The Second Hydrophobic Domain Contributes to the Kinetic Properties of Epithelial Sodium Channels*

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The epithelial sodium channel (ENaC) is the prototype of a new class of ion channels known as the ENaC/Deg family. The hallmarks of ENaC are a high selectivity for Na+, block by amiloride, small conductance, and slow kinetics that are voltage-independent. We have investigated the contribution of the second hydrophobic domain of each of the homologous subunits α, β, and γ to the kinetic properties of ENaC. Chimeric subunits were constructed between α and β subunits (α-β) and between γ and β subunits (γ-β). Chimeric and wild-type subunits were expressed in various combinations in Xenopus oocytes. Analysis of whole-cell and unitary currents made it possible to correlate functional properties with specific sequences in the subunits. Functional channels were generated without the second transmembrane domain from α subunits, indicating that it is not essential to form functional pores. The open probability and kinetics varied with the different channels and were influenced by the second hydrophobic domains. Amiloride affinity, Li+/Na+ selectivity, and single channel conductance were also affected by this segment.

The epithelial sodium channel (ENaC) mediates Na+ reabsorption in many epithelial tissues including the distal nephron, colon, lung, and secretory glands. ENaC channels are heteromultimeric proteins formed by the association of homologous subunits: α, β, and γ. The second transmembrane domain (M2) from each subunit participates in forming the ion pathway. α subunits can generate functional channels, but β or γ cannot; nonetheless, they impart specific properties to the heterologimeric complex. For instance, α in combination with either β or γ forms channels with levels of expression intermediate between α alone and αβγ (1). The need to express α subunits in order to induce amiloride-sensitive currents has suggested that they are essential to form the channel pore. The subunits of ENaC are 70–80-kDa glycoproteins. A hydropathy profile of the amino acid sequence, gene fusion experiments, identification of glycosylation sites, and partial proteolysis have confirmed a simple structure for the subunits characterized by two transmembrane segments (M1 and M2), a large extracellular domain with multiple N-glycosylation sites, and the amino and carboxyl termini in the cytoplasmic side (2–4).

Several lines of evidence indicate that the M2 domains of the subunits determine the properties of the ion pore. This is the most conserved region among all of the channels that form the ENaC/Deg family of ion channels. Members include the mammalian acid-sensitive ion channels (5), the peptide-gated Na+ channel from the snail Helix aspersa (6), the degenerins from Caenorhabditis elegans (7), and two channels cloned from Drosophila: pickpocket and ripped pocket (8).

According to the predicted secondary structure, M2 includes a typical α-helix, long enough to traverse the plasma membrane, and a less structured segment of hydrophobic residues preceding the α-helix. Mutations of residues located in the initial segment of M2 alter the affinity of channels for the blocker amiloride and the ion selectivity (9–11).

We previously showed that channels formed by α with β (αβ) and α with γ subunits (αγ) differ in many functional properties such as in affinity for amiloride, ion selectivities, unitary conductances, and single channel kinetics (12, 13). Here, we constructed chimeric subunits between α and β (α-β) and between β and γ subunits (γ-β) and expressed them in various combinations in Xenopus oocytes in order to examine the properties of whole-cell and unitary currents of the chimeric channels. Taking advantage of the differences in their functional properties, we have correlated specific amino acid sequences in the subunits to functional properties in order to gain insight into the structure-function of these channels.

MATERIALS AND METHODS

Construction of Chimeras and cRNA Synthesis—Two types of chimeras were made: γ-β containing the amino terminus of γ and the carboxyl terminus of β, designated (γ-βS581), (γ-βS535), and (γ-βE540); and α-β containing the amino terminus of α and the carboxyl terminus of β, designated (α-βS580). The letters and numbers indicate the first residue that belongs to the β subunit in each chimera. Chimeras were made using polymerase chain reaction according to the protocol previously described (12) and were subcloned in pSPORT vector (Life Technologies, Inc.). Point mutations in the β subunit were also generated by polymerase chain reaction: βS529S and βS530C. A stop codon was introduced in the carboxyl terminus of the β subunit at position Arg1984. This truncation has been shown before to increase the level of expression of channels at the plasma membrane without altering other properties (14). All constructs were verified by DNA sequencing. Capped cRNAs from linearized cDNAs were synthesized in vitro using T7 mMESSAGEmACHINE (Ambion, Austin, TX) according to the provider’s instructions.

Preparation and Injection of Oocytes—Xenopus laevis oocytes were surgically removed from adult females and prepared with standard procedures. Stage V and VI oocytes were injected with 1 ng each of the following cRNAs: α and (γ-βS581), α and (γ-βS529), α and (γ-βS535), α and (γ-βE540), (α-βS580) with γ, and (α-βS508) with (γ-βS481). After injection, oocytes were incubated at 19 °C for 2–4 days in amphibian Ringer solution supplemented with 10 μM amiloride.

Electrophysiology and Data Evaluation—Channel activity was recorded using either two-microelectrode voltage clamp or patch clamp techniques. For two-microelectrode recordings, current and voltage electrodes were pulled from borosilicate glass, had resistances <1 megaohm, and were filled with 3 M KCl. ENaC activity was calculated

This work was supported by National Institutes of Health Grants 5-R01-DK54062 and I-P50HL55007. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: ENaC, epithelial sodium channel; M1 and M2, first and second transmembrane domain, respectively; EVL file, events list file.
from the difference in whole-cell current before and after the addition of 50 μM amiloride to the bathing solution. Currents were recorded with an OC-725B oocyte clamp (Warner Instrument Corp., Hamden, CT), digitized at 0.1 kHz (ITC-16; HEKA, Lambrecht, Germany), and stored on a hard disc. Membrane potential was held at 260 mV. Current-voltage relations were generated by changing the membrane potential from −180 to 80 mV in 20-mV incremental steps of 200-ms duration. I/V curves were fitted to the constant field equation. The standard bath solution was composed as follows: 100 mM sodium gluconate, 4 mM KCl, 2 mM CaCl₂, and 10 mM HEPES, pH adjusted to 7.4. In some experiments, 100 mM Na⁺ was replaced by 100 mM Li⁺ or K⁺. The Kᵢ values of amiloride were calculated by measuring the fractional inhibition of whole-cell currents produced by increasing concentrations of amiloride in the bath solution. The data were fitted to the equation,

\[ \frac{I}{I_{\text{max}}} = \frac{1}{1 + A/K_i} \]  

(Eq. 1)

where A represents the amiloride concentration and Kᵢ is the half-inhibition constant. For patch clamp recordings, patch pipettes were pulled from borosilicate glass and typically had tip resistances of about 5 megaohms when filled with pipette solution. All recordings were made in the cell-attached configuration. The compositions of pipette solutions were as follows: 150 mM NaCl or 150 mM LiCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH adjusted to 7.4. The bath solution in all experiments was as follows: 150 mM KCl, 5 mM EDTA, 10 mM HEPES, pH adjusted to 7.4.

An Axopatch 200A amplifier and Digidata 1200A (Axon Instruments, Foster City, CA) interfaced to a PC were used to acquire data at 5 kHz. The data were filtered at 200 Hz during acquisition using an eight-pole Bessel filter (Frequency Devices, Inc., Haverford, MA) and stored directly on the hard drive of a PC. Axon’s pClamp 6 software was used for data analysis. Data were filtered digitally at 50 Hz for analysis and display.

I/V relations were constructed by measuring current passing through channels between 0 and −80 mV, and the single channel conductance was subsequently estimated by linear regression between −20 and −80 mV.

Fetchan was used to generate events list files (EVL files) from the data files. A 50% threshold was set to determine transitions between the open and closed levels from at least 10 min of data recorded at −40 mV. Channel open probability (Pₒ) was calculated from the EVL files generated by Fetchan using a specialized NP{o} program written by Jinliang Sui (Mount Sinai School of Medicine, New York, NY) (15) and available on the World Wide Web. The NP{o} program reads Fetchan EVL files of any size and expresses these files as the product of N (number of channels in the membrane patch; maximum N = 5) and Pₒ versus time.

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M2 Contributes to Kinetic Properties of ENaCs

The resolution of changes in NP, determined by a user-selectable bin width ranging from 0.1 to 30 s/bin. To express the NP, of each patch, we used the mean of successive 30-s measurements made over the entire EVL file. Patches containing single and multiple channels were used to calculate $P_o$.

Open and closed times were calculated from histograms generated using Petchan EVL files and the Pstat program within pClamp in logarithmic binning mode (i.e., a plot of number of observations versus log dwell time in ms). The fitting method was simple-least squares. Results are expressed as mean ± S.E. Differences between groups were assessed using Student’s t test. $p < 0.05$ was considered to be statistically significant.

RESULTS

Expression of Chimeras in Xenopus Oocytes—In order to define the domains responsible for conferring the functional differences between $\alpha$ and $\gamma$, we generated several chimeric subunits that contained the amino terminus of $\gamma$ and the second hydrophobic domain (M2) of the $\beta$ subunit. We made four $\gamma$-$\beta$ chimeras, ($\gamma$-E8481), ($\gamma$-E540), ($\gamma$-L535), and ($\gamma$-E540), each of which contained progressively fewer sequences from $\beta$. The letter and number indicate the first residue from the $\beta$ subunit at the junction of the chimera. An $\alpha$-$\beta$ chimera was also made; it contained most of the $\alpha$ sequences and the M2 from $\beta$, (a-$\beta$-S508). A schematic representation of the chimeras is shown in Fig. 1.

None of the $\gamma$-$\beta$ chimeras injected alone or cojected with $\beta$ or $\gamma$ subunits induced a significant increase in oocyte whole-cell conductance. However, cojection with wild-type $\alpha$ subunits induced several $\mu$A of amiloride-sensitive current that were in the range of 2–6 $\mu$A/oocyte.

Injection of $\alpha$-$\beta$ chimera with wild-type $\gamma$ induced currents in the range of 3–8 $\mu$A (4.8 ± 1.2 $\mu$A/oocyte), but no currents were observed when the $\alpha$-$\beta$ chimera was injected alone or when cojected with wild-type $\beta$ or $\gamma$ subunits.

Two different chimeras injected together, $\alpha$-$\beta$ with $\gamma$-$\beta$, induced functional channels with cells expressing 2–4 $\mu$A of whole-cell current. The magnitude of the amiloride-sensitive currents expressed by these channels appears in the right column of Fig. 1.

These experiments showed that sodium channels, sensitive to amiloride block, can be formed by combinations of chimeras and subunits other than $\alpha$, $\beta$, and $\gamma$. Most significantly, $\gamma$ with $\alpha$-$\beta$ chimera and two chimeras $\alpha$-$\beta$ with $\gamma$-$\beta$ induced sodium currents although these channels do not have the M2 from $\alpha$ subunits.

Table I

| Channel $P_o$ | Open time | Closed time |
|---------------|-----------|-------------|
| $\alpha$-$\gamma$-S481 | 0.94 ± 0.03 | 3360 ± 1240 | 20 ± 15 |
| $\alpha$-$\gamma$-G529 | 0.62 ± 0.04 | 215 ± 33 | 82 ± 48 |
| $\alpha$-$\gamma$-L535 | 0.21 ± 0.02 | 232 ± 30 | 560 ± 50 |
| $\gamma$-$\gamma$-E540 | 0.12 ± 0.04 | 224 | 14.0 |
| $\gamma$-$\alpha$-S508 | 0.84 ± 0.07 | 4610 ± 916 | 104 ± 8 |
| ($\alpha$-$\beta$-S508) ($\gamma$-$\beta$-S481) | 0.77 ± 0.07 | 520 ± 370 | 84 ± 18 |

* Significantly different from $\alpha$-$\gamma$-S481 ($p < 0.05$).
* Significantly different from $\alpha$-$\gamma$-G529 ($p < 0.001$).
* Significantly different from $\alpha$-$\gamma$-E540 ($p < 0.01$).
* Significantly different from $\gamma$-$\beta$-E540 ($p < 0.001$) and $\alpha$-$\gamma$-L535 ($p < 0.02$).
* Values pooled from three patches.

Fig. 3. Dwell time histograms of open and closed events recorded from $\alpha$-$\gamma$-S481 (A), $\alpha$-$\gamma$-G529 (B), $\alpha$-$\gamma$-L535 (C), and $\alpha$-$\gamma$-E540 (D) obtained from 7–9 patches containing single channels. Data were collected at −40 mV. The solid lines represent the fit of each dataset with a single exponential probability density function. The time constants (in ms) from each exponential are shown for the open ($\tau_o$) and closed ($\tau_c$) states.

Open Probability and Kinetics of Single Channels—The level of expression of all of the functional constructs was consistently above 1 $\mu$A/oocyte, making possible the characterization of the properties of unitary currents of these channels.

We examined the kinetics and $P_o$ of all of the functional channels generated by injection of various combinations of subunits and chimeras. For kinetic studies, only patches containing single channels were included in the analysis, while for calculation of $P_o$, patches with single and multiple channels were considered.

Fig. 2 shows representative examples of patches containing single channels for the four different $\alpha$-$\gamma$-$\beta$ channels. $\alpha$-$\gamma$-S481 channels exhibited very high $P_o$ (0.94 ± 0.03) that was indistinguishable from our previous report of $\alpha$-$\beta$ channels ($P_o < 1$). The next three $\gamma$-$\beta$ chimeras, that contain progressively less sequence from the $\beta$ subunit, had significantly lower $P_o$ values that were 0.62 ± 0.04 for $\alpha$-$\gamma$-G529, 0.21 ± 0.02 for $\alpha$-$\gamma$-L537, and 0.12 ± 0.04 for $\alpha$-$\gamma$-E540 (Table I).

The four types of channels containing $\gamma$-$\beta$ chimeras had kinetics described by only one open and one closed state. Fig. 3 shows histograms of the distribution of dwell times of open and closed events. The histograms were constructed with data from several patches containing single channels in order to accumulate a large enough number of events. In some cases, such as for $\alpha$-$\gamma$-E540 channels, the long duration of the closed state prevented the accumulation of a large number of events. All histograms were well fitted with a single exponential probability density function. The time constants for the open ($\tau_o$) and closed ($\tau_c$) states are also shown in Fig. 3. The values of the
Fig. 4A, representative example of a cell-attached patch containing a single γ(α-βS508) channel. B, IV relationship of unitary currents obtained within 150 mM Na+ in the pipette. Data points represent the mean of four different patches. Error bars represent S.E. C, IV of the amiloride-sensitive component of whole-cell currents in the presence of 100 mM Na+ or Li+ in the bath solution. Values were normalized to the measurement made with 100 mM Na+ at −100 mV. Lines represent the fit of the data to the constant field equation. Symbols show the mean ± S.E. of measurements obtained from six oocytes.

Fig. 5A, representative example of a cell-attached patch containing a single (α-βS508)(γ-βS481) channel. B, IV relationships of unitary currents obtained in the presence of 100 mM Na+ or Li+ in the pipette. Data points represent the mean of three (Li+) and seven (Na+) different patches. Error bars represent S.E. C, IV of the amiloride-sensitive component of whole-cell currents in the presence of 100 mM Na+ or Li+ in the bath solution. Values were normalized to the measurement made with 100 mM Na+ at −100 mV. Lines represent the fit of the data to the constant field equation. Symbols show the mean ± S.E. of measurements obtained from eight oocytes.

durations of open and closed events were also calculated from individual patches, and the mean ± S.E. of all of these values appear in Table I. The progressive decrease in lower P_o exhibited by the γ-β chimeric channels was mainly due to longer closed times, from 48 ± 15 ms for α(γ-βS481) channels to 14.5 s for (γ-βE540) channels.

Figs. 4A and 5A show representative examples of patches containing single γ(α-β) or (α-β)(γ-β) channels. The P_o of γ(α-βS508) channels was 0.84 ± 0.07, and P_o of (α-βS508)(γ-βS481) channels was 0.77 ± 0.07. The mean open and closed times are shown in Table I and in the histograms of Fig. 6. The values for τ_o and τ_c were 4610 ± 916 and 520 ± 270 ms, and for τ_g they were 104 ± 8 and 84 ± 18 ms, respectively. The differences in the values for τ_o, τ_g, and τ_c reported in Table I and Fig. 6 result from taking the mean ± S.E. from each individual patch (Table I) or from pooling the data from all patches to construct the dwell time histograms (Fig. 6).

Amiloride Affinity of Chimeric Channels—In addition to the differences in P_o and kinetics, other properties were noticed to be different among the chimeric channels. The affinity for amiloride was examined by measuring the fractional block of whole-cell currents by increasing concentrations of amiloride in the perfusate. Measurements were done with the two-electrode voltage clamp in the presence of 100 mM Na+ in the bath and at a membrane potential of −60 mV. The K_i values for α(γ-βS481) and α(γ-βG529) were 1.75 ± 0.24 and 1.2 ± 0.11 μM, respectively (Table II). For α(γ-βE540) and α(γ-βE540), the K_i values were 0.25 ± 0.05 and 0.2 ± 0.02, respectively. For the first two chimeric channels, the K_i values were similar to that of αβ, whereas the K_i values of the last two channels were equal to that of αγ channels. These results are in agreement with the notion that residues located in the segment preceding M2 determine amiloride affinity. Since the first two channels have the preceding M2 segment from β subunits, they had the K_i of αβ channels (1 μM); in contrast, the last two chimeras have the preceding M2 segment from γ, and therefore they exhibit a K_i similar to αγ channels (0.1 μM).

The affinities of γ(α-βS508) and (α-βS508)(γ-βS481) channels were lower, with K_i of 4.3 ± 0.2 and 5.5 ± 0.4 μM, respectively (Table II).

Ion Selectivity and Single Channel Conductance—Ion selectivity and single channel conductance were also affected by the
M2 contributes to kinetic properties of ENaCs

**Fig. 6.** Dwell time histograms of open and closed events recorded from γ(α-β)S508 (A) and (α-β)S508/γ-βS481 (B) single channel patches. Data were collected at ~40 mV. The solid lines represent the fit of each data set with a single exponential probability density function. The time constants (in ms) from each exponential are shown for the open (τo) and closed (τc) states.

**Table II**
Summary of single channel conductance, Li⁺/Na⁺ selectivity, and amiloride Kᵢ from chimeric channels

| Conductance | Li⁺ | Na⁺ | Li⁺/Na⁺ selectivity | Amiloride Kᵢ |
|-------------|-----|-----|----------------------|-------------|
|             |     |     | Patch | TEVC | |
| α(γ-β)S481 | 4.8 ± 0.2 (3) | 4.7 ± 0.3 (3) | 1:1 | 1:1 | 1.75 ± 0.15 |
| α(γ-β)S529 | 4.9 ± 0.2 (4) | 4.7 ± 0.4 (6) | 1.1 | 1.1 | 1.2 ± 0.04 |
| α(γ-β)535 | 5.0 ± 0.3 (4) | 4.0 ± 0.1 (4) | 1.2:1 | 1.1:1 | 0.25 ± 0.01 |
| α(γ-β)540 | 5.2 ± 0.2 (4) | 3.7 ± 0.3 (4) | 1:4:1 | 1:3:1 | 0.2 ± 0.06 |
| γ(α-β)S508 | ND | 3.2 ± 0.2 (4) | ND | 0.6:1 | 4.3 ± 0.82 |
| (α-β)S508/γ-βS481 | 3.8 ± 0.1 (3) | 7.9 ± 0.5 (7) | 0.5:1 | 0.5:1 | 5.5 ± 0.65 |

a Two electrode voltage clamp.
b p < 0.02 for α(γ-β)537.
c p < 0.01 for α(γ-β)540.
d ND, not determined. Significantly larger Li⁺ than Na⁺ conductance.

TABLE II

M2 subunit composition. The conductance and selectivities for Na⁺ and Li⁺ were determined by the ratio of unitary currents in the presence of 150 mM Li⁺ or Na⁺. The amiloride Kᵢ was calculated from the fractional inhibition of whole-cell currents produced by increasing concentrations of amiloride in the perfusate. Data were fitted to Equation 1. All values are mean ± S.E. The number of observations for the different experimental conditions is given in parentheses.

M2 subunit composition affects the selectivity of ENaC. For instance, channels formed by α, β, and γ subunits are highly selective for Na⁺ over K⁺ (Na⁺/K⁺ ratio of 1.9:1) and slightly more permeable to Li⁺ than to Na⁺ (Li⁺/Na⁺ ratio of 1.5:1). αγ channels also exhibit Li⁺/Na⁺ permeabilities of 1.5:1, whereas αβ channels show Li⁺/Na⁺ permeabilities of 1.1 (13).

All the functional chimeric channels conducted in the presence of Na⁺ or Li⁺ but none in the presence of K⁺. α(γ-β)S481 and α(γ-β)5529 channels were equally permeable to both cations (I_{Li⁺/Na⁺} of 1:1). α(γ-β)535 and α(γ-β)540 channels were more permeable to Li⁺ with I_{Li⁺/Na⁺} of 1.2:1 and 1.4:1, respectively (Fig. 7 and Table II). Differences in the I_{Li⁺/Na⁺} were due mainly to a decrease in the Na⁺ conductance from 4.7 to 3.7 picoisemins with little change in the Li⁺ conductance, which remained close to 5 picoisemins for all α(γ-β) channels. γ(α-β)S508 and (α-β)S508/γ-βS481 channels were more permeable to Na⁺ than to Li⁺; the I_{Li⁺/Na⁺} ratios were smaller, 0.6:1 and 0.5:1, respectively (Figs. 4C and 5C and Table II). The conductance of γ(α-β)S508 and (α-β)S508/γ-βS481 channels with Na⁺ in the pipette was 3.2 and 7.9 picoisemins, respectively. We could not measure the unitary currents of α(γ-β)S508 channels with Li⁺ because of its small magnitude.

**Discussion**

The Role of a Subunit M2 in Channel Function—Until now, the presence of the α subunit M2 was thought to be critical for expression of functional amiloride-sensitive Na⁺ channels. However, we have shown that the chimeric channels, composed of γ(α-β)S508 and (α-β)S508/γ-βS481, both of which lacked the M2 from the α subunit, gave rise to functional channels. When compared with wild-type channels, the properties of γ(α-β)S508 and (α-β)S508/γ-βS481 were different. Nonetheless, these channels still possessed the general basic properties of ENaC in that they were selective for Na⁺ and not for K⁺ (but I_{Na⁺} > I_{Li⁺}) and in that channel activity could still be inhibited by amiloride, although at slightly higher doses.

Our observations indicate that the pore forming region of the α subunit is not a prerequisite for the expression of functional
There have been numerous studies to demonstrate the putative roles of various residues in ion permeation and amiloride block (10, 11). However, none of the studies attempted to characterize if the various mutations altered channel kinetics or ionic selectivity, increased channel activity and ionic selectivity, or altered channel gating (13). Most likely, the cytoplasmic region preceding M1 can also alter the kinetics of ENaC channels. Mutations of residues β3G37S and α9G95S in this region decrease the \( P_o \) (18). However, it is unlikely that the N-terminal domain constitutes the gate. The amino acid sequence of the N-terminal domain is one of the least conserved among all members of the ENaC/degenerin family. In addition, a large segment from the N terminus of α, β, and γ can be deleted without affecting activity (2). Most likely, α-Gly37 and β-Gly525 are not located in the gate, but their mutations produce an allosteric effect that alters the \( P_o \). On the other hand, we can rule out the gate being located at the level of the C terminus, because deletions of the C terminus of β and γ do not alter channel gating (13).

Determinants of Amiloride Affinity and Selectivity—Amiloride blocks all of the channels belonging to the ENaC/degenerin family, although the affinities for amiloride vary widely among each of the individual members. Amiloride blocks the open channel by occluding the pore in a voltage-dependent manner; it senses approximately 15% of the membrane electrical field in wild-type ENaC. Therefore, the site of action of amiloride is most likely at the outer part of the pore, which is formed by the first segment of M2 (10). The residues α-Ser583, β-Gly525, and γ-Gly536 all contribute to form a high affinity site \( K_i = 0.1 \mu M \), maybe by forming a ring to accommodate the amiloride molecule. In agreement with this notion, our analysis of the amiloride affinity of α(γ-β) channels showed that the \( K_i \) was influenced by the first segment of M2. However, the results also showed that, in addition, other factors contribute to define the amiloride affinity. αβ, αγ, and all four chimeric α(γ-β) channels have rings formed by identical residues, yet their amiloride affinities are different. Among the α(γ-β) channels, we found that the transition from low to high amiloride \( K_i \) occurred contained only part of the M2 from γ and also channels that did not have any sequences from γ M2 (α-β508/γ-β508). The results showed that the M2 region of the subunits markedly influenced the \( P_o \) and kinetics of channels. However, regardless of the subunit composition, all channels exhibited closures (although the \( \tau_c \) was of different duration for each type of channel), indicating that the gating mechanism is not exclusively located in any one subunit but that most likely all three subunits contribute to it.

The cytoplasmic region preceding M1 can also alter the kinetics of ENaC channels. Mutations of residues β3G37S and α9G95S in this region decrease the \( P_o \) (18). However, it is unlikely that the N-terminal domain constitutes the gate. The amino acid sequence of the N-terminal domain is one of the least conserved among all members of the ENaC/degenerin family. In addition, a large segment from the N terminus of α, β, and γ can be deleted without affecting activity (2). Most likely, α-Gly37 and β-Gly525 are not located in the gate, but their mutations produce an allosteric effect that alters the \( P_o \). On the other hand, we can rule out the gate being located at the level of the C terminus, because deletions of the C terminus of β and γ do not alter channel gating (13).

Determinants of Amiloride Affinity and Selectivity—Amiloride blocks all of the channels belonging to the ENaC/degenerin family, although the affinities for amiloride vary widely among each of the individual members. Amiloride blocks the open channel by occluding the pore in a voltage-dependent manner; it senses approximately 15% of the membrane electrical field in wild-type ENaC. Therefore, the site of action of amiloride is most likely at the outer part of the pore, which is formed by the first segment of M2 (10). The residues α-Ser583, β-Gly525, and γ-Gly536 all contribute to form a high affinity site \( K_i = 0.1 \mu M \), maybe by forming a ring to accommodate the amiloride molecule. In agreement with this notion, our analysis of the amiloride affinity of α(γ-β) channels showed that the \( K_i \) was influenced by the first segment of M2. However, the results also showed that, in addition, other factors contribute to define the amiloride affinity. αβ, αγ, and all four chimeric α(γ-β) channels have rings formed by identical residues, yet their amiloride affinities are different. Among the α(γ-β) channels, we found that the transition from low to high amiloride \( K_i \) occurred contained only part of the M2 from γ and also channels that did not have any sequences from γ M2 (α-β508/γ-β508). The results showed that the M2 region of the subunits markedly influenced the \( P_o \) and kinetics of channels. However, regardless of the subunit composition, all channels exhibited closures (although the \( \tau_c \) was of different duration for each type of channel), indicating that the gating mechanism is not exclusively located in any one subunit but that most likely all three subunits contribute to it.
between chimera (γ-βG529) \((K_\gamma = 1.2 \pm 0.04)\) and chimera (γ-βL537) \((K_\gamma = 0.25 \pm 0.01)\). The main difference between these chimeras is the presence of residues Gly529 and Gly530 in β and of Ser541 and Cys542 in γ. To examine whether these two residues were responsible for conferring the differences in \(K_\gamma\), we expressed the mutant channels αβG529S and αβG530C, expecting to obtain channels with low \(K_\gamma\). Unfortunately, these mutants were not functional when expressed with α subunits alone but induced large currents when coexpressed with wild-type α and γ. The amiloride \(K_\gamma\) values of αβG529Sγ and αβG530Cγ were \(-0.1\) μM, indicating that these residues, although essential for ion permeation, do not change the amiloride affinity. The observations suggest that, in addition to the composition of the side chains forming the putative binding site for amiloride, other surrounding amino acids influence the structure of the site and thus alter amiloride affinity.

As expected from the small differences between αβ and αγ channels, all chimeric channels were permeable to Na\(^+\) and Li\(^+\) but not to K\(^+\). Experiments with γ-β chimeras showed that M2 changes the \(I_{\text{Li}}^{\alpha}/I_{\text{Na}}^{\alpha}\) ratio from 1:1 (α channels) to 1.5:1 (αγ channels). The change was due to a progressive decrease in Na\(^+\) currents without a significant change in the magnitude of Li\(^+\) currents as indicated in Table II. These results show that the critical residues that determine selectivity and conductance are located in the initial segment of M2 as has recently been shown by Kellenberger et al. (11, 19). The corresponding residues in the three subunits (α-Gly587, β-Gly529, and γ-Ser541) and a ring of conserved serines (α-Ser589, β-Ser531, and γ-Ser543) form the putative selectivity filter. However, differences in \(I_{\text{Li}}^{\alpha}/I_{\text{Na}}^{\alpha}\) ratio and in single channel conductance were observed among channels that had identical residues in the selectivity filter. For instance, pores formed only by α subunits (homeric α channels) have \(I_{\text{Li}}^{\alpha}/I_{\text{Na}}^{\alpha}\) of >1, whereas pores formed only by β subunits ((α-βS508)/(γ-βS481)) have \(I_{\text{Li}}^{\alpha}/I_{\text{Na}}^{\alpha}\) of <1. The same arguments presented for the amiloride affinity seem to apply for the determinants of selectivity in that other elements besides these residues affect the structure of the selectivity filter.

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