4}/I_{\text{Na}} = \sim 0.2$), to guanidine and to some of its derivatives but not to methyl derivatives of ammonium (Hille, 1992; Sun et al., 1997). From these permeation data, the minimum pore size of voltage-gated Na^+ channels was estimated to a rectangle of 3.1×5.1 Å (Hille, 1992). The permeability of K^+ channels to organic cations is more restricted, and the largest molecule passing through K^+ channels is NH_4^+ (Hille, 1992). The minimum pore size of voltage-gated K^+ channels was estimated to be a circle with a diameter of 3.3 Å (Hille, 1992). The ENaC wt pore is narrower than that of either of these channel

types and the shape of the cross-sectional area of ENaC α S589 mutants appears to be closer to that of voltage-gated Na^+ than K^+ channels.

Model of the ENaC Pore

There is good evidence that the preM2 segments of ENaC subunits line the outer part of the channel pore. The preM2 segments contain the binding site for the pore blocker amiloride, S583 in α and homologous residues in β and γ ENaC (Schild et al., 1997). Cys residues introduced at this position can bind extracellular zinc ions and can be modified by extracellular sulfhydryl reagents resulting in channel block (Schild et al., 1997; Firsov et al., 1998; Kosari et al., 1998; Snyder et al., 1999). The outer channel pore is relatively wide at the amiloride binding site (diam ~ 5 Å; Kellenberger et al., 1999b) and gets narrower toward the selectivity filter. Mutations of the Gly and Ser residues at α 587 and corresponding positions in β and γ subunits have dramatic effects on single channel conductance and ENaC affinity for Na^+ and Li^+ ions, indicating that these residues tightly interact with the permeating ions. The residue α S589 that determines the channel permeability cutoff is in the continuation of the amiloride binding site (position α S583) and the Na^+/Li^+ binding site (position α G587). The 583–589 sequence might represent a β sheet structure that precedes the putative transmembrane spanning M2 helix and lines the outer ion channel pore. It is quite understandable that introducing large residues such as His at position α 589 in-

duce steric changes in the pore with consequences on channel block by amiloride. All other functional α S589 mutants preserved the high affinity of ENaC wt for amiloride block, indicating that the selectivity filter does not overlap with the amiloride binding site.

How do substitutions of α S589 by large residues increase the diameter of the pore and facilitate the passage of large organic cations such as ammonium or guanidine? This effect on the pore size is only possible if the side chain of residue α S589 points away from the pore lumen. Thus, most likely the carbonyl oxygens of the peptide backbone face the pore lumen to accommodate permeating cations. This orientation of the carbonyl oxygens is also suggested by the high permeability of NH_4^+ in α S589 mutants. This orientation of the polypeptide is similar to the KcsA channel in which the carbonyl oxygens are exposed to the pore lumen and the side chains of the amino acids point away from the pore. The side chain of α S589 and of the homologous Ser residues in β and γ subunits may point toward the interface between the subunits. Fig. 8 illustrates possible steric changes that may occur when α S589 is replaced by larger residues. This figure shows schematically a cross-section of the channel pore at residue α S589 in wt ENaC (Fig. 8, left) and the α S589N mutant (Fig. 8, right). The carbonyl oxygens of the polypeptide backbone, shown as hatched circles, face the pore lumen. In our model, the side chains of the residues at position α 589 and the homologous positions in β and γ ENaC, identified by the dark border, are oriented toward the subunit interface. Increasing the size of the residue at position α 589 pushes the backbone of the β and γ subunits away from the pore lumen, leading to an enlargement of the pore diameter (Fig. 8, right). Due to the α - β - α - γ subunit arrangement around the channel pore, large amino acid substitutions at the α - β and α - γ subunit interface may result in changes in the pore diameter predominantly in the direction of one axis (Firsov et al., 1998). Such a rectangular geometry of the ion-selective region of the pore could account for the higher channel permeability to the planar guanidine compared with bulky organic cations in α S589 mutants. We have shown that larger substituting residues of α S589 lead to a wider pore at the selectivity filter and that the enlargement depends on the size of the residue (Table I). The only exception from this rule is the α S589Q mutant that shows a lower permeability to large cations such as K^+ and NH_4^+ than expected from its size, although this mutant is still highly permeant to K^+ and NH_4^+ . This indicates that the size of the residue at position α 589 is not the only determinant of the pore diameter. Other factors such as interactions between α S589 and adjacent residues may also control the diameter of the channel pore at the selectivity filter. These factors are not considered in our

model. Substitution of α S589 by smaller residues such as Gly and Ala resulted in an increased K^+ permeability. This observation suggests that other structures in the selectivity filter prevent the pore from collapsing when α S589 is replaced with smaller residues.

Mutations at position 529 of the β subunit resulted in a smaller pore enlargement compared with α S589 mutations. This can be explained by the fact that there is one β subunit in an ENaC tetramer compared with two α subunits (Firsov et al., 1998). Alternatively, the smaller effects of β G529 mutations on K^+/Na^+ selectivity may be a function of the position of this residue in the pore. β G529 is two residues more NH_2 -terminal in the sequence than α S589 (Fig. 1).

In this context, it is interesting to note that the substitution of α S589 by amino acids with a van der Waals volume $\geq 118 \text{ \AA}^3$ is not compatible with channel function at the cell surface (Table I). Given the steric effects of the α S589 mutations on the pore geometry it is possible that substitution by large amino acids prevents correct folding and assembly of subunits in the ER. Such changes in the ENaC biosynthetic process would direct the channel to degradation by a quality control in the ER. Mutation of the Ser homologous to α S589 has been shown to result in nonfunctional channels in other ENaC/DEG family members, DEG-1 (Garcia-Anoveros et al., 1995), MEC-4 (Hong and Driscoll, 1994), and ASIC2a (Waldmann et al., 1996). Recently, a mutation of the homologous residue in human α ENaC, S562, to leu was found in a patient with pseudohypoaldosteronism type 1, which is consistent with loss of channel function (Schaedel et al., 1999).

We use here a model of the channel pore that is based on the tetrameric stoichiometry of ENaC (Coscoy et al., 1998; Firsov et al., 1998; Kosari et al., 1998). An alternative ENaC subunit stoichiometry of eight or nine subunits also has been proposed (Snyder et al., 1998; Eskandari et al., 1999). The present work together with previous studies provides evidence that the carbonyl oxygens of all subunits that form the channel line the pore at its narrowest part (Kellenberger et al., 1999a,b). K^+ channels are tetramers and form a selectivity filter that is slightly wider than that of wt ENaC. It seems very unlikely that nine subunits can be arranged around a unique ion permeation pathway with all nine subunits lining the pore to form a narrow pore of a diameter smaller than K^+ channels.

It is not clear what the structures are that interact with the external channel pore to maintain the selectivity filter at the proper diameter and position. It is conceivable that mutations within these neighboring structures also affect channel ionic selectivity. The predicted transmembrane segment M2 could be part of this scaffold, or it might form the continuation of the pore. Our mutagenesis screen shows that M2 is not involved in ion discrimi-

nation. This transmembrane domain of the ENaC subunit is not accessible to extracellular sulfhydryl reagents. We extended our mutational analysis to the cytoplasmic COOH terminus of the α subunit because truncation of this region was recently claimed to influence ionic selectivity and single-channel conductance (Jovov et al., 1999). As for the truncation in the β subunit (Schild et al., 1996), the α COOH terminus truncation has no effect on the permeation properties of the channel. Together, these observations indicate that the ion-selective region of ENaC is restricted to a short sequence of three residues in the preM2 segment that represents the narrowest part of the external channel pore.

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