The epithelial Na\(^+\) Channel (ENaC) mediates Na\(^+\) reabsorption in a variety of epithelial tissues. ENaC is composed of three homologous subunits, termed \(\alpha\), \(\beta\), and \(\gamma\). All three subunits participate in channel formation as the absence of any one subunit results in a significant reduction or complete abrogation of Na\(^+\) current expression in Xenopus oocytes. To determine the subunit stoichiometry, a biophysical assay was employed utilizing mutant subunits that display significant differences in sensitivity to channel blockers from the wild type channel. Our results indicate that ENaC is a tetrameric channel with an \(\alpha_2\beta\gamma\) stoichiometry, similar to that reported for other cation selective channels, such as Kv, Kir, as well as voltage-gated Na\(^+\) and Ca\(^{2+}\) channels that have 4-fold internal symmetry.

Epithelial Na\(^+\) channels are expressed in apical plasma membranes of principal cells in the distal nephron, airway and alveolar epithelia in the lung, surface cells in the distal colon, urinary bladder epithelia, and other tissues including ducts of salivary and sweat glands (1–3). These channels mediate reabsorptive Na\(^+\) transport across epithelial cell layers (2–5) and are selectively inhibited by submicromolar concentrations of the diuretic amiloride (6). Epithelial Na\(^+\) channels have a key role in the regulation of urinary Na\(^+\) reabsorption, extracellular fluid volume homeostasis, and control of blood pressure, and are a major site of action of volume regulatory hormones, including aldosterone (2, 7, 8). The role of Na\(^+\) channels in blood pressure regulation has been illustrated in recent studies that have identified mutations in ENaC as the basis of the pathogenesis of Liddle’s disease, a disorder characterized by volume depletion and hypotension (11).

The epithelial Na\(^+\) channel consists of at least three structurally related subunits, termed \(\alpha\)-ENaC, \(\beta\)-ENaC, and \(\gamma\)-ENaC (epithelial Na\(^+\) channel) (12). The primary and predicted secondary structures of these ENaCs have been described (12–15). Each subunit has two predicted \(\alpha\)-helical membrane spanning regions separated by a large extracellular domain. Significant amino acid sequence similarities across species have been observed for individual subunits (on the order of \(-60\%\) to greater than \(90\%\) amino acid homology), although regions are present that are more highly conserved. A family of genes identified in Caenorhabditis elegans based on mutations that result in mechanosensation defects (mecs) and degeneration of selected neuronal cells (degs) are structurally related to ENaCs (16–18). Several of these genes, including mec-4, mec-6, and mec-10, are thought to form an ion channel in a manner analogous to the three ENaC subunits (16, 19). These observations suggest that ENaCs and mecs (and degs) are members of a new gene superfamily. Members of this family include ENaCs, mecs and degs, FaNaCh (a peptide-gated channel cloned from the marine snail Helix aspersa), \(\delta\)-ENaC, and BNaC (cloned from human brain), ASIC (an acid-sensing ion channel), DRASIC (dorsal root ganglia acid-sensing ion channel) (20–24), and likely includes mechanosensitive cation channels present on cochlear hair cells and oocytes (2, 3, 5, 18). Epithelial Na\(^+\) channels are composed of at least three structurally distinct subunits (12). We have used a biophysical approach to assess number of each ENaC subunit that assembles to form the Na\(^+\) channel complex (i.e. subunit stoichiometry).

Our results suggest that ENaC has a tetrameric structure and is composed of two \(\alpha\)-, one \(\beta\)-, and one \(\gamma\)-subunit.

**EXPERIMENTAL PROCEDURES**

Materials—Reagents were purchased from vendors listed below or from Sigma.

Preparation of Mouse ENaC (mENaC) Mutants—All three mutants were made using a polymerase chain reaction (PCR)-based mutagenesis technique (25). For each mutant, two rounds of PCR amplification were performed using Pfu DNA polymerase (Stratagene Corp., La Jolla, CA) and an MJ Research thermal cycler (Watertown, MA). Two overlapping fragments were generated in the first round of PCR amplification using the internal mutagenic forward primer with the 3’ end external reverse primer and, in a separate reaction, using the internal mutagenic reverse primer with the 5’ end external forward primer. In the second round of PCR amplification, the two overlapping fragments generated in the first PCR were first spliced together by two rounds of thermocycling, followed by 22 rounds of amplification with added external primers. The PCR product from the second step was ligated into the original mENaC clone, using unique restriction sites at the two ends. All sequences were confirmed by automated DNA sequence analysis performed at the University of Pennsylvania’s DNA sequencing facility.

Expression of Na\(^+\) Channels in Xenopus Oocytes—The mutant and the wild type cRNAs were prepared using a cRNA synthesis kit (mMESSAGE mMACHINE, Ambion Inc., Austin, TX). cRNA concentration was measured spectrophotometrically. For optimum ENaC expression, equal amounts (3 ng) of each of the three subunits were injected into oocytes and electrophysiological measurements were performed 1 to 5 days after injection. Two-electrode voltage clamp experiments were performed by clamping oocytes to \(-100\) mV (with reference to bath) for 500 ms and 0 mV for 450 ms. For all measurements, the current difference between the two command potentials was used for data analysis. The difference in current measured in the presence and absence of amiloride (100 \(\mu\)M for wild type mENaC and 1 or 5 \(\mu\)M amiloride for mutant mENaCs) was used to define the amiloride-sensitive current. Amiloride titration measurements were performed under continuous flow (~4 ml/min) of buffers containing varying concentra-
tions of amiloride. Single channel recordings were performed in the cell attached configuration. All data were collected at room temperature and were filtered at 100 Hz. The applied voltage to the membrane patch represents the voltage deflection from the resting membrane potential. Inward Na+ current is represented by a downward deflection in single channel recordings.

Solutions—Following injection, oocytes were incubated in modified Barth's saline solution containing (in mM): 88 NaCl, 1 KCl, 2.4 CaCl₂, 0.82 MgSO₄, pH 7.2. The buffer was supplemented with 10 μg/ml penicillin, 10 μg/ml streptomycin sulfate, 100 μg/ml gentamycin sulfate, and 10 μg/ml nystatin.

The bath media for two-electrode voltage clamp experiments contained (in mM): 100 NaCl, 1.8 CaCl₂, 2 KCl, 10 HEPES, 5 BaCl₂, 10 TEA-Cl, pH 7.2. Measurements of single channel conductance were performed with a buffer containing (in mM): 100 NaCl, 1.8 CaCl₂, 2 KCl, 10 HEPES, pH 7.2, in the pipette and in the bath.

In patch-clamp studies performed to examine the effects of (2-aminoethy1)methanethiosulfonate (MTSEA, Toronto Research Chemicals Inc., Toronto, Ontario, Canada), the bath solution contained (in mM): 100 NaCl, 2 KCl, 1.8 CaCl₂, 10 HEPES, 5 BaCl₂, 10 TEA-Cl, pH 7.2. To investigate the effects of inhibitors on the expressed channels allows for the selective and/or inhibitor-insensitive subunits. Analyses of the single channel conductances of mENaC expressed in Xenopus oocytes: amiloride dose-response relationships.

RESULTS

Wild type and mutant mENaC subunits that express different sensitivities to channel blockers were used to determine mENaC stoichiometry. The injection of oocytes with varying ratios of wild type and mutant mENaC cRNAs results in expression of oligomeric channels, each containing either inhibitor-sensitive and/or inhibitor-insensitive subunits. Analyses of the effects of inhibitors on the expressed channels allows for determination of subunit stoichiometry (27–29). mENaC is blocked by amiloride in the submicromolar range (IC₅₀ of 0.1 μM). All three subunits appear to participate in amiloride binding, as single point mutations in the putative pore forming regions of any of these subunits results in altered amiloride binding (30).

The mutants αS583C, βG525C, and γG542C were used for these studies, as they differ from wild type ENaC in their sensitivities to channel blockers (30). αβG525C, γmENaC and αβγG542C-mENaC are inhibited by amiloride with IC₅₀ values of 73 and 43 μM, respectively (Fig. 1 and Table I). αS583CβγmENaC is blocked by amiloride with an IC₅₀ of 0.6 μM (Table I), sufficiently close to the IC₅₀ of wild type mENaC to preclude the use of amiloride in the determination of the stoichiometry of the α-subunit. However, wild type mENaCs are insensitive to the sulphydryl reagent MTSEA (0.5 mM), whereas αS583Cβγ-ENaC is blocked by MTSEA (Fig. 2A).

The analysis of subunit stoichiometry assumes that the functional properties of mutant and wild type channels are similar, other than differential sensitivities to inhibitors. We examined the single channel conductances of the mutant mENaCs (αS583CβγmENaC; αβG525C, γmENaC; and αβγG542C-mENaC) expressed in Xenopus oocytes (Fig. 3). The slope conductance of αS583CβγmENaC was 4.7 pS, essentially indis-

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**TABLE I**

| mENaC species | Kᵢ (amiloride) (nM) | Hill coefficient conductance (pS) | Single channel |
|---------------|---------------------|-----------------------------------|---------------|
| Wild type     | 92.8 (n = 7)        | 0.99                              | 4.7           |
| αS583Cβγγ     | 600 (n = 2)         | ND*                               | 4.7           |
| αβG525C, γ    | 73 (n = 8–10)       | 0.75                              | 2.8           |
| αβγG542C      | 43 (n = 6–7)        | 0.84                              | 3.2           |

* ND, not determined.

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Fig. 1. Expression of αβG525C, γ-mENaC or αβγG542C-mENaC in oocytes: amiloride dose-response relationships. Sodium currents were measured in oocytes expressing αβG525C, γ-mENaC (open circles) or αβγG542C-mENaC (closed circles) using a two-electrode voltage clamp (see “Experimental Procedures”). Currents were corrected for the values measured in the presence of 5 mM amiloride and were normalized to the current measured in the absence of amiloride. IC₅₀ values for amiloride and Hill coefficients were determined by curve fitting the amiloride dose-response data to the Langmuir inhibition isotherm (Equation 1). Points represent data obtained from six to 10 experiments. Error bars indicate the standard errors.

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FIG. 2.α-Subunit stoichiometry. A, responses to MTSEA of oocytes injected with wild type α,β,γ-mENaC or with the mutant αSS83C,β,γ-mENaC cRNAs. Currents were corrected for the values measured in the presence of 1 mM amiloride and were normalized to the current measured in the absence of amiloride (see “Experimental Procedures”). Wild type α,β,γ-mENaC (open bar) is insensitive to 0.5 mM MTSEA (5 ± 2% inhibition) whereas αSS83C,β,γ-mENaC mutant (closed bar) is largely blocked (92 ± 2% inhibition) by the drug. Points represent data obtained from six to nine experiments. Error bars indicate the standard errors. B, response to 0.5 mM MTSEA of oocytes co-injected with a 1:1 ratio of wild type α,β,γ-mENaC and mutant αSS83C,β,γ-mENaC cRNAs (open bar). Currents were corrected for the values measured in the presence of 1 mM amiloride and normalized to currents measured in the absence of amiloride. The right side of the panel illustrates the predicted current response to 0.5 mM MTSEA, assuming an α-subunit stoichiometry (n) of one, two, or three. The response to MTSEA (69 ± 2% inhibition) is consistent with a stoichiometry of 2 (predicted response of 71% inhibition). Points represent data obtained from six experiments. Error bar indicates the standard error.

FIG. 3. Expression of αSS83C,β,γ-mENaC,αβG525CG542C-mENaC, or α,β,γG542C-mENaC in oocytes, single channel current/voltage relationships. Currents were measured while varying the holding potential between −100 and +20 (or +100) mV (20 mV steps), using a cell attached patch-clamp. Pipette and oocytes bath solutions contained 100 mM NaCl. Linear current voltage relationships were observed. The slope conductance for αSS83C,β,γ-mENaC was 4.7 pS, for αβG525C,γ- mENaC was 2.8 pS, and for α,β,γG542C-mENaC was 3.2 pS. Points represent data obtained from three to 10 experiments. Error bars indicate the standard errors.

cytes co-injected with a 1:1 ratio of α,β,γ-mENaC and αSS83C,β,γ-mENaC cRNAs (Fig. 4). Wild type α,β,γNa⁺ channels detected in the absence of MTSEA were also observed following MTSEA infusion into the patch pipette (n = 26) (26). In contrast, all αSS83C,β,γ mNa⁺ channels detected in the absence of MTSEA were completely blocked following MTSEA infusion (n = 17). Of the 28 mNa⁺ channels observed in oocytes injected with the 1:1 mixture of α,β,γ-mENaC and αSS83C,β,γ-mENaC cRNAs, no prolonged subconductance state, suggestive of a partial mNa⁺ channel block, was observed following MTSEA infusion. In addition, no flickering response suggestive of a rapid, reversible block of Na⁺ channels was observed.

β-Subunit Stoichiometry—The differential sensitivities of wild type mENaC and αβG525C,γ-mENaC to amiloride were used to determine β-subunit stoichiometry. The responses to amiloride of oocytes injected with a 1:1 or a 1:4 mixture of wild type (α, β, γ) and mutant (αβG525C,γ) mENaC cRNAs are illustrated in Fig. 5. This figure also illustrates the predicted responses of oocytes injected with a 1:1 or 1:4 mixture of wild type and mutant cRNAs, for β-subunit stoichiometries (n) of 1 or 2. A random assembly resulting in a binomial distribution of wild type and mutant β-subunits expressed in channels was assumed. In generating the predicted responses, the conductance difference between the mutant and the wild type channels has been taken into account (see Fig. 5, legend). If channels are composed of 2 β-subunit, three distinct populations of channel will be present: (i) wild type channels, (ii) fully mutant channels, and (iii) channels that have both a wild type and a mutant β-subunit (i.e. hybrid channels). The values for the K̄ for amiloride, Hill coefficient, and single channel conductance for the hybrid channels were obtained by minimizing the χ² error of the predicted response for a stoichiometry of 2 to the experimental data. Optimized parameters from nonlinear regression were utilized in likelihood ratio analysis. This analysis indicated that n = 1 is e⁻25 times more likely to produce the experimental results than n = 2. Therefore, the experimental data is most consistent with the n = 1 stoichiometry of one.

β-Subunit stoichiometry can also be illustrated graphically
by transforming the amiloride titration data (Fig. 5A) according to Equation 2 (see Fig. 5B, legend). Fig. 5B illustrates the outcome of such a transformation using data collected from oocytes injected with 1:4 mixture of wild type (α, β, γ) and mutant (α, β, γ542C) mENaC cRNAs as illustrated in Fig. 6A. The predicted responses for γ-subunit stoichiometries of 1 and 2 are also plotted in the figure. In generating the predicted response for a stoichiometry of 2, the Hill coefficient, K, for amiloride and single channel conductance for the hybrid species (channels containing both wild type γ and the γ542C mutant) were inferred from minimizing χ² error by nonlinear regression. Likelihood analysis indicate that n = 1 fits the experimental data e⁰¹ times better than n = 2.

Fig. 6B displays the analysis of transformed data obtained from 1:4 mixture of wild type (α, β, γ) and mutant (α, β, γ542C). Similar to the β-subunit, as amiloride concentration increases, the transformed data approach a value of 1 for γ-subunit stoichiometry. Therefore, this analysis indicates that a single γ-subunit takes part in formation of ENaC.

**DISCUSSION**

Our analyses of inhibition of wild type and mutant mENaCs suggest that ENaCs are composed of two α-subunits, one β-subunit, and one γ-subunit. This channel has a quaternary structure, similar to that reported for K, K, and voltage-gated Na⁺ and Ca²⁺ channels (Na⁺ and Ca²⁺ channels are composed of a large polypeptide with 4-fold internal symmetry) (27, 29, 31, 32). As mentioned above, the analysis of subunit stoichiometry rests on the assumption that the integration of wild type or mutant subunits into Na⁺ channels during assembly is a random event. The validity of this assumption is supported by analyses of currents expressed by oocytes injected with mixtures of wild type and mutant subunits that demonstrated that the distribution of these subunits was indeed binomial. Our analysis also rests on the assumption that a single drug-sensitive subunit confers blocker sensitivity to the channel. Again, this is a valid assumption. We demonstrated that channels that have one wild type and one mutant (i.e. α5S83C α-subunit, or channels that have two mutant α-subunits, are completely blocked by MTSEA (Figs. 2, B and C, and 4). As Na⁺ channels have only one β-subunit and one γ-subunit, the presence (or lack) of a mutant β-subunit (αβG525C, γ) or mutant γ-subunit (αβ, γG542C) determines the sensitivity to amiloride.

Firsov and co-workers (33) have recently reported an ENaC subunit stoichiometry in agreement with what we observed, using both a biophysical approach as well as analysis of ENaC subunit concatamers, although limitations in the use of concatamers to determine stoichiometry of ion channels have been described (34). Snyder and co-workers (35) have recently reported an entirely different ENaC stoichiometry of 3 α-, 3 β-, and 3 γ-subunits. This proposed stoichiometry of 9 subunits would be unique for an ion channel. In utilizing a biophysical approach to determine subunit stoichiometry, a major assumption is that other than differential sensitivities to inhibitors, functional properties of mutant and wild type channels are similar (27, 28). These authors utilized 2 different mutants to determine γ-subunit stoichiometry, one of which was used in our study (γG542C-mENaC) (35). mENaCs that have this mutant have a single channel conductance that is 69% of wild type. If we did not include a correction for the reduced conductance of the mutant channel, our results would indicate a γ-subunit stoichiometry of between 1 and 2. The effects of the other subunit mutations (αS549C, βS520C, γS529C) on human ENaC functional (single channel) properties were not reported. These mutations are within largely hydrophobic regions preceding the predicted second membrane spanning domain of the channel that appear to participate in the formation of the channel pore. If these mutations introduce changes in human hENaC (hENaC) functional properties in the presence (or absence) of the methanethiosulfonate derivatives used in their study, analyses of the response to inhibitors might lead to an error in determination of subunit stoichiometry. In addition, a determination of stoichiometry requires that the relative amounts of Na⁺ subunits expressed in oocytes are precisely known. The authors injected hENaC subunit cDNAs into oocytes and examined subsequent functional expression. In this regard, both transcription and translation efficiencies among the different subunits must be similar. The use of cDNAs, rather than cRNAs, introduces an independent variable that is not present in our expression studies.

Snyder and co-workers (35) also examined the mass of hENaC expressed in COS-7 cells and synthesized in vitro in the presence of microsomal membranes by sucrose density sedimentation. A predicted mass of ~950 kDa was observed, and
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FIG. 5. β-Subunit stoichiometry. A, unblocked response to amiloride of oocytes co-injected with a 1:1 (open circles) or a 1:4 mixture (closed circles) of wild type (i.e. α, β, γ) and mutant (i.e. αβG525C, γ) mENaC cRNAs. Currents were corrected for the values measured in the presence of 5 mM amiloride and normalized to currents measured in the absence of amiloride. Solid lines illustrate the predicted amiloride dose-response assuming a 1:1 stoichiometry of one. Similar data points were observed if a Cs+, assuming a stoichiometry of 1. Similar data points were observed if a 1:4 mixture of wild type (i.e. αβG525C, γ) mENaC cRNAs. Currents were corrected for the values measured in the presence of 5 mM amiloride and normalized to currents measured in the absence of amiloride. Solid lines illustrate the predicted amiloride dose-response assuming a 1:1 stoichiometry of one. Dashed lines illustrate the predicted amiloride dose-response assuming a 1:2 stoichiometry of two. The conductance difference between the wild type and the mutant channels is taken into consideration in calculating the predicted dose-response curve by weighting the probability of occurrence of each species of channel by its conductance. In generating the predicted curve for n = 2, the Kc, and Hill coefficient for amiloride and the single channel conductance for hybrid channels (i.e. channels with one wild type and one mutant β-subunit) were obtained by minimizing the χ² error of the predicted response for n = 2 to the experimental results. Points represent data obtained from four to 11 experiments. Error bars indicate the standard errors. B, the amiloride titration data points (A) were transformed according to the following equation,

\[ \frac{1}{f^{\text{mRT}}} \ln \left( \frac{U_{\text{mix}}}{U_{\text{mut}}} \right) = n - \frac{1}{f^{\text{mRT}}} \ln \left( 1 - \frac{R}{U_{\text{mix}}} \right), \quad (\text{Eq. 2}) \]

where $U_{\text{mix}}$ represents unblocked response from oocytes injected with a 1:4 mixture of wild type (i.e. α, β, γ) and mutant (i.e. αβG525C,γ) mENaC cRNAs. $U_{\text{mut}}$ denotes unblocked response of αβG525C,γ mENaC (Fig. 1). R is the unblocked response due to all species containing one or more wild type β-subunits. f represents the effective cRNA ratio, that includes a correction for the differences in single channel conductances between wild type and mutant (i.e. αβG525C,γ) mENaC, assuming a stoichiometry of 1. Similar data points were observed if f was optimized for a stoichiometry of 2. As amiloride concentration increases, R diminishes and the right side of Equation 2 approaches n, the stoichiometry. The solid lines represent the theoretical curves for subunit stoichiometry of 1 and 2. Data points are from four to seven experiments. Error bars indicate the standard errors. These data are consistent with a β-subunit stoichiometry of one.

the authors suggest that this size is consistent with an ENaC stoichiometry of 3 α, 3 β, and 3 γ-subunits. However, ENaC is likely associated with cytoskeletal proteins, and these associated proteins may contribute to the apparent size of the ENaC complex, assessed by sucrose density sedimentation. For example, we have observed that ankyrin, α-spectrin, and the protein Apx (Apical protein Xenopus) (36) co-immunoprecipitate with
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α-Subunits by themselves can form ENaC channels (12, 17). Recent studies, presented in abstract form, suggest that these α-subunit channels are composed of four subunits (37), consistent with the tetrameric structure that we report for α, β, γ Na⁺ channels. These studies used an α-subunit that has a deletion of a 6-residue tract (rat α-ENaCΔ278–283 (α-rENaCΔ278–283)) that results in a loss of sensitivity of the channel to submicromolar concentrations of amiloride (38). Oocytes expressing αΔ278–283, β, γ-rENaCs have current levels that are markedly lower than currents observed in oocytes expressing wild type α, β, γ-rENaCs (38). This precluded the use of α-ENaC Δ278–283 in our analysis of α, β, γ-ENaC stoichiometry. We previously observed that α-subunit channels formed by the mutant α-ENaC H282D was insensitive to submicromolar concentrations of amiloride (38). Interestingly, αH282D, β, γ-rENaCs is inhibited by amiloride with an IC₅₀ similar to wild type α, β, γ-rENaC (data not shown), again precluding the use of this mutant in our analysis of α, β, γ-ENaC stoichiometry. α, β, γ, Or α, β-subunits expressed in oocytes form functional Na⁺ channels (12). McNicholas and Canessa (39) suggested that α, β-rENaC and α, γ-rENaC are composed of equal numbers of α- and β-subunits or of α- and γ-subunits. As our results suggest that ENaCs have a tetrameric structure, we propose that these channels are composed of 2 α- and 2 β-subunits or of 2 α- and 2 γ-subunits.

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