External Cu$^{2+}$ Inhibits Human Epithelial Na$^+$ Channels by Binding at a Subunit Interface of Extracellular Domains*

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Epithelial Na$^+$ channels (ENaCs) play an essential role in the regulation of body fluid homeostasis. Certain transition metals activate or inhibit the activity of ENaCs. In this study, we examined the effect of extracellular Cu$^{2+}$ on human ENaC expressed in Xenopus oocytes and investigated the structural basis for its effects. External Cu$^{2+}$ inhibited human $\alpha\beta\gamma$ ENaC with an estimated IC$_{50}$ of 0.3 $\mu$M. The slow time course and a lack of change in the current-voltage relationship were consistent with an allosteric (non pore-plugging) inhibition of human ENaC by Cu$^{2+}$. Experiments with mixed human and mouse ENaC subunits suggested that both the $\alpha$ and $\beta$ subunits were primarily responsible for the inhibitory effect of Cu$^{2+}$ on human ENaC. Lowering bath solution pH diminished the inhibition by Cu$^{2+}$. Mutations of two $\alpha$, two $\beta$, and two $\gamma$ His residues within extracellular domains significantly reduced the inhibition of human ENaC by Cu$^{2+}$. We identified a pair of residues as potential Cu$^{2+}$-binding sites at the subunit interface between thumb subdomain of $\alpha$hENaC and palm subdomain of $\beta$hENaC, suggesting a counterclockwise arrangement of $\alpha$, $\beta$, and $\gamma$ ENaC subunits in a trimeric channel complex when viewed from above. We conclude that extracellular Cu$^{2+}$ is a potent inhibitor of human ENaC and binds to multiple sites within the extracellular domains including a subunit interface.

The epithelial Na$^+$ channel (ENaC)$^2$ mediates Na$^+$ transport across apical membranes of high resistance epithelia in kidney, colon, and lung. ENaC has important roles in the maintenance of extracellular fluid volume and the regulation of airway surface liquid volume (1). Alterations in ENaC activity have been associated with several human diseases. For example, enhanced ENaC activity is responsible for the hypertension seen in Liddle’s syndrome, contributes to the mucociliary dysfunction seen in cystic fibrosis, and is believed to contribute to hyperolemia associated with nephrotic syndrome (2, 3).

A variety of intracellular and extracellular factors regulate ENaC activity by distinct mechanisms (4). External amiloride analogs, cations, anions, nucleotides, serine proteases, and lamellar shear stress inhibit or stimulate endogenous or exogenous ENaCs (5–12). All of these extracellular regulators appear to directly alter the activity of ENaCs in plasma membranes rather than affect channel subunit trafficking (1). Their primary targets likely reside within the characteristically large extracellular domains (ECDS) of ENaC subunits. This notion is in line with the well defined subdomains within the ECDS of the chicken acid-sensing ion channel 1 (cASIC1), a member of the ENaCdegenerin family, revealed in a crystal structure and the identification of proton binding sites within the ECDS (13).

We have previously examined the effects of the transition metals, Ni$^{2+}$ and Zn$^{2+}$, on ENaC activity. External Ni$^{2+}$ inhibits and Zn$^{2+}$ activates mouse ENaCs in Xenopus oocytes by directly interacting with the channels and altering channel gating (6, 7). Some of these metal effects are thought to be related to Na$^+$ self-inhibition, a down-regulation of open probability ($P_o$) by extracellular Na$^+$ (7, 14). Yu et al. (14) have also examined the effects of several transition metals on the single channel activity of native Xenopus ENaCs in A6 cells. These metals differentially affect Xenopus ENaC Po and channel number in membrane patches without changing the single channel conductance. However, the exact binding sites and detailed mechanisms for the metal effects on ENaCs remain largely unknown.

Copper is the third most abundant trace metal in humans and has a variety of important biological functions. Excessive Cu$^{2+}$ is highly toxic to cells, and its content in cells is carefully maintained at low levels. Indeed, Cu$^{2+}$ is implicated in several human diseases such as Wilson disease, Menkes disease, neurodegenerative disorders, and cancers (15, 16). The therapeutic potential of copper chelators and copper complexes is being intensively investigated (16). In addition, particulate matters contain high amounts of transitional metals including copper. Soluble metals in airborne particles contribute to pulmonary and cardiovascular toxicity (17, 18). Recent studies suggest that copper nanoparticles are highly toxic (19). The underlying mechanisms for the harmful effects of Cu$^{2+}$ are not fully understood. Many studies have suggested that certain metals exert their toxic effects in part by altering functions of ion channels or transporters (20, 21). Clearly, a better understanding of the interactions between copper and biological molecules is crucial to an elucidation of its physiological, pathological, and toxicological roles in human health.

In this report, we examined the effects of external Cu$^{2+}$ on amiloride-sensitive Na$^+$ currents in oocytes expressing $\alpha\beta\gamma$ human ENaC (hENaC) and probed the structural basis by site-directed mutagenesis. We found that external Cu$^{2+}$ is a potent inhibitor of hENaC. The inhibitory effect of Cu$^{2+}$ on hENaC

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2 The abbreviations used are: ENaC, epithelial Na$^+$ channel; hENaC, human ENaC; mENaC, mouse ENaC; ASIC, acid sensing ion channel; ECD, extracellular domain; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; TTM, tetrathiomolybdate.
depends on the α and β subunits. The most important site for Cu²⁺ inhibition was identified at the α/β subunit interface.

**EXPERIMENTAL PROCEDURES**

*DNA Constructs and Site-directed Mutagenesis*—Wild-type α, β, and γ hENaC cDNAs (22, 23) were in pSPORT, plBluestrip K5+, and pC DNA3 vectors, respectively. Point mutations were introduced into hENaC cDNAs using the QuikChange II XL site-directed mutagenesis kit (Stratagene). The presence of intended mutations and the absence of unwanted mutations were verified by direct DNA sequencing. Mouse α, β, and γ ENaC (mENaC) cDNAs were in plBluestrip SK− vector. Wild-type and mutant hENaC cRNAs were made using SP6 (dhENaC) or T7 (β and γ hENaC) RNA polymerase (Ambion, Inc.). All of the mENaC cRNAs were made using T3 RNA polymerase (Ambion, Inc.). The synthesized cRNAs were purified with an RNA purification kit (Qiagen) and quantified by spectrophotometry.

**ENaC Expression and Two-electrode Voltage Clamp**—hENaC expression in *Xenopus* oocytes and current measurements by two-electrode voltage clamp were performed as previously reported (6). Stage V and VI oocytes with the follicle cell layer removed were injected with 50 nL/cell of mixed cRNAs composed of 2 ng of each hENaC subunit or 1 ng of each mENaC subunit. Injected oocytes were incubated at 18 °C in modified Barth’s solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 15 mM HEPES, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 10 μg/ml of sodium penicillin and streptomycin sulfate, 100 μg/ml gentamycin sulfate, pH 7.4). All of the experiments were performed at room temperature (20–24 °C) 20–30 h following injection. The oocytes were placed in a recording chamber from Warner Instruments (Hamden, CT) and perfused with bath solutions at a constant flow rate of 12–15 ml/min. Voltage clamp was performed using Axoclamp 900A amplifier and DigiData 1440A interface controlled by pClamp 10 (Molecular Devices Corporation, Sunnyvale, CA). The oocytes were either continuously clamped to −100 mV to monitor current change over a period of time or stepwise clamped to a series of voltages (−140 to 60 mV) to determine the current-voltage relationship.

**Examination of the Effects of Cu²⁺ on ENaCs in Oocytes**—The oocytes were perfused with normal bath solution (NaCl-110, containing 110 mM NaCl, 2 mM KCl, 2 mM CaCl₂, pH 7.4) while clamped to −100 mV. Bath solution was buffered with either 10 mM HEPES or 5 mM MES and 5 mM MOPS. Although HEPES reportedly forms complex with Cu²⁺ (24), we observed similar responses to Cu²⁺ using bath solution buffered with either HEPES or MES and MOPS that do not complex Cu²⁺. inward currents were continuously recorded, whereas bath solutions supplemented with or without Cu²⁺ were exchanged. Following Cu²⁺ washout, bath solution was switched to NaCl-110 with 10 μM amiloride to determine the amiloride-insensitive current. The effects of Cu²⁺ on ENaCs were analyzed by comparison of the amiloride-sensitive currents prior to and after Cu²⁺ application. A Cu²⁺ stock solution of 1 M was prepared by dissolving CuSO₄·5H₂O (purity of 99.999%; Sigma-Aldrich) in deionized water and was diluted to its final concentration in NaCl-110. The highest concentration of Cu²⁺ solution that could be made in this bath solution and remain relatively stable was 100 μM, and precipitates appeared at higher concentration. Bath solutions containing Cu²⁺ were prepared fresh prior to experiments to minimize the reduction of Cu²⁺ concentration because of the slow formation of insoluble Cu(OH)₂ at neutral pH. Nominal concentrations of Cu²⁺ were used. Oocytes with unstable currents were not used in these experiments.

**Examination of Na⁺ Self-inhibition**—The Na⁺ self-inhibition responses were examined as previously reported (25, 26). Briefly, Na⁺ self-inhibition was examined by rapidly replacing a low [Na⁺] bath solution (NaCl-1; containing 1 mM NaCl, 109 mM N-methyl-D-glutamine (NMDG), 2 mM KCl, 2 mM CaCl₂, 10 mM HEPES, pH 7.4) with a high [Na⁺] bath solution (NaCl-110), whereas the oocytes were continuously clamped to −100 mV. Bath solution exchange was done with a Teflon valve perfusion system controlled by computer (AutoMate Scientific Inc, Berkeley, CA). Upon completion of the experiment, 10 μM amiloride was added to the bath solution so as to determine the amiloride-insensitive portion of the whole cell current. To avoid complications from the observable variability in the Na⁺ self-inhibition response of WT ENaCs among different batches of oocytes (27), the response of WT channels was always tested in an alternating manner with mutants in the same batch of oocytes. Steady state current (Iₛₛ) was measured at 40 s after I_peak. The amiloride-insensitive currents were subtracted from Iₛₛ and I_peak currents to determine the amiloride-sensitive current ratio of Iₛₛ/I_peak, the index for the magnitude of Na⁺ self-inhibition.

**Examination of the Effect of Cu²⁺ on hENaCs in Human Airway Epithelial Monolayer**—Primary human airway epithelial cells were cultured from excess pathological tissue following lung transplantation and organ donation under a protocol approved by the University of Pittsburgh Investigational Review Board. Human airway epithelial cells were cultured on human placental collagen-coated Costar Transwell filters (0.33 cm²) as described previously (28) and used for experimentation following 4–6 weeks of culture at an air-liquid interface. Short circuit currents (Iₛₛ) were measured as previously described (28). In brief, cells cultured on filter supports were mounted in modified Ussing chambers, and the cultures were continuously short circuited with an automatic voltage clamp (Physiologic Instruments). The bathing Ringer’s solution was composed of 120 mM NaCl, 10 mM HEPES, 3.3 mM KH₂PO₄, 0.8 mM K₂HPO₄, 0.6 mM MgCl₂, 0.6 mM CaCl₂, and 10 mM glucose (pH, 7.4). Chambers were constantly gassed with a mixture of 95% O₂ and 5% CO₂ at 37 °C, which maintained a pH of 7.35. Simultaneous transepithelial resistance was recorded by applying a 10-mV pulse/s via an automated pulse generator. Acquire and Analyze 2.3 (Physiological Instruments) was used to control the voltage clamp and analyze the Iₛₛ data. Iₛₛ recordings included a 30-min equilibration period, followed by the addition of Cu²⁺ from a 1 M Cu²⁺ stock solution in water, followed by the addition of 20 μM amiloride.

**Statistical Analysis**—The data are presented as the means ± S.E. Significance comparisons between groups were performed using Student’s t test. Curve fittings were performed with Origin Pro 8.0 (OriginLab Corporation, Northampton, MA).
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RESULTS

External Cu\(^{2+}\) Inhibits Human αβγ ENaC—We examined the effect of extracellular Cu\(^{2+}\) on whole cell currents in Xenopus oocytes expressing αβγ hENaCs. Oocytes were continuously clamped at −100 mV to monitor inward current changes before, during (10 min), and after Cu\(^{2+}\) applications. Each trace represents at least five independent observations. The oocytes were clamped at −100 mV. Cu\(^{2+}\) was added to the bath solution (NaCl-110 with 110 mM Na\(^{+}\), buffered with 5 mM MES and 5 mM MOPS) at 0.1, 0.3, 1, 3, 10, and 30 μM. Control oocytes were clamped in the same way except no Cu\(^{2+}\) application. Inward currents were shown in negative values by convention. The durations of Cu\(^{2+}\) and amiloride (10 μM) applications are indicated by black and open bars, respectively. Black arrows point to the transient increases in currents after initiation of Cu\(^{2+}\) applications. B, time courses of the current changes in the absence (control) or presence of Cu\(^{2+}\). The relative currents were the ratios of the amiloride-sensitive currents measured every minute after the beginning of the Cu\(^{2+}\) applications and the amiloride-sensitive currents prior to Cu\(^{2+}\) additions (at time 0). The data were from 17 oocytes for control 3 and 10 μM Cu\(^{2+}\) applications. The black arrowheads indicate the times when datum points were chosen for dose-response analysis (2 min for 3 and 10 μM Cu\(^{2+}\), 5 min for 1 μM Cu\(^{2+}\), and 10 min for 0.1 and 0.3 μM Cu\(^{2+}\)). C, dose response of Cu\(^{2+}\) on αβγ hENaCs. The relative currents from B were adjusted to deduct rundown contribution from the observed current decreases at the corresponding time with the formula: \(I_R = \frac{I_{n-1}}{I_{n}}\). \(I_R\) and \(I_R\) control were the relative currents with and without Cu\(^{2+}\), respectively. The line was from a best fit with the Hill equation. The parameters were: \(IC_{50} = 0.31 \mu M\); n, 1.28, B, 0.23; and R\(^2\), 0.99.

We generated a dose-response curve by plotting the relative currents against Cu\(^{2+}\) concentrations (Fig. 1C). Measurements were taken at times equivalent to approximately three time constants after the addition of Cu\(^{2+}\) (Table 1 and black arrowheads in Fig. 1B). Time constant for 0.1 μM Cu\(^{2+}\) could not be determined; values at 10 min were used. The data for 30 μM Cu\(^{2+}\) were taken at 2 min from experiments with Cu\(^{2+}\) applied

Table 1

| [Cu\(^{2+}\)] (μM) | n | Tau | Oocytes |
|-------------------|---|-----|--------|
| 0 (control)       | 13| 736.6 ± 67.2 | 0.1 μM | ND |
| 0.3 μM            | 7 | 199.9 ± 8.5  | 1 μM   | 921.1 ± 4.4 |
| 3 μM              | 8 | 35.7 ± 2.3   | 3 μM   | 31.8 ± 2.7 |
| 10 μM             | 9 | 19.9 ± 2.7   | 10 μM  | ND |

ND, not determined.

FIGURE 1. External Cu\(^{2+}\) inhibits human ENaC. A, representative recordings of whole cell currents from oocytes expressing αβγ hENaCs before, during, and after Cu\(^{2+}\) applications. Each trace represents at least five independent observations. The oocytes were clamped at −100 mV. Cu\(^{2+}\) was added to the bath solution (NaCl-110 with 110 mM Na\(^{+}\), buffered with 5 mM MES and 5 mM MOPS) at 0.1, 0.3, 1, 3, 10, and 30 μM. Control oocytes were clamped in the same way except no Cu\(^{2+}\) application. Inward currents were shown in negative values by convention. The durations of Cu\(^{2+}\) and amiloride (10 μM) applications are indicated by black and open bars, respectively. Black arrows point to the transient increases in currents after initiation of Cu\(^{2+}\) applications. B, time courses of the current changes in the absence (control) or presence of Cu\(^{2+}\). The relative currents were the ratios of the amiloride-sensitive currents measured every minute after the beginning of the Cu\(^{2+}\) applications and the amiloride-sensitive currents prior to Cu\(^{2+}\) additions (at time 0). The data were from 17 oocytes for control and 9 or 10 oocytes for Cu\(^{2+}\) applications. The black arrowheads indicate the times when datum points were chosen for dose-response analysis (2 min for 3 and 10 μM Cu\(^{2+}\), 5 min for 1 μM Cu\(^{2+}\), and 10 min for 0.1 and 0.3 μM Cu\(^{2+}\)). C, dose response of Cu\(^{2+}\) on αβγ hENaCs. The relative currents from B were adjusted to deduct rundown contribution from the observed current decreases at the corresponding time with the formula: \(I_R = \frac{I_{n-1}}{I_{n}}\). \(I_R\) and \(I_R\) control were the relative currents with and without Cu\(^{2+}\), respectively. The line was from a best fit with the Hill equation. The parameters were: \(IC_{50} = 0.31 \mu M\); n, 1.28, B, 0.23; and R\(^2\), 0.99.
for 3 min (Fig. 1A). The relative currents were adjusted to deduct rundown contribution from the observed current decreases at the corresponding time as described in details in the figure legend. Fitting the data with the Hill equation \( I_{\text{Cu}^2+} / I = IC_{50}/(C^n + IC_{50}^n) + B \), \( n \) for Hill coefficient, \( C \) for \( \text{Cu}^2+ \) concentration, and \( B \) for bottom plateau yielded the following parameters: \( IC_{50} \), 0.31 \( \mu M \); \( n \), 1.28; and \( B \), 0.23 with the coefficient of determination (\( R^2 \)) of 0.98.

Prior to the inhibitory effect of \( \text{Cu}^2+ \) on hENaC, a small transient increase in current was typically seen (arrows in Fig. 1A). The transient change in current was not an artifact, although its magnitude varied among different batches of oocytes. It likely resulted from a rapid activation of the channel prior to the inhibitory effect of \( \text{Cu}^2+ \).

The inhibitory effect of \( \text{Cu}^2+ \) on hENaCs was not readily reversible. Washout of \( \text{Cu}^2+ \) for 1 min only moderately restored the inhibited currents (Figs. 1 and 2A). This slow reversal could result from either tight binding of \( \text{Cu}^2+ \) to the channel complex or permanent inactivation of channels. To distinguish these two possibilities, we utilized a high affinity \( \text{Cu}^2+ \) chelator, tetraethiomylobdate (TTM), to facilitate \( \text{Cu}^2+ \) removal from its binding site(s). Inhibition of hENaC by 10 \( \mu M \) \( \text{Cu}^2+ \) was nearly completely reversed following the addition of 10 \( \mu M \) TTM (Fig. 2, B and D). TTM alone did not change the current (Fig. 2C). The results suggested that the slow reversibility of \( \text{Cu}^2+ \) inhibition reflects tight binding of \( \text{Cu}^2+ \) to hENaC.

External \( \text{Cu}^2+ \) Inhibits hENaC by Acting on Sites outside of the Pore—Because \( \text{Cu}^2+ \) was applied in bath solution in our study, its action site is likely within either the ECD or the transmembrane domain. We carried out experiments to examine these two possibilities. First, we examined the effect of \( \text{Cu}^2+ \) on the current-voltage (I-V) relationship of hENaC. As shown in Fig. 3A, the I-V curve remained linear following a 3-min application of 10 \( \mu M \) \( \text{Cu}^2+ \). The voltage independence of \( \text{Cu}^2+ \) inhibition is inconsistent with a pore blocking effect. A positively charged pore blocker such as amiloride preferentially blocks the inward current causing an outward rectification in the I-V curve (Fig. 3A, curve for amiloride). Second, we tested whether pore blocker amiloride could protect the channels from inhibition by \( \text{Cu}^2+ \). ENaC currents completely recovered after washout of 10 \( \mu M \) amiloride (Fig. 3, B and D). However, when oocytes were pretreated with 10 \( \mu M \) amiloride followed by 10 \( \mu M \) \( \text{Cu}^2+ \), currents only partially recovered after washout of both \( \text{Cu}^2+ \) and amiloride (Fig. 3, C and D). Apparently, \( \text{Cu}^2+ \) inhibited hENaC current after the pore had been occupied by amiloride. These results suggest that \( \text{Cu}^2+ \) acts at a site that is external to the amiloride-binding site within the pore. Based on the above observations, we conclude that external \( \text{Cu}^2+ \) inhibits human ENaCs likely by binding to sites outside of the pore.

Human \( \alpha \) and \( \beta \) Subunits Are Necessary to Confer the Response of hENaC to \( \text{Cu}^2+ \)—We tested whether \( \text{Cu}^2+ \) had a similar effect on mouse ENaC and observed that 10 \( \mu M \) \( \text{Cu}^2+ \) did not significantly inhibit the current in oocytes expressing \( \alpha \beta \gamma \) mENaC (Fig. 4E). To determine the hENaC subunits (\( \alpha, \beta, \) and/or \( \gamma \)) that are necessary for \( \text{Cu}^2+ \) inhibition, we expressed hybrid channels containing mixed human and mouse subunits in Xenopus oocytes. Replacing human \( \alpha \) or \( \beta \) subunits with their mouse counterparts significantly reduced the inhibitory effect of 10 \( \mu M \) \( \text{Cu}^2+ \), whereas replacing the \( \gamma \) subunit did not alter the effect of 10 \( \mu M \) \( \text{Cu}^2+ \) (Fig. 4). These results suggest that both the \( \alpha \) and \( \beta \) subunits are required for the inhibitory effect of \( \text{Cu}^2+ \) on hENaC activity.

Mutations of Multiple His Residues within the ECDs Reduce \( \text{Cu}^2+ \) Inhibition—We hypothesized that the ECDs of the \( \alpha \) and \( \beta \) hENaCs contain residues that are required to confer the inhibitory response to \( \text{Cu}^2+ \). Because \( \text{Cu}^2+ \) is often coordinated by side chains of His, Cys, Met, Glu, and Asp (31), we replaced His, Met, Glu, and Asp residues of \( \alpha \) or \( \beta \) hENaC to their counterparts seen in mENaC at equivalent sites. All 16 Cys residues within \( \alpha \) ECDs and 18 Cys residues within \( \beta \) ECDs are conserved between human and mouse ENaCs and therefore were not included in this analysis. Among nine \( \alpha \) mutants, \( \alpha H468S \) significantly reduced \( \text{Cu}^2+ \) inhibition (Fig. 5A). Substitution of \( \alpha H468G \) with Cys, Asp, Glu, Lys, or Ala similarly attenuated \( \text{Cu}^2+ \) inhibition (Fig. 5, C and D). Moreover, these mutations also rendered the \( \text{Cu}^2+ \) inhibition reversible. Certain mutations at \( \alpha H468 \) also decreased the rates of \( \text{Cu}^2+ \) inhibition or increased the rates of current recovery following washout (Table 2). Interestingly, \( \alpha H468C \) dramatically increased
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FIGURE 3. Cu²⁺ binding site is likely not located within hENaC pore. A, I-V curves before and after 10 μM Cu²⁺. Oocytes expressing αβγ hENaCs (n = 5) were clamped to a series of voltages (−140 to 60 mV) for 500 ms, and the currents were measured at 400 ms. B, recording trace showing current changes before, during, and after washout of 10 μM amiloride (80 s). Oocytes were clamped at −100 mV. The current decay from a peak current following amiloride washout reflects Na⁺ self-inhibition response (25). C, recording from a similar experiment to the one in B except that 10 μM Cu²⁺ was co-applied with 10 μM amiloride for 60 s. Cu²⁺ was added 10 s after amiloride and withdrawn 10 s before amiloride to ensure that Cu²⁺ was only applied in the presence of amiloride. Bath solution was buffered with 5 mM MES and 5 mM MOPS. D, current recovery after amiloride washout with (open bar) or without (black bar) Cu²⁺. The values were the ratios of the amiloride-sensitive currents measured after amiloride washout for 60 s and before amiloride application. The current recovery following co-application of amiloride and Cu²⁺ was significantly lower than that following amiloride application alone (p < 0.001, n = 10 for amiloride and 7 for amiloride + Cu²⁺).

Both α and β subunits contribute to the distinct responses of hENaC and mENaC to external Cu²⁺. The effects of 10 μM Cu²⁺ on whole cell currents were examined in oocytes expressing αβγ hENaC (A, HaHβHγ), α mENaC with β and γ hENaC (B, MuHβHγ), α and γ hENaC with β mENaC (C, HuMβHγ), and β hENaC and γ mENaC (D, HuHβHγ), or αβγ mENaC (E, MuMβMγ). Each recording represents five observations. The gray bars show the presence of 10 μM amiloride, F, relative current (I / I_n = 5). Bath solution was buffered with 10 mM HEPES. The I / I_n values were the ratios of amiloride-sensitive currents measured after and before 10 μM Cu²⁺ applications. Rundown was not deducted from the observed current decreases in this and all subsequent figures. The solid bars indicate that the values are significantly different from that of αβγ hENaC obtained in the same batches of oocytes (p < 0.05 for MuHβHγ, n = 3). Therefore examined the effect of 10 μM Cu²⁺ in pH 6.0 bath solution on hENaC currents. Changing the pH of the bath solution from 7.4 to 6.0 moderately increased currents in oocytes expressing αβγ hENaCs (relative current = 1.19 ± 0.01, n = 7, p < 0.001; Fig. 6A) as reported by Collier and Snyder (8). At pH 6.0, we observed a minimal inhibitory effect of 10 μM Cu²⁺ (IC₅₀/I = 0.86 ± 0.01, n = 7, p < 0.001), in contrast to the large inhibitory effect at pH 7.4 (p < 0.001; Fig. 6B). These results demonstrate that Cu²⁺ inhibition of hENaC is pH-dependent and are consistent with His side chains mediating the interaction of Cu²⁺ with hENaC.

There are 10, 13, and 14 His residues in the ECDs of α, β, and γ hENaC, respectively. We individually mutated each His residue to identify other sites for Cu²⁺ interaction with the chan-

Histidine has an ionizable imidazole ring with an average pKₐ of 6.6 ± 1.0 in folded proteins (32). If His residues are indeed involved in Cu²⁺ inhibition of hENaC, lowering the pH of the bath solution containing Cu²⁺ should reduce Cu²⁺ inhibition because of increased protonation of imidazole nitrogens. We
nel. In addition to the αHis468 mutants (Fig. 5), substitution of αHis233 with Ala, Cys, Asp, or Arg significantly reduced channel inhibition by 10 μM Cu²⁺ (Fig. 7A). For the β subunit, two mutations (βH159A and βH160A) significantly reduced Cu²⁺ inhibition (Fig. 7B). The change in Cu²⁺ inhibition by βH159A was similar to that of βH159D (Fig. 5B). Two γ His mutants (γH88A and γH277A) showed a significantly reduced inhibitory effect of 10 μM Cu²⁺ (Fig. 7C). We also noted that substitutions at two γECD sites (γHis323 and γHis325) increased the inhibitory effect of 10 μM Cu²⁺ (p < 0.01).

We examined whether mutations of His residues in multiple subunits would enhance the loss of the inhibitory effect of 10 μM Cu²⁺, as we observed with the αH468S/βH159D mutant (Fig. 5). Double mutations such as αH468A/βH160A (ICu/I = 1.01 ± 0.02, n = 5, p < 0.0001 versus WT) and αH468A/γH277A (ICu/I = 0.97 ± 0.03, n = 4, p < 0.0001 versus WT) eliminated inhibitory effect of 10 μM Cu²⁺. In contrast, βH159A/γH277A greatly reduced but did not eliminate 10 μM Cu²⁺ inhibition (ICu/I = 0.48 ± 0.03, n = 5, p < 0.0001 versus WT; Fig. 8). These data suggest that αHis468 is

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| hENaC                      | Application Washout | Oocytes       |
|----------------------------|---------------------|---------------|
| WT                         | 27.1 ± 0.8          | 50.0 ± 7.3    | 67 |
| αH255A                     | 21.3 ± 1.7          | 36.0 ± 6.0    | 5  |
| αH255C                     | 33.9 ± 3.4          | 35.9 ± 6.0    | 6  |
| αH255D                     | 29.4 ± 1.5          | 21.5 ± 4.7    | 6  |
| αH255R                     | 27.0 ± 2.1          | 44.3 ± 7.7    | 5  |
| αH468A                     | 32.4 ± 3.3          | 14.7 ± 1.4    | 5  |
| αH468C                     | 10.2 ± 1.5<sup>a</sup> | 15.6 ± 1.3    | 5  |
| αH468D                     | 46.4 ± 4.6<sup>a</sup> | 31.0 ± 3.4    | 5  |
| αH468E                     | 64.6 ± 6.7<sup>a</sup> | 17.3 ± 3.2    | 4  |
| αH468K                     | 49.6 ± 3.2<sup>a</sup> | 32.4 ± 3.7    | 7  |
| αH468S                     | 23.7 ± 3.8          | 27.6 ± 2.5    | 5  |
| βH159A                     | 29.7 ± 2.5          | 18.3 ± 2.6    | 6  |
| βH159D                     | 34.1 ± 2.0          | 17.7 ± 3.2    | 5  |
| βH160A                     | 28.9 ± 1.3          | 64.1 ± 5.9    | 5  |
| βE254A                     | 29.5 ± 2.3          | 24.8 ± 3.9    | 5  |
| βE254C                     | 38.1 ± 1.9          | 22.7 ± 2.5    | 5  |
| βE254D                     | 33.7 ± 3.8          | 22.0 ± 3.3    | 5  |
| βE254H                     | 33.5 ± 2.0          | 22.0 ± 1.7    | 5  |
| βE254Q                     | 28.5 ± 2.1          | 20.6 ± 2.6    | 5  |
| γH88A                      | 32.2 ± 2.9          | 16.9 ± 2.6    | 6  |
| γH233A                     | 30.0 ± 3.1          | 44.0 ± 4.6    | 5  |
| γH233C                     | 18.3 ± 2.1          | ND            | 5  |
| γH233D                     | 21.6 ± 2.2          | ND            | 5  |
| γH233F                     | 13.0 ± 1.8<sup>a</sup> | ND            | 5  |
| γH233R                     | 22.5 ± 2.4<sup>a</sup> | ND            | 5  |
| γH277A                     | 35.9 ± 1.1          | 18.3 ± 1.7<sup>a</sup> | 5  |
| γH332A                     | 17.1 ± 0.4<sup>a</sup> | ND            | 5  |

<sup>a</sup> The values were significantly different from that of WT in the same batch of oocytes (p < 0.01).

The table shows the time constants for current changes during application and washout of Cu<sup>2+</sup> in hENaCs. The values were obtained from WT and mutants obtained in the same batches of similar numbers of oocytes. ND, not determined.

**FIGURE 6.** Lowering pH of bath solution diminishes the inhibitory effect of Cu<sup>2+</sup> on hENaCs. The I<sub>o2</sub>/I values reflecting the magnitudes of the inhibitory effect of 10 μM Cu<sup>2+</sup> were obtained from oocytes expressing mutant α together with WT β and γ hENaCs (A), mutant β with WT α and γ hENaCs (B), or mutant γ with WT α and β hENaCs (C). Black bars in A–C indicate the values that were significantly greater than that of WT αβγ hENaCs obtained in the same batches of oocytes (p < 0.01, n = 3 for both mutants and WT). The values in Fig. 5 <sup>H233R</sup>, <sup>γH233A</sup>, <sup>γH233D</sup>, and <sup>γH332A</sup> (gray bars) were significantly less than that of WT (p < 0.01). The dashed line shows the average I<sub>o2</sub>/I value from all oocytes expressing WT αβγ hENaCs used in this experiment (0.15 ± 0.00, n = 164) for reference only but not for statistical significance analyses. I<sub>o2</sub>/I for αH468A was from Fig. 5C and is shown for comparison. D, representative recordings of currents with 3-min applications of 10 μM Cu<sup>2+</sup>. The gray bars in D indicate the presence of 10 μM amiloride. Bath solution was buffered with 10 mM HEPES.

A Cu<sup>2+</sup> Binding Site Is Located at a Subunit Interface—In certain ligand-gated channels, transition metal-binding sites are located at subunit interfaces (33, 34). We reasoned that external Cu<sup>2+</sup> might bind at a contact site between two subunits. Residue αH88<sup>680</sup> aligned with Lys<sup>355</sup> of cASIC1. The cASIC1 structure shows that the side chain of Lys<sup>355</sup>, located at the carboxyl end of an α helix (α5) in the thumb domain interacts with side chain of Glu<sup>178</sup> within the β3-β loop of the palm domain of an adjacent subunit (Fig. 9A) (13). The distance between NZ of Lys<sup>355</sup> and OE2 of Glu<sup>178</sup> is 3.2 Å, well within the range of distance for ion pairs in proteins (35). Lys<sup>355</sup> of cASIC1 aligns with hENaC αHis<sup>408</sup>, βArg<sup>437</sup>, and γGlu<sup>446</sup>, whereas Glu<sup>178</sup> of cASIC1 aligns with hENaC αVal<sup>397</sup>, βGlu<sup>254</sup>, and γVal<sup>205</sup> (Fig. 9C). Of these six hENaC residues, only αHis<sup>408</sup> and βGlu<sup>254</sup> could form an ion pair. If a His residue resides at the position of Lys<sup>355</sup> in subunit A of cASIC1, the distances between an imidazole nitrogen of Lys<sup>355</sup> and a carboxyl oxygen of Glu<sup>178</sup> in subunit C of cASIC1 would be 3.4–5.5 Å, close to the sum of average distances for Cu<sup>2+</sup>-N-His (2.0–2.1 Å) and Cu<sup>2+</sup>-O-Glu (2.2 Å) in proteins (36). We therefore hypothesized that αHis<sup>408</sup> and βGlu<sup>254</sup> coordinate the same Cu<sup>2+</sup> at the α and β subunit interface. To test the hypothesis, we mutated βGlu<sup>254</sup> to Ala, Arg, Glu, His, Asp, and Asp. All of the substitutions except βE254D led to a modest but significant reduction of inhibition by 10 μM Cu<sup>2+</sup> (Fig. 9D). The double mutant αH468A/BE254A nearly eliminated the inhibitory effect of 10 μM Cu<sup>2+</sup> (I<sub>o2</sub>/I = 0.83 ± 0.01, n = 5, p < 0.0001 versus WT, αH468A or BE254A; Fig. 9E). Our observations are consistent with the above hypothesis that αHis<sup>408</sup> and βGlu<sup>254</sup> contribute to coordination of the same Cu<sup>2+</sup> ion. The close proximity of αHis<sup>408</sup> and βGlu<sup>254</sup> requires an obligate counterclockwise subunit arrangement of α-β-γ when viewed from above (Fig.
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A (αH468AβH160A)

10 μM Cu²⁺

|    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|
| Iₐ |    |    |    |    |    |    |
| -3.5 | -3.0 | -2.5 | -2.0 | -1.5 | -1.0 | -0.5 | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 |
| Time (min) | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |

B (αH468AγH277A)

10 μM Cu²⁺

|    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|
| Iₐ |    |    |    |    |    |    |
| -3.5 | -3.0 | -2.5 | -2.0 | -1.5 | -1.0 | -0.5 | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 |
| Time (min) | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |

C (βH159AγH277A)

10 μM Cu²⁺

|    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|
| Iₐ |    |    |    |    |    |    |
| -3.5 | -3.0 | -2.5 | -2.0 | -1.5 | -1.0 | -0.5 | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 |
| Time (min) | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |

D

|    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|
| Iₐ |    |    |    |    |    |    |
| -3.5 | -3.0 | -2.5 | -2.0 | -1.5 | -1.0 | -0.5 | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 |
| Time (min) | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |

FIGURE 8. Double mutations of αHis⁴⁶⁸ and βHis⁴⁶⁰ or γHis²⁷⁷ eliminate the inhibitory effect of 10 μM Cu²⁺ on hENaCs. Oocytes expressing two mutant hENaC subunits and one WT hENaC subunit were clamped to −100 mV. The effects of 10 μM Cu²⁺ were examined together with WT αβγ hENaCs in the same way as previous figures. A–C, representative recordings showing the responses of the double mutants to 10 μM Cu²⁺. Gray bars show the presence of 10 μM amiloride. D, 1/ᵢₒ obtained from four or five oocytes expressing the double mutants. Black bars show that the values were significantly different from that of WT obtained in the same batches of oocytes (p < 0.001). The dashed line shows the average 1/ᵢₒ from all WT expressing oocytes used in this particular experiment (0.16 ± 0.01, n = 19) for the purpose of reference. Bath solution was buffered with 10 mM HEPES.

9F). A clockwise arrangement of α, β, and γ subunits would place γGlu¹⁴⁶ and βGlu²⁵⁴ in close proximity (Fig. 9G). Charge reversal mutation γE₄₄₆R did not alter the inhibitory effect of 10 μM Cu²⁺ (Iᵢₒ/I = 0.08 ± 0.01, n = 4, p > 0.7 versus WT). The result was not consistent with an involvement of γGlu⁴⁴⁶ in Cu²⁺ binding and a pairing between γGlu⁴⁴⁶ and βGlu²⁵⁴ that would otherwise require a clockwise organization of α, β, and γ subunits.

Cu²⁺ Inhibition and Na⁺ Self-inhibition—We studied the effect of Cu²⁺ on hENaC in a bath solution containing 110 mM Na⁺, which is typically used by investigators so as to produce easily measurable currents. At this concentration, extracellular Na⁺ reduces the Pₐₙ of ENaC via a process of Na⁺ self-inhibition (25, 26, 37, 38). The degree of Na⁺ self-inhibition dramatically affects the other extracellular regulators on ENaCs such as Zn²⁺, H⁺, and Cl⁻ (7–9). We suspected that some of the six His mutations might alter the Na⁺ self-inhibition response, and as a secondary effect Cu²⁺ inhibition appeared to be reduced. We therefore examined the Na⁺ self-inhibition responses of these mutant ENaCs.

As shown in Fig. 10, αH₂₅₅₅A greatly enhanced Na⁺ self-inhibition, whereas βE₂₅₄Q and γH₂₇₇₇A moderately increased the magnitude of Na⁺ self-inhibition. In contrast, αH₄₆₈₈A, βH₁₅₉₉A, βH₁₆₀₀₉A, and γH₈₈₈₈A did not significantly

FIGURE 9. Identification of αHis⁴⁶⁸ and βGlu²⁵₄ pair at subunit interface. A, a structural model of chicken ASIC1. The trimeric cASIC1 structure (4S) was rendered as three colored ribbons (subunit A, B, and C in green, red, and blue, respectively) with PyMol (version 1.3) using coordinates from Protein Data Bank (identification code 3HGC). The ECDs on the top part of the structure are linked to the transmembrane (TM) domain via six short coiled segments termed the wrist (13). The boxed area is enlarged on the right to show the contact between the thumb domain Lys⁵⁵⁵ of subunit A and the palm domain Glu¹⁴⁶ of subunit C in the cASIC1 structure. For clarity the area is shown with semitransparent surface and ribbon rendering. The side chains of Lys and Glu are shown as sticks with carbon, oxygen, and nitrogen atoms colored in cyan, red, and blue, respectively. B, nontransparent surface rendering of the same region as in A with subunit A in red and subunit C in blue to highlight the subunit interface. Lys⁵⁵⁵ was mutated to a His. Homologous hENaC residues to K555H and Glu¹⁴⁶ are shown in parentheses. The surface near the two residues are omitted. C, sequence alignments of human, mouse ENaCs, and cASIC1 in regions surrounding βGlu²⁵₄ (−) and αHis⁴⁶⁸ (+). Secondary structures are shown according to the cASIC1 structure (13). D, 1/ᵢₒ from oocytes expressing β mutant and WT α and γ hENaCs. Black bars indicate that the values were significantly different from that of WT in the same batches of oocytes (p < 0.001, n = 5). The dashed line shows the average value from pooled WT expressing oocytes in this experiment (0.16 ± 0.01, n = 10). Bath solution was buffered with 10 mM HEPES. E, superimposed traces showing the responses of WT (black), BE254A (blue), αH468A (red) and αH468A/BE254A (purple) and 1/ᵢₒ values in corresponding colors. All of the mutant values were significantly greater than that of WT (p < 0.001), and the asterisk shows that the value of the double mutant was significantly greater than that of the either single mutant (p < 0.001, n = 5). F, an illustration showing a counterclockwise arrangement of three ENaC subunits in a top view. Three rectangles represent the approximate spaces occupied by α (red), β (blue), and γ (green) subunits. Overlapped regions highlight inter-subunit contacts including three-way interaction at the center and two-way interactions at more distal regions. Subunit-subunit interactions happen primarily along the two inner laterals. We designated the long inner sides as ± and the short inner side as − by convention used in other ligand-gated channels. G, an alternative (clockwise) arrangement of α, β, and γ ENaC subunits.
change the Na\(^+\) self-inhibition response. Na\(^+\) self-inhibition was largely eliminated in the \(\alpha H255A\) mutant, as previously reported (8). These observations suggest that His468, His159, His160, and His88 have specific roles in Cu\(^{2+}\) inhibition of hENaC.

Mutations that altered the Na\(^+\) self-inhibition response also changed the magnitude of the transient activation of current that appeared before the inhibitory effect of Cu\(^{2+}\) (Figs. 1A and 7D). The transient activation was absent in mutants with Na\(^+\) self-inhibition eliminated (for example, \(\beta H159A\); Fig. 7D). It suggests that the transient activation by Cu\(^{2+}\) was caused by a relief of Na\(^+\) self-inhibition, in a manner similar to that of ENaC activation by external Zn\(^{2+}\) and H\(^+\) (7, 8). Accordingly, \(\alpha H255D\) and \(\gamma H277A\) that enhanced the Na\(^+\) self-inhibition response increased the magnitude of the transient Cu\(^{2+}\) activation by 5.3- and 3.1-fold, respectively, compared with WT (\(p < 0.001\)). These observations suggest that the reduced Cu\(^{2+}\) inhibition in \(\alpha H255\) and \(\gamma H277\) mutants (i.e. greater \(I_{\text{Cu}}/I_{\text{peak}}\); Fig. 7) was related to the augmented stimulatory effect of Cu\(^{2+}\), which resulted from enhanced Na\(^+\) self-inhibition. Other mutations including \(\alpha H668\) mutations (Fig. 5D), \(\beta H159A\) (Fig. 7D), \(\beta H160A\) (Fig. 7D), \(\beta Glu254\) mutations (Fig. 9E), and \(\gamma H88A\) (Fig. 7D) did not significantly change the transient Cu\(^{2+}\) stimulation, consistent with their specific role in the inhibitory effect of Cu\(^{2+}\).
Copper Inhibits Human ENaC

**Discussion**

In this study, we found that external Cu\(^{2+}\) inhibited human αβγ ENaC in both *Xenopus* oocytes and human airway epithelia. External Cu\(^{2+}\) at 10 μM does not inhibit mouse (Fig. 4) or rat ENaC.\(^3\) Native *Xenopus* ENaCs in A6 cells are activated by extracellular Cu\(^{2+}\) (14). Therefore, in the context of previous reports and our current observations, external Cu\(^{2+}\) appears to be a specific inhibitor of human ENaC among cloned ENaCs. The inhibitory effect of Cu\(^{2+}\) on the hENaC current in the human airway epithelia appeared to be smaller and weaker than that in oocytes (Fig. 11). We do not know the exact cause for the different responses to Cu\(^{2+}\). They could be related to certain experimental conditions utilized in the two systems, such as temperatures (i.e., 20–24 °C in oocytes and 37 °C in epithelia) and oxygen tensions, both of which regulate ENaC activity (25, 39, 40). The differences in the response to Cu\(^{2+}\) could also reflect the inherent differences between native and heterologously expressed channels. It has been reported that ENaCs in oocytes and epithelial monolayers display different sensitivities to the peptide inhibitors derived from the inhibitory domains of α and γ mouse ENaCs (41, 42).

The estimated IC\(_{50}\) of 0.31 μM for Cu\(^{2+}\) inhibition suggests that external Cu\(^{2+}\) is a potent hENaC inhibitor. A high affinity ENaC inhibitor may be useful in treating diseases associated with elevated ENaC-mediated Na\(^+\) absorption such as Liddle syndrome and cystic fibrosis (43, 44).

Recent studies have established a critical role for ENaCs in the regulation of airway surface liquid volume and excessive activity of ENaCs in airways contributes to the pathogenesis of cystic fibrosis (44). Impaired Na\(^+\) transport in alveoli leads to pulmonary edema (40). Airborne particles contain a considerable amount of transition metals including copper. Upon contact with biological fluids, free metal ions can be released from the particles and cause local and even remote toxic effects (17, 18). We speculate that the inhibitory effect of Cu\(^{2+}\) on human ENaCs in lung epithelia may contribute to the toxicological symptoms caused by inhaled particulate matters. Cu\(^{2+}\), released from particulate matters, may worsen particle-induced pulmonary edema by inhibition of Na\(^+\) absorption in airways and alveoli.

The main goal of this study was to probe the structural basis for hENaC inhibition by external Cu\(^{2+}\). Experiments with mixed human and mouse ENaC subunits demonstrate that α and β hENaC subunits are necessary for the specific response of hENaCs to Cu\(^{2+}\) (Fig. 4). Initial mutational screening of the hENaC-specific residues within α and β ECDs identified αHis\(^{468}\) and βHis\(^{159}\) as residues involved in Cu\(^{2+}\) inhibition (Fig. 5). The double mutant (αH468S-βH159D) converted the response to Cu\(^{2+}\) of the human channel to that of the mouse channel (Figs. 4 and 5), suggesting that these two hENaC-specific residues are primarily responsible for the distiction to 10 μM Cu\(^{2+}\) of human versus mouse ENaC. Subsequently, systematic screening of His residues within ECDs of α, β, and γ subunits identified additional four sites (αHis\(^{255}\), βHis\(^{160}\), γHis\(^{388}\), and γHis\(^{277}\)) where mutations significantly reduced the inhibitory effect of 10 μM Cu\(^{2+}\) (Fig. 7). However, further analyses showed that αHis\(^{255}\) and γHis\(^{277}\) mutations significantly increased the magnitudes of Na\(^+\) self-inhibition and Cu\(^{2+}\)-induced transient activation preceding the inhibitory effect (Figs. 7D and 10), suggesting indirect roles for both His residues in the Cu\(^{2+}\) inhibition. Another residue, γHis\(^{388}\), does not appear to have an essential role in Cu\(^{2+}\) inhibition, given the small effect of its mutation on Cu\(^{2+}\) inhibition (Fig. 7). On the contrary, αHis\(^{468}\), βHis\(^{159}\), and βHis\(^{160}\) mutations specifically reduced Cu\(^{2+}\) inhibition, without affecting the Na\(^+\) self-inhibition response and the transient activation by Cu\(^{2+}\). We conclude that these three His residues are involved in Cu\(^{2+}\) inhibition.

Our data suggest that αHis\(^{468}\) has a key role in mediating Cu\(^{2+}\) inhibition. Taking advantage of the structural information for CASIC1 (13, 45), we predicted that αHis\(^{468}\) and βGlu\(^{254}\) could contribute to a Cu\(^{2+}\)-binding site at the α/β subunit interface. Mutational analyses confirmed the prediction (Fig. 9). The identification of αHis\(^{468}\)/βGlu\(^{254}\) pair suggests a counterclockwise configuration of α, β, and γ subunits when viewed

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\(^3\) J. Chen and S. Sheng, unpublished observations.

**Figure 11.** Cu\(^{2+}\) inhibits native human ENaCs in human airway epithelial cells. A, superimposed short circuit recordings showing the responses of the currents to 1, 10, 30, and 100 μM Cu\(^{2+}\) applied in apical chamber. B, \(I_{sc}\)/\(I_{c}\), ratio of amiloride-sensitive current in the presence of Cu\(^{2+}\) and amiloride-sensitive current prior to Cu\(^{2+}\) application. The asterisks indicate that the values in the presence of 30 and 100 μM Cu\(^{2+}\) were significantly less than that of base-line currents (\(p < 0.001, n = 6\)).
from above the channel (Fig. 9F). This subunit arrangement is in agreement with a recent report by Collier and Snyder (46). However, we cannot rule out the presence of both the counter-clockwise and clockwise subunit arrangements. Another limitation of the notion is that it is based on the assumption that ENaC, like ASIC1, has a trimeric architecture, which remains to be established experimentally.

ENaC P∞ is regulated by a variety of extracellular factors that may share common pathways in their regulation of ENaC gating. Indeed, the effects of external Zn²⁺, H⁺, and Cl⁻ on ENaC activity rely on the existence of Na⁺ self-inhibition (7–9). However, Cu²⁺ inhibition of hENaC does not depend on the existence of Na⁺ self-inhibition. In fact, the magnitude of Cu²⁺ inhibition was increased by Na⁺ self-inhibition eliminating mutation (γH233A) and reduced by Na⁺ self-inhibition enhancing mutation (αH255A). Therefore, Cu²⁺ likely inhibits the hENaC via a pathway distinct from that of Na⁺ self-inhibition. In contrast, the transient activation of hENaC by Cu²⁺ appears to result from a relief of Na⁺ self-inhibition, because Na⁺ self-inhibition eliminating (γH233A) or enhancing (αH255A) mutations diminished or enhanced its magnitude accordingly.

In summary, we found that external Cu²⁺ is a high affinity inhibitor of human ENaC and identified a Cu²⁺-binding site at subunit interface within the extracellular domains. Structure-assisted mutational analyses suggest that a thumb domain His residue of α subunit and a palm domain Glu residue of the β subunit interact with a Cu²⁺ ion. This pairing (αHis⁶⁸α/βGlu₂⁵⁴α) requires a counterclockwise arrangement of α, β, and γ ENaC subunits when viewed from above.

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REFERENCES

1. Sheng, S., Johnson, J. P., and Kleyman, T. R. (2007) in Seldin and Giebisch’s The Kidney: Physiology & Pathophysiology (Alpern, R. J., and Hebert, S. C., eds.) 4th Ed., pp. 743–768, Academic Press, New York
2. Bhatta, V., and Hallows, K. R. (2008) J. Am. Soc. Nephrol. 19, 1845–1854
3. Passero, C. J., Hugh, R. P., and Kleyman, T. R. (2010) Curr. Opin. Nephrol. Hypertens 19, 13–19
4. Garty, H., and Palmer, L. G. (1997) Physiol. Rev. 77, 359–396
5. Van Driessche, W., and Zeiske, W. (1985) Physiol. Rev. 65, 833–903
6. Sheng, S., Perry, C. J., and Kleyman, T. R. (2002) J. Biol. Chem. 277, 50098–50111
7. Sheng, S., Perry, C. J., and Kleyman, T. R. (2004) J. Biol. Chem. 279, 31687–31696
8. Collier, D. M., and Snyder, P. M. (2009) J. Biol. Chem. 284, 792–798
9. Collier, D. M., and Snyder, P. M. (2009) J. Biol. Chem. 284, 29320–29325
10. Nie, H. G., Zhang, W., Han, D. Y., Li, Q. N., Li, J., Zhao, R. Z., Su, X. F., Peng, J. B., and Ji, H. L. (2010) Am. J. Physiol. Renal Physiol. 298, F323–F334
11. Kleyman, T. R., Carattino, M. D., and Hugh, R. P. (2009) J. Biol. Chem. 284, 20447–20451
12. Carattino, M. D., Sheng, S., and Kleyman, T. R. (2004) J. Biol. Chem. 279,