The Role of Pre-H2 Domains of α- and δ-Epithelial Na\(^+\) Channels in Ion Permeation, Conductance, and Amiloride Sensitivity*

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Epithelial Na\(^+\) channels (ENaC) regulate salt and water re-absorption across the apical membrane of absorptive epithelia such as the kidney, colon, and lung. Structure-function studies have suggested that the second transmembrane domain (M2) and the adjacent pre- and post-M2 regions are involved in channel pore formation, gating selectivity, and amiloride sensitivity. Because Na\(^+\) selectivity, unitary Na\(^+\) conductance \((\gamma_{Na})\), and amiloride sensitivity of δ-ENaC are strikingly different from those of α-ENaC, the hypothesis that the pre-H2 domain may contribute to these characteristics has been examined by swapping the pre-H2, H2, and both (pre-H2+H2) domains of δ- and α-ENaCs. Whole-cell and single channel results showed that the permeation ratio of Li\(^+\) and Na\(^+\) \((P_{Li}/P_{Na})\) for the swap α chimeras co-expressed with βγ-ENaC in Xenopus oocytes decreased significantly. In contrast, the ratio of \(P_{Li}/P_{Na}\) for the swap δ constructs was not significantly altered. Single channel studies confirmed that swapping of the H2 and the pre-H2+H2 domains increased the \(\gamma_{Na}\) of α-ENaC but decreased the \(\gamma_{Na}\) of δ-ENaC. A significant increment in the apparent inhibitory dissociation constant \(K_{amil}\) was observed in the α chimeras by swapping the pre-H2, H2, and pre-H2+H2 domains. In contrast, a striking decline of \(K_{amil}\) was obtained in the chimeric δ constructs with substitution of the H2 and pre-H2+H2 domains. Our results demonstrate that the pre-H2 domain, combined with the H2 domain, contributes to the \(P_{Li}/P_{Na}\) ratio, single channel Na\(^+\) conductance, and amiloride sensitivity of α- and δ-ENaCs.

The epithelial Na\(^+\) channel (ENaC)\(^3\) was the first subgroup of the ENaC/DEG superfamily cloned from mammals. The topology of ENaC/DEG comprises two short N- and C-terminal intracellular tails, two hydrophobic membrane-spanning domains (M1 and M2), and a large, extracellular loop with two (or three) cysteine-rich domains (1, 2). There is an overall ∼37% amino acid identity between α-, δ-, β-, and γ-subunits. Both the α- and δ-subunits can form independent conducting channels with similar amiloride sensitivities and ion selectivities to those co-expressed with βγ-subunits. The β- and γ-subunits are modifying subunits that regulate the trafficking and conductance of α- and δ-ENaCs (1, 2). The \(\alpha\beta\gamma\)-ENaC has a higher permeability to Na\(^+\) and Li\(^+\) compared with the other alkali metals. For example, \(\alpha\beta\gamma\)-ENaC has a permeability ratio of \(P_{Li}/P_{Na}\) up to 2, but the channel is virtually impermeant to K\(^+\) ions (1, 2). Similar to other ENaC/DEG superfamily members, ENaC is very sensitive to amiloride, displaying an apparent inhibitory dissociation constant \(K_{amil}\) in the nanomolar range (1, 2).

Previous publications (1, 2) have demonstrated that the pre-M2 region of α-ENaC, more precisely, the second hydrophobic domain (H2) preceding the M2 region, serves as the outer mouth of the ENaC pore and is involved in channel gating. An amiloride-binding site has also been identified functionally in the H2 domain in addition to the one more proximal to this domain (3, 4). The H2 domain also forms part of the ion selectivity filter, as deduced by mutation analysis of the pre-M2 region (5–12).

The 20 amino acids comprising the M2 domain mainly function as part of the conductive pathway. Studies on mice, rat, and human α-ENaC support this hypothesis (8, 9, 13, 14). We have shown previously (15) that positively charged residues downstream of the M2 region of α-hENaC (post-M2 domain) also contributed to ion permeation and gating behavior and that the functional diameter of the channel pore, in constructs in which these post-M2 positively charged amino acids were mutated to negatively charged glutamic acids, was altered.

There is 63% diversity in the predicted amino acid sequences between α- and δ-ENaC subunits. δ- and α-ENaCs have three essential biophysical and pharmacological differences (16). First, the whole-cell amiloride-sensitive Na\(^+\) current of δγ-ENaC is greater than the amiloride-sensitive Li\(^+\) current, yielding a Li\(^+/Na^+\) permeation ratio \((P_{Li}/P_{Na})\) of 0.6 rather than 2.0 (for \(\alpha\beta\gamma\)-ENaC). There is no difference in \(P_{Na}/P_{Li}\). Second, the value of \(K_{amil}\) for δγ-ENaC (up to 2.6 μM) is 30-fold greater than that for \(\alpha\beta\gamma\)-ENaC (16). Third, the unitary Na\(^+\) conductance \((\gamma_{Na})\) measured for δγ-ENaC in cell-attached patches is 2.4-fold greater than that of wt \(\alpha\beta\gamma\)-ENaC, but the unitary Li\(^+\) conductance \((\gamma_{Li})\) is unchanged (16). Nothing is known about the molecular basis for the biophysical and pharmacological differences between α- and δ-ENaC.

The ectodomains of Na/K-ATPase, gastric H/K-ATPase (18), inward-rectified and hERG K\(^+\) channel (19, 20), and C5a receptor (21) have been shown recently to mediate ion selectivity, channel conductance, and agonist/ion affinity. The contribu-
tions of the extracellular pre-H2 domains of ENaC to the ion selectivity filter and amiloride inhibition are unknown. The aim of the present study was to test the hypothesis that the pre-H2 domains of α- and δ-ENaC subunits may be integrally associated with amiloride affinity, ion selectivity, and conductance, therefore contributing to the different pharmacological and biophysical properties between α- and δ-ENaC. Our results show that swapping the pre-H2 and/or H2 regions of α- and δ-ENaC carried over the features associated with their parental wild type constructs partly in cation selectivity, single channel conductance, and almost entirely amiloride sensitivity to the chimeric mutations.

EXPERIMENTAL PROCEDURES

Site-directed and Swap Mutagenesis and Functional Expression—Swap and triple mutants were generated in rat α- and human δ-ENaC cRNAs cloned into pSP70 vector (Promega, WI) using the Clameleon Double-stranded Mutagenesis kit (Stratagene) (15). cRNAs were synthesized using the T7 promoter (Ambion, TX) and dissolved in nucleic-free water. Defolliculated oocytes were injected with 50 nl of cRNAs containing α- (or δ-), β-, and γ-ENaC (ratio 1:1:1) for each subunit. Injected oocytes were incubated in 3% strength L-5 medium at 18 °C (22, 23). The triple mutants and swap chimeras were engineered as follows (Fig. 1): the pre-H2 chimeras (αH2 and δH2), swapping α545–568 with δ459–518; the H2 chimeras (αH2 and δH2), exchanging α569–587 with δ519–537; the pre-H2+H2 chimeras (αH2δH2 and δH2αH2), swapping α545–568 with δ459–518 and δ519–537, respectively. These oocytes were injected with 50 nl of cRNAs containing α- (or δ-), β-, and γ-ENaC (ratio 1:1:1) for each subunit. Injected oocytes were incubated in 3% strength L-5 medium at 18 °C (22, 23). The triple mutants and swap chimeras were engineered as follows (Fig. 1): the pre-H2 chimeras (αH2 and δH2), swapping α545–568 with δ459–518; the H2 chimeras (αH2 and δH2), exchanging α569–587 with δ519–537; the pre-H2+H2 chimeras (αH2δH2 and δH2αH2), swapping α545–568 with δ459–518 and δ519–537, respectively. The voltage dependence of amiloride inhibition was also characterized by fitting the bi-ionic I-V curves with the modified Goldman-Hodgkin-Katz current Equation 2,

\[
I_{amil} = \frac{P_{amil} \cdot z^2 \cdot E_{amil}}{RT} \cdot e^{-\frac{zF \cdot E_{amil}}{RT}}
\]

where \( I_{amil} \) represents the amiloride-sensitive current carried by the cation \( X \); \( P_{amil} \) is the amiloride permeability value for the cation \( X \); and \( E_{amil} \) is the energy of the amiloride; and \( F \) have their usual meanings. The corresponding permeability coefficients were expressed as \( P_{amil} \) and \( F \), respectively.

To analyze the affinity of amiloride to the channel, perfusates containing different concentrations of amiloride (ranging from 0 to 1 mM) were switched into the chamber. Both the \( K_{amil}^{exp} \) and Hill coefficient were retrieved by fitting the dose-response curves of amiloride with the Hill Equation 3,

\[
I = \frac{1}{1 + \left(\frac{X_{amil}}{K_{amil}}\right)^n}
\]

where \( K_{amil}^{exp} \) is the concentration required for inhibiting half of the maximal current; I is the measured amiloride-sensitive current at the concentration of Amil; and \( n \) represents the Hill coefficient.

The voltage dependence of amiloride inhibition was also characterized by fitting the plot of \( K_{amil}^{exp} \) as function of membrane voltage with the Woodhull Equation 4 (24),

\[
K_{amil}^{exp}(E_{amil}) = K_0 \cdot \exp\left(\frac{\delta \cdot z \cdot F \cdot E_{amil}}{R \cdot T}\right)
\]

where \( K_{amil}^{exp}(E_{amil}) \) is the equilibrium inhibitory dissociation constant at a test potential \( E_{amil} \); \( K_{amil}^{exp}(0) \) is the \( K_{amil}^{exp} \) at 0 mV; \( \delta \) is the fraction of distance across the electric field that can be sensed by amiloride; \( z \) is +1 for amiloride; and \( R \), \( T \), and \( F \) have their usual meanings.

RESULTS

The purpose of the study was to test the role of the pre-H2 regions of α- and δ-ENaC subunits in ion permeation, conductance, and amiloride inhibition. Because these properties of α- and δ-ENaC are different significantly from each other at the macroscopic and microscopic current levels, these particular subunits were chosen for analysis. The chimeras...
were engineered by swapping the pre-H2 region as well as the H2 domain. Fig. 1 shows that there is 45.8 and 52.6% identity (37% identity for full-length rENaC and δ-ENaC) between the pre-H2 and H2 regions, respectively.

Ion Selectivity, Whole-cell and Single Channel Studies

Whole-cell Studies—To test the role of the pre-H2 region in ion selectivity, whole-cell amiloride-sensitive currents carried by extracellular Na\(^+\) or Li\(^+\) (101 mM) were recorded in oocytes with the conventional two-electrode voltage clamp technique. Wild type (wt) rat β2-ENaC subunits were co-expressed with wt α-ENaC and its swap chimeras, whereas δ-ENaC and the swapped constructs were expressed with wt human β2-ENaC subunits. As shown in Fig. 2, the activation of the amiloride-sensitive currents evoked at membrane potentials was not time-dependent between −120 mV to +80 mV. Oocytes were held at 0 mV, which was close to the resting membrane potential as ENaC expression caused an overloading of the cytosolic Na\(^+\) and Li\(^+\) concentration, leading to a depolarization of the resting membrane potential. Both wt and chimeric constructs had a slightly inward rectified I-V curve with a reversal potential (E\(_{\text{rev}}\)) of more than +10 mV (Fig. 3). The macroscopic amiloride-sensitive Na\(^+\) and Li\(^+\) currents were −1520.37 ± 361 and −3524.33 ± 606 nA, respectively, at a membrane potential of −120 mV for wt αβγ-rENaC (n = 33). Current carried by Li\(^+\) was markedly greater than that of Na\(^+\) (p < 0.001). On the other hand, the whole-cell amiloride-sensitive Na\(^+\) and Li\(^+\) currents of αβδ2βγ-rENaC were −2970.99 ± 832 (p < 0.01 compared with that of wt αβγ-rENaC) and −4011.51 ± 1178 nA, respectively (n = 25). Conversely, the amiloride-sensitive Na\(^+\) current in oocytes expressing δβγ-ENaC was greater than the Li\(^+\) current (−2602.61 ± 530 versus −1660.94 ± 386 nA for Na\(^+\) and Li\(^+\), respectively, n = 34). The current amplitudes of both amiloride-sensitive Na\(^+\) and Li\(^+\) currents associated with δβδ2βγ-ENaC were greater than wt construct (−3302.67 ± 670 nA for Na\(^+\)) and −2490.11 ± 528 nA for Li\(^+\), respectively, n = 26, p < 0.05). These results for wt αβγ-rENaC and δβγ-ENaC were consistent with those reported previously (16, 25, 26).

The whole-cell Na\(^+\) and Li\(^+\) current levels associated with the pre-H2 swapped chimeras (Figs. 2 and 3) were not identical to those of wt constructs, indicating that their permeabilities to Na\(^+\) (P\(_{\text{Na}}\)) and Li\(^+\) (P\(_{\text{Li}}\)) have been altered. To address whether the pre-H2 domain regulates Na\(^+\) or Li\(^+\) permeation, we calculated the absolute permeation constant. The retrieved values were listed in Table I. Only αβδ2 and αβδ2βδ chimeras shifted the E\(_{\text{rev}}\) significantly to the hyperpolarizing direction. The value of P\(_{\text{Na}}\) for wt rat ENaC was about half of P\(_{\text{Li}}\), yielding a P\(_{\text{Li}}\)/P\(_{\text{Na}}\) ratio of 1.8. However, the P\(_{\text{Na}}\) of wt human ENaC was greater and the P\(_{\text{Li}}\)/P\(_{\text{Na}}\) ratio was less compared with its rENaC counterpart. In contrast, the P\(_{\text{Li}}\) for wt δ-ENaC was smaller (approximately up to 50% that of rENaC), whereas P\(_{\text{Na}}\) was greater than those of wt rENaC, yielding a P\(_{\text{Li}}\)/P\(_{\text{Na}}\) ratio of 0.62. The selectivity ratios of wt rat and human ENaC constructs calculated from the permeation constant were completely consistent with previous studies (16, 25).

An essential G(S)XS tract has been identified in the H2 region of ENaC, which controls Na\(^+\) permeability (10, 12). As shown in Fig. 1, there is a serine-rich domain (564–568, SESP3) within the H2 region of α-ENaC, which is not present in the equivalent stretch of δ-ENaC subunit. To determine the role of this SXS tract in ion selectivity, Na\(^+\) and Li\(^+\) permeabilities of triple mutants αδβ4E,δ566A,δ568V βγ-rENaC (αδβ4E) and δE514S,A516S,V518S βγ-ENaC (δδβ4E) were examined. For comparison, chimeras swapping the H2 and pre-H2+H2 domains were constructed. A significant increment in P\(_{\text{Na}}\) was observed for the αδβ4E, αδδ4E, and αδβδH2 chimeras, but there was a significant decrease in P\(_{\text{Li}}\) for the αδH2 chimera (Table I). The P\(_{\text{Li}}\)/P\(_{\text{Na}}\) ratio reduced markedly for all four chimeric α-ENaC constructs. In contrast, only the δβ4E mutation exhibited an increment in P\(_{\text{Li}}\), whereas the P\(_{\text{Li}}\)/P\(_{\text{Na}}\) ratio was not statistically different from that of wt δ-ENaC.

Single Channel Studies—In order to re-confirm the macroscopic results under strictly controlled ionic conditions, we performed single channel studies under bi-ionic conditions. The on-cell and inside-out patches were obtained from injected oocytes, and single channel traces were digitized (Fig. 4). The average unitary currents were plotted as I-V curves shown in Figs. 5 and 6. Wild type αβγ-rENaC and αβδ2βγ chimera function as slight outward rectifiers compared with the macroscopic I-V relationships (Figs. 3 and 5A). Replacement of the pre-H2 region with the corresponding regions of δ-ENaC shifted the E\(_{\text{rev}}\) to −5 from −15 mV. By comparison, the I-V relationship of
δβγ-hENaC revealed an inwardly rectified curve with an \( E_{rev} \) of 15 mV (Fig. 5B).

We also applied the Goldman-Hodgkin-Katz equation to the I-V relationships generated under bi-ionic conditions to determine the permeability constant and ratio. The permeability ratio was also recalculated using the \( E_{rev} \) (Table II). Consistent with the whole-cell observations, the \( P_L \) was greater than \( P_{Na} \) for wt αβγ-rENaC, yielding a \( P_L/P_{Na} \) of 1.82 (Table II). Substitution of the pre-H2 and pre-H2+H2 domains of α-ENaC with the corresponding regions of δ-ENaC resulted in an increment in \( P_{Na} \), whereas swapping of the H2 domain with that of δ-ENaC led to a decrease in \( P_{Na} \). Both the pre-H2 and/or H2 domain substitution of α-ENaC resulted in reduced discrimination between Na\(^+\) and Li\(^+\). With respect to wt δ-hENaC, the \( P_L \) was less than its \( P_{Na} \), with a calculated \( P_L/P_{Na} \) ratio of 0.55. The chimeric δ-ENaC constructs with replacement of the pre-H2 and/or H2 domains of α-ENaC showed no marked change in Na\(^+\) and Li\(^+\) permeabilities.

**Single Channel Conductance**

The results of on-cell single channel experiments further verified the whole-cell observations (Fig. 4A). The unitary current level recorded at -100 mV in on-cell patches displayed differences when the pipette was filled with Na\(^+\) or Li\(^+\) (100 mM). For the wt αβγ-rENaC and swap chimeric constructs, the Na\(^+\) current was much smaller than Li\(^+\) current (Fig. 4A, top panel and Table III). In contrast, the single channel Na\(^+\) current of wt δβγ-hENaC was much greater (12 versus 4 pS for wt αβγ-ENaCs), whereas the Li\(^+\) current associated with δβγ-hENaC was almost identical to that of αβγ-ENaCs (7 pS). Swapping the H2 and pre-H2+H2 domains increased \( \gamma_{Na} \) of α chimeras, but the \( \gamma_{Li} \) was significantly reduced for the swap δ-ENaC constructs in on-cell patches.

For wt αβγ-rENaC, there was no significant difference in \( \gamma_{Na} \) and \( \gamma_{Li} \) between the on-cell and inside-out configurations. In contrast, both the \( \gamma_{Na} \) and \( \gamma_{Li} \) of δ-ENaC in the inside-out patches were strikingly smaller than those from the cell-attached patches (57 and 66% of the on-cell conductance, respectively, see Table III). With regard to wt αβγ-hENaC, the \( \gamma_{Li} \) was reduced by 44%. Consistent with the results in the on-cell patches, an increment for the \( \delta^{H2} \) and a decrease for the \( \delta^{H2\alpha^H2} \) constructs in \( \gamma_{Na} \) were observed in the inside-out patches (Table III). Inconsistent with data obtained from the on-cell configuration, both the \( \gamma_{Na} \) and \( \gamma_{Li} \) for the other constructs declined significantly in the inside-out patches (Table III).

**Amiloride Sensitivity**

To test the hypothesis that the pre-H2 region preceding the amiloride-binding site located in the H2 domain modified the kinetics of amiloride inhibition of the ENaC channel, we used the whole-cell mode to record amiloride-sensitive Na\(^+\) currents in the presence of various amiloride concentrations (range, 0–1 mM). Fig. 7A shows representative traces of the time course and concentration dependence of the amiloride inhibitory effect for wt and pre-H2 swap constructs. Generally speaking, stable currents could be achieved within a few seconds following the application of amiloride. Fig. 7B and C shows the dose-response curves at -100 mV fitted with the Hill equation. The value of \( K_i^{amil} \) for wt αβγ rENaC was 54.7 ± 19 nM (\( n = 7 \)). For the αβγδβγ chimeric mutation, the \( K_i^{amil} \) increased approximately by 3-fold to 150.0 ± 6.5 nM (\( n = 5 \), \( p < 0.01 \)). Consistent with a previous report (16), the \( K_i^{amil} \) for δβγ-hENaC was 52-fold greater than that of αβγ-rENaC (2860 ± 501 nM, \( n = 12 \)). Exchanging the pre-H2 region of δ-hENaC with that of α-rENaC significantly decreased the \( K_i^{amil} \) to 745 ± 247 nM for the \( \delta^{H2\alpha^H2} \)-hENaC (\( n = 6 \)).

We constructed swap chimeras by switching the H2 domain and the pre-H2+H2 domains, in which an amiloride-binding site is located as positive controls (1, 2). Fig. 8 presents the computed \( K_i^{amil} \) and Hill coefficient as a function of membrane potential. The voltage dependence of \( K_i^{amil} \) was fitted with the Woodhull equation (24). The retrieved parameters including the fractional distance across the electrical field sensed by amiloride (\( \delta \)), the experimental \( K_i^{amil} \) at 0 mV, and the esti-
The experimental $K_{\text{amil}}$ at 0 mV for the $\delta^{\text{H2}}$ construct decreased significantly as compared with that for wt $\delta$-ENaC, whereas an increment was observed for the $\alpha^{\text{H2}}$ chimera (Table IV). Swapping of the H2 domains between $\alpha$- and $\delta$-ENaCs almost completely exchanged the values of $K_{\text{amil}}$ for each other (Fig. 8, A and B). However, the pre-H2+H2 domain swap channels did not show an additional shift in amiloride affinity to the others, instead the change in $K_{\text{amil}}$ was smaller compared with those of the H2 swap chimeras. These results suggest that there are intramolecular domain-domain interactions between the pre-H2 and H2 domains and that their interactions with amiloride are not synergistic.

**Fig. 3.** Macroscopic current-voltage (I-V) relationships. A, I-V curves of $\alpha\beta\gamma$-rENaC (left) and $\alpha^{\text{H2}}\beta\gamma$-rENaC (right). Amiloride-sensitive (AS) Na$^+$ and Li$^+$ currents are as indicated by the inset legend. $V_m$, membrane potential. The reversal potential is shown by arrow and number. The dotted lines between the symbols are created by fitting average data with the Goldman-Hodgkin-Katz equation (see “Experimental Procedures” for details). B, I-V curves of $\delta\beta\gamma$-hENaC (left) and $\delta^{\text{H2}}$ chimera (right). C, I-V curves of $\alpha$-triple ($\alpha^{\text{tri}}$, left) and $\delta$-triple mutants ($\delta^{\text{tri}}$, right). D, I-V curves of swap $\alpha^{\text{H2}}$ (left) and $\alpha^{\text{H2tri}}$ mutants (right). E, I-V curves of swap $\delta^{\text{H2}}$ (left) and $\delta^{\text{H2tri}}$ mutants (right). F, I-V curve of wild type $\alpha\beta\gamma$-ENaC.
In comparison to hyperpolarizing membrane potentials, the $K_{\text{amil}}$ was more voltage-dependent at depolarizing membrane potentials from $-20$ to $+80$ mV (Fig. 8, A and B). All estimated values of the $K_{\text{amil}}$ at $0$ mV ($K_{\text{amil}}(0)$) were pretty close to those computed from the experimental data. For example, the calculated $K_{\text{amil}}(0)$ for wt $\alpha\beta\gamma$-rENaC was 354 and 330 nM for the experimental data. The value of $\delta$ was 0.35 for wt rENaC, close to those yielded by noise analysis of the macroscopic current.
Ion permeation ratio estimated by fitted the macroscopic currents with the Goldman-Hodgkin-Katz Equation 1

The pre-H2 (αβγ) and δβγ, H2 (αβ) and δβγ, and pre-H2H2 (αδβγ) and δδβγ swap chimeras as well as triple mutants (αδγ and δδγ) are compared with wild type constructs. $E_{rev}$: reversal potential of Na\(^+\) current; $P_{Na}$ and $P_{Li}$, absolute permeation constants for Na\(^+\) and Li\(^+\); and $P_{Li}/P_{Na}$, permeation ratio of Na\(^+\) and Li\(^+\). Numbers in parentheses in the 1st column are oocytes tested.

| Constructs       | $E_{rev}$ (mV) | $P_{Na}$ ($\text{cm}^2\text{s}^{-1} \times 10^{-6}$) | $P_{Li}$ ($\text{cm}^2\text{s}^{-1} \times 10^{-6}$) | $P_{Li}/P_{Na}$ |
|------------------|----------------|-----------------------------------------------|-----------------------------------------------|-----------------|
| αβγ-ENaC         | 33             | 5.85 ± 0.2                                    | 9.23 ± 0.2                                    | 1.58            |
| αδβγ-ENaC        | 6              | 6.12 ± 0.1†                                   | 9.23 ± 0.2                                    | 1.58            |
| αδβγβ-ENaC (25)  | 10 ± 1.2*      | 6.85 ± 0.5§                                   | 8.30 ± 0.6                                    | 1.21§           |
| αδβγβδ-ENaC (6)  | 14 ± 2.2§      | 7.52 ± 0.3§                                   | 7.76 ± 0.3                                    | 1.04§           |
| δβγ-ENaC         | 11             | 4.73 ± 0.7                                    | 6.24 ± 0.5                                    | 1.34            |
| δδβγ-ENaC (34)   | 15 ± 1.4       | 6.19 ± 0.1                                    | 3.84 ± 0.2                                    | 0.62            |
| δδβγ-ENaC (6)    | 12 ± 1.5       | 6.67 ± 0.4                                    | 5.01 ± 0.4§                                   | 0.75§           |
| δδβγ-ENaC (26)   | 13 ± 1.1       | 6.21 ± 0.4                                    | 4.75 ± 0.2                                    | 0.76            |
| δδβγ-ENaC (9)    | 13 ± 1.1       | 6.10 ± 0.2                                    | 4.36 ± 0.2                                    | 0.72            |
| δδβγ-ENaC (6)    | 14 ± 1.5       | 6.55 ± 0.2                                    | 4.55 ± 0.2                                    | 0.73            |

† $p < 0.05$ compared with the corresponding wild type channels.

As shown in the right panel in Fig. 8A, all swap chimeric α-ENaC constructs had an increased Hill coefficient. On the other hand, the Hill coefficient reduced markedly for all chimeric δ constructs at depolarizing membrane potentials (Fig. 8B, right panel).

### DISCUSSION

The goal of the present study was to examine whether the pre-H2 domain regulates ion selectivity, unitary conductance, and amiloride sensitivity of ENaC. Our experimental strategy was to swap 24 amino acid residues preceding the H2 regions of α- and δ-ENaCs. The main findings of our studies are as follows: 1) The pre-H2 and H2 domains and an SXSXS motif of α- and δ-ENaCs are involved in Na\(^+\) and/or Li\(^+\) permeation. 2) Single channel studies confirmed that the $γ_{Na}$ for δβγ-ENaC increased for the αH2 and αδH2-ENaC chimera, supportive of the whole-cell results; the $γ_{Na}$ for chimeric δ-ENaC constructs was reduced in on-cell patches. 3) The $γ_{Na}$ of all chimeric α and δ constructs decreased in the inside-out mode with the exception of $γ_{Na}$ for the αH2 and δδβγ-ENaC chimeras. 4) The pre-H2 clusters combined with the H2 domains of α- and δ-subunits contribute to amiloride inhibition by modifying the $K_i^{amil}$, the Hill coefficient, and voltage dependence.

**Ion Permeation**—Our results suggest that the swap chimeric αδβγ, αδH2γ, and δδβγH2-ENaC channels discriminate less between Na\(^+\) and Li\(^+\) due to the elevated Na\(^+\) permeation, which was inherited from the wild type δβγ-ENaC (Tables I and II). These results for wild type δβγ-ENaC are consistent with the observations Waldmann et al. (17). Replacement of hydrophobic region 2 (H2) of α-ENaC with the corresponding sequence of MEC-4 significantly increased $γ_{Na}$ to 14 pS and the $γ_{Na}/γ_{Li}$ ratio increased to 1.6 (17). Series 589 and 593 located in the pore region of α-ENaC were further identified as modifying $γ_{Na}$ to the same extent (17). Additionally, mutations of the pre-M2 region revealed that the H2 domain regulated ion permeation; in particular, $γ_{Na}$ was altered (4, 8, 9, 11, 14). However, these two key residues are highly conserved in both α- and δ-ENaC isoforms. The present studies with an α-triple mutant suggested that an SXSXS motif partially contributes to the increased Na\(^+\) permeability of the αδβγH2-ENaC chimera. In comparison to the αδβγ-chimera, the swap δ constructs did not significantly affect Na\(^+\) permeability.

Our results suggest that multiple domains integrally control Li\(^+\) and Na\(^+\) permeation. They support the idea that the selectivity filter consists of a chain of amino acid residues (i.e.
FIG. 5. Unitary current-voltage (I-V) relationships for wild type and swap ENaC constructs from on-cell and inside-out patches. 

A, I-V curves of wt and the swap rENaC from the on-cell patches at pipette voltage ($V_p$) from −120 to −20 mV. The dotted line was drawn by fitting the data with a linear fitter to calculate single channel conductance of Na$^+$ (square) and Li$^+$ (circle). Bottom panel shows I-V curves obtained from the inside-out patches for wt ($n = 11$) and the swap rENaC ($n = 10$). Reversal potential is labeled by arrow and number. The dotted lines were created by fitting the current data with the Goldman-Hodgkin-Katz equation strictly for bi-ionic conditions (Equation 2). The unitary Na$^+$ ($\gamma_{\text{Na}}$) and Li$^+$ ($\gamma_{\text{Li}}$) conductances under bi-ionic conditions were computed by linear regression of inward and outward currents, respectively. 

B, I-V curves for wt and the swap δ-hENaC constructs from the on-cell and inside-out patches.
the pre-H2, H2, M2, and post-M2 domains) and that the selectivity to Li⁺, Na⁺, and K⁺ is determined by different residues. Moreover, more than one residue can influence the permeation for each individual alkali cation species (8–10, 12). This idea explains why the Na⁺ permeation of the swap chimeric δH2H2βγ-hENaC channels was not changed and why the $P_{Na}$ of the αH2H2βγ-hENaC is not identical to that of wt δβγ-hENaC. Our observations suggested that the pre-H2 and H2 domains of δ-ENaC are not the only key regions for its greater Na⁺ permeability over α-ENaC.

Why are the $γ_{Na}$ and $γ_{Li}$ of wt and chimeric constructs in the inside-out patches lower than those of the on-cell patches (Table III)? Our previous studies have demonstrated that cytoskeletal elements physically interact with and functionally regu-
late αβγ-ENaC activity (34, 35). In the excised patches, the
precise architectural arrangement of the cytoskeleton is un-
doubtedly disrupted, and the channel conformation and/or in-
teractions with these cytoskeletal proteins may be changed.
Also, tension on δ-ENaC during the formation of the inside-out
patches may contribute to an alteration in cation conductance.
We cannot rule out the involvement of soluble intracellular
elements and salt components. In the on-cell patches, swapping
the pre-H2 domain with the corresponding sequence of α-ENaC
resulted in a decrease in γNa of δ-ENaC, suggesting that the
pre-H2 region of δ-ENaC may be an interactive motif, which
directly cross-talks with the extracellular loop of other mem-
brane proteins or serves as a regulator of other protein-protein
interacting motifs. Finally, the cytosolic Li⁺ concentration (100
mM) used for the inside-out configuration is much greater than
the concentrations for the cell-attached mode. As predicted by
the reversal potential of the macroscopic I-V curves, intra-
ocytic Na⁺ content is approximately 60 mM for α-ENaC and
less for δ-ENaC. The decreased γNa and γLi in the inside-out
patches excised from δ-ENaC-expressing cells may thus be due
to the feedback regulation and self-regulation, respectively (2).
If so, wt δβγ-hENaC is more sensitive to the inhibitory regu-
lation than wt αβγ-hENaC.

As indicated by the Goldman-Hodgkin-Katz current equa-
tion (Equation 1), the permeability to cations positively corre-
lates to whole-cell current. Whole-cell current is determined by
the number of electrically detected active channels (N), channel
open probability (Po), as well as single channel conductance.
Our results, namely that the PoNa of the αββγ-hENaC is dou-
bled whereas the single channel conductance is not changed,
imply that the αββγ-hENaC may increase either or both
active channel number or channel open probability.

As shown in Figs. 1F and 6E, as well as Table III, wt αβγ-
hENaC differs from the rat counterpart in their biological
properties including the PNa/PNa ratio, Erev of the macroscopic
Na⁺ current, and γLi in the inside-out patches. If these differ-
es are mainly determined by wt β- and γ-hENaC subunits,
then the properties of the swap δ-ENaC constructs may be
affected by β-hENaC subunits.

Amiloride Inhibition—Multiple amiloride-binding sites at the
extracellular loops of αβγ subunits have been biochemi-
cally and functionally identified (3, 4). However, no amiloride
binding or regulatory sites have yet been localized to the
pre-H2 region. Our observations on amiloride inhibition of
the wt and swap constructs suggested that the pre-H2 do-
main is also important for amiloride interaction with the
channel, particularly for δ-ENaC. The values of Kₐ⁰ for the
chimeras that swap the H2 and pre-H2 domains beside these two regions influence amiloride
interaction with the
channel, particularly for δ-ENaC. The values of Kₐ⁰ of the
chimeras that swap the H2 and pre-H2 domains are
not identical to that of the other parental constructs, suggesting
that domains beside these two regions influence amiloride
affinity. Because β- and γ-subunits also contribute to amilo-
ride affinity, co-expression of wt βγ-hENaC subunits may
compensate for the effects of swapping pre-H2 and H2 regions on
amiloride inhibition.

Our observations also confirm previous studies (16) that
the Kₐ⁰ of δβγ-ENaC is in the micromolar rather than the
nanomolar range. Amiloride inhibition of both α- and
δ-ENaCs is voltage-dependent, especially at depolarizing
membrane potentials above 0 mV (Fig. 7). Amiloride is a
positively charged molecule when protonated and is thus
sensitive to membrane potential (36). Cations compete with
amiloride for at least one of these two binding sites. Although
amiloride affinity is regulated by extracellular cation concen-
tration, external protons, membrane potential, and intracel-

#### Table II

| Constructs          | PNa/cmS × 10⁻⁶ | PNa/mL | PNa/PNa | Erev/mV | PNa/PNa α |
|---------------------|----------------|--------|---------|---------|-----------|
| αβγ-ENaC (12)       | 4.99 ± 0.3     | 10.0   | 1.2     | 2.00    | 16 ± 1.4  | 1.82      |
| αββγ-ENaC (11)      | 8.50 ± 0.4*    | 9.85   | 1.1     | 1.18a   | 5 ± 1.6   | 1.23a     |
| αββγ-ENaC (7)       | 5.28 ± 0.4     | 5.07   | 0.3*    | 0.96a   | 0.51 ± 0.1| 0.97a     |
| αββγ-ENaC (4)       | 8.84 ± 0.2*    | 9.28   | 0.2     | 1.05*   | 0.5 ± 0.1 | 1.02*     |
| δβγ-hENaC (10)      | 10.0 ± 0.6     | 7.32   | 0.6     | 0.73    | 15 ± 1.2  | 0.55      |
| δβγ-hENaC (3)       | 8.87 ± 0.3     | 6.02   | 0.3     | 0.68    | 13.5 ± 0.7| 0.61      |
| δβγ-hENaC (5)       | 9.21 ± 0.2     | 6.81   | 0.4     | 0.74    | 12.9 ± 0.5| 0.73      |

* PNa/PNa permeability ratio that is computed with the Erev.
* p < 0.05 compared with wild type αβγ-hENaC.

#### Table III

| Constructs          | On-cell patch (4–15) | Inside-out patch (5–9) |
|---------------------|----------------------|-----------------------|
|                      | γNa                  | γNa                  |
| αβγ-ENaC             | 4.03 ± 0.11          | 4.31 ± 0.22          |
| αββγ-ENaC (11)       | 4.22 ± 0.16          | 3.42 ± 0.12          |
| αββγ-ENaC (7)        | 7.92 ± 1.22*         | 5.32 ± 0.31*         |
| αββγ-ENaC (4)        | 5.68 ± 0.49*         | 3.35 ± 0.36*         |
| αβγ-hENaC            | 4.00 ± 0.18          | 4.30 ± 0.37          |
| δβγ-hENaC (10)       | 12.09 ± 0.53         | 6.92 ± 0.33          |
| δβγ-hENaC (3)        | 8.16 ± 0.44*         | 7.01 ± 0.41          |
| δβγ-hENaC (5)        | 6.98 ± 0.15b         | 5.08 ± 0.61b         |
| δβγ-hENaC (5)        | 7.03 ± 0.17           | 7.01 ± 0.41          |
| δβγ-hENaC (5)        | 7.14 ± 0.34           | 4.55 ± 0.56b         |
| δβγ-hENaC (5)        | 7.38 ± 0.32           | 2.93 ± 0.22          |

* p < 0.05.
* p < 0.01 compared with wt constructs.
lular amiloride accumulation, the fact that the identical experimental conditions were used regardless of the ENaC constructs under investigation excluded contributions from these other factors (36).

Comparing data from /H9251/H9252/H9253-rENaC and /H9251/H9252-rENaC revealed that /H9251/H9252-ENaC channel was more permeable to Na
/H11001 than Li
/H11001 (P
/H11022 P
/H11021), had an identical fractional distance across the electrical field sensed by amiloride, and an increased Ki
/amil (4
/M, Refs. 6 and 28). These results raised the question that the chimeric /H9251-pH2-ENaC may assemble a channel with only a /H9252-subunit (and not a /H9253-subunit). However, the /H9251-pH2/H9252/H9253-ENaC has a greater permeability to Li (Na 4.2 versus Li 6.9 pS) and a smaller Ki
/amil (0.13 μM), which were similar to those of wt /H9251/H9252/H9253-ENaC. Our studies also showed that the whole-cell Na
 current for the /H9251-pH2-ENaC at a holding potential of −120 mV was less than 200 nA (not shown), ~10% of the amplitude of /H9251-pH2-ENaC (Fig. 2). Thus, the possibility that chimeric α-construct may assemble with only a β-subunit is unlikely.

The voltage dependence of amiloride inhibition in toad urinary bladder (31, 32) and cloned ENaC (27, 33) has been confirmed. Furthermore, depolarization led to an increment in amiloride k
 on but a decrease in k
 off for the native epithelial Na
 conductance (31, 32), whereas only a weak voltage dependence of k
 on and/or k
 off was observed for αβγ- and αβ-rENaC (28, 33). A simple plug-type blocking model was proposed whereby the positively charged protonated amiloride is attracted by negatively charged amino acids located in the vestibule of the channel (31). Because the negative charges in the M2 domain did not contribute to amiloride affinity as evidenced by pore-region truncation mutants (37), and the two amiloride-binding sites identified by Ismailov et al. (3) and Snyder et al. (12) are not negatively charged, it was speculated that the negative charges of the pre-H2 region preceding the H2 cluster contributed to amiloride affinity. However, because the pre-H2 domain of δ-hENaC contains more negative charges than the corresponding region within α-rENaC and yet has a lower Ki
/amil, it is unlikely that these negative charges are important in amiloride-ENaC interactions.

We also found that the fractional distance across the electrical field sensed by amiloride for δ-ENaC is greater (0.48) than that of α-ENaC (0.35). As summarized in Table IV, the value for αβγ-ENaC is close to the result by Segal et al. (33) and similar to other studies (26–32) on endogenous and cloned ENaC/DEG channels. Like the observation by Woodhull (24) on the proton block of voltage-dependent Na
 channels, the steep-

\[\text{Fig. 7. Amiloride sensitivity of wt and the pre-H2 swap ENaC channels. A, current traces of time course for amiloride inhibition from 0 nM to 10 nM of macroscopic Na}\textsuperscript{+} \text{current at −100 mV are shown in the top panel. B, dose-response curves for wild type and swap α-rENaC at membrane potential of −100 mV. Circle, wt ENaC; square, the swap mutant. Both solid and dotted lines are created by fitting the data with the Hill equation to calculate the } K_{amil} \text{ and Hill coefficients. C, dose-response curves for wild type and swap δ-hENaC.}\]
FIG. 8. Voltage dependence of amiloride inhibition for wild type and swap constructs. A, the $K_{amil}$ (left) and Hill coefficient (right) of wt and swap $\alpha$-rENaC plotted against membrane potential ($V_m$). $K_{amil}$ was calculated by fitting the dose-response curves with the Hill equation. Lines on the left panel are created by fitting the data with the Woodhull equation. B, the $K_{amil}$ (left) and Hill coefficients (right) of wt and swap $\delta$-hENaC are plotted against membrane potential ($V_m$).

**Table IV**

| Constructs | $\delta/1^{\text{st}}$ | Estimated $K_{amil}(0)$ | Experimental $K_{amil}(0)$ |
|------------|-------------------------|--------------------------|---------------------------|
| $\alpha\beta\gamma\delta$-rENaC (7) | 0.35 ± 0.03 | 354 ± 27 | 330 ± 54 |
| $\alpha\beta\gamma\delta$-rENaC (5) | 0.30 ± 0.02 | 550 ± 26 | 563 ± 46 |
| $\alpha\beta\gamma\delta$-rENaC (5) | 0.45 ± 0.02 | 10981 ± 356 | 11618 ± 2909 |
| $\alpha\beta\gamma\delta$-rENaC (7) | 0.46 ± 0.03 | 3098 ± 250 | 3400 ± 192 |
| $\delta\beta\gamma\delta$-hENaC (6) | 0.48 ± 0.02 | 13515 ± 354 | 13089 ± 3110 |
| $\delta\beta\gamma\delta$-hENaC (5) | 0.43 ± 0.03 | 3738 ± 279 | 3416 ± 2296 |
| $\delta\beta\gamma\delta$-hENaC (3) | 0.32 ± 0.03 | 1087 ± 79 | 699 ± 186 |
| $\delta\beta\gamma\delta$-hENaC (8) | 0.31 ± 0.02 | 1724 ± 76 | 1665 ± 342 |

$^a$ $\delta$ is 0.15–0.48 for $\alpha\beta\gamma\delta$-rENaC (4, 27), 0.3 for $\alpha\beta\gamma\delta$-rENaC (28), 0.54–0.62 for MEC4/MEC10 (29), 0.65–0.68 for UNC-105d (30), and 0.12–0.23 for toad urinary bladder (31, 32).

$^b$ $p < 0.05$.

$^c$ $p < 0.01$.

ess of the voltage dependence of block increases, whereas the $\delta$ value elevates for wt ENaC. Their results suggested that amiloride indeed blocks the channel pore. An alternative interpretation is that the amiloride-binding site is not in the channel pore (3). A depolarizing membrane potential may act on intracellular cations as well as on amiloride. The shorter electrical distance from the inside for $\delta$-ENaC (0.52 versus 0.65 for $\alpha$-ENaC) may facilitate intracellular cation binding for outward transport, competitively decreasing amiloride affinity and showing more voltage dependence. However, our observations for the swap chimeric constructs, where a different $K_{amil}$ can be obtained for the pre-H2 swap chimeras with an almost
unaltered electrical distance (Table IV), do not support either of these two interpretations. Swapping of the H2 domains with or without combination of the pre-H2 segment suggested that some key residues within the H2 domain contribute to 90% of the difference in amiloride affinity, because the identified amiloride-binding site is identical between α- and δ-ENaCs.

Changes in the fractional distance sensed by amiloride of the H2 swap chimeras also suggested that the H2 domain determines the diversity in amiloride sensitivity between α- and δ-ENaCs. The pre-H2 domain secondarily mediates amiloride inhibition.

It is well known that ENaC has a different closing conformation from DEG members (1, 2). Swapping of the M2 domain of α-ENaC with that of MEC-4 decreased amiloride affinity accompanied by an alteration in closing conformation (17). Regulation of channel conformation and ligand binding has been found in other channels as evidenced by our pre-H2 domain functionally interacts with the H2 domain as evidenced by our pre-H2+H2 domain swap studies.

Physiological Relevance—The possible physiological relevance for the pre-H2 region involvement of amiloride sensitivity, unitary conductance, and ion selectivity is not clear. The extraordinarily large M1-M2 connecting loop, comprising over 70% of the mass of the entire channel sequence, implies that the extracellular loop may function as a receptor or sensor of different stimuli. The ENaC/DEG channels are regulated by many external ligands, for example, channel-activating proteins, channel structure, and external cation affinity, cannot be excluded by our results. Therefore, whether the changes in ion selectivity, single channel conductance, and amiloride affinity associated with chimeric α- and δ-ENaC constructs are due to direct effects or conformation alterations is not clear.

In conclusion, our studies demonstrate that the pre-H2 regions of both α-ENaC and δ-ENaCs contribute to ion selectivity, single channel conductance, and amiloride inhibition. The pre-H2 domain functionally interacts with the H2 domain as evidenced by our pre-H2+H2 domain swap studies.

Acknowledgments—We thank Hannah Mebane for preparing oocytes. We are grateful for the kind gifts of the δ-ENaC clone provided by Drs. R. Waldman and M. Lazzunski (Institut de Pharmacologie Moleculaire et Cellulaire, CNRS, France) and εENaC clones from Drs.

H.-L. Ji, L. R. Bishop, S. J. Anderson, C. M. Fuller, and D. J. Benos, unpublished data.

C. Caneas (Molecular and Cellular Physiology, Yale University) and B. Rossier (Institute of Pharmacology and Toxicology, Lausanne, Switzerland). We acknowledge Isabel Quinones for superb secretarial assistance.

REFERENCES

1. Benos, D. J., and Stanton, B. A. (1999) J. Physiol. (Lond.) 520, 631–644
2. Kellenberger, S., and Schild, L. (2002) Physiol. Rev. 82, 735–767
3. Ismailov, I. I., Kiefer-Emmons, T., Lin, C., Berdiev, B. K., Shlyonsky, V. G., Patton, H. K., Fuller, C. M., Worrell, R., Zuckerman, J. B., Sun, W., Eaton, D. C., Benos, D. J., and Kleyman, T. R. (1997) J. Biol. Chem. 272, 21075–21083
4. Schild, L., Schneebberger, E., Gautschi, I., and Firsov, D. (1997) J. Gen. Physiol. 109, 15–26
5. Adams, C. M., Snyder, P. M., Price, M. P., and Welsh, M. J. (1998) J. Biol. Chem. 273, 50204–50207
6. Pyke, G. K., Zhang, P., and Caneas, C. M. (1999) J. Biol. Chem. 274, 36415–36421
7. Kellenberger, S., Auberson, M., Gautschi, I., Schneebberger, E., and Schild, L. (2001) J. Gen. Physiol. 118, 679–692
8. Kellenberger, S., Gautschi, I., and Schild, L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4170–4175
9. Kellenberger, S., Hoffmann-Pochon, N., Gautschi, I., Schneebberger, E., and Schild, L. (1999) J. Gen. Physiol. 114, 13–30
10. Sheng, S., Li, J., McNulty, K. A., Kiefer-Emmons, T., and Kleyman, T. R. (2004) J. Biol. Chem. 279, 1326–1334
11. Sheng, S., Li, J., McNulty, K. A., Avery, D., and Kleyman, T. R. (2000) J. Biol. Chem. 275, 8572–8581
12. Snyder, P. M., Olson, D. R., and Bucher, D. B. (1999) J. Biol. Chem. 274, 28484–28490
13. Langholf, A. L., Berdiev, B., Ji, H. L., Keyser, K., Stanton, B. A., and Benos, D. J. (2002) Am. J. Physiol. 283, C64–C74
14. Sheng, S., McNulty, K. A., Harvey, J. M., and Kleyman, T. R. (2001) J. Biol. Chem. 276, 44091–44098
15. Ji, H. L., Parker, S., Langholf, A. L., Fuller, C. M., and Benos, D. J. (2001) Am. J. Physiol. 281, C64–C74
16. Waldmann, R., Champigny, G., Bassilana, F., Vuillery, N., and Lazarduski, M. (1995) J. Biol. Chem. 270, 27411–27414
17. Weihmann, R., Champigny, G., and Lazarduski, M. (1995) J. Biol. Chem. 270, 11735–11737
18. Mense, M., Rajendran, V., Blaustein, R., and Caplan, M. J. (2002) Biochemistry 41, 9963–9962
19. Choe, H., Sackin, H., and Palmer, L. G. (2000) J. Gen. Physiol. 115, 391–404
20. Dun, W., Jiang, M., and Tseng, G. N. (1999) Pflügers Arch. 439, 141–149
21. Cain, S. A., Woudruff, T. M., Taylor, S. M., Fairlie, D. P., Sanderson, S. D., and Monk, P. N. (2001) Biochem. Pharmacol. 61, 1571–1579
22. Ji, H. L., Fuller, C. M., and Benos, D. J. (1998) Am. J. Physiol. 275, C1182–C1190
23. Ji, H. L., Fuller, C. M., and Benos, D. J. (1999) J. Biol. Chem. 274, 37693–37704
24. Woodhull, A. M. (1973) J. Gen. Physiol. 61, 867–708
25. Caneas, C. M., Horisberger, J. D., and Rossier, B. C. (1993) Nature 361, 467–470
26. Caneas, C. M., Schild, L., Buell, G., Thoren, B., Gautschi, I., Horisberger, J. D., and Rossier, B. C. (1994) Nature 367, 463–467
27. McNicholas, C. M., and Caneas, C. M. (1997) J. Gen. Physiol. 109, 681–692
28. Pyke, G. K., and Caneas, C. M. (1998) J. Gen. Physiol. 112, 423–432
29. Goodman, B. M., Gourdie, G. G., Chelur, D. S., O’Hagan, R., Yao, C. A., and Challe, M. (2002) Nature 415, 1039–1042
30. Garcia-Anoveros, J., Garcia, J. A., Liu, J. D., and Corey, D. P. (1998) Neuron 20, 1231–1241
31. Warncke, J., and Lindemann, B. (1985) J. Membr. Biol. 86, 255–265
32. Palmer, L. G. (1985) J. Membr. Biol. 87, 191–199
33. Segal, A., Awadya, M. S., Eggermont, J., Van Driessche, W., and Weber, W. M. (2002) Pflügers Arch. 443, 882–897
34. Berdiev, B. K., Latorre, R., Benos, D. J., and Ismailov, I. I. (2001) Biophys. J. 80, 2176–2186
35. Smith, P. R., Saccamani, G., Joe, E. H., Angelides, K. J., and Benos, D. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6971–6975
36. Kleyman, T. R., and Crague, E. J., Jr. (1988) J. Membr. Biol. 105, 1–21
37. Li, X.-J., Xu, R.-H., Guggino, W. B., and Snyder, S. H. (1995) Mol. Pharmacol. 47, 1135–1140
38. Valla, G., Chraib, A., Gaeggerl, H. P., Horisberger, J. D., and Rossier, B. C. (1997) Nature 389, 607–610
39. Ji, H. L., Jorov, B., Pu, J. Bishop, L. R., Mebane, H. C., Fuller, C. M., Stanton, B. A., and Benos, D. J. (2002) J. Biol. Chem. 277, 8395–8405