External Nickel Inhibits Epithelial Sodium Channel by Binding to Histidine Residues within the Extracellular Domains of \( \alpha \) and \( \gamma \) Subunits and Reducing Channel Open Probability*

Received for publication, September 30, 2002, and in revised form, October 18, 2002
Published, JBC Papers in Press, October 22, 2002, DOI 10.1074/jbc.M209975200

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Epithelial sodium channels (ENaC) are regulated by various intracellular and extracellular factors including divalent cations. We studied the inhibitory effect and mechanism of external Ni\(^{2+}\) on cloned mouse \( \alpha \)-\( \beta \)-\( \gamma \) ENaC expressed in Xenopus oocytes. Ni\(^{2+}\) reduced amiloride-sensitive Na\(^{+}\) currents of the wild type mouse ENaC in a dose-dependent manner. The Ni\(^{2+}\) block was fast and partially reversible at low concentrations and irreversible at high concentrations. ENaC inhibition by Ni\(^{2+}\) was accompanied by moderate inward rectification at concentrations higher than 0.1 mM. ENaC currents were also blocked by the histidine-reactive reagent diethyl pyrocarbonate. Pretreatment of the oocytes with the reagent reduced Ni\(^{2+}\) inhibition of the remaining current. Mutations at \( \alpha \)His\(^{282}\) and \( \gamma \)His\(^{239}\) located within the extracellular loops significantly decreased Ni\(^{2+}\) inhibition of ENaC currents. The mutation \( \alpha \)H282D or double mutations \( \alpha \)H282R/\( \gamma \)H239R eliminated Ni\(^{2+}\) block. All mutations at \( \gamma \)His\(^{239}\) eliminated Ni\(^{2+}\)-induced inward rectification. Ni\(^{2+}\) block was significantly enhanced by introduction of a histidine at \( \alpha \)Arg\(^{230}\). Lowering extracellular pH to 5.5 and 4.4 decreased or eliminated Ni\(^{2+}\) block. Although \( \alpha \)H292C/\( \beta \)-\( \gamma \) channels were partially inhibited by the sulphydryl-reactive reagent 2-(trimethylammonium)methyl methanethiosulfonate bromide (MTSET), \( \alpha \)-\( \beta \)-\( \gamma \) H293C channels were insensitive to MTSET. From patch clamp studies, Ni\(^{2+}\) did not affect unitary current but decreased open probability when perfused into the recording pipette. Our results suggest that external Ni\(^{2+}\) reduces ENaC open probability by binding to a site consisting of \( \alpha \)His\(^{282}\) and \( \gamma \)His\(^{239}\) and that these histidine residues may participate in ENaC gating.

Epithelial Na\(^{+}\) channels (ENaC\(^{\dagger}\)) mediate Na\(^{+}\) transport across high resistance epithelia and participate in the regulation of extracellular fluid volume and blood pressure. Molecular cloning has revealed that ENaC subunits (\( \alpha \), \( \beta \), and \( \gamma \)) belong to a super gene family which is now often referred to as ENaC/Deg family and consist of ENaCs, degenerins (DEG and MEC), acid-sensing ion channels (ASIC), and FMRFamide-gated Na\(^{+}\) channels (1–5). These channel proteins are composed of subunits that share a common membrane topology with two transmembrane domains, intracellular N and C termini and large extracellular loops (ECL). Members of the ENaC/Deg family are Na\(^{+}\)-selective and sensitive to the epithelial Na\(^{+}\) channel blocker amiloride, although differences in factors that activate, or gate, these channels have been reported (1). Combined application of site-directed mutagenesis and oocyte expression has yielded important findings regarding structure and function relationships of ENaC (6–9). Although the exact subunit stoichiometry of \( \alpha \)-\( \beta \)-\( \gamma \) ENaC is still controversial (10–12), all three ENaC subunits (\( \alpha \), \( \beta \), and \( \gamma \)) contribute to formation of the core structure, the ion conduction pore (13). Two amiloride-binding sites have been identified within ENaC subunits; one within the pore regions and a second within the ECL of \( \gamma \)ENaC (13–15). The main selectivity filter resides at a 3-residue tract (G(S)KXS) within the putative pore region (or equivalently termed as pre-M2 region) (16–20). Mutations that alter ENaC gating have been found in the N termini (21, 22), pore regions (23, 24), and nearby regions (25), M2 domains (26), and the C termini (27).

Variability in the ionic selectivity, conductance, and gating of ENaCs in both native tissues and expression systems has been observed (1). In part, the functional diversity of the Na\(^{+}\) channels may reflect the influence of intracellular and extracellular factors that regulate channel activity. Divalent cations are important regulators of many ion channels, especially voltage-gated and ligand-gated channels (28). Several cations (Ca\(^{2+}\), Mg\(^{2+}\), and Ba\(^{2+}\)) were reported to block Na\(^{+}\) currents from the external side with low affinity and complex features in native tissues (29). Recently, it was reported that external Ni\(^{2+}\) stimulated short circuit currents in A6 monolayers (30) and blocked whole-cell currents in oocytes expressing rat \( \alpha \)-\( \beta \)-\( \gamma \) ENaC (31). We report the inhibitory effects of extracellular Ni\(^{2+}\) on the whole-cell and single channel currents in Xenopus oocytes expressing rat \( \alpha \)-\( \beta \)-\( \gamma \) ENaC (31). We report the inhibitory effects of extracellular Ni\(^{2+}\) on the whole-cell and single channel currents in Xenopus oocytes expressing rat \( \alpha \)-\( \beta \)-\( \gamma \) ENaC (31).

**Experimental Procedures**

Site-directed Mutagenesis and Functional Expression of \( \alpha \)-\( \beta \)-\( \gamma \) mENaC in Xenopus oocytes—Point mutations were generated in \( \alpha \), \( \beta \), or \( \gamma \) mENaC cRNAs (34) cloned into pBluescript SK – vector (Stratagene, La Jolla, CA) using two-step PCR methods as described previously (19). cRNAs were synthesized with T3 RNA polymerase (Ambion Inc.,

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* This work was supported by National Institutes of Health Grants DK51391 and DK54354. 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 Na\(^{+}\) channel; mENaC, mouse epithelial Na\(^{+}\) channel; I-V, current-voltage relationship; \( K_{\text{I}} \), inhibitory constant; DEPC, diethyl pyrocarbonate; MTSET, 2-(trimethylammonium)methyl methanethiosulfonate bromide; WT, wild type; MES, 2-(N-morpholino)ethanesulfonic acid; ASIC, acid-sensing ion channels; CNG, cyclic nucleotide-gated channel; ECL, extracellular loops.
Austen, TX) and dissolved in nuclease-free water (Ambion Inc.). Stage V–VI Xenopus oocytes were treated with collagenase type IV (Sigma) to remove follicle cell layers and injected with 1–4 ng of cRNA for each mENaC subunit per cell in a total volume of 50 µl. Injected oocytes were maintained at 18°C in modified Barth’s saline.

**Two-electrode Voltage Clamp**—Two-electrode voltage clamp was performed 24–72 h after cRNA injection at room temperature (20–24°C) as described previously (19). The bath solution contained 110 mM NaCl, 4 mM KCl, 2 mM CaCl2, 10 mM HEPES, and the pH was adjusted to 7.40 with NaOH. Pipettes filled with 3 M KCl had a resistance of 0.5–2 mehmohms. Typically, oocytes were clamped from −140 to 60 mV in increments of 20 mV for 500 ms every 2 s. Amiloride at the concentration of 100 µM was added to the bath at the end of each experiment to determine the amiloride-insensitive current that was used to calculate amiloride-sensitive currents. Data acquisition and analyses were performed with pClamp 7.1 for Windows (Axon Instruments, Union City, CA).

**Patch Clamp**—Vitelline membranes of oocytes were removed manually following incubation of the oocytes at room temperature in a hypertonic solution containing 200 mM sucrose. Oocytes were then transferred to a recording chamber with bath solution and allowed to recover for 30 min before clamping. The bath solution contained 110 mM NaCl, 2 mM CaCl2, 10 mM HEPES, pH 7.40. For Na+ current recordings the pipette solution was identical to the bath solution, and for Li+ current recordings NaCl was replaced by LiCl. Glass pipettes with tip resistances of 5–20 mehmohms were used. Single channel currents were recorded in cell-attached configuration using PC ONE Patch Clamp amplifier (Dagan Corp., Minneapolis, MN), DigiData 1322A interface, and Clampex 8.1 software (Axon).

**Examination of Effects of Inhibitors**—Inhibitors used in two-electrode voltage clamps (NiCl2, MgCl2, amiloride, diethyl pyrocarbonate, and sulfhydryl reagents) were prepared in the bath solution and delivered to the bath by gravity perfusion. To avoid hydrolysis, diethyl pyrocarbonate (DEPC) and sulfhydryl reagents were prepared immediately prior to use. Whole-cell Na+ currents were recorded before and after external application of an inhibitor. The effects of a certain inhibitor on ENaC currents were defined by comparison of the amiloride-insensitive currents with calculated amiloride-sensitive currents. Dose-response relationship for Ni2+ inhibition of mENaC currents was obtained by plotting the relative currents measured at −100 mV and in increasing concentrations of NiCl2 (0.01, 0.1, 1, 10, 25, and 50 µM) against Ni2+ concentrations using a semi-logarithmic scale. The relative currents represent the ratios of whole-cell amiloride-sensitive Na+ currents in the presence of Ni2+ in both solutions relative to the current measured immediately before application of Ni2+. The osmolality of the bath solution containing NiCl2 was not adjusted. Non-linear least squares curve fitting was used to obtain the dose-response parameters.

**RESULTS**

**External Ni2+ Inhibited Amiloride-sensitive Na+ Currents in Oocytes Expressing α-β-γ mENaC**—The effect of externally applied Ni2+ on whole-cell amiloride-sensitive Na+ currents was studied in Xenopus oocytes expressing α-β-γ mENaC. Ni2+ inhibited amiloride-sensitive Na+ currents in a dose-dependent manner in the concentration range of 0.01–50 mM (Fig. 1A). At 50 mM, Ni2+ inhibited about 80% of the amiloride-sensitive current. The dose-response data were fitted reasonably well with the one-site equation described under “Experimental Procedures” (Fig. 1C, dashed line). The estimated inhibitory constant (K) and Hill coefficient were 0.58 ± 0.05 (n = 6) and 0.37 ± 0.01 mM (n = 6), respectively (Table 1). However, the dose-response curve appeared to contain more than one component. Data were fitted with the two-site equation as described under “Experimental Procedures,” assuming that two classes of binding sites with different apparent affinities for Ni2+ account for the inhibitory effect of Ni2+ on ENaC currents (Fig. 1C, solid line). The non-linear least square fitting resulted in a higher correlation coefficient with the two-site equation (0.9958) than that obtained from fitting with the one-site equation (0.9545).

The current reduction by Ni2+ was accompanied by a moderate inward rectification as the concentration of Ni2+ exceeded 0.1 mM (Fig. 1A and B). The current-voltage relationship curves (I-V) indicate that Ni2+ inhibition of ENaC currents was enhanced when oocyte membranes were depolarized. The Ni2+-induced current rectification is different from that induced by amiloride, which is generally considered as a pore blocker for ENaC.

The Ni2+ inhibition of mENaC currents was fast with a maximal effect observed around 1 min after initiation of Ni2+ perfusion (Fig. 1D). Removal of Ni2+ from the bath solution partially restored the Na+ currents when Ni2+ was applied at 1 mM. However, at a high concentration (50 mM) Ni2+ inhibition was not reversed by removal of Ni2+ from the bath for a period of 2 min (Fig. 1E).

It was reported that external Ca2+, Mg2+, Ba2+, and Sr2+ blocked Na+ channels in toad urinary bladder with complex voltage dependence. The blocking effects were rather weak with calculated concentrations for half-maximal inhibition of greater than tens of millimolar (29). To test whether the relatively high affinity Ni2+ inhibition of α-β-γ mENaC was unique among divalent cations, we examined several other divalent cations for their effects on α-β-γ mENaC. As shown in Fig. 2, external Mg2+ produced a weak inhibition of amiloride-sensitive Na+ currents with an estimated KI of 73.5 ± 10.9 mM (n = 5) and a Hill coefficient of 0.47 ± 0.05 (n = 5). Furthermore, current rectification was not observed in the presence of external Mg2+. The inhibition potency of external Mg2+ on mENaC is similar to that observed in toad bladder (29). We previously reported that external Cd2+ moderately increased amiloride-sensitive Na+ currents at a concentration of 5 mM (24). Schild et al. (13) reported that inward Li+ currents in oocytes expressing α-β-γ rat ENaC were insensitive to external Zn2+ even in the millimolar range, and a small current reduction (~20%) was shown with 10 mM Zn2+. We observed no inhibition of amiloride-sensitive Na+ currents with 5 mM Zn2+ in the bath solution. However, at low concentrations (0.01–1 mM), Zn2+ produced a moderate potentiation of the Na+ currents, similar in amplitude to the increase in Na+ currents observed with Cd2+. Therefore, among divalent cations (Ca2+, Mg2+, Ba2+...S. Sheng, C. J. Perry, and T. R. Kleyman, unpublished data.
Nickel is often coordinated by nitrogen atoms from histidine residues, although sulfur and oxygen can also coordinate Ni$^{2+}$ as a transition ion with polarizability between those of hard ions and soft ions. Nitrogen is considered as its most preferred ligand, although sulfur and oxygen can also coordinate Ni$^{2+}$. Nickel is —39), and it is conceivable that Ni$^{2+}$ binds to histidine residue(s) within ENaC subunits to exert its inhibitory effect on the channel. We therefore examined if the histidine-modifying reagent DEPC had any effect on α-β-γ mENaC expressed in oocytes and whether DEPC-modified channels exhibited an altered response to Ni$^{2+}$. Perfusion with 0.1 mM amiloride prepared in the bath solution. Scales are identical and shown at the bottom of each panel. A, representative recordings in an oocyte expressing α-β-γ mENaC. A, representative recordings in an oocyte expressing α-β-γ mENaC by two-electrode voltage clamp. Na$^{+}$ currents were measured by clamping the oocyte from −140 to 60 mV in the presence of 0 (control), 0.01, 0.1, 1, 10, 25, and 50 mM NiCl$_2$ in the bath solution. Scales are identical and shown at the bottom of each panel. A, representative recordings in an oocyte expressing α-β-γ mENaC. B, current-voltage relationship (I-V) curves were generated by plotting amiloride-sensitive currents against clamping voltages from the above recordings. For clarity, only I-V curves obtained in 0 (●), 0.01 (○), 0.1 (△), 1 (▲), 10 (○), 25 (×), and 50 (+) mM NiCl$_2$ are shown. C, dose-response relationship of Ni$^{2+}$ inhibition of the Na$^{+}$ currents. Relative currents represent currents in the presence of Ni$^{2+}$ normalized to the control currents. Data points displayed as open circles with vertical bars represent mean ± S.E. from six oocytes. The dashed line is from curve fitting with a one-site equation (fitting parameters: $K_i = 0.54$, Hill coefficient = 0.37, $R^2 = 0.955$), and the solid line is from fitting with a two-site equation (fitting parameters: $K_i = 0.05$ mM, $K_i = 10.23$ mM, $A = 0.63$, $B = 0.2$) as described under “Experimental Procedures.” D, time course of Ni$^{2+}$ inhibition. Oocytes were clamped to −60 mV and 60 mV for 450 ms every 5 s during experiments. Relative currents were obtained by normalizing the currents at −60 mV to the current level immediately before Ni$^{2+}$ reached bath. Data are presented as mean ± S.E. (vertical lines) from five oocytes. Solid and open bars indicate the periods of application of 1 mM Ni$^{2+}$ and 0.1 mM amiloride, respectively. E, lack of reversibility of Ni$^{2+}$ inhibition at high concentration. Relative currents are shown in the absence and presence of 50 mM Ni$^{2+}$, and after a 2-min washout. Vertical bars are S.E. (n = 8).

**Table 1**

| Channel     | n  | $K_i$ (mM) | Hill coefficient | $R^2$    |
|-------------|----|------------|------------------|----------|
| α-β-γ       | 6  | 0.58 ± 0.09| 0.37 ± 0.01      | 0.9545 ± 0.0116 |
| αH381R-β-γ  | 6  | 0.73 ± 0.15| 0.42 ± 0.02      | 0.9891 ± 0.0022 |
| α-βH319R-γ  | 8  | 0.82 ± 0.12| 0.33 ± 0.02      | 0.9894 ± 0.0025 |
| α-β-γH338R  | 6  | 0.97 ± 0.22| 0.39 ± 0.03      | 0.9895 ± 0.0022 |
| α-βQ220H-γ  | 6  | 1.03 ± 0.10| 0.33 ± 0.01      | 0.9529 ± 0.0054 |

* p < 0.01 from Student’s t tests between WT and the mutant channel.
of oocytes with bath solutions containing DEPC at concentrations of 0.1, 1, and 10 mM blocked amiloride-sensitive Na\(^+\) currents in a dose-dependent manner. The dose-response relationship was satisfactorily fitted with the one-site equation (\(R^2 = 0.998\)) (Fig. 3A). The estimated \(K_i\) was 0.45 ± 0.07 mM and the Hill coefficient was 0.99 ± 0.05 (n = 4). The Na\(^+\) currents were essentially insensitive to Ni\(^{2+}\) in the absence (○) and presence of 0.01 (●), 0.1 (▲), 1 (▲), 10 (□), 25 (×), and 50 (+) mM MgCl\(_2\) were generated the same way as Fig. 1B.

The above results encouraged us to examine whether an equivalent histidine residue in \(\gamma mENaC\) (\(\gamma His^{283}\)) was also involved in Ni\(^{2+}\) inhibition. The mutations \(\gamma H239C\), \(\gamma H239R\), and \(\gamma H239D\) significantly attenuated Ni\(^{2+}\) inhibition (Fig. 5). Surprisingly, Na\(^+\) currents in oocytes expressing the mutant channels did not exhibit rectification. The experiments with DEPC suggested that histidine residues might be involved in Ni\(^{2+}\)-induced current rectification. The double mutations (\(\alpha H282R\) and \(\gamma H239R\)) eliminated Ni\(^{2+}\)-induced inhibition of Na\(^+\) currents (Fig. 6A); these channels did not display current rectification (Fig. 6B). These results provided strong evidence that \(\alpha His^{282}\) and \(\gamma His^{239}\) provide Ni\(^{2+}\)-binding sites and are primarily responsible for Ni\(^{2+}\) inhibition of ENaC currents. Introduction of a histidine residue at the corresponding site in \(\beta mENaC\) (\(\beta Q220H\)) resulted in a modest increase in Ni\(^{2+}\) \(K_i\), suggesting that this residue does not have an important role in Ni\(^{2+}\) inhibition of ENaC (Table 1).

**Introduction of Additional Histidine Residues at Sites Neighboring \(\alpha His^{282}\) Altered Ni\(^{2+}\) Inhibition of ENaC Currents**—In nickel-binding proteins, a Ni\(^{2+}\) can be coordinated by one (41), two (42–44), three (45, 46), or four (47) histidine residues, and two histidine residues are often separated by one or three residues (HXH or HXXHX) (37, 48–50). A sequence “HEXXH” located in an α-helix has been considered as a signature metal-binding motif for metalloproteins (45, 46, 51, 52). To gain more knowledge about the putative Ni\(^{2+}\)-binding site, we examined whether the introduction of additional histidine residue(s) in the vicinity of \(\alpha His^{282}\) enhanced Ni\(^{2+}\) inhibition of ENaC currents, presumably by altering Ni\(^{2+}\) binding affinity. Histidine residues were individually introduced at positions \(\alpha Trp^{278}\), \(\alpha Tyr^{279}\), \(\alpha Arg^{280}\), \(\alpha Phe^{281}\), \(\alpha Tyr^{283}\), \(\alpha lle^{284}\), and \(\alpha Asn^{285}\). In addition, two α-subunit mutants with consecutive 4-histidine tracts, \(\alpha Y279H/R280H/F281H/H282\) (referred to as \(\alpha His^{279–282}\)) and \(\alpha H282/Y283H/I284H/N285H\) (referred to as \(\alpha His^{282–285}\)), were also generated. The
effects of Ni\textsuperscript{2+} on these mutant channels are shown in Fig. 7. The mutant channel αR280H-β-γ showed a significant increase in Ni\textsuperscript{2+} sensitivity, greatly shifting the high affinity component in the dose-response curve to the left with less effect on the low affinity component compared with WT dose response (Table II). Interestingly, double mutations (αR280H and αY239H) shifted the high affinity component in the dose-response curve to the right compared with αR280H-β-γ but to the left compared with WT mENaCs. The low affinity component was shifted to the right compared with both αR280H-β-γ and WT mENaCs (Fig. 7E and Table II). Like other mutations at αHis\textsuperscript{282}, αR280H did not affect the Ni\textsuperscript{2+}-induced current rectification (Fig. 7F). Three other mutations (ωY278H, αY283H, and αN285H) slightly decreased Ni\textsuperscript{2+} block of the Na\textsuperscript{+} currents, whereas three other mutations (ωY279H, αP281H, and αI284H) did not significantly change Ni\textsuperscript{2+} block (Fig. 7A). As observed with αR280H-β-γ channels, the mutant channels with a consecutive 4-histidine tract including αR280H exhibited a large decrease in the Ni\textsuperscript{2+} K\textsubscript{i} (αHis\textsuperscript{279-282}, K\textsubscript{i} = 0.12 ± 0.03, n = 8). In contrast, the α-subunit mutant with a consecutive 4-histidine tract C-terminal to (and including) αHis\textsuperscript{282} exhibited a large increase in the Ni\textsuperscript{2+} K\textsubscript{i} (αHis\textsuperscript{282-285}, K\textsubscript{i} = 10.37 ± 0.92 mM, n = 5). These data suggest that αR280H, in addition to αHis\textsuperscript{282} and αHis\textsuperscript{239}, participates in Ni\textsuperscript{2+} binding. Fig. 7G provides three models illustrating the coordination of Ni\textsuperscript{2+} by these residues, generated based on the changes in Ni\textsuperscript{2+} inhibition of ENaC currents observed with mutant channels and the preferred coordination geometries of Ni\textsuperscript{2+} complexes and Ni\textsuperscript{2+}-binding peptides and proteins (see “Discussion”).

**MTSET Inhibited Amiloride-sensitive Na\textsuperscript{+} Currents in Oocytes Expressing αH282C-β-γ mENaCs**—The above results suggested that a Ni\textsuperscript{2+}-binding site consisting of αHis\textsuperscript{282} and γHis\textsuperscript{239} has a key role in Ni\textsuperscript{2+} inhibition of ENaC currents. These histidine residues may also have an important functional role. To explore further the role of αHis\textsuperscript{282} in channel activity, we examined the response of the mutant channels (αH282C-β-γ) to external sulphydryl reagents. The positively charged reagent MTSET irreversibly inhibited amiloride-sensitive Na\textsuperscript{+} currents by 45% when applied externally at the concentration of 1 mM (Fig. 8, A and B). As observed with Ni\textsuperscript{2+}, inward rectification was observed following MTSET modification of the channel (Fig. 8D). The remaining currents following MTSET modification were less sensitive to Ni\textsuperscript{2+} (Fig. 8C). MTSET at 1 mM did not alter amiloride-sensitive Na\textsuperscript{+} currents in oocytes expressing WT α-β-γ mENaCs (24) or α-β-γ-H239C mENaCs (Fig. 8A), and pretreatment of the oocytes with the reagent did not alter Ni\textsuperscript{2+} inhibition of these channels. The negatively charged reagent MTSES [sodium (2-sulfonatoethyl) methanethiosulfonate] at 5 mM also inhibited about 40% of the Na\textsuperscript{+} currents of αH282C-β-γ channels, whereas it did not change the currents of WT channels (24). A possible explanation of why MTSET failed to inhibit α-β-γ-H239C mENaC is illustrated in Fig. 8D. We propose that γHis\textsuperscript{239} is located deeper within a putative Ni\textsuperscript{2+}-binding pocket than αHis\textsuperscript{282} and is not accessible to MTSET that requires a cylindrical space of 6 × 10 Å (53).

In summary, we observed that amiloride-sensitive Na\textsuperscript{+} currents were blocked by three different reagents. Ni\textsuperscript{2+} and DEPC blocked WT ENaC. MTSET and MTSES blocked αH282Cβγ.
Moreover, pretreatment of channels with either DEPC or MTSET resulted in a reduced response to Ni²⁺, suggesting that these three reagents interact with a common site within the channel. Furthermore, these results suggest that αHis²⁸² and γHis²³⁹ have an important role in channel function.

**Ni²⁺ Block Was Reduced by Extracellular Acidification but Not by Amiloride**—The imidazole rings from histidine residues have pHₐ values of 6.0 and 14 and may exist in different protonation states including a protonated imidizolium, a neutral imidazole, and a negatively charged imidazolate. Prototated imidizolium cannot coordinate a metal ion (54). Therefore, we examined whether Ni²⁺ inhibition of ENaC currents was affected by acidification of the bath solution. The Ni²⁺ inhibitory effect on Na⁺ currents was examined with bath solutions buffered to pH 7.4 with HEPES (buffer range 6.8–8.2), 5.5 with MES (buffer range 5.5–6.5), or 4.4 with citric acid (buffer range 2.2–6.5). Fig. 9 shows that lowering the bath pH reduced (at pH 5.5) or eliminated (at pH 4.4) the Na⁺ current inhibition by Ni²⁺. We observed a biphasic effect of extracellular acidification on amiloride-sensitive Na⁺ currents, a brief stimulation (10% for pH 5.5 and 70% for 4.4) with peak effect at 1–2 min followed by a return to basal current or below basal current.² These results are consistent with a recent report (55) and inconsistent with others (56, 57). Because of the biphasic pH effects, a Ni²⁺ dose response could not be accurately obtained. Instead, we compared the effects of 1 mM Ni²⁺ on the Na⁺ currents at different pH values. As different effects of extracellular acidification were observed in native tissues and oocytes expressing cloned ENaC, the physiological significance of these pH effects is not clear (55).

Residue αHis²⁸² is located within a previously identified amiloride-binding domain in the ECL of the α subunit (14, 40). We have demonstrated that αHis²⁸², together with γHis²³⁹, is involved in Ni²⁺ inhibition of ENaC. We performed two experiments to examine whether Ni²⁺ and amiloride compete for the same binding site when both inhibitors were present in the bath solution. First, we determined the response of WT α-β-γ mENaC to increasing concentrations of Ni²⁺ and amiloride.² Second, the response of WT channels to increasing concentrations of Ni²⁺ was examined in the presence or absence of an intermediate concentration of Ni²⁺ (1 mM). Ni²⁺ did not significantly alter the sensitivity of the channel to amiloride.² Second, the response of WT channels to increasing concentrations of Ni²⁺ was examined in the presence or absence of an intermediate concentration (100 nM) of amiloride. Amiloride did not significantly alter the sensitivity of the channel to Ni²⁺.² These results suggest that Ni²⁺ and amiloride do not compete for the same site in α-β-γ ENaC.

**Ni²⁺ Reduced ENaC Open Probability without Altering Single Channel Current Levels**—Reduction of whole-cell Na⁺ currents by external Ni²⁺ could be due to a decrease in single channel conductance, open probability (Pₒ) of individual channels, number of functional channels on the cell surface, or electrochemical driving force, or by a combination of these factors. We performed single channel recordings to distinguish these possibilities. The unitary Na⁺ currents were not altered...
ENaC and Ni$^{2+}$

**Fig. 5. Mutations at $\gamma$His$^{399}$ reduced Ni$^{2+}$ inhibition.** A, dose-response curves of Ni$^{2+}$ on $\alpha$-$\beta$-H239R ( ), $\alpha$-$\beta$-H239D ( ), and $\alpha$-$\beta$-H239C ( ) mENaCs were generated by fitting the data with the one-site equation (dashed lines) and two-site equation (solid lines) as described under “Experimental Procedures.” WT dose-response curve (○) is shown for comparison. Data are shown as mean ± S.E. Numbers of clamped oocytes are 6, 5, and 7 for $\alpha$-$\beta$-H239R, $\alpha$-$\beta$-H239D, and $\alpha$-$\beta$-H239C, respectively. B–D, I–V curves in the absence and presence of varying concentrations of external Ni$^{2+}$ were obtained by plotting the amiloride-sensitive Na$^+$ currents against clamping voltages. Mutant channels are identified as point mutation names. Symbols used in the I–V curves are the same as in Fig. 4. The curves represent the results from 5 to 7 oocytes.

by including 10 mM Ni$^{2+}$ in the pipette solution (Fig. 10, A and B). As ENaC open probability is highly variable from patch to patch, it is difficult to determine whether Ni$^{2+}$ reduces ENaC $P_o$, by comparing the $P_o$ values from patches performed in the absence and presence of Ni$^{2+}$ in the pipette solution. We therefore examined channel transitions while Ni$^{2+}$ was being added to the patch pipette by pipette perfusion. The results from pipette perfusion experiments indicated that Ni$^{2+}$ reduced $N_{Po}$ of mENaCs without effect on unitary Li$^+$ current (Fig. 10, C–F).

Single channel conductances before and after pipette perfusion of Ni$^{2+}$ were 8.3 ± 0.4 pS (n = 4) and 8.1 ± 0.3 pS (n = 4), respectively. Since Ni$^{2+}$ inhibition was not accompanied by a decrease in the reversal potential in oocytes, it was unlikely that external Ni$^{2+}$ in the millimolar range significantly altered the driving force for Na$^+$ influx. Moreover, the density of channel proteins was not likely reduced by external Ni$^{2+}$ within 1 min. Therefore, our results suggest that Ni$^{2+}$ inhibition of ENaC currents is due to a reduction of channel $P_o$.

**DISCUSSION**

Ni$^{2+}$ Is an External Inhibitor of ENaC—In this study, we found that external Ni$^{2+}$ inhibited amiloride-sensitive whole-cell Na$^+$ currents from oocytes expressing $\alpha$-$\beta$-$\gamma$ mENaC in a dose-dependent manner. The blocking effect of Ni$^{2+}$ is in good agreement with that observed by Segal and colleagues (31) who reported that 2.5 mM Ni$^{2+}$ inhibited 60% of $\alpha$-$\beta$-$\gamma$ rENaC currents. Another group recently reported (58) that Ni$^{2+}$ and other Ca$^{2+}$ channel blockers partially blocked amiloride-sensitive short circuit currents in fetal rat alveolar type II epithelia. Apical membranes of this type of epithelia are believed to have two types of Ca$^{2+}$-activated and amiloride-sensitive channels (28 pS nonselective cation channel and 12 pS Na$^+$ channel) whose properties are clearly different from those of $\alpha$-$\beta$-$\gamma$ ENaC (59, 60). Therefore, the identity of the Ni$^{2+}$-blocked channels is not clear, although under certain cultural conditions the predominant channels expressed in these cells show single channel properties similar to those of ENaCs (61). It appears that Ni$^{2+}$ is the only divalent cation that externally blocks ENaC at submillimolar concentrations. Why is Ni$^{2+}$ a high affinity blocker of ENaC? A simple explanation is that metal ion-binding site(s) are located in functional domains within the ENaC complex that exhibit higher affinity for Ni$^{2+}$ than other divalent cations. In metalloproteins, formation of a metal-ligand complex is dependent on many factors including the size, relative charge, polarizability (softness and hardness), preferential coordination geometry of the ion, the liganding donor atoms, and solvent effects (62, 63). The ionic radius of the divalent cation does not appear to be the most important determinant in blocking ENaC. A smaller cation Mg$^{2+}$ (0.65 Å, Pauling (64)) is a much weaker inhibitor of ENaC than Ni$^{2+}$ (0.72 Å), and a larger cation Cd$^{2+}$ (0.97 Å) does not inhibit ENaC at a concentration of 5 mM (24). Moreover, Zn$^{2+}$ (0.74 Å) with very close radius to Ni$^{2+}$ has no inhibitory effect on ENaC.

**Fig. 6. Double mutations ($\alpha$H282R/H239R) eliminated Ni$^{2+}$ inhibition.** A, dose response of Ni$^{2+}$ on amiloride-sensitive Na$^+$ currents was examined in oocytes expressing $\alpha$H282R-$\beta$-$\gamma$H239R mENaCs. Relative currents in the presence of varying concentrations of external Ni$^{2+}$ are displayed as filled squares (mean ± S.E., n = 5). Dashed line was from fitting the data with the one-site equation. The data could not be fitted with the two-site equation. B, I–V curves in the absence and presence of Ni$^{2+}$ were generated same way as above, and symbols are identical to those in Fig. 4. The curves are representative of five oocytes.
Fig. 7. Effect of histidine substitution at αHis282 neighboring sites altered Ni²⁺ sensitivity. Histidine residues were introduced at varying sites within the tract WYRFHYIN (α278–285). Sequences of the tract with the introduced histidine (boldface letter) are shown in C. A naturally occurring histidine (αHis285) is boxed throughout the sequences. WT sequence is underlined, and mutant channels are identified by the point mutations. His279–282 and His282–285 represent two multiple mutations: αY279H/R280H/F281H and αY283H/I284H/N285H. The dose responses of the mutant channels were examined as above. Data were fitted with both the one-site and two-site equations. For ease of comparison, Ki values and Hill coefficients from one-site fittings are shown in A and B. Data are displayed as mean ± S.E. from 5 to 12 oocytes. Open bars indicate no significant difference between mutant and WT channels (p > 0.05), and filled bars indicate the values of mutant channels that are statistically different from those of WT channels (p < 0.01). D, inward amiloride-sensitive Na⁺ currents measured at −100 mV in oocytes expressing WT or mutant mENaCs are shown as mean ± S.E. from 5 to 12 oocytes.

TABLE II

Fitting parameters for Ni²⁺ dose-response curves with the two-site equation

Parameters were obtained from the best fitting of the dose-response data by non-linear least square method using Equation 2. Data are mean ± S.E., and n represents number of oocytes used in the experiments.

| Channel     | n  | A      | K₁   | B      | K₂   | B²   |
|-------------|----|--------|------|--------|------|------|
| αβ-γ        | 8  | 0.58 ± 0.02 | 0.029 ± 0.003 | 0.22 ± 0.01 | 6.93 ± 1.59 | 0.9968 ± 0.0008 |
| αR280H-β-γ  | 6  | 0.57 ± 0.02 | 0.002 ± 0.0000 | 0.16 ± 0.015 | 0.94 ± 0.199 | 0.9939 ± 0.0019 |
| αR280H-β-γH239R | 7  | 0.48 ± 0.01 | 0.003 ± 0.0000 | 0.33 ± 0.015 | 8.04 ± 1.14  | 0.9933 ± 0.0009 |

* Values are p < 0.05 from Student’s t test between WT and mutant channels.

* Values are p < 0.01 from Student’s t test between WT and mutant channels.

* Values are p < 0.001 from Student’s t test between WT and mutant channels.

at millimolar concentrations (13). As transition metal ions, Ni²⁺ and Zn²⁺ have a number of common properties including size, charge, intermediate polarizability (not as “hard” as Ca²⁺ and Mg²⁺, and not as “soft” as Cd²⁺ and Hg²⁺), and favor nitrogen and sulfur atoms as their binding ligands (62). It is not clear why Zn²⁺ and Ni²⁺ do not have similar inhibitory effects on ENaC. It was recently reported that Zn²⁺ might be a physiological co-activator of the acid-sensing ion channels (111). Therefore, the observed ENaC current stimulation by low concentrations of external Zn²⁺ suggests that Zn²⁺ might be a
MTSET reduced Na⁺ currents in oocytes expressing αH282C-β-γ mENaC and further reduced Ni²⁺ inhibition of the remaining currents. A, effects of MTSET on αH282C-β-γ and α-β-γH239C mENaCs. Amiloride-sensitive Na⁺ currents were measured at −100 mV before, 2 min after perfusion of 1 mM MTSET prepared in the bath solution, and 2 min after washout of MTSET from the bath. Relative currents were obtained by normalizing the currents to the levels before application of MTSET. Open, filled, and shaded bars are the relative currents (mean ± S.E., n = 5 for αH282C-β-γ and n = 13 for α-β-γH239C) obtained before and after MTSET and after washout of MTSET, respectively. B, I-V curves of amiloride-sensitive Na⁺ currents before (○) and after (■) MTSET and after washout of MTSET (△) were generated as before. Data are mean ± S.E. from 21 oocytes. C, Ni²⁺ dose responses on αH282C-β-γ mENaC currents without (○) and with (■) treatment of the oocytes with 1 mM MTSET. The dose response of Ni³⁺ on αH282C-β-γ mENaCs without MTSET is the same as in Fig. 5A. Relative currents in solid circles (mean ± S.E., n = 5) were obtained by normalizing the currents in the presence of Ni²⁺ to the current levels following 1 mM MTSET perfusion for 3 min and washout of the reagent for 2 min, and immediately prior to Ni³⁺ application. Dashed lines are from fitting the data with the one-site equation. D shows one of the possible reasons why MTSET inhibited αH282C-β-γ mENaC without effect on α-β-γH239C mENaC. The shaded area represents a bound Ni²⁺-binding site. The three open rectangles represent three Ni²⁺-binding ligands out of a possible total number of four or six ligands. Side chains of αHis²⁸² and γHis²⁸⁹ provide three of the ligands. A circle inside the pocket represents a bound Ni²⁺ ion to WT channels. One molecule of MTSET is drawn with space-filled model, and its thiol head is placed at the entrance of the pocket. Attachment of MTSET to sulphhydryl group of αH282C would prevent Ni²⁺ from entering the binding site to cause inhibition of the channel currents. The −SH group of γH239C may be too deep for interaction with MTSET.

Fig. 8. MTSET reduced Na⁺ currents in oocytes expressing αH282C-β-γ mENaC and further reduced Ni²⁺ inhibition of the remaining currents. A, effects of MTSET on αH282C-β-γ and α-β-γH239C mENaCs. Amiloride-sensitive Na⁺ currents were measured at −100 mV before, 2 min after perfusion of 1 mM MTSET prepared in the bath solution, and 2 min after washout of MTSET from the bath. Relative currents were obtained by normalizing the currents to the levels before application of MTSET. Open, filled, and shaded bars are the relative currents (mean ± S.E., n = 5 for αH282C-β-γ and n = 13 for α-β-γH239C) obtained before and after MTSET and after washout of MTSET, respectively. B, I-V curves of amiloride-sensitive Na⁺ currents before (○) and after (■) MTSET and after washout of MTSET (△) were generated as before. Data are mean ± S.E. from 21 oocytes. C, Ni²⁺ dose responses on αH282C-β-γ mENaC currents without (○) and with (■) treatment of the oocytes with 1 mM MTSET. The dose response of Ni³⁺ on αH282C-β-γ mENaCs without MTSET is the same as in Fig. 5A. Relative currents in solid circles (mean ± S.E., n = 5) were obtained by normalizing the currents in the presence of Ni²⁺ to the current levels following 1 mM MTSET perfusion for 3 min and washout of the reagent for 2 min, and immediately prior to Ni³⁺ application. Dashed lines are from fitting the data with the one-site equation. D shows one of the possible reasons why MTSET inhibited αH282C-β-γ mENaC without effect on α-β-γH239C mENaC. The shaded area represents a bound Ni²⁺-binding site. The three open rectangles represent three Ni²⁺-binding ligands out of a possible total number of four or six ligands. Side chains of αHis²⁸² and γHis²⁸⁹ provide three of the ligands. A circle inside the pocket represents a bound Ni²⁺ ion to WT channels. One molecule of MTSET is drawn with space-filled model, and its thiol head is placed at the entrance of the pocket. Attachment of MTSET to sulphhydryl group of αH282C would prevent Ni²⁺ from entering the binding site to cause inhibition of the channel currents. The −SH group of γH239C may be too deep for interaction with MTSET.

Fig. 9. Ni²⁺ inhibition of ENaC was reduced by extracellular acidification. The effects of 1 mM external Ni²⁺ on amiloride-sensitive Na⁺ currents at three external pH values (7.4, 5.5, and 4.4) were examined in oocytes expressing α-β-γ mENaCs. Bars represent relative currents calculated by normalizing the amiloride-sensitive Na⁺ currents in the absence (open bars) and presence (solid bars) of 1 mM Ni²⁺ to the current levels in the absence of Ni²⁺ in each bath solution with the corresponding pH value. Data are mean ± S.E., and the numbers of observations are indicated in the parentheses. The * indicates the absolute currents in the presence of 1 mM Ni²⁺ are significantly lower than the absolute control currents with pH 7.4 and 5.5 (p < 0.001). The difference between the control currents and currents in the presence of 1 mM Ni²⁺ at pH 4.4 is not statistically significant (p > 0.05). Triangles indicate that the relative currents with 1 mM Ni²⁺ at pH 5.5 and 4.4 are significantly higher than the relative current with 1 mM Ni²⁺ at pH 7.4.

high affinity stimulator of ENaC. It has been known for a long time that sulfhydryl group-reactive reagents, including Cd²⁺, stimulate epithelial Na⁺ transport, possibly by relieving Na⁺ self-inhibition; however, its physiological significance and mechanism are still unknown (1).

The inhibition of ENaC currents by external Ni²⁺ could be due to a direct blocking mechanism (pore plugging) or an indirect one, often referred to as an allosteric mechanism. The inward current rectification observed in the presence of Ni²⁺ does not fit with a typical voltage-dependent block of a cation channel from the external side. For example, current-voltage relationship curves in the presence of submaximal concentrations of amiloride show outward rectification, indicating a reduced block of ENaC when the membrane is depolarized and consistent with the idea that amiloride is an external pore blocker of ENaC (1). The poor reversibility of Ni²⁺ inhibition of the ENaC currents suggests a complex feature of the blocking mechanism and is inconsistent with the notion of a direct pore plugging. We favor the idea that Ni²⁺ is an external inhibitor of ENaC but does not directly interact with the pore.

It has been known that external Ni²⁺ affects a variety of ion channels including voltage-gated Ca²⁺ channels (65–68), voltage-gated K⁺ channels (69–71), voltage-gated Na⁺ channels (72), voltage-gated H⁺ channels (73–75), CNG channels (76), P2X receptors (77, 78), γ-aminobutyric acid type A receptors...
Ni$^{2+}$ to elicit voltage-dependent currents. Second, we evaluated the Ni$^{2+}$ effect on ENaC currents at a clamp potential of $-100$ mV, a potential at which many voltage-gated channels should not be activated. The linear I-V relationship in the range of $-140$ to $60$ mV of the currents measured in ENaC-expressed oocytes indicated a lack of voltage-gated current. Moreover, the observed Ni$^{2+}$ inhibition of ENaC currents was specifically eliminated by $\alpha$H282D mutation and by the double ($\alpha$H282R/H239R) mutation, which strongly suggests that the whole-cell current reduction is due to Ni$^{2+}$ interaction with ENaC rather than with other channels. It was recently reported that external Ni$^{2+}$ (less potent than Cd$^{2+}$ and Zn$^{2+}$) blocked a slowly activating Na$^+$ current elicited by sustained depolarization of the $Xenopus$ oocyte membrane, a current that is clearly different from ENaC currents (86).

Whether Ni$^{2+}$ has any physiological role in channel regulation in humans is unclear, although it is known that Ni$^{2+}$ is essential for bacteria and plant growth (87–89). As nickel is considered a hazardous ion to human, the inhibitory effect of Ni$^{2+}$ on ENaC activity may contribute to its toxic effects on kidneys, lungs, and digestive system where ENaC is expressed. We anticipate that Ni$^{2+}$ will be a useful tool to study the structure and function of epithelial Na$^+$ channels. Nickel has been used successfully as a pharmacological tool to distinguish different types of voltage-gated Ca$^{2+}$ channels and to gain useful information regarding gating mechanisms of CNG channels (90, 91).

**Histidine Residues $\alpha$His$^{282}$ and $\gamma$His$^{239}$ within the Extracellular Loops of ENaCs Form the Ni$^{2+}$-binding Site**—The histidine-reactive reagent DEPC inhibited amiloride-sensitive Na$^+$ currents with an estimated $K_i$ of 0.45 mM, suggesting that histidine residue(s) in ENaC complex are located in a functional domain. The potential role of histidyl groups in amiloride-sensitive Na$^+$ channels was proposed nearly 20 years ago based on the identification of a titratable group with a $pK_a$ of 6.7 and DEPC inhibition of short circuit current in toad urinary bladder (92). Moderate DEPC treatment (1 mM) of oocytes expressing WT mENaCs significantly reduced Ni$^{2+}$ inhibition on the remaining currents, suggesting that Ni$^{2+}$ and DEPC act at a common site.

Our mutagenesis studies suggest that $\alpha$His$^{282}$ and $\gamma$His$^{239}$ are the primary structural determinants for Ni$^{2+}$ inhibition of mENaC currents. However, there were distinct changes associated with mutations at these two sites. The $\gamma$-mutation-induced changes in Ni$^{2+}$ inhibition followed the order: $\gamma$H239R $> \gamma$H239D $> \gamma$H239C, consistent with the preferential coordination of Ni$^{2+}$. Cysteine residues coordinate Ni$^{2+}$ in hydrogenase, dehydrogenase, and deformylase enzymes (38, 39, 42, 44). Aspartic acid participates in Ni$^{2+}$ coordination in urease (37). The relationship between substituted residues at $\alpha$His$^{282}$ and the degrees of alteration in Ni$^{2+}$ inhibition did not follow the order observed with $\gamma$His$^{239}$. The largest change in Ni$^{2+}$ dose response on mENaC currents was observed with $\alpha$H282D (Fig. 4A). The most

**Fig. 10.** Ni$^{2+}$ reduced $P_e$ without changing single channel current. Patch clamp recordings were performed in cell-attached configuration in oocytes expressing $\alpha$$\beta$$\gamma$ mENaCs. Oocytes were bathed in the same bath solution as used in the two-electrode voltage clamp studies. Na$^+$ currents were recorded at $-100$ mV (membrane potential) in a pipette solution either same as the bath solution (A) or with the bath solution containing 1 mM NiCl$_2$ (B). Li$^+$ current was recorded at $-60$ mV before pipette perfusion (C) with a pipette solution containing 110 mM LiCl, 2 mM CaCl$_2$, 10 mM HEPES with pH of 7.4. Traces D–F were from the same patch as in C but recorded at 2, 5, or 8 min following pipette perfusion of 10 mM NiCl$_2$. All recordings are shown on the same scale (right to each trace) with total length of 120 s. Dashed lines indicate the closed state, and solid lines indicate open levels. Current traces were filtered at 100 Hz with Clampfit 8.1 (Axon). All-points amplitude histograms (G) were generated using Fetchan 6 (Axon) from the current recording before (0–400 s, left panel) or after pipette perfusion (600–1500 s, right panel). Letters C, $O_1$, $O_2$, and $O_3$ indicate closed, or the first, second, or the third open state, respectively. The dashed lines were from least square fitting with the Levenberg-Marquardt Method using Pstat 6 (Axon).

(79), and glutamate receptors (80, 81). These channels display different sensitivities to Ni$^{2+}$ with inhibitory constants in the range of micromolar to millimolar. The mechanism of Ni$^{2+}$ effects on these channels is not clear, although both direct and allosteric blocking mechanisms have been suggested (67, 69, 70, 77, 82). $Xenopus$ oocytes express several kinds of endogenous channels whose activity is normally small compared with that of overexpressed foreign channels (83). However, expression of selected exogenous proteins has been reported to activate certain endogenous channels that are normally rather “silent” (84, 85). Therefore, it is possible that the expressed whole-cell currents in oocytes may contain a significant level of endogenous channel activity that is sensitive to both external Ni$^{2+}$ and amiloride if ENaC expression activates these channels. This is unlikely the case under our experimental conditions. First, there is no clear evidence that ENaC expression in $Xenopus$ oocytes activates endogenous channels. When extracellular Na$^+$ is replaced by $K^+$ or Ca$^{2+}$, no inward currents were detected in oocytes expressing ENaC that are clamped at $-100$ mV (16, 18, 19). These data indicated that endogenous channels do not provide a significant contribution to the whole-cell currents measured in ENaC-expressed oocytes under our typical clamping protocols (i.e., protocols that are not designed to elicit voltage-dependent currents).
FIG. 11. Sequence alignments, structure prediction, and working models for the Ni\(^{2+}\)-binding domain and mechanism of Ni\(^{2+}\) inhibition. 

**A**, sequence alignments were performed with Vector NTI 7.0 (InforMax Inc., Bethesda) from sequences as follows: \(\alpha\), \(\beta\), and \(\gamma\) mouse ENaCs (GenBank\textsuperscript{TM} accession numbers AF112185, AF112186, and AF112187); \(\alpha\), \(\beta\), and \(\gamma\) rat ENaCs (GenBank\textsuperscript{TM} accession numbers X70497, X77932, and X77933); \(\alpha\), \(\beta\), and \(\gamma\) human ENaCs (GenBank\textsuperscript{TM} accession numbers L29007, L36593, and L36592); \(\delta\) bovine ENaC (GenBank\textsuperscript{TM} accession number U14944); and \(\varepsilon\) human ENaC (GenBank\textsuperscript{TM} accession number U38254). The first amino acid residue number for each sequence is listed in parentheses. Homology is presented in the following colors codes: identical, \textcolor{red}{red} background; similar, \textcolor{yellow}{yellow} background; and block of similar residues, \textcolor{green}{green} background. The histidine residues studied in this report are in \textcolor{red}{boldface} type.

**B**, a linear model is shown to indicate the location of the histidine residues (\(\alpha\)His\textsuperscript{282} and \(\gamma\)His\textsuperscript{239}, as a \textcolor{red}{red} sphere) in one ENaC subunit. Secondary structural predictions were performed on the entire amino acid sequence of \(\alpha\)mENaC (C) or \(\varepsilon\)mENaC (E) with five different methods and only predictions of \(\alpha\)Cys\textsuperscript{263}–Pro\textsuperscript{291} and \(\gamma\)Cys\textsuperscript{216}–247
ENaC and Ni²⁺

striking difference observed with the mutations αHis282 and γHis239 was that all channels with mutations at γHis239 showed no Ni²⁺-induced current rectification (Fig. 5, B–D), whereas all channels with α-subunit mutations (αHis282 or αArg280) showed inward rectification in the presence of Ni²⁺ (Fig. 4, B–E, and Fig. 7F). These results suggest that the interaction of Ni²⁺ with γHis239 is required for Ni²⁺-induced current rectification.

Our results suggest that αHis282 and γHis239 are involved in Ni²⁺ binding to the channel complex. However, other residues may be needed to complete the Ni²⁺ coordination geometry as Ni²⁺ is coordinated by 4, 5, or 6 ligands in most Ni²⁺-binding proteins (89). In order to identify other residues involved in Ni²⁺ binding and to probe the secondary structure of residues near αHis282, one or three additional histidines were engineered in the tract αWYRFHYIN (residues 278–285) of mENaC. Among 7 single histidine substitutions in this tract, αR280H was the only mutation associated with enhanced Ni²⁺ inhibition of channel currents, suggesting that the introduced histidine at α280 contributed to Ni²⁺ coordination (Fig. 7G).

The enhanced Ni²⁺ inhibition by αR280H was attenuated by mutation of γHis239 (αR280H-β-γH239R). Although these data suggest that αArg280 and αHis282 might be located in a β-sheet structure, other secondary structures can also place these two residues in close proximity favoring metal coordination by both residues, such as π helix, 3₁₀ helix, and random coil. An α-helical structure containing both residues would place αArg280 and αHis282 on opposing faces of the helix (Fig. 11D) and is not consistent with the observed enhancement of Ni²⁺ inhibition by αR280H. Another possibility is that αArg280 and αHis282 are not located in the same secondary structure, in agreement with structural predictions that place αArg280 within an α-helix and αHis282 immediately adjacent to the α-helix (Fig. 11C). We generated a structural model of a Ni²⁺-binding site (Fig. 11G) based on consensus structural predictions of the regions flanking αHis282 and γHis239 (Fig. 11, C and E) and our results from mutagenesis studies. The Ni²⁺-binding domain in the α-subunit was modeled as an N-terminal α-helix followed by a β-sheet with αHis282 at the transition point. Our model places αR280H and αHis282 at positions that allow both residues to participate in Ni²⁺ coordination. This proposed arrangement of αR280H and αHis282 is strikingly similar to the arrangement of a pair of metal-coordinating histidine residues, His112 within an α-helix and His110 adjacent to the helix in the Ni²⁺-binding metallochaperone UreE (48). The corresponding region in γ mENaC was modeled as an α-helix based on secondary structure predictions.

The dose-response relationship of Ni²⁺ inhibition of Na⁺ currents of WT mENaC was best described with an equation providing two classes of binding sites for Ni²⁺ (Fig. 1). For several mutations, such as αR280H, αH282C, and γH239C, the high affinity component of the Ni²⁺ dose-response curve appeared to be shifted more than the low affinity component (Figs. 4A, 5A, and 7). Ni²⁺-dose response curves for other mutant channels (except αH282D) showed similar shifts in both the high and low affinity binding sites. Although these data do not allow us to distinguish whether αHis282 and γHis239 form independent Ni²⁺-binding sites or form a single binding site, we favor the view that these histidine residues contribute to a common Ni²⁺-binding site. Substitution of αHis282 or γHis239 with cysteine or arginine produced similar shifts in the Ni²⁺-dose response curves, and channels with mutations at both sites (αH282R-β-γH239R) were Ni²⁺ insensitive. These data suggest collaborative coordination of Ni²⁺ binding by these histidine residues. The elimination of Ni²⁺ inhibition by point mutation αH282D, the enhanced Ni²⁺ inhibition by αR280H, and partial reversal of the enhancement by a mutation in γENaC (γH239R) also support this view. Inter-subunit coordination of Ni²⁺ has been demonstrated in CNG channels and the Ni²⁺-binding metallochaperone UreE (47, 48, 76, 91). Alternatively, the high and low affinity sites for Ni²⁺ may be formed by αHis282 (or more than one αHis282) and γHis239, respectively. The relative shift of the high affinity component of the dose-response relationship by mutations at αHis282 appeared to be smaller than shift of the low affinity component (Fig. 5A), whereas a greater shift of the high affinity component of the dose-response relationship was observed with αHis282 mutations (Fig. 4A). As the osmolarity of Ni²⁺-containing bath solutions was not adjusted in this study and hypertonicity may affect ENaC currents (93, 94), we cannot rule out the possibility of osmotic effects on ENaC or endogenous channels at high concentrations of Ni²⁺ (25 and 50 mM). The observations that αH282D or αH282R/γH239R eliminated both high and low affinity inhibition are not consistent with the possibility.

Based on above results, preferences of Ni²⁺ coordination, and assuming that the channel complex contains more than one α-subunit (10–12), we propose a Ni²⁺-binding site within WT ENaC where Ni²⁺ is coordinated by four ligands, three nitrogen atoms from two αHis282 residues and one from γHis239. A fourth unknown ligand could be an oxygen atom from solvent (water) or an oxygen-bearing residue (Figs. 7G and 11G). Although we do not have direct evidence for the involvement of two αHis282 residues in Ni²⁺ coordination, the loss of Ni²⁺ block observed with αH282D-β-γ channels, compared with the modest change in Ni²⁺ sensitivity observed with α-β-γH239D channels, is consistent with the notion that more than one α-subunit participates in the coordinated binding of Ni²⁺. Our proposed Ni²⁺-coordination pattern is similar to that of theendonuclease domain of the bacterial toxin colicin E9 and the...
zinc endopeptidase astacin. Both proteins utilize three nitrogen atoms from histidine residues and one oxygen atom from either a solvent or a tyrosine residue as Ni\(^{2+}\) ligands (45, 49).

**External Ni\(^{2+}\) Inhibits ENaC by Reducing Channel Open Probability**—Our single channel results indicate that external Ni\(^{2+}\) decreased the open probability of \(\alpha-\beta-\gamma\) mENaC without affecting single channel currents. This notion is consistent with the recent observation that Ni\(^{2+}\) decreased apparent open channel density without changing the single channel current as determined by noise analysis (31). A decrease in channel number is less likely a mechanism for Ni\(^{2+}\) inhibition of ENaC currents, given the rapidity of the current response to Ni\(^{2+}\) (Fig. 1D) and the lack of change in oocyte capacitance in response to Ni\(^{2+}\) (31).

The open probability of ENaC has been shown to be highly variable in different systems (1, 95). ENaCs display slow gating with long open and close times, and in some cases two or more distinct gating modes with high and low \(P_o\) values were observed. ENaC may naturally transition among different open states that can be regulated by various factors (96, 97). We observed that Ni\(^{2+}\) inhibition was saturated at the concentrations near 50 mM (−100-fold greater than the estimated \(K_i\) for Ni\(^{2+}\)) and did not eliminate amiloride-sensitive Na\(^+\) currents. These data are consistent with the view that Ni\(^{2+}\) binding to ENaC results in a conformational change that shifts channels to a gating mode with a lower \(P_o\) or, alternatively, that Ni\(^{2+}\) stabilizes a closed conformation and thus reduces ENaC \(P_o\). Ni\(^{2+}\)-induced conformational changes in proteins have been observed in Ni\(^{2+}\)-dependent enzymes and other native proteins or synthetic peptides (41, 98, 99).

Fig. 11F illustrates two potential mechanisms by which Ni\(^{2+}\) binding leads to a decrease in ENaC \(P_o\), based on our presumption that the Ni\(^{2+}\)-binding site is located within the extracellular region outside of the conduction pore. One model (the “gate” model) places the Ni\(^{2+}\)-binding site within a putative gate that swings into the outer vestibule of the pore during channel closure. Ni\(^{2+}\) binding stabilizes the gate in the closed channel state. An extracellular gate has been proposed to explain state-dependent accessibility of an introduced cysteine within the pore region to sulphydryl reagents (23). The second model (the “transduction” model) proposes that Ni\(^{2+}\) binding induces a local conformational change near the binding site that is transmitted to the outer vestibule of the channel pore through a “linker” region connecting the Ni\(^{2+}\)-binding site and the pore. Ni\(^{2+}\) binding to the ECLs ultimately results in conformational changes in the outer pore that favor the closed state, which is associated with a decreased \(P_o\). The second model is similar to the gating mechanism proposed for ionotropic glutamate receptors (100, 101).

\(\alpha\)His\(^{282}\) and \(\gamma\)His\(^{235}\) May Be Located within a Gating Domain in the ECLs of ENaC Subunits—A number of observations suggest that \(\alpha\)His\(^{282}\) and \(\gamma\)His\(^{235}\) are located within domains that participate in the control of ENaC gating. Ni\(^{2+}\) decreased ENaC open probability (see above and Ref. 31) through interactions with \(\alpha\)His\(^{282}\) and \(\gamma\)His\(^{235}\). MTSET inhibition of \(\alpha\)H282C-\(\beta\)H239R mENaC has a high \(P_o\) and Applebury et al. (102, 103) have reported that mutations within the WYRFHY tract (residues 278–283 in rat ENaC) altered gating kinetics of \(\alpha\)-subunit channels expressed in Chinese hamster ovary cells. Primary structure analyses also support the notion that \(\alpha\)His\(^{282}\) and \(\gamma\)His\(^{235}\) are located in important functional domains. These tracts are among the most conserved domains within ENaC subunits (Fig. 11A) but are not found in the ECLs of other members of the ENaC/DEG family. Secondary structural predictions suggest that these domains exhibit a high degree of organization, including regions that are predicted to form \(\alpha\)-helices or \(\beta\)-sheets (Fig. 11, C and E). Helical wheel analyses of the predicted \(\alpha\)-helical regions revealed that charged or highly polar residues are located on one face of the helix, whereas hydrophobic residues are located on the opposing face (Fig. 11, D and F), a structure capable of interacting with different environments (i.e. aqueous or lipophilic) or domains.

The potential role of histidine residues in ENaC gating is consistent with the role of histidine residues in mediating conformational changes in enzymes (104–107) or ion channels. Okada et al. (108) proposed protonation of a histidine (His\(^{17}\)) in the M2 ion channel protein from influenza A virus, and its interaction with Trp\(^{41}\) is a key step in channel activation. The role of histidine residues in CNG gating has been well characterized (91).

The mechanism by which domains containing \(\alpha\)His\(^{282}\) and \(\gamma\)His\(^{235}\) affect ENaC gating is unclear and deserve further investigation with methods involving structural approaches. Information regarding ENaC gating mechanisms is emerging through mutagenesis studies. Previous studies have shown that ENaC gating is affected by mutations in several different domains in epithelial Na\(^+\) channel subunits including the N-terminal domain (21, 22, 109), the pore region (23, 24), the C-terminal part of the ECL (25), the M2 domain (26), and the intracellular C-terminal domain (27, 110). However, it is unclear whether these different regions exert effects on channel gating in an independent manner or whether interactions between these domains influence channel gating through a common pathway.

The proposed role of ECLs in ENaC gating is consistent with the notion that ECL domains within ENaC-related channels are involved in channel gating. ASIC are activated by extracellular acidification, and a histidine (His\(^{17}\)) in the ECL of ASIC-2a has been identified as a putative H\(^+\) sensor (111). FaNaCh is a peptide-gated Na\(^+\) channel, and the peptide (FM-RFamide) binding domain was proposed to reside within the ECL (112). Residues or domains within the ECLs of subunits of mechanotransducing channels in Caenorhabditis elegans (i.e. Deg-1 and Mec-4) were proposed to modulate channel gating (113, 114). ENaC may share a similar design in gating machinery with other members in the ENaC/DEG family, although it is clear that they are gated by different factors.

In summary, we have characterized Ni\(^{2+}\) inhibition of ENaC activity at whole-cell and single channel levels, identified \(\alpha\)His\(^{282}\) and \(\gamma\)His\(^{235}\) as primary Ni\(^{2+}\)-binding residues, and demonstrated that Ni\(^{2+}\) reduces channel \(P_o\). Our results also suggest that these two residues are present in domains within the extracellular loops that are associated with channel gating.

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