Identification of an Amiloride Binding Domain within the α-Subunit of the Epithelial Na⁺ Channel*

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Limited information is available regarding domains within the epithelial Na⁺ channel (ENaC) which participate in amiloride binding. We previously utilized the anti-amiloride antibody (BA7.1) as a surrogate amiloride receptor to delineate amino acid residues that contact amiloride, and identified a putative amiloride binding domain WYRFHY (residues 278–283) within the extracellular domain of αrENaC. Mutations were generated to examine the role of this sequence in amiloride binding. Functional analyses of wild type (wt) and mutant αrENaCs were performed by cRNA expression in Xenopus oocytes and by reconstitution into planar lipid bilayers. Wild type αrENaC was inhibited by amiloride with a $K_i$ of 189 nM. Deletion of the entire WYRFHY tract (αrENAC Δ278–283) resulted in a loss of sensitivity of the channel to submicromolar concentrations of amiloride ($K_i$ = 26.5 μM). Similar results were obtained when either αrENAC or αrENAC Δ278–283 were co-expressed with wt β- and γrENAC ($K_i$ values of 155 nM and 22.8 μM, respectively). Moreover, αrENAC H282D was insensitive to submicromolar concentrations of amiloride ($K_i$ = 6.52 μM), whereas αrENAC H282R was inhibited by amiloride with a $K_i$ of 29 nM. These mutations do not alter ENaC Na⁺-H⁺ selectivity nor single-channel conductance. These data suggest that residues within the tract WYRFHY participate in amiloride binding. Our results, in conjunction with recent studies demonstrating that mutations within the membrane-spanning domains of αrENaC and mutations preceding the second membrane-spanning domains of α-, β-, and γrENaC alters amiloride’s $K_i$, suggest that selected regions of the extracellular loop of αrENaC may be in close proximity to residues within the channel pore.

The diuretic amiloride is a prototypic inhibitor of epithelial Na⁺ channels (ENaCs)¹ (1), although amiloride and its various derivatives inhibit many Na⁺-selective transport proteins. Several laboratories have recently identified domains within the epithelial Na⁺ channel and the Na⁺/H⁺ exchanger that appear to participate in amiloride binding. Residues within the second membrane-spanning domain of αrENaC may interact with amiloride, as mutations of a serine residue at position 589 result in a large decrease of the apparent $K_i$ for amiloride and the amiloride analog benzamil, as well as alter cation selectivity (2). Selected mutations of residues within a hydrophobic region, termed H2 (3), immediately preceding the second membrane-spanning domains of the α-, β-, and γ-subunits of rENaC (i.e. Trp-α582, Ser-α583, Gly-β585, Gly-γ587) and the α-subunit of bovine ENaC (Lys-504, Lys-515) affect the $K_i$ for amiloride, and several of these mutations affect single-channel conductance (4, 5). Snyder and co-workers have identified splice variants of αrENaC in which the C-terminal 199 or 216 amino acid residues, including the second membrane-spanning domain, are truncated (6). These splice variants are not functional when expressed in Xenopus oocytes, but retain amiloride and phenamil binding activity, suggesting that at least a portion of the amiloride and phenamil binding domain is proximal to the C-terminal 216 residues of αrENaC.

Pouyssegur and co-workers generated a mutant NHE1 that had an apparent 30-fold decrease in its affinity for methylpropanilamidol. Sequence analysis identified a single point mutation within the putative fourth transmembrane domain, changing a leucine in position 167 to a phenylalanine. Further analysis of this region by site-directed mutagenesis identified phenylalanine residues at positions 165 and 168 that may participate in amiloride binding (7). Analysis of this leucine residue within the putative fourth transmembrane domain of NHE2 yielded similar results (8).

We have previously raised both polyclonal and monoclonal antibodies to amiloride (9–11). Amiloride was conjugated to carrier protein with linking groups located at different positions on the amiloride molecule to allow distinct sites of the amiloride molecule to be exposed following immunization (10). One amiloride derivative was coupled to bovine serum albumin through a hydrocarbon spacer arm on a terminal nitrogen of its guanidinium moiety (9). This strategy was based on previous observations that several amiloride analogs with hydrophobic substituents at this site are potent inhibitors of epithelial Na⁺

¹ The abbreviations used are: ENaC, epithelial Na⁺ channel; PCR, polymerase chain reaction; pS, picosiemen(s); wt, wild type; GHK, Goldman-Hodgkin-Katz; MOPS, 3-(N-morpholino)propanesulfonic acid; FaNaCh, FMRF amide peptide-gated Na⁺ channel.
channels (1). The binding of anti-amiloride antibodies to amiloride was examined by solid phase immunosassay using amiloride-bovine serum albumin conjugates adsorbed onto a solid support. Polyclonal and several monoclonal anti-amiloride antibodies recognized both benzamil and amiloride, but did not bind ethyl isopropylamiloride (9, 10), consistent with the rank order of potency of inhibition of high amiloride affinity epithelial Na⁺ channels (i.e., benzamil > amiloride > ethyl isopropylamiloride (1)). We utilized one monoclonal anti-amiloride antibody (BA7.1) as a surrogate amiloride receptor (12) to identify the amino acid residue types that may form an amiloride binding site, as well as their topologic orientation. Analysis of structural features of this anti-amiloride antibody led to identification of a structurally related 6-residue tract within the extracellular loop of αENaC (13). We now provide evidence that this 6-amino acid residue tract within the extracellular loop of αENaC, identified as a putative amiloride binding site by its homology with the amiloride binding domain within the anti-amiloride antibody BA7.1 (12), is required to express an epithelial Na⁺ channel that is sensitive to nanomolar concentrations of amiloride.

**EXPERIMENTAL PROCEDURES**

**Materials—**Amiloride was a gift from Merck. Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Moloney murine leukemia virus reverse transcriptase and DNA polymerase were obtained from Life Technologies, Inc. T4 polymerase from Perkin Elmer, dNTPs from Pharmacia Biotech Inc., Geneclean kit from Bio101, Sequenase II DNA polymerase from U. S. Biochemical Corp., T4 ligase from Boehringer Mannheim, Escherichia coli strains from Stratagene (La Jolla, CA), restriction enzymes and cap analog from New England Biolabs (Beverly, MA), and pALTER-1 restriction enzymes and cap analog from New England Biolabs (Beverly, MA). Ribonuclease, RNase, Ag-AgCl, and were connected to the chamber via 3% agar bridges filled with 3 M KCl. The recording and references electrodes were connected to a four-electrode voltage clamp (TEV-200, Dagan, Minneapolis, MN). Oocytes were voltage-clamped to 0 mV, and their membrane voltage was measured for 450 ms from −100 to +100 mV in 20-mV increments to measure whole-cell currents. The potential was returned to 0 mV for 50 ms between each voltage step. Current-voltage (IV) curves were constructed as described previously (16). Increasing concentrations of amiloride were sequentially added to the bath solution, and current was allowed to stabilize for 5 min between bath additions. Currents measured in the presence of varying concentrations of amiloride were normalized to the currents obtained in the absence of amiloride.

**Xenopus Oocyte Membrane Vesicle Preparations—**Membrane vesicles from oocytes injected with αENaC, αENaC Δ278–283, αENaC H282D, or αENaC H282R cRNA, vesicles from oocytes injected with α,β,γENaC cRNAs, and vesicles from oocytes injected with α,β,γENaC cRNAs were made essentially as described previously (17). Briefly, 90 oocytes in each group were rinsed and homogenized in high K⁺/sucrose medium containing multiple protease inhibitors. Membranes were isolated by discontinuous sucrose gradient centrifugation and resuspended in 300 mM sucrose, 100 mM KCl, and 5 mM MOPS (pH 6.8). This material was aliquoted into 50-μl fractions and stored at −80 °C until use.

**Planar Lipid Bilayer Experiments and Channel Expression—**Planar lipid bilayer experiments are described previously (17). A thin glycolipid bilayer was formed for a 5-s gap between injection of αENaC or control cRNA. The membrane potential was monitored with a Keithley 181 nanovoltmeter, and changes in membrane currents were monitored with a high gain differential amplifier (WPI Instruments, Sarasota, FL). The extracellular compartment was connected to a high impedance measuring bridge which was connected to the chamber, which was connected to a high gain amplifier circuit, as described previously (17). Under these experimental conditions, the channels were oriented with their amiloride-sensitive (extracellular) surface exposed to the trans compartment in over 90% of the incorporations. The probability of successful incorporation of single channels versus multi-channel incorporations depends upon the density of channels in oocyte membranes. In our experience, these probabilities were highly variable, and we have developed a simple method to estimate the number of empty oocyte vesicles yielding predominately single-channel incorporations (17). The experiments when no channels were evident in the membrane were considered unsuccessful incorporations as described previously. The rate of successful incorporations from oocyte membrane was 1 in 50–200 attempts. Amiloride was added to the trans chamber at concentrations indicated in the figures.
Amiloride Binding Domain within αENaC

Data Analysis— Acquisition and analysis of single-channel recordings were performed using pCLAMP software and hardware (Axon Instruments) as described previously (17). Data were stored digitally, and were filtered at 300 Hz with a 8-pole Bessel filter prior to acquisition at 1 ms/point. All the analyses were performed for membranes containing only single Na⁺ channels. The single-channel open probability was calculated for at least 3 min of continuous recording using Equation 1.

\[ P_o = \frac{I}{N \cdot i} \]  

(Eq. 1)

\( N \) is total number of channels (always equal to 1 in these experiments, as determined by activating all of the channels present in the bilayer by imposing a hydrostatic pressure gradient (see Ref. 17 for details)), \( I \) is the mean current over the period of observation, and \( i \) is the main (highest observed) state unitary current determined from all points current amplitude histograms produced by pCLAMP. The mean current \( (I) \) over the period of observation was calculated using the events list generated by pCLAMP software and Equation 2.

\[ \sum_{n=-\infty}^{\infty} \frac{I}{n} \]  

(Eq. 2)

\( i_n \) is an event current (all levels, including the zero current level); \( t_n \) is an event dwell time, and \( M \) is the total number of events. Data are expressed as mean value ± 1 standard deviation for n experiments.

RESULTS

Limited information is available regarding ENaC domains that participate in amiloride binding. We previously utilized the anti-amiloride antibody BA7.1 as a surrogate amiloride receptor (12) to delineate amino acid residues that contact amiloride. We observed that the sequence YYGHY contained in the CDR3 domain of the heavy chain of mAB BA7.1 aligned with the sequence tract WYRFHY, corresponding to residues 278–283 within αENaC (13, 19). It is likely that the α-subunit of the epithelial Na⁺ channel possesses an amiloride binding site, as expression of the α-subunit alone is sufficient to induce expression of a Na⁺ current in Xenopus oocytes, which is inhibited by amiloride with apparent inhibitory constants \( (K_i) \) nearly identical to that observed with expression of αβγENaC heterotrimers \( (K_i \text{ values of 100 and 104 nM, respectively}) \). Similar results have been reported with expression of αENaC alone or expression of αβγENaC heterotrimers in planar lipid bilayers \( (K_i \text{ values of 170 nM for both channels}) \) (3, 15, 17, 19). The putative amiloride binding tract WYRFHY is within the extracellular domain of αENaC (20–22). To examine the role of this sequence in amiloride binding, we generated several mutants of αENaC. One mutant, αENaC Δ278–283, has a deletion of residues 278–283 (i.e. WYRFHY). Our analysis of the binding of amiloride to BA7.1 suggested that the histidine within the CDR3 region of the heavy chain stabilized amiloride binding via electrostatic interactions with the halide (Cl) on the pyrazine ring of amiloride (12). Therefore, two site-directed mutants were generated: αENaC H282D, in which the histidine at position 282 was mutated to aspartic acid, and αENaC H282R, in which the histidine at position 282 was mutated to arginine. An additional site-directed mutant, αENaC R280G, was also generated.

Functional Properties of Mutations within the Sequence Tract WYRFHY— A major technical difficulty in examining the functional properties of αENaC Δ278–283 in the Xenopus oocyte expression system is the low level of Na⁺ current observed when the α-subunit is expressed alone (i.e. without co-expression of β- and γENaC) (3). Therefore, experiments using this system were performed with the heterotrimeric channel. Fig. 1 shows the results of measurements of macroscopic currents in Xenopus oocytes expressing wt αβγENaC or αΔ278–283, βγENaC. Interestingly, oocytes expressing αΔ278–283, βγENaC displayed a current that was ~40% smaller than that in oocytes expressing wt αβγENaC. The deletion of residues 278–283 within αENaC may affect the association of subunits forming the channel and alter subsequent transit of the channel to the cell surface, or alter single-channel properties. The amiloride-sensitive portion of the cur-
rent (10 μM amiloride added to the bath) was much larger in the ooocytes expressing wt αβγENaC (63 ± 8%) than in oo-
cytes expressing Δ278–283β,γENaC (19 ± 3%). These ob-
servations are consistent with those reported by Busch et al. (23). A portion of the base-line current in water-injected oo-
cytes was also found to be amiloride-sensitive (17 ± 4%). Inhibition
of wt αβγENaC by amiloride can be described in terms of
Michaelis-Menten kinetics (Fig. 2) with a $K_i$ of 23 ± 6 nM
($n = 4$), in reasonable agreement with previous observations
(3). The low current induced by Δ278–283β,γENaC and the
limited inhibition by 10 μM amiloride precluded the detailed
analysis of amiloride-induced inhibition of Δ278–283β,γENaC expressed in ooocytes. A rough estimation of
Michaelis-Menten kinetics, based on analyses of the normal-
ized current and assuming that 60% of the total current is
mediated by the Na$^+$ channel, gave a $K_i$ of 35 ± 10 μM ($n = 9$)
for the mutant channel. In view of the low levels of macroscopic
current observed in Xenopus ooocytes expressing αENaC alone,
Further studies examining the properties of αENaC Δ278–
283γENaC, and of αENaC with selected mutations within the
WYRFHY tract (residues 278–283) were performed in planar
lipid bilayers.

When reconstituted in planar lipid bilayers, αENaC Δ278–
283, αENaC R280G, αENaC H282D, and αENaC H282R formed Na$^+$ channels essentially indistinguishable by conduct-
earce or gating from those produced by wt αENaC (Fig. 3). All
mutated αENaC channels display a concerted type gating
between 13 and 39 pS states consistent with what was reported
previously (17). We have previously observed this gating pattern
for αENaC alone, and for αβENaC or αγENaC hetero-
dimers, which also display a concerted type gating between
the 13 pS and 39 pS states and which is quite distinct from the

\[ \text{Fig. 3. Single-channel current recordings of αENaC and αENaC mutants reconstituted into planar lipid bilayers.} \]

\[ \text{Bilayers were bathed with 100 mM NaCl containing 10 mM MOPS-Tris buffer (pH 7.4). Holding potential was +100 mV referred to the virtually grounded trans chamber. Records shown were digitally filtered at 100 Hz using pCLAMP software subsequent to the acquisition of analog signal filtered at 300 Hz with an 8-pole Bessel filter at 1 ms/point. Amiloride was added at concentrations indicated in the figure to the trans compartment. Records are representative of at least 7 separate experiments.} \]

FIG. 4. Amiloride dose-response curves of αENaC and αENaC mutants reconstituted into planar lipid bilayers. Data
points and error bars represent mean ± S.D. $P_o$ computed from at least 6 independent experiments. The lines through the data points represent
fits of the data obtained using the Michaelis-Menten equation re-written as follows:

\[ P_o = P_{o,max}(1 - \frac{[\text{amiloride}]}{K_i + [\text{amiloride}]}) \]

(Equation 6), where $P_o$ is the single-channel open probability at a given
amiloride, $P_{o,max}$ is the single-channel open probability in the nominal
absence of amiloride, $n$ is the Hill coefficient, and $K_i$ is the equilibri-
mum inhibitory constant for amiloride (see Table I). Hill coefficients ($n$) were
obtained using a best fit approach and are indicated for each plot.

A \\
\[ \text{gating pattern observed with heterotrimeric channels (17). However, similar to the experiments with heterotrim-
eric channels containing αENaC Δ278–283, the mutant channels ex-
hibited altered sensitivities to amiloride (Figs. 3 and 4). Wild}
\]

\[ \text{type αENaC was inhibited by amiloride with a } K_i \text{ of 169 ± 15} \]
Both the response data to the first order Michaelis–Menten equation.

ative mutation of His-282 (a 4.9-fold increase when compared with wt a

283, the apparent Ki

expression of epithelial Na+ channels that residues within the tract WYRFHY are required for ex-

amirolide dose-response data to either the first order Michaelis–Menten equation

Ki values were obtained by fitting the amiloride dose-response data to the Michaelis–Menten equation with the Hill coefficient as follows:

\( P_o = P_o^{\text{max}} \times K_i / (K_i + [\text{amiloride}]) \)

Determined by fitting the amiloride dose-response data to the Michaelis–Menten equation with the Hill coefficient as follows:

\( P_o = P_o^{\text{max}} \times K_i / (K_i + [\text{amiloride}]) \)

where \( n \) is the Hill coefficient.

TABLE I

| rENaCs expressed in lipid bilayers | Amiloride inhibitory constant (Ki)\(^a\) | Hill coefficient (n) | Amiloride inhibitory constant (Ki)\(^b\) |
|------------------------------------|------------------------------------------|---------------------|------------------------------------------|
| wt a,β,γrENaC                     | 155 ± 14 nM (n = 12)                     | 2.36                | 189 ± 28 nM                               |
| aΔ278–283,β,γrENaC                | 22.8 ± 3.1 μM (n = 8)                    | 0.7                 | 20.1 ± 2.2 μM                             |
| wt arENaC                          | 169 ± 15 nM (n = 13)                     | 2.37                | 199 ± 39 nM                               |
| arENaC 278–283                     | 26.5 ± 3.5 μM (n = 7)                    | 0.72                | 25.1 ± 4.9 μM                             |
| arENaC R280G                       | 830 ± 70 nM (n = 6)                      | 1.35                | 902 ± 45 nM                               |
| arENaC H282D                       | 6.52 ± 0.45 μM (n = 10)                  | 1.22                | 6.66 ± 0.61 μM                            |
| arENaC H282R                       | 29 ± 3 nM (n = 8)                        | 3.08                | 46.4 ± 7.1 nM                             |

\( ^a \) Determined by fitting the amiloride dose-response data to the first order Michaelis–Menten equation as follows:

\( P_o = P_o^{\text{max}} \times K_i / (K_i + [\text{amiloride}]) \)

\( ^b \) Determined by fitting the amiloride dose-response data to the Michaelis–Menten equation with the Hill coefficient as follows:

\( P_o = P_o^{\text{max}} \times K_i / (K_i + [\text{amiloride}]) \)

nm (n = 13), as determined by fitting the amiloride dose-response data to the first order Michaelis–Menten equation. Both the arENaC 278–283 mutant and arENaC H282D were largely insensitive to submicromolar concentrations of amiloride, with Ki values of 26.5 ± 3.5 μM (n = 7) and 6.52 ± 0.45 μM (n = 10), respectively (Fig. 4 and Table I). However, a conservative mutation of His-282 (arENaC H282R) led to a decrease in the apparent Ki for amiloride by 5.8-fold. The arENaC mutant R280G had an apparent Ki for amiloride of 830 ± 70 nm (n = 6), a 4.9-fold increase when compared with wt arENaC. Similar apparent Ki values for amiloride were obtained by fitting the amiloride dose-response data to either the first order Michaelis–Menten equation or the Michaelis–Menten equation with the Hill coefficient (Table I). These data support the hypothesis that residues within the tract WYRFHY are required for nanomolar concentrations of amiloride, and suggest that amiloride binds to, or interacts with, residues within this tract.

Single-channel properties of arENaC channels expressed in planar lipid bilayers under these conditions differ from heterotrimeric rENaC channels expressed in Xenopus oocytes and studied by the patch-clamp technique (3, 17). Therefore, we examined the properties of wt a,β,γrENaC and of aΔ278–283,β,γrENaC in planar lipid bilayers. Incorporation of membrane vesicles obtained from oocytes co-expressing wt a,β,γrENaC or aΔ278–283,β,γrENaC in planar lipid bilayers generated channels which displayed an essentially identical gating pattern with a predominant residence in a 13-pS state and occasional openings to 39 pS (Fig. 5, top traces) and consistent with our previous findings of the heterotrimeric ENaC currents observed in bilayers (17). However, the sensitivity of these channels to inhibition by amiloride was significantly different. The heterotrimeric channel containing arENaC 278–283 was inhibited by amiloride at concentrations more than 2 orders of magnitude higher than wt a,β,γrENaC (Table I). Amiloride dose-response curves are illustrated in Fig. 6. The amiloride inhibitory constants (Ki values) for the deletion mutant (i.e. aΔ278–283,β,γrENaC) and wt a,β,γrENaC were 22.8 ± 3.1 μM (n = 8) and 155 ± 14 nm (n = 12), respectively, determined by fitting the amiloride dose-response data to the first order Michaelis–Menten equation. Similar apparent Ki values were obtained by fitting the amiloride dose-response data to the Michaelis–Menten equation with the Hill coefficient (Ki values of 20.1 ± 2.2 μM and 189 ± 28 nm, respectively; see Table I). These results are similar to the apparent Ki for amiloride of wild type a,β,γrENaC and the estimated apparent Ki for amiloride of aΔ278–283,β,γrENaC expressed in oocytes.

We have previously demonstrated that the addition of actin...
to the cis compartment alters properties of wt α,β,γrENaC reconstituted into planar lipid bilayers, by reducing single-channel conductance to 6 pS and by increasing mean open and closed times, characteristics similar to those observed for ENaCs expressed in native tissues and analyzed by patch-clamp (18, 24). The sensitivities to amiloride of wt α,β,γrENaC and of αΔ278–283,β,γrENaC were not altered when actin was added to the cis compartment (Figs. 6 and 7), although single-channel conductance was reduced to 6 pS.

Additional studies were performed to examine whether deletion of the WYRFHY tract (residues 278–283) or mutations we have generated within this tract affect selectivity properties of the channel. The Na⁺:K⁺ selectivity of αrENaC A278–283, αrENaC R280G, αrENaC H282D, and αrENaC H282R did not differ from wt αrENaC (Fig. 8), as determined in symmetrical NaCl and bi-ionic (NaCl trans:KCl cis) conditions in planar lipid bilayers. In addition, Na⁺:K⁺ selectivity ratio of the heterotrimetric Na⁺ channel was not altered by deletion of residues 278–283, as determined by the incorporation of channels in planar lipid bilayers (Fig. 9), or by expression of channels in Xenopus oocytes using the two electrode voltage clamp technique (Fig. 10). The plots in these graphs represent fit of the data obtained in Na⁺ to K⁺ substitution experiments using the Goldman-Hodgkin-Katz (GHK) equation,

$$I = I_{Na} + I_{K}$$ (Eq. 3)

where

$$I_{Na} = P_{Na} P_{K} e^{E_{K} (V) - E_{Na} (V)} / (1 - e^{-E_{K} / R T})$$ (Eq. 4)

and

$$I_{K} = P_{Na} P_{K} e^{E_{Na} (V) - E_{K} (V)} / (1 - e^{-E_{Na} / R T})$$ (Eq. 5)

As the intracellular Na⁺ and K⁺ concentrations in Xenopus oocytes were not directly measured, these concentrations were set as adjustable parameters when determining Na⁺:K⁺ selectivity of rENaC expressed in oocytes by the GHK equation (equations 3–5). The control IV curves for wt α,β,γrENaC and mutant αΔ278–283,β,γrENaC rENaC were obtained in a bath solution containing 96 mM NaCl and 2 mM KCl. Under these conditions the best curve fit was achieved with the ratios of $P_{Na}:P_{K}$ of 4:1 and 8:1 for wt α,β,γrENaC and for the WYRFHY tract deletion mutant, respectively (Fig. 10). The intracellular Na⁺ and K⁺ concentrations of 14 mM and 148 mM for wt α,β,γrENaC, respectively, and 31 mM and 158 mM for αΔ278–283,β,γrENaC, respectively, were computed as the parameters for best fit to the GHK equation (equations 3–5). Substitution of 96 NaCl in the bath with 15 mM Na⁺/33 mM K⁺ shifted the IV curve for both wt and the WYRFHY tract deletion mutant (Fig. 10). The best fit to GHK equation under these conditions was obtained with $P_{Na}:P_{K}$ ratios of 31:1 and 20:1 for wt α,β,γrENaC and αΔ278–283,β,γrENaC, respectively. Intracellular Na⁺ and K⁺ concentrations of 11 mM and 186 mM for wt α,β,γrENaC, respectively, and of 12 mM and 223 for αΔ278–283,β,γrENaC, respectively, were computed as the parameters for best fit to the GHK equation. These results are consistent with the findings in planar lipid bilayers, suggesting that the mutations we have generated within αENaC do not change its Na⁺ to K⁺ permeability ratio. Substituting all the Na⁺ in the bath for K⁺ (100 mM KCl buffer) resulted in a dramatic shift of the IV curve for both wt α,β,γrENaC and for the WYRFHY tract deletion mutant (Fig. 10). Fitting these curves to GHK results in estimation of the Na⁺ to K⁺ permeability ratios of $1.6 \times 10^{10}$ for wt α,β,γrENaC, and $3.1 \times 10^{10}$ for αΔ278–283,β,γrENaC, values that are close to infinity. Intracellular Na⁺ and K⁺ concentrations computed as parameters for these fits were found to be 1.2 mM and 198 mM for wt α,β,γrENaC, respectively, and 0.9 mM and 167 mM for αΔ278–283,β,γrENaC, respectively. Ion concentration dependence of the selectivity properties has been previously shown for channels that can accommodate multiple ions at the time (25–27), and α,β,γrENaC indeed is a multi-ion channel (28). In this case a close proximity in the of ion concentration dependence of the selectivity properties of the wt and mutant channel may suggest that deletion of the WYRFHY tract does not affect the number of ions that channel can accommodate at the same time. On the other hand these computer-generated estimates are rough, especially for the complete Na⁺ to K⁺ substitution experiments. Nonetheless, the relative changes in cation selectivity associated with changes of the ionic composition are similar among wt and mutant channels, consistent with previous measurements made with the use of bilayer system.

**DISCUSSION**

Amiloride is the prototypic inhibitor of epithelial Na⁺ channels. Amiloride analogs have been used by a number of investigators as tools to isolate and characterize amiloride-sensitive Na⁺ channels, and may have an important role as Na⁺ channel inhibitors in the treatment of selected forms of hypertension (29, 30). Previous studies demonstrated that it is the charged, protonated species of amiloride that inhibits Na⁺ channels (31–34). This channel block is dependent, in part, on the apical membrane potential. Analysis of the kinetics of amiloride binding to the Na⁺ channel in the presence of a varying apical plasma membrane potential suggests that amiloride senses between 10% and 45% of the membrane electric field (31, 34). This observation, when taken together with studies utilizing voltage-clamped cells to demonstrate that amiloride binding kinetics are altered by Na⁺ or Li⁺ loading cells to generate an outward current through the channel (35, 36), supports the idea that amiloride interacts within the channel pore. This hypothesis is further supported by recent studies of Waldmann et al. demonstrating that substitutions of the first or second putative transmembrane region of αrENaC with the corresponding domains within Mec-4 led to a decrease amiloride sensitivity by 3- and 14-fold, respectively (2). Mec-4 has significant sequence similarity with ENaCs and is a member of the Caenorhabditis elegans degenerin family that is associated...
with mechanotransduction (19). Mutation of a serine to phenylalanine in position 589 of the second membrane-spanning domain of \( \alpha \)rENaC increases the \( K_i \) for amiloride and alters cation selectivity and single-channel conductance, suggesting that serine 589 participates in amiloride binding and resides within the channel pore (2). In addition, residues preceding the second membrane-spanning domains of \( \alpha \)-, \( \beta \)-, and \( \gamma \)-rENaC and of bovine \( \alpha \)-ENaC may also form part of the channel pore, and selected mutations within these regions dramatically affect amiloride sensitivity (4, 5).

Organic cations other than amiloride and related analogs, such as 2,4,6-triaminopyrimidine, also function as epithelial Na\(^+\) channel inhibitors, although with \( K_i \) values much greater than that of amiloride (37), suggesting that it is the guanidine moiety of amiloride that is interacting with the channel pore. However, the substituted pyrazine ring of amiloride is required for the drug to inhibit ENaCs with a submicromolar \( K_i \), and may have a critical role in stabilizing amiloride bound to the channel (1, 38). The putative amiloride binding site on the anti-amiloride antibody BA7.1 that we previously characterized primarily interacts with the substituted pyrazine ring moiety of amiloride (12). By analogy, the 6-amino acid track within the extracellular loop of \( \alpha \)-ENaC we identified based on its homology with the amiloride binding site on BA7.1 likely binds amiloride via interactions with the substituted pyrazine moiety of amiloride, and is not necessarily associated with the pore region of the channel. Our results demonstrating that the single-channel conductances and Na\(^+\):K\(^+\) selectivity ratios of

FIG. 7. Single-channel current recordings of \( \text{wt } \alpha,\beta,\gamma-r\)ENaC and \( \alpha_{\Delta 278-283},\beta,\gamma-r\)ENaC following addition of actin to the cis compartment. Recording conditions, holding potential, and data treatment were as indicated for Fig. 3, with the addition of actin to the cis compartment. Records are representative of at least 5 separate experiments.

FIG. 8. Single-channel current-voltage relations of \( \alpha\)rENaC and \( \alpha\)rENaC mutants reconstituted into planar lipid bilayers under bi-ionic or symmetric conditions. Data points and error bars represent the mean ± S.D. from at least 6 independent experiments. Bathing solutions contained 100 mM NaCl cis/100 mM NaCl trans, 10 mM MOPS (pH 7.5) (open symbols) and 100 mM KCl cis/100 mM NaCl trans, 10 mM MOPS (pH 7.5) (filled symbols).

FIG. 9. Single-channel current-voltage relations of \( \text{wt } \alpha,\beta,\gamma-r\)ENaC and \( \alpha_{\Delta 278-283},\beta,\gamma-r\)ENaC under bi-ionic or symmetric conditions. Data points and error bars represent the mean ± S.D. from at least 6 independent experiments. Bathing solutions contained 100 mM NaCl cis/100 mM NaCl trans, 10 mM MOPS (pH 7.5) (open symbols) and 100 mM KCl cis/100 mM NaCl trans, 10 mM MOPS (pH 7.5) (filled symbols).
Amiloride Binding Domain within αENaC

**FIG. 10.** Currents measured in *Xenopus* oocytes expressing wt α,β,γ-rENaC and αΔ278–283,β,γ-rENaC bathed in solutions with different ionic compositions. Oocytes were injected with a total of 25 ng of wt or mutant rENaCs, the oocytes were bathed sequentially in solutions containing (in mM) 96 Na+, 2.4 K+, 2.4 CaCl2, 1.8 MgCl2, and 5 HEPES (pH 7.4). The ND96 solution contained (in mM): 0 NaCl, 100 KCl, 1.8 CaCl2, 1.0 MgCl2, and 5 HEPES (pH 7.4). Currents were measured in each oocyte with the ND96, 15 mM Na+, and 100 mM K+ buffers. Oocytes were sequentially bathed in the following buffers: ND96, 15 mM Na+ or 100 mM K+. The chamber was washed for 10 min with the buffer and a voltageclamp protocol performed. Ten μM amiloride was added, and after 4 min, the voltageclamp protocol was repeated. Data points represent amiloride-sensitive currents. The measurements were performed in *Xenopus* oocytes expressing wt α,β,γ-rENaC (n = 3) and in *Xenopus* oocytes expressing αΔ278–283,β,γ-rENaC (n = 4). Lines through data points represent best fits of the Goldman-Hodgkin-Katz equation (Equations 3–5) with intracellular ion concentrations and Na+/K+ permeability ratios set adjustable.

αENaC Δ278–283, αrENaC R280G, αrENaC H282D, and αrENaC H282D are indistinguishable from wt αrENaC suggest that residues 278–283 do not form part of the Na+ channel pore. Li and co-workers have identified splice variants of αrENaC, in which the C-terminal 199 or 216 amino acid residues are truncated, including the second membrane-spanning domain (6). These splice variants are not functional when expressed in *Xenopus* oocytes, but retain amiloride and phenamil binding activity, suggesting that part of the amiloride and phenamil binding site is proximal to the C-terminal 216 residues of αrENaC, again consistent with our findings. The previously published results suggest that residues within the second membrane-spanning domain of αrENaC and within a hydrophobic (putative pore) region preceding the second membrane-spanning domains of α-, β-, and γENaC bind amiloride, and our results, indicating that residues within the extracellular loop of αrENaC (i.e. residues 278–283) bind amiloride, suggest that amiloride contact residues are derived from different regions of rENaC and that selected regions of the extracellular loop of αrENaC may be in close proximity to residues within the Na+ channel pore.

Our previous analysis of the amiloride binding site on the anti-amiloride antibody BA7.1 suggested that a histidine residue within the CDR3 region of the heavy chain primarily interacts with the Cl atom on the pyrazine ring moiety of amiloride through an electrostatic interaction (12). Therefore, we examined the effects of mutations of the histidine residue (His-282) present within the 6 amino acid putative amiloride binding domain on the extracellular loop of αENaC. Mutagenesis of this histidine to aspartic acid (H282D) resulted in a change in charge of this residue from cationic to anionic and was associated with a 30-fold increase in the apparent K_i for amiloride. Alternatively, mutagenesis of this histidine to arginine (H282R) with conservation of the cationic charge was associated with a 6-fold decrease in the apparent K_i for amiloride. H282R is the first mutation of αENaC described that is associated with a large decrease in the amiloride K_i.

These data suggest that His-282 may have an important role in stabilizing the binding of amiloride to the Na+ channel. Although it is possible that His-282 primarily interacts with the Cl atom on the pyrazine ring moiety of amiloride, there is no direct evidence to support this hypothesis. H282R and H282D have a difference in their apparent K_i values for amiloride of 225-fold. The αrENaC mutant R280G was inhibited by amiloride with a K_i of 30 mM, a 4.9-fold increase in the apparent K_i for amiloride when compared with wt αrENaC suggesting that residues within the tract 278–283, other than His-282, may bind amiloride or affect the topology of this site.

The Hill coefficient of 2.4 that we observed in our studies of amiloride inhibition of wt α,β,γ-rENaC and wt αrENaC reconstituted into planar lipid bilayers is in reasonable agreement with previous observations (17). αENaC stoichiometry has not been determined. If amiloride interacts primarily with the α-subunit, a Hill coefficient of 2.4 indicating cooperative binding suggests that ENaCs have more than one α-subunit or, alternatively, that there are multiple sites, or domains, within the α-subunit which participate in amiloride binding. Interestingly, the Hill coefficient decreased with αrENaC mutations that increased amiloride’s K_i, suggesting that the amiloride binding domain we have identified may affect subunit-subunit interactions, may affect intramolecular interactions within the α-subunit, or alternatively may alter α-subunit stoichiometry.

The amiloride binding domain WYRFHY is conserved within αENaC in all species that have been cloned and sequenced to date, including rat, human, bovine, *Xenopus*, and mouse (15, 19, 39–41). A nearly identical tract, WYRFHY, is present in the recently cloned δ subunit of a Na+ channel that appears to be expressed in both epithelial and nonepithelial tissues (42). Both αENaC and δENaC are sufficient, by themselves, to induce the expression of Na+-selective, amiloride-sensitive channels in *Xenopus* oocytes. The current levels observed with expression of αENaC or δENaC increase by approximately 1000-fold when co-expressed with β- and γENaC. An amiloride- and benzamil-sensitive FMRFamide peptide-gated Na+ channel (FaNaCh) was recently cloned from marine snail neurons (43). Interestingly, FaNaCh does not have a WYRFHY tract, although a related tract WLRFIQKF is present in the putative extracellular domain of FaNaCh that shares some features.
with the amiloride binding domain we have identified within αENaC, including the presence of planar and cationic amino acid residues. Further studies are required to examine whether residues within the tracts WYKHFLY in δENaC and WLR-FIQKF in FaNaCh participate in amiloride binding.

The sensitivity of αENaC to amiloride does not appear to be dependent upon co-expression with β- and γENaC, as wt αENaC and wt α,β,γENaC reconstituted into planar lipid bilayers have similar \( K_\text{m} \) values for amiloride (155 nM and 169 nM, respectively (Table I)). This was also observed with αENaC \( \Delta278-283 \), as αENaC \( \Delta278-283 \) and α\( \Delta278-283,β,γ \)ENaC have nearly identical sensitivities to amiloride (\( K_\text{m} \) values of 22.8 μM and 26.5 μM, respectively, Table I). These data support the hypothesis that residues required to form high affinity amiloride binding domains reside within the α-subunit. However, β- and γENaC also participate in amiloride binding (4). Interestingly, a tract WYKLHY (residues 230–235) within the extracellular loop of δENaC bears striking similarity to the amiloride binding domain we identified within the extracellular domain of αENaC (3). Additional studies are required to determine whether this region within γENaC (i.e. residues 230–235) participates in amiloride binding. It is conceivable that mutations we have generated alter the stoichiometry of subunit association, which might affect amiloride binding. Previous studies from our laboratory suggest that αENaC, reconstituted alone or as α/β or α/γ heterodimers, primarily exhibits 13-pS and 39-pS conductance states, whereas the α/β/γ heterotrimer primarily exhibits a 13-pS conductance state (17). The conductance states observed with both wt α,β,γENaC and with α\( \Delta278-283,β,γ \)ENaC (Fig. 3A) indicate that α\( \Delta278-283,β,γ \)ENaC is reconstituted into the lipid bilayer as a heterotrimer.

In summary, the analysis of an anti-amiloride antibody resulted in the identification of an amiloride binding domain on αENaC. Although there are other sites within αENaC, as well as within other ENaC subunits that participate in amiloride binding, our data clearly suggest that residues 278–283 within αENaC, particularly His-282, are part of an amiloride binding site. In addition, these studies support the hypothesis that selected anti-ligand antibodies may serve as surrogate ligand receptors, and that in selected systems these antibodies may provide useful tools to develop models of tertiary structural features of naturally occurring ligand receptors.

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