Charged residues in the M2 region of α-hENaC play a role in channel conductance

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Langloh, Anne Lynn B., Bakhrom Berdiev, Hong-Long Ji, Kent Keyser, Bruce A. Stanton, and Dale J. Benos. Charged residues in the M2 region of α-hENaC play a role in channel conductance. Am. J. Physiol. Cell Physiol. 278: C277–C291, 2000.—The epithelial Na+ channel (ENaC) is a low-conductance channel that is highly selective for Na+ and impermeable to anions. The molecular basis underlying these conduction properties is not well known. Previous studies with the ENaC subunits demonstrated that the M2 region of α-ENaC is critical to channel function. Here we examine the effects of reversing the negative charges of highly conserved amino acids in α-subunit human ENaC (α-hENaC) M1 and M2 domains. Whole cell and single-channel current measurements indicated that the M2 mutations E568R, E571R, and D575R significantly decreased channel conductance but did not affect Na+ -K+ permeability. We observed no functional perturbations from the M1 mutation E108R. Whole cell amiloride-sensitive current recorded from oocytes injected with the M2 α-hENaC mutants along with wild-type (wt) β- and γ-hENaC was low (46–93 nA) compared with the wt channel (1–3 µA). To determine whether this reduced macroscopic current resulted from a decreased number of mutant channels at the plasma membrane, we coexpressed mutant α-hENaC subunits with green fluorescent protein-tagged β- and γ-subunits. Confocal laser scanning microscopy of oocytes demonstrated that plasma membrane localization of the mutant channels was the same as that of wt. These experiments demonstrate that acidic residues in the second transmembrane domain of α-hENaC affect ion permeation and are thus critical components of the conductive pore of ENaC.

site-directed mutagenesis; Xenopus oocytes; dual-electrode voltage clamp; planar lipid bilayers; green fluorescent protein; biotinylation; confocal microscopy; channel pore

SINCE THE CLONING OF THE epithelial amiloride-sensitive Na+ channel (ENaC), many of its biochemical and electrophysiological characteristics have been elucidated. Human ENaC (hENaC), which is predominantly found in the epithelia of the colon, lung, and kidney, is composed of three subunits, α, β, and γ (18, 19, 27). The α-subunit alone forms an amiloride-sensitive Na+ channel when expressed in Xenopus oocytes. Coexpression of all three subunits yields a whole cell current ~20-fold larger than that observed with α-subunit only (18). The hENaC homologue δ-NaCh, which is expressed mainly in brain, pancreas, testis, and ovary, also produces a small amiloride-blockable conductance in oocytes that is potentiated by coexpression of the β- and γ-hENaC subunits. However, the biophysical properties of the δγ-channel are different from those of the αβγ-channel (28). Therefore, it has been proposed that α-subunits (or δ-subunits) form the conductive moiety and control the conductive characteristics of the multimeric channel and that the β- and γ-subunits are auxiliary proteins that augment channel function (4). The α-subunit is also a key target for the channel-blocking drug amiloride. Specific amino acid residues in the predicted extracellular loop of this subunit are important to amiloride binding and block of channel activity (10, 12). One feature of the channel that is not well defined is the conductive pore. By analogy to inwardly rectifying (Kir) and voltage-gated K+ channels, which share a homologous pore region, it has been postulated that amino acids in specific positions in the extracellular and transmembrane domains of ENaC are important for determination of ion selectivity, permeability, and conductance.

The putative pore region of Kir channels occurs in the small extracellular loop between the two membrane-spanning domains of each subunit in the channel. It contains the P loop, a critical feature of which is the highly conserved K+ channel signature sequence (Gly-Tyr-Gly) that determines ion selectivity. This channel is a tetramer in which the second transmembrane domain (inner helix) of each subunit is arranged symmetrically around the pore. The positioning of these helices, as well as the location of specific residues in the helices, controls the characteristics of ion conduction in the pore (20). Also, P loops have been characterized in voltage-gated K+ channels, where they occur in the segment connecting the fifth and sixth membrane-spanning regions of the constituent subunits (17, 30, 31). In all cases, P loops serve as the selectivity filter that attracts and concentrates K+ (16).

Hydropathy analysis of the cloned members of the ENaC/degenerin superfamily has shown them to be structurally similar to renal outer medulla K+ channel and Kir K+ channels, with two large hydrophobic regions connected by an extracellular segment. Stretches of amino acids that attracts and concentrates K+ (16).
acids within each hydrophobic region are long enough to span the membrane and are predicted to have α-helical structure (transmembrane domains M1 and M2). The hydrophobic residues downstream of M1 (H1 domain) and upstream of M2 (pre-M2 or H2 domain) are extracellular and assume β-sheet or β-barrel conformations (3). Recent studies with α-subunit rat ENaC (α-rENaC) support a K⁺ channel-like P loop model in which the pre-M2 region dips into the membrane, possibly contributing to the pore of the channel (22). Previous studies of α-ENaC splice variants and chimeras have indicated that the second transmembrane domain is clearly important for Na⁺ channel function (15, 26, 29). Schill et al. (24) demonstrated that mutating a serine residue (S583C) within the predicted H2 domain is critical for Na⁺ channel function. Schild et al. (24) demonstrated that mutating a serine residue (S583C) within the predicted H2 domain is critical for Na⁺ channel function. Schild et al. (24) demonstrated that mutating a serine residue (S583C) within the predicted H2 domain is critical for Na⁺ channel function. Schild et al. (24) demonstrated that mutating a serine residue (S583C) within the predicted H2 domain is critical for Na⁺ channel function. Schild et al. (24) demonstrated that mutating a serine residue (S583C) within the predicted H2 domain is critical for Na⁺ channel function. Schild et al. (24) demonstrated that mutating a serine residue (S583C) within the predicted H2 domain is critical for Na⁺ channel function.

Previously, a point mutation that results in reduced single-channel conductance (to Na⁺ and Li⁺) and increased sensitivity to external Ca²⁺ block. We have performed α-helical wheel analysis of the two hydrophobic domains of α-hENaC. The analysis indicated that there are three negatively charged residues that occur on the hydrophilic face of the M2 helix and one such residue in the M1 helix. We hypothesize that these negative charges are part of the conduction pore of the multimeric channel and are therefore critical to channel function. To test this hypothesis, we used site-directed mutagenesis to reverse these charges in α-hENaC. We then assessed the effects of such mutations by examining the whole cell and single-channel Na⁺ current produced by Xenopus oocytes injected with mutant α-hENaC subunits along with wild-type (wt) β- and γ-hENaC subunits. To determine the localization of wt and mutant hENaC channel proteins in the oocytes, we injected enhanced green fluorescent protein (EGFP)-tagged ENaC constructs and examined their localization with confocal laser scanning microscopy.

**MATERIALS AND METHODS**

**Preparation of Site-Directed Mutants**

The full-length α-, β-, and γ-hENaC cDNAs were a kind gift of Dr. M. J. Welsh (University of Iowa). Site-directed mutants were created using the Quick Change mutagenesis kit (Stratagene, La Jolla, CA). Primers were designed to be complementary to the sense and antisense sequences of a particular region of α-hENaC. Each set of primers contained the necessary base changes required to code for an arginine instead of the wt glutamic or aspartic acid. In the cases reported here, two adjacent bases in each primer were altered from the wt sequence. Plasmid DNA was subjected to the following PCR protocol, using one set of mutagenic primers: 1 cycle at 95°C for 1 min to denature the DNA and then 16–17 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 12–13 min. Parental (nonmutated) DNA was removed from completed PCR reactions by digestion with Dpn I at 37°C for 1 h. This enzyme is specific for methylated DNA and will not digest the nonmethylated PCR products. These products were then transformed into supercompetent Escherichia coli (XL1 BLUE, Stratagene) and grown on Luria-Bertani (+50 μg/ml ampicillin) agar overnight at 37°C. Colonies were picked and grown up overnight, and the DNA was isolated according to a standard alkaline lysis miniprep procedure. Positives for the specific mutation encoded by the PCR primers were confirmed by dideoxy sequence analysis.

**In Vitro Transcription**

Mutated DNA samples were in vitro transcribed using the SP6 and T7 mMessage mMACHINE kits (Ambion, Austin, TX). Briefly, ~1 μg of DNA was combined with appropriate reaction buffer, a mixture of all ribonucleotide triphosphates and m(7)G(5)ppp(5)’G analog, and SP6 or T7 RNA polymerase. Transcription proceeded at 37°C for 5–6 h. Template DNA was digested with DNase at 37°C for 15 min and then extracted and precipitated. The quality and size of the cRNA was confirmed by denaturing formaldehyde-agarose gel electrophoresis. RNA concentration was approximated by ultraviolet spectrophotometric measurement of optical density (λ = 260 nm).

**Oocyte Preparation and Microinjection**

Oocytes were surgically removed from ice-water-anesthetized adult female Xenopus laevis by standard techniques. Surrounding follicle cells were removed by digestion with 3 mg/ml collagenase (Boehringer Mannheim, Indianapolis, IN) in Ca²⁺-free OR-2 medium (in mM: 82.5 NaCl, 2.4 KCl, 1.8 MgCl₂, and 5 HEPES, pH 7.4) for 45–90 min at room temperature with constant agitation. Defolliculated oocytes were washed several times with OR-2 and allowed to recover for 24 h in half-strength Leibovitz medium (0.5× L-15; Sigma, St. Louis, MO) at 18°C. Groups of stage V and VI eggs were injected via a microinjector (World Precision Instruments, Sarasota, FL) with 50 nl (12.5 ng) of the following cRNAs (all subunit mixtures were 1:1:1): 1) wt α-hENaC + wt β-hENaC + wt γ-hENaC (wt γ; 2) M1 mutant α-hENaC (αE108R) + wt β + wt γ; 3) M2 E568R mutant α-hENaC (αE568R) + wt β + wt γ; 4) M2 E571R mutant α-hENaC (αE571R) + wt β + wt γ; 5) M2 D575R mutant α-hENaC (αD575R) + wt β + wt γ; 6) E568R + E571R + D575R mutant α-hENaC (α triple mutant) + wt β + wt γ.

When injected oocytes were to be processed for membrane vesicles to be incorporated into planar bilayers, 80–100 eggs were injected with the appropriate cRNA. Otherwise, 10–20 eggs were injected for dual-electrode voltage-clamp recordings. In both cases, injected oocytes were maintained for 2 days in 0.5× L-15 at 18°C before processing or recording.

To demonstrate that the EGFP-rENaC constructs that were used for confocal laser scanning fluorosence microscopy experiments produced whole cell amiloride-sensitive Na⁺ current similar to that produced by the constructs above, normal (not albino) oocytes were injected with 12.5 ng of the following combinations of cRNAs (in a 1:1:1 ratio): wt α-hENaC + GFP-β-rENaC + GFP-γ-rENaC; and D575R α-hENaC + GFP-β-rENaC + GFP-γ-rENaC.

**Oocyte Membrane Vesicle Preparation**

Oocytes injected with cRNA were washed three times with 3 ml of high-K⁺ 300 mM sucrose buffer (in mM: 400 KCl, 5 Pipes, and 300 sucrose), and homogenized in 600 μl of the same buffer, on ice for 5 min. The homogenate was centrifuged through a sucrose gradient (high K⁺-20% sucrose and high K⁺-50% sucrose) at 18,500 rpm for 30 min. The resulting interface between the gradients was drawn off, diluted with 3–4 ml of high-K⁺ buffer, and centrifuged at 23,500 rpm for 45
Electrophysiological Recording

Whole cell. Membrane currents in oocytes were evaluated at 20°C by double-electrode voltage clamp. Oocytes were impaled with two 3 M KCl-filled electrodes with resistances of 0.5–2.0 MΩ, connected to a TEV-200 voltage-clamp system (Dagan, Minneapolis, MN). Two reference electrodes were connected to the bath by 3 M KCl-3% agar bridges. The bathing solution (ND-96; in mM: 96 NaCl, 1 MgCl₂, 1.8 CaCl₂, 2 KCl, and 5 HEPES, pH 7.4) was perfused by gravity at a rate of 1.5 ml/min. The voltage clamp was controlled by pCLAMP 5.5 software (Axon Instruments, Burlingame, CA), and current was constantly monitored on a strip chart recorder. Oocytes were clamped at a holding potential of 0 mV. Current/voltage (I-V) relations were acquired by stepping the holding potential at 500-ms intervals in 20-mV increments from −100 to +80 mV. I-V data were recorded 4–5 min after impalement of the oocyte and then again 3 min after the addition of 10 μM amiloride to the bath. Data analysis was performed with pCLAMP 5.5 software.

Planar lipid bilayers. Oocytes expressing the different mutant or wt α-hENaC cDNAs (along with wt β- and wt γ-hENaC) were processed to yield enriched membrane vesicles that were fused with artificial planar lipid bilayers. Bilayers were composed of a phospholipid solution containing a 2:1 mixture of diphytanoyl-phosphatidylethanolamine and diphytanoyl-phosphatidylserine in n-octane. Bilayers were bathed with 100 mM NaCl and 10 mM MOPS-Tris (pH 7.4) solutions. Applied voltage was referred to the virtually grounded trans chamber. Amiloride was added to the trans compartment to give a final concentration of 0.3 μM. Ion selectivity of the channels incorporated into the bilayer was examined by substituting 100 mM KCl for 100 mM NaCl in the cis compartment. Records were digitally filtered at 100 Hz using pCLAMP software, subsequent to acquisition of the analog signal filtered at 300 Hz with an 8-pole Bessel filter before acquisition at 1 ms/point.

Cell-attached patch clamp. Oocytes were shrunken in hypertonic medium, and the vitelline membranes were removed before patch clamping. The cell-attached configuration was used to record single-channel current with an Axopatch 1B amplifier (Axon Instruments). The borosilicate glass pipettes were made with a PP-83 vertical puller (Narishige, Foster City, CA). The fusion cDNA pEGFP-β-rENaC was constructed by digesting the subcloned PCR product with SalI/BsmI and ligating the gel-purified 316-bp PCR fragment into the SalI/BsmI-digested pEGFP-β-rENaC cDNA. EGFP-β-rENaC was subcloned from pEGFP-β-rENaC into pcDNA3.1 (Invitrogen) using NheI and KpnI (to generate pcDNA3.1/EGFP-β-rENaC). The sequence of both strands was confirmed by ABI PRISM dye terminator cycle sequencing. planar lipid bilayers. GFP-γ-rENaC was constructed by excising γ-rENaC from pSport/γ-rENaC with SalI/KpnI and ligating the excised fragment into SalI/KpnI-digested pGFP-C2 (Clontech). GFP-γ-rENaC was subcloned from pGFP-C2/GFP-γ-rENaC into pcDNA3.1 (Invitrogen) using NheI. pcDNA3.1 was digested with NheI and treated with calf intestinal alkaline phosphatase to prevent self-ligation (to generate pcDNA3.1/GFP-γ-rENaC). The sequence of both strands was confirmed by ABI PRISM dye terminator cycle sequencing. These GFP constructs are referred to throughout as GFP-β-rENaC and GFP-γ-rENaC.

Oocyte Preparation for Confocal Microscopy

Adult female albino X. laevis frogs were obtained from Xenopus I (Dexter, MI) and maintained in the same conditions as normal X. laevis frogs. Stage V and VI oocytes were isolated from an ice/cetraine-anesthetized frog, as described above. Eggs were defolliculated with 3 mmg/ml collagenase (Boehringer Mannheim) in Ca²⁺-free OR-2 medium for 1 h at room temperature with constant agitation. They were washed several times in OR-2 and then stored in 0.5× L-15 at 18°C. Oocytes were injected 24 h postisolation with 12.5 ng (in 50 nl) of the following dRNAs (in a 1:1:1 ratio): wt α-hENaC + GFP-β-rENaC + GFP-γ-rENaC; mutant α-hENaC + GFP-β-rENaC + GFP-γ-rENaC; and GFP-β-rENaC + GFP-γ-rENaC. A group of 10 oocytes was also injected with 50 nl of water, as a control for background fluorescence. α-hENaC and EGFP dRNAs were generated with the mMessage mMachine in vitro transcription kits, as described above. Maximum hENaC channel activity was observed in oocytes ~48 h after dRNA injection. Therefore, oocytes were processed for confocal microscopy 2 days postinjection. To identify the plasma membrane, 10 eggs from each injection group were surface biotinylated. The eggs were equilibrated in ND-48 (in mM: 48 NaCl, 48 N-methyl-D-glucamine chloride, 1 MgCl₂, 1.8 CaCl₂, 2 KCl, and 5 HEPES, pH 7.4). Oocytes were incubated 24 h postisolation with 12.5 ng (in 50 nl) of the following dRNAs (in a 1:1:1 ratio): wt α-hENaC + GFP-β-rENaC + GFP-γ-rENaC; mutant α-hENaC + GFP-β-rENaC + GFP-γ-rENaC; and GFP-β-rENaC + GFP-γ-rENaC. A group of 10 oocytes was also injected with 50 nl of water, as a control for background fluorescence. α-hENaC and EGFP dRNAs were generated with the mMessage mMachine in vitro transcription kits, as described above. Maximum hENaC channel activity was observed in oocytes ~48 h after dRNA injection. Therefore, oocytes were processed for confocal microscopy 2 days postinjection. To identify the plasma membrane, 10 eggs from each injection group were surface biotinylated. The eggs were equilibrated in ND-48 (in mM: 48 NaCl, 48 N-methyl-D-glucamine chloride, 1 MgCl₂, 1.8 CaCl₂, 2 KCl, and 5 HEPES, pH 7.4). Oocytes were incubated 24 h postisolation with 12.5 ng (in 50 nl) of the following dRNAs (in a 1:1:1 ratio): wt α-hENaC + GFP-β-rENaC + GFP-γ-rENaC; mutant α-hENaC + GFP-β-rENaC + GFP-γ-rENaC; and GFP-β-rENaC + GFP-γ-rENaC. A group of 10 oocytes was also injected with 50 nl of water, as a control for background fluorescence. α-hENaC and EGFP dRNAs were generated with the mMessage mMachine in vitro transcription kits, as described above. Maximum hENaC channel activity was observed in oocytes ~48 h after dRNA injection. Therefore, oocytes were processed for confocal microscopy 2 days postinjection. To identify the plasma membrane, 10 eggs from each injection group were surface biotinylated. The eggs were equilibrated in ND-48 (in mM: 48 NaCl, 48 N-methyl-D-glucamine chloride, 1 MgCl₂, 1.8 CaCl₂, 2 KCl, and 5 HEPES, pH 7.4). Oocytes were incubated 24 h postisolation with 12.5 ng (in 50 nl) of the following dRNAs (in a 1:1:1 ratio): wt α-hENaC + GFP-β-rENaC + GFP-γ-rENaC; mutant α-hENaC + GFP-β-rENaC + GFP-γ-rENaC; and GFP-β-rENaC + GFP-γ-rENaC. A group of 10 oocytes was also injected with 50 nl of water, as a control for background fluorescence. α-hENaC and EGFP dRNAs were generated with the...
ated were treated with the Texas red-streptavidin as above. Eggs were washed several times with ND-48 and stored in the same solution for the duration of the confocal microscopy.

Confocal Microscopy

Images were acquired using an Olympus Fluoview BX50 upright confocal laser scanning microscope, equipped with a UplanF1 \( \times 10, 0.30 \) numerical aperture air objective and air-cooled krypton and argon lasers. The 488-nm argon laser line and the 568-nm krypton laser line excited the EGFP and Texas red, respectively. EGFP fluorescence was collected through the 510-nm and 550-nm barrier filters and Texas red fluorescence through the 610-nm filter. X-Y scans were obtained at 12-bit resolution at approximately the midsection of each oocyte. Acquired images were imported into Adobe Photoshop 5.0 for processing.

RESULTS

Generation of \( \alpha \)-hENaC Mutants

Results of the \( \alpha \)-helical wheel analysis of the M1 and M2 sequences are shown in Fig. 1A, and the residues
examined in this study are indicated by gray shading. According to this analysis, these gray residues occur on the hydrophilic sides of the helices. Figure 1B shows sequence alignments of just the second large hydrophobic regions (H2 and M2) of several of the cloned ENaCs. It demonstrates the relative positions and conserved nature of the M2 residues that we have mutated and analyzed. We used site-directed mutagenesis to change these glutamic and aspartic acids to arginines (point mutants αE108R, αE568R, αE571R, and αD575R). In this manner, the specific negatively charged amino acids in M1 and M2 were changed to positively charged residues. Additionally, the following combination mutants were generated: E568R + E571R, E568R + D575R, and E568R + E571R + D575R (triple). All mutant constructs were confirmed by sequence analysis.

**Functional Studies With wt and Mutant α-hENaCs**

Oocytes were injected, as described under Oocyte Preparation and Microinjection, with 12.5 ng of wt αβγ-hENaC or mutant α-hENaC + wt β-hENaC + wt γ-hENaC cRNA. Channel activity was recorded 48 h after injection. Channel activity expressed by the M1

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**Fig. 2.** Channel activity recorded from Xenopus oocytes expressing wild-type (wt) αβγ-hENaC or M1 mutant α-hENaC (αE108R; with wt β- and γ-subunits). A: whole cell current traces and current-voltage (I-V) relationships. Each set of traces represents current measured when eggs were clamped from -100 to +80 mV, in 20-mV increments. Recordings were made in 96 mM NaCl solution (ND-96). Top and middle traces depict whole cell currents recorded before and 3-4 min after addition of 10 µM amiloride. Amiloride-sensitive component of whole cell current is shown in bottom sets of traces. I-V relationships plotted below current traces are based on amiloride-sensitive whole cell currents. The wt and M1 mutant proteins produced channels with linear I-V relations and reversal potentials of +15 and +10 mV, respectively. Slopes of two I-V relations are almost identical, indicating that both channels had similar whole cell Na⁺ conductance. B: average amiloride-sensitive whole cell current measured in wt and M1 mutant-expressing oocytes. Current amplitude was measured 400 ms after eggs were clamped at -100 mV. The wt current was -3,017.6 ± 769.4 nA (n = 8). The α M1 mutant channel displayed an average current of -2,157 ± 465.2 nA (n = 7), which was not significantly different from that for wt channel recorded in same oocytes on same day.
mutant α-hENaC is seen in Fig. 2A. These results demonstrate a magnitude of whole cell current that was similar to that seen for the wt αβγ-hENaC channel. The inward Na\(^{+}\) currents recorded during the voltage-clamp protocol, before and after the addition of 10 µM amiloride, for the wt and the mutant were the same. The average amiloride-sensitive component of the whole cell current in both cases was equivalent (Fig. 2B). The I-V curves for the two channels were very similar. The reversal potentials were +15 mV for the wt and +10 mV for the mutant. These data indicate that there was little or no effect of this M1 point mutation on Na\(^{+}\) channel function at the whole cell level. Oocytes injected with these same combinations of αβγ cRNAs were processed to obtain membrane vesicles that were then fused to an artificial planar lipid bilayer for single-channel analysis. Single-channel records for wt α-hENaC and for the M1 mutant α-hENaC are shown in Fig. 3A. The apparent unitary conductance of both channels was 13 pS. Their open probabilities (P\(_{o}\)) were essentially the same at 0.13 ± 0.02 (n = 3) for wt and 0.15 ± 0.02 (n = 4) for the mutant αE108R. The amiloride dose-response curves (Fig. 3B) indicate that the M1 mutation did not alter the amiloride sensitivity.
of the channel, and the apparent equilibrium dissociation constant of amiloride ($K_{\text{ami}}$) for both channels was 0.2 µM. The wt and αE108R channels were recorded in symmetrical 100 mM NaCl solutions, and the plots of the resulting I-V relationships are shown in Fig. 3C. The curves were both linear with reversal potentials of 0 mV. I-V curves for the channels recorded in the bi-ionic conditions of 100 mM NaCl trans and 100 mM KCl cis are also plotted. The wt and mutant channels were both more selective for Na$^+$ over K$^+$ and became slightly inwardly rectified with a reversal potential of +60 mV under the asymmetrical conditions.

When the M2 α-hENaC mutant constructs were co-injected with wt β-hENaC and wt γ-hENaC into oocytes, the resulting whole cell currents were markedly smaller than those of the wt α-hENaC-injected eggs (Figs. 4 and 5). The average amiloride-sensitive current for wt was $-3,017.6 \pm 769.4$ nA ($n = 8$). The average amiloride-sensitive currents expressed by the M2 mutants were $-60.9 \pm 17.7$ nA ($n = 5$), $-93.2 \pm 26.9$ nA ($n = 4$), $-46.3 \pm 7.8$ nA ($n = 7$), and $-22.0 \pm 8.1$ nA ($n = 5$) nA for αE568R, αE571R, αD575R, and the α triple mutant, respectively (Fig. 5). The I-V plots in Fig. 4 demonstrate a significantly smaller amiloride-sensitive current for the M2 mutants than for the wt channel ($P < 0.05$). They also indicate a slight rectification of the whole cell currents from the mutant-expressing oocytes. The single-channel characteristics of the M2 point mutant, αD575R, were examined in the planar lipid bilayer system and also by cell-attached patch clamp of hENaC-expressing oocytes. As seen in Fig. 6A, in the bilayer, the αD575R hENaC + wt β-hENaC + wt γ-hENaC channel had a smaller unitary conductance (9 pS) than did wt (13 pS). The $P_o$ values of the two channels were similar: 0.14 ± 0.02 for wt ($n = 3$) and 0.13 ± 0.02 for αD575R ($n = 3$). The αD575R mutation appeared to have a small effect on the amiloride sensitivity of the channel determined from the amiloride dose-response curves shown in Fig. 6B. The $K_{\text{ami}}$ for the mutant channel was twofold greater (0.45 ± 0.035 µM, $n = 3$) than that of wt (0.20 ± 0.018 µM, $n = 3$). The I-V relationships plotted in Fig. 6C demonstrate the decreased conductance of the mutant, as well as inward rectification of the single-channel current, under symmetrical NaCl conditions. Conductances of both channels decreased when KCl was substituted for NaCl in the cis chamber. The shift in reversal potential for the αD575R mutant in asymmetrical conditions was not significantly different from the shift in the wt curve under the same conditions. These results indicate that the Na$^+$:K$^+$ selectivity of hENaC was not affected by the M2 mutation. The M2 mutants, αE568R and αE571R, were examined in the same manner in the bilayer and gave identical results (not shown). Addition-

![Fig. 4. Dual-electrode voltage-clamp studies of Xenopus oocytes expressing wt or M2 mutant α-hENaC cRNAs. Traces represent whole cell amiloride-sensitive current recorded in 96 mM NaCl. Cells were clamped between −100 mV and +80 mV in 20-mV increments. Corresponding I-V relationships for amiloride-sensitive current are plotted to the right of wt traces and below mutant traces. Wt αβγ-hENaC channels had a linear I-V relationship and a reversal potential of +30 mV. Macroscopic amiloride-sensitive current produced by each of α mutants [αE568R, αE571R, αD575R, or αE568R + αE571R + αD575R (triple)] were significantly reduced compared with wt. I-V relationships for these mutant channels demonstrate slight inward rectification, and their reversal potentials were similar to that of wt. However, slopes of curves are almost negligible and indicate a Na$^+$ conductance that was significantly smaller than wt.](image-url)
rENaC or γ-rENaC insert. The corresponding cRNAs for these constructs were coinjected with the various α-hENaC constructs. We first measured the whole cell current produced by the expression of α-hENaC with GFP-tagged β- and γ-rENaC subunits. Figure 7 shows that the wt α-hENaC combination produced whole cell amiloride-sensitive current that was equivalent to the current shown in Fig. 2A for wt α-hENaC + wt β-hENaC + wt γ-hENaC. The αD575R mutant, coexpressed with GFP-tagged β- and γ-subunits, produced the same low-level amiloride-sensitive current that was observed in Fig. 4. Average amiloride-sensitive currents were −2,690.5 ± 1,262.1 (n = 4) and −24.4 nA for the wt and mutant, respectively (Fig. 5).

These data established that the channels formed by the GFP-tagged subunits were functionally identical to the channels formed by wt β- and γ-hENaC constructs.

For confocal laser scanning fluorescence microscopy, oocytes were injected and processed as described under Oocyte Preparation for Confocal Microscopy. As a control, oocytes injected with 50 nl of water and surface labeled were imaged and compared with eggs injected with wt α-hENaC + GFP-β-rENaC + GFP-γ-rENaC. Each set of three images in Fig. 8 shows a similar optical section of oocytes obtained by a dual-laser scan at approximately the midpoint of the egg. The biotinylation protocol labeled proteins in the plasma membrane of the eggs with Texas red. As seen in Fig. 8, A1 and B1, the membrane of the cells was clearly defined by a distinct ring of Texas red fluorescence. The compact band of GFP fluorescence seen around the perimeter of the αβ-injected egg (Fig. 8A2) represents the localization of the ENaC protein. The water-injected oocyte showed no GFP fluorescence (Fig. 8B2) compared with the wt, indicating that the background fluorescence of the oocytes in this emission spectrum was negligible. Figure 8A3 is an overlay of the A1 and A2 images and shows the colocalization of the GFP and Texas red fluorescence, which appears as yellow. This confirms that some of the wt hENaC channel expressed by the oocyte was successfully inserted into the plasma membrane, accounting for the 1–3 µA of whole cell current. Figure 8B3 is the overlay of images B1 and B2; there is no yellow fluorescence, since there was no detectable GFP fluorescence in the water-injected oocyte.

Oocytes that were injected with a 1:1 mixture of the GFP-β-rENaC and GFP-γ-rENaC cRNAs were surface biotinylated 2 days after injection and viewed with the confocal imaging system in the same manner as described for Fig. 8. Figure 9 compares the fluorescence of these oocytes to that of wt hENaC-expressing oocytes. Figure 9, A1 and B1, shows the Texas red-labeled plasma membrane of the two eggs. The αβ channel GFP fluorescence is shown in Fig. 9A2, and its localization in the membrane is demonstrated by the yellow fluorescence in Fig. 9A3. The expression of the α- and β-subunits alone produced a pattern of GFP fluorescence that appeared as a diffuse band of green under the plasma membrane (Fig. 9B2). There was negligible colocalization of the β- and γ-rENaCs with the red
Fig. 6. Single-channel recordings of wt α-hENaC and αD575R reconstituted into planar lipid bilayers. A: current traces represent single-channel activity observed after vesicles were incorporated into bilayers bathed in symmetrical 100 mM NaCl solutions and held at −100 mV. $P_o$ for wt and mutant channels were $0.14\pm0.02$ ($n=3$) and $0.13\pm0.02$ ($n=3$), respectively. Unitary conductance of mutant channel (9 pS) was lower than wt conductance (13 pS). Effect of addition of 0.2 µM amiloride to trans compartment of bilayer is shown in bottom set of traces. Similar reduced conductances were observed when other α-subunit M2 point mutants (αE568R or αE571R) were coexpressed in oocytes with wt β- and γ-subunits and incorporated into planar lipid bilayers. B: single-channel amiloride dose-response curves for wt and αD575R. Holding potential was −100 mV. Mutant channel affinity for amiloride was slightly less (apparent $K_{am}$ = 0.45 µM) than that of wt channel (apparent $K_{am}$ = 0.2 µM). C: single-channel I-V relationships of wt and αD575R channels incorporated into planar lipid bilayers under bi-ionic and symmetrical conditions. D: cell-attached patch-clamp recordings from oocytes expressing wt or αD575R mutant channels. Bathing solution contained 100 mM LiCl, and membrane potential was held at −60 mV. Dashed line represents closed state. Single-channel conductances were 7 and 5 pS for wt ($n=3$) and mutant ($n=1$) channel, respectively.
subunits in Fig. 4. were identical to those of oocytes expressing all hENaC currents in oocytes expressing GFP-rENaC constructs of the neuronal channel formed by these ENaC homodimers (8).

We examined the cellular localization of two of the point mutants, αE571R and αD575R. These constructs were coinjected into oocytes with GFP-β-rENaC and GFP-γ-rENaC. Figure 10, A1, A2, B1, and C1, shows the red-labeled plasma membrane of wt-, αE571R-, and αD575R-expressing oocytes. The GFP fluorescence pattern of the same oocytes are seen in Fig. 10, A2, B2, and C2. Compared with the wt channel (Fig. 10A2), the GFP localization of both mutants was very similar (Fig. 10, B2 and C2). Their tight green bands of fluorescence colocalized with the red fluorescence of the membrane, as seen by the yellow in Fig. 10, B3 and C3. Thus it appears that the mutants were localized in the plasma membrane, just like the wt. On some occasions, when these experiments were repeated, the relative strength of the GFP fluorescence signal at the membrane of the mutant-injected oocytes was weaker than that of the wt.

DISCUSSION

The cloning of ENaC has aided the study of the epithelial Na⁺ channel conductance and gating characteristics, ion selectivity, and inhibition by amiloride. Identification of the conductive pore of the channel will help further the understanding of its functional properties, as well as clarify the nature of the interaction of its constitutive subunits in the membrane. In terms of their location in the transmembrane α-helices and their distinct conservation throughout the ENaCs (Fig. 1), we hypothesized that the few charged amino acids in the membrane-spanning domains of α-hENaC are potentially important pore residues.

By site-directed mutagenesis, we reversed the charge of Glu-108 in M1, changing the glutamate to arginine. Coexpression of this mutated form of α-hENaC with wt β- and γ-hENaC and wt γ-hENaC in oocytes produced whole cell amiloride-sensitive current of the same magnitude as that seen in oocytes expressing wt α-hENaC together with the wt β- and γ-subunits. This result indicates that the point mutation did not affect macroscopic channel function. When examined at the single-channel level in the bilayer, the mutant α-hENaC had the same unitary conductance (13 pS) as the wt. The mutation did not appear to alter amiloride sensitivity of the channel or Na⁺:K⁺ selectivity. The negative results for this point mutant serve as a control to show that the more drastic effects of the M2 mutants on channel function are not a result of the mutagenesis and in vitro transcription procedures or a problem of heterologous expression in the oocyte. These data indicate that the one negatively charged residue in the M1 domain of α-hENaC is not critical for movement of Na⁺ through the channel. In the K⁺ channels, the second transmembrane domains (the inner helices) of the four subunits that come together to form the channel are oriented to face the center of the pore, whereas the M1 domain of each subunit is situated away from the pore, facing the membrane (6). It is possible that α-hENaC orients itself around the conductive pore in a similar fashion. On the other hand, Coscoy et al. (5) recently identified a nine-amino acid region preceding the first transmembrane domain of ASIC2 and its splice variant ASIC2b that affects the ion selectivity and pH dependence of the neuronal channel formed by these ENaC homo-
logues. Thus it seems premature to rule out some role of α-hENaC M1 region in the ion pore.

The M2 point mutations αE568R, αE571R, and αD575R had a much more significant effect on the amiloride-sensitive Na\(^+\) channel activity. In the oocyte, the mutant channels demonstrated reduced levels (50–100 nA) of amiloride-sensitive whole cell current. This was significantly lower than the 1–3 µA seen in wt αβγ-ENaC-injected oocytes. Also, the I–V relationships plotted for the mutant channels (Fig. 4) indicate that representative whole cell currents were inwardly rectified. Such inward rectification was seen as well in the single-channel I–V relationship for the point mutant αD575R (Fig. 6C). This finding suggests that these specific residues are likely part of the conductive pore of the channel, since alteration of them affects both the magnitude and the voltage dependence of the Na\(^+\) conductance. The mechanism by which these charge reversals actually invoke the inward rectification of the current is unknown. The α double mutants, E568R + E571R and E568R + D575R, and the α triple mutant, E568R + E571R + D575R, all produced amiloride-sensitive whole cell current that was at least 20-fold less than wt (data for the double mutants are not shown).

Several laboratories have determined that charged residues in the pore region and inner helix (M2) of various Kir channels are important for channel conductance and/or ion selectivity. Krapivinsky et al. (14) recently cloned a new member of the Kir family, Kir 7.1. This channel demonstrates lower single-channel conductance than the other Kir channels and a decreased sensitivity to block by external Ba\(^{2+}\) and Cs\(^+\). There are three amino acids in the pore region of Kir 7.1 that are thought to contribute to the observed functional differences, as they differ from the conserved corresponding residues in the other members of the family. Mutation of one of these, Met-125, to the arginine that is conserved in other Kir channels produced a channel with a much higher conductance than the wt and an increased sensitivity to Ba\(^{2+}\). Studies of IRK1 show that the acidic residue, Asp-172, in the second hydrophobic segment affects channel function and block by internal Mg\(^{2+}\), indicating that it is positioned in the permeation pathway. Additionally, its size and/or charge contribute to channel selectivity (23). Experiments with a different type of channel, the nicotinic ACh receptor, have demonstrated that reversing the negatively charged residues occurring on both sides of the M2 regions of the constituent α-, β-, γ-, and δ-subunits reduces channel...
conductance significantly. Proposed explanations for this finding include perturbation of electrostatic forces, charge mutation-induced changes in pore structure, or alteration in ionic energy in the narrow region of the pore (13).

Waldmann et al. (29), who made α-rENaC/Mec-4 chimeras to confirm that the M2 region plays an important role in characteristic ENaC function, also demonstrated that the α-subunit point mutations S588I and S592I cause an increase in the channel conductance for Na⁺ and fast voltage-dependent gating. According to α-helical wheel analysis, the serines involved in these mutations occur on the same side of the M2 helix as the glutamate and aspartate residues that we examined. These data support the idea that the M2 region of α-hENaC is oriented in the membrane such that its hydrophilic face is an integral part of the conductive pore and that certain amino acids along that side of the helix are critical to ion permeation. Whether the size of the residue side chains or their polarity is more important remains to be determined. Interestingly, Waldmann et al. (29) constructed a chimera that exchanged residues 597–602 in α-rENaC with the homologous region of Mec-4. This included an E599F switch. Glu-599 in α-rENaC corresponds to Asp-571 in α-hENaC, which we mutated in these experiments. The properties of the chimera are indistinguishable from wt α-rENaC, whereas our α-hENaC D571R point mutant demonstrated reduced whole cell and single-channel conductance compared with wt hENaC channels. One explanation for these findings could be that the E599F change is not severe enough to evoke the conductance change that we saw with the D571R change. Alternatively, replacing the other residues around the α-rENaC Glu-599 with the corresponding Mec-4 residues could help maintain the channel properties, because it is thought that degenerins may function as channel proteins (7).

Kellenberger et al. (11) analyzed the monovalent and divalent cation permeability of heterotrimeric channels formed with α-rENaC Ser-589 mutants and determined that the geometry and size of the pore at the selectivity filter region influence what ions pass through the channel. Their experiments indicate that residue side chains affect the size and shape of the pore and thereby create a "molecular sieving" effect. Similarly,

Fig. 9. Laser scanning confocal images of oocytes injected with wt α-hENaC + GFP-β-rENaC + GFP-γ-rENaC (A) or GFP-β-rENaC + GFP-γ-rENaC (B). Injection, labeling, and microscopy protocols used to acquire these images were same as those described for Fig. 8. Each set of images (in A or B) depicts same laser scanning section of oocyte, which was at approximately middle of egg. Column 1 shows Texas red fluorescence, column 2 shows GFP-ENaC fluorescence, and column 3 shows images in columns 1 and 2 superimposed. Colocalized fluorescence appears yellow. As seen in B3, GFP fluorescence pattern in eggs expressing only β- and γ-rENaC subunits was a diffuse band that did not colocalize with red fluorescence at membrane (n = 9). This indicated that most of βγ protein was in cytoplasmic compartment of oocytes, as would be expected. Most of GFP fluorescence associated with wt αβγ channel (A) was localized in plasma membrane of oocyte and not peripheral endoplasmic reticulum (n = 7). Scale bar, 85 µm.
X-ray crystallographic studies of the KcsA K⁺ channel suggest that the arrangement of residues, specifically the main chain carbonyl oxygen atoms in the selectivity filter of the pore region, creates sites that accommodate K⁺ in a size-specific manner (6). On the basis of these results and earlier work by Palmer (21), Kellenberger et al. (11) propose a model of the ENaC pore in which the H2 segments of the constituent subunits form a funnel-shaped outer channel vestibule that narrows down to a very constricted selectivity filter that is composed of the conserved serine residues at the start of the M2 region of each subunit. The M2 segments, arranged such that they gradually open up to the cytoplasm, form the intracellular mouth of the pore.

Examination of our α-hENaC M2 single point mutants in the planar lipid bilayer revealed that each of the individual charge reversals produced channels with a 30% lower unitary conductance than the wt (9 pS for the mutants vs. 13 pS for the wt). The amiloride sensitivity of the mutant channel was slightly less than the wt, and its selectivity for Na⁺ over K⁺ was not altered. According to our analysis, residues 568, 571, and 575 occur in the middle of the M2 helix. On the basis of the model described above and that proposed for the conductive pore of K⁺ channels, this location is near the inner mouth of the pore, slightly removed from the putative P loop/selectivity filter that lies at the outer mouth. It is also >300 residues downstream from a putative amiloride-binding sequence (10, 12). Thus we predict that the role of these residues in the channel pore would be to attract or bind Na⁺ and aid in their movement through the pore. A role in selectivity or channel block seems less likely because of their location. They may create electrostatic energy wells for the diffusing ions, or their negative charges may help increase the concentration of cations at the intracellular entryway of the channel. In the case of the KcsA K⁺ channel, the intracellular and extracellular openings are negatively charged by acidic amino acids (6).

It is interesting that when measured by dual-electrode voltage clamp, our M2 mutant channels produced extremely low amiloride-sensitive whole cell current in the oocytes, and yet when recorded in the planar lipid bilayer the same channels had substantial unitary conductances of 9 pS. One explanation for this apparent discrepancy is that many of the mutant channels expressed at the oocyte plasma membrane were functioning transiently or were nonfunctional due
to the mutations in the α-subunit. The mutations could have caused enough of a conformational or steric change in the pore-lining M2 domains that, the majority of the time, they created a nonconductive pore. This is despite the fact that the three subunits did associate to the degree required for normal trafficking to the plasma membrane. A phenomenon such as this was observed by Krapivinsky et al. (14), who reported that a point mutation (G129E) in the pore region of Kir 7.1 produced no measurable whole cell current. However, localization of the mutant channel at the plasma membrane was the same as wt.

Regarding the differences in the conductances of the hENaC channels measured in the bilayer and the oocyte, our laboratory has studied the effect of actin on the channel in the bilayer. When actin was added to the cytoplasmic side of the bilayer, the unitary conductance of the wt channel decreased to 6–7 pS (a single-channel conductance consistent with that measured in the cell-attached patch-clamp experiments) and the conductance of the αD575R mutant decreased to 4 pS. This conductance is more representative of the channel, since it is expressed in conjunction with the actin cytoskeleton in the oocyte (see Ref. 2).

An obvious explanation for the small macroscopic current observed with the mutant α-subunits is that the channels were not processed or trafficked correctly, such that their insertion into the plasma membrane was hindered. To determine whether this was the case, we examined the localization of the hENaCs in live oocytes. We coexpressed our mutant and wt α-hENaC subunits with GFP-tagged β- and γ-rENaC subunits. Laser scanning confocal sections from the middle of an oocyte injected with wt α-hENaC + GFP-β-rENaC + GFP-γ-rENaC showed a distinct band of GFP fluorescence that colocalized with the plasma membrane. This same wt channel produced 1–3 μA of whole cell amiloride-sensitive current. Together, these data indicate that the three subunits formed functional channels that were predominantly localized in the plasma membrane of the oocyte.

In contrast, oocytes expressing only the GFP-tagged β- and γ-rENaCs showed a pattern of fluorescence that was consistent with previous findings of Firsov et al. (8). They expressed epitope-tagged α-, β-, and γ-subunits and combinations thereof in oocytes and used antibody binding assays to demonstrate that β- or γ-subunits alone are not present at the cell surface. They also found that α + β, α + γ, and β + γ demonstrate no surface antibody binding. These data corroborate our results that the GFP-tagged β- and γ-subunits showed intracellular fluorescence, rather than plasma membrane fluorescence, and that the GFP fluorescence in wt αβγ-expressing oocytes demonstrated the association of the α-subunit with the β- and γ-subunits in the plasma membrane. Visualizing specific endoplasmic reticulum (ER) localization of our GFP-tagged proteins in whole oocytes was difficult, due to the size of the cell and the limitations of the microscope objective. It is likely that there was GFP fluorescence in the dense ER network surrounding the nucleus and also in the cytoplasmic ER extending to the plasma membrane. Unfortunately, the oocyte is relatively large, and the working distance of the ×10 objective used is relatively short. Consequently, the fluorescence emission from perinuclear regions and cytoplasm was undetectable. This is why the oocytes in Figs. 8–10 showed no GFP fluorescence in the middle. The green fluorescent emission in and proximal to the plasma membrane was much more distinguishable due to the thinness of the membrane and the fact that the fluorophores there were more accessible to direct laser excitation.

The localization of GFP fluorescence in oocytes expressing the α-hENaC mutants αE571R and αD575R was similar to or only slightly less than that observed in wt α-hENaC-injected eggs. These results provide strong evidence that the mutant α-subunits successfully associated with the β- and γ-rENaC subunits and that this complex was trafficked to the plasma membrane. Thus the reduced whole cell current expressed by these point mutants was not solely due to a decrease in channel expression at the cell surface. These data support our single-channel measurements that indicate that a critical effect of reversing any or all of the negatively charged residues in the second transmembrane segment of ENaC α-subunit was to reduce channel conductance. These studies thereby demonstrate that the M2 domain forms part of the conductive pore of the channel and that acidic residues near the internal mouth of the pore are important for the movement of Na⁺ through the channel.

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