Probing the Structural Basis of Zn\textsuperscript{2+} Regulation of the Epithelial Na\textsuperscript{+} Channel\textsuperscript{*}

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Background: Extracellular Zn\textsuperscript{2+} regulates epithelial Na\textsuperscript{+} channel (ENaC) activity.

Results: Specific mutations selectively weaken either the stimulatory or inhibitory effect of Zn\textsuperscript{2+}.

Conclusion: External Zn\textsuperscript{2+} regulates ENaC by interacting with multiple extracellular sites within the γ-subunit.

Significance: This report provides novel insights into the structural basis of Zn\textsuperscript{2+} regulation of ENaC.

Extracellular Zn\textsuperscript{2+} activates the epithelial Na\textsuperscript{+} channel (ENaC) by relieving Na\textsuperscript{+} self-inhibition. However, a biphasic Zn\textsuperscript{2+} dose response was observed, suggesting that Zn\textsuperscript{2+} has dual effects on the channel (i.e., activating and inhibitory). To investigate the structural basis for this biphasic effect of Zn\textsuperscript{2+}, we examined the effects of mutating the 10 extracellular His residues of mouse γENaC. Four mutations within the finger subdomain (γH193A, γH200A, γH202A, and γH239A) significantly reduced the maximal Zn\textsuperscript{2+} activation of the channel. Whereas γH193A, γH200A, and γH202A reduced the apparent affinity of the Zn\textsuperscript{2+} activating site, γH239A diminished Na\textsuperscript{+} self-inhibition and thus concealed the activating effects of Zn\textsuperscript{2+}. Mutation of a His residue within the palm subdomain (γH88A) abolished the low-affinity Zn\textsuperscript{2+} inhibitory effect. Based on structural homology with acid-sensing ion channel 1, γAsp\textsuperscript{316} was predicted to be in close proximity to γHis\textsuperscript{88}. Ala substitution of the residue (γD516A) blunted the inhibitory effect of Zn\textsuperscript{2+}. Our results suggest that external Zn\textsuperscript{2+} regulates ENaC activity by binding to multiple extracellular sites within the γ-subunit, including (i) a high-affinity stimulatory site within the finger subdomain involving His\textsuperscript{193}, His\textsuperscript{200}, and His\textsuperscript{202} and (ii) a low-affinity Zn\textsuperscript{2+} inhibitory site within the palm subdomain that includes His\textsuperscript{88} and Asp\textsuperscript{316}.

ENaC activity is regulated by a variety of extracellular factors, including proteases, shear stress, pH, anions, nucleotides, and transition metals (8–15). Although some metals are essential for normal physiological function, excessive environmental exposure can be toxic (16, 17). For instance, particulate matter and airborne particles containing high amounts of transitional metals, including zinc and copper, contribute to pulmonary and cardiovascular toxicity (18, 19). Although recent studies have suggested that ion channels are potential molecular targets of transition metals (20), the mechanisms that confer the harmful effects of heavy metals are poorly understood.

Zinc is the second most abundant transition metal in living organisms and is thought to complex with ~10% of the human proteome (21). It has catalytic, structural, or regulatory roles in proteins that are involved in diverse biological processes (22). For example, zinc regulates voltage- and ligand-gated ion channels and may function as a signaling ion in brain (23, 24). Zinc may play a pathophysiological role in several disorders such as Alzheimer disease, cancer, diabetes, and depression (22).

Previous studies have demonstrated that low concentrations of extracellular Zn\textsuperscript{2+} activate ENaC. Zn\textsuperscript{2+} increases short-circuit current in amphibian epithelia expressing native Na\textsuperscript{+} channels (8). Zn\textsuperscript{2+} activates ENaCs in heterologous expression systems by directly interacting with the channel and altering channel gating (25). Single channel recordings revealed that external Zn\textsuperscript{2+} increased the number of observed channels within a patch without changing single channel conductance (10). The response of ENaC to extracellular Zn\textsuperscript{2+} is biphasic. Low concentrations of Zn\textsuperscript{2+} activate the channel, with a maximal response at 100 μM Zn\textsuperscript{2+}. Further increases in [Zn\textsuperscript{2+}] reduce channel activity. This bell-shaped dose-response relationship suggests that Zn\textsuperscript{2+} enhances channel activity at low concentrations and is inhibitory at high concentrations (25). The stimulatory effect of Zn\textsuperscript{2+} on ENaC has been attributed to relief of Na\textsuperscript{+} self-inhibition (25). Na\textsuperscript{+} self-inhibition is a rapid down-regulation of ENaC open probability when the extracellular Na\textsuperscript{+} concentration increases (9, 26). However, the Zn\textsuperscript{2+}-binding sites within ENaC have not been identified, and the biphasic dose response to the heavy metal has not been explained.

ENaCs are typically composed of three homologous subunits, each of which contains short intracellular N and C ter-

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2 The abbreviations used are: ENaC, epithelial Na\textsuperscript{+} channel; γENaC, mouse γENaC; cASIC1, chicken acid-sensing ion channel 1; ECD, extracellular domain.
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mini, two transmembrane domains, and a large extracellular region. The crystal structure of the chicken acid-sensing ion channel 1 (cASIC1; homologous to ENaC) reveals a trimeric channel complex with a large extracellular domain (ECD) connected to the transmembrane domain via a wrist region (27). The extracellular region of a subunit is organized into five subdomains, referred to as palm, β-ball, knuckle, finger, and thumb. Recent studies suggest that the extracellular regions of ENaC subunits have a similar overall design to that of cASIC1 and that the ECD may sense various extracellular signals (12, 14, 28–31). However, the exact locations where most extracellular factors interact with ENaC and the mechanistic details of how the initial contacts lead to altered channel activity remain elusive.

The ECDs of ENaC subunits contain numerous residues, such as His, Cys, Glu, and Asp, which are capable of serving as Zn²⁺-binding ligands (32). We hypothesized that some of these residues mediate Zn²⁺ interaction with ENaC. Several lines of evidence suggest that the γ-subunit has a particularly important role in regulating channel gating in response to external factors (11, 33, 34). Mutations were introduced into His residues within the γ-subunit ECD to identify sites that have a role in the regulation of ENaC by Zn²⁺. Three His residues were identified within the finger subdomain that participate in a high-affinity Zn²⁺ activating site. Additionally, mutation of a palm subdomain His residue altered the low-affinity inhibitory response. These His residues are unique to the γ-subunit, as they are not found at the homologous sites in the α- and β-subunits.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Point or multiple mutations were generated in mouse γENaC (γmENaC) cDNA in the pBluescript SK− vector (Stratagene, La Jolla, CA) using the QuickChange II XL site-directed mutagenesis kit (Stratagene). Following mutagenesis, cDNAs were validated by sequencing. Wild-type ENaC α-, β-, and γ-subunit and mutant ENaC γ-subunit cRNAs were made using T3 RNA polymerase (Ambion), purified using an RNA purification kit (Qiagen), and quantified by spectrophotometry.

**ENaC Expression and Two-electrode Voltage Clamp**—ENaC expression in *Xenopus* oocytes and two-electrode voltage clamp were performed as reported previously (35). Stage V and VI oocytes free of follicle cell layers were injected with 1 ng of cRNA for each mENaC subunit per oocyte and incubated at 18 °C in modified Barth’s saline (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 sodium penicillin, 10 μg/ml streptomycin sulfate, and 100 μg/ml gentamycin sulfate, pH 7.4).

All experiments were carried out at room temperature (20–24 °C). Two-electrode voltage clamp was performed using either an Axoclamp 900A amplifier (Molecular Devices, Sunnyvale, CA) or a TEV-200 voltage clamp amplifier (Dagan Corp., Minneapolis, MN) and a DigiData 1440A interface (Molecular Devices). Data acquisition and analyses were done with pCLAMP 8 or 9 (Molecular Devices). 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. Oocytes were continuously clamped at −60 or −100 mV as specified in the figure legends.

**Na⁺ Self-Inhibition Response**—Na⁺ self-inhibition responses were examined as described previously (36, 37). Briefly, Na⁺ self-inhibition was determined by measuring the decrease in current from the peak (I_peak) to a steady state (I_min) elicited by a rapid increase in extracellular Na⁺ concentration from 1 to 110 mM. The [Na⁺] jump was done by rapidly replacing a 1 mM Na⁺ bath solution (NaCl-1 containing 1 mM NaCl, 109 mM N-methyl-D-glucamine, 2 mM KCl, 2 mM CaCl₂, and 10 mM HEPES, pH 7.4) with a 110 mM Na⁺ bath solution (NaCl-110 containing 110 mM NaCl, 2 mM KCl, 2 mM CaCl₂, and 10 mM HEPES, pH 7.4). Rapid solution exchange was performed with a 16-channel Teflon valve perfusion system (AutoMate Scientific, Inc., Berkeley, CA). The magnitude of Na⁺ self-inhibition was represented by the ratio of amiloride-sensitive I₄₋ and I_peak.

**Zn²⁺ Dose Responses**—A 1 mM ZnCl₂ stock solution (super pure, >99.999%, Sigma-Aldrich) was prepared in water and diluted to the desired concentrations in NaCl-110 bath solution. To examine Zn²⁺ dose responses, oocytes expressing mENaCs were continuously clamped at −60 mV while Zn²⁺ was applied at increasing concentrations in the range of 0.1 μM to 5 mM. Due to the limited solubility of ZnCl₂ in NaCl-110 bath solution, Zn²⁺ concentrations >5 mM were not tested. At the end of an experiment, 10 μM amiloride was added to the bath to determine the amiloride-sensitive current. The changes in amiloride-sensitive currents in response to Zn²⁺ applications were used to analyze the dose-response relationship.

Because the amiloride-sensitive current demonstrated a biphasic dose-response relationship with [Zn²⁺], we assumed that ENaCs possess a high-affinity activating site and a low-affinity inhibitory site for Zn²⁺. Thus, the normalized currents in the presence of increasing concentrations of Zn²⁺ were fitted with Equation 1,

\[
I_R = (I_{max} - I_{min})(C/(C + EC_{50}))(IC_{50}/(C + IC_{50})) + I_{min}
\]

(Eq. 1)

where I_R is the relative current at certain [Zn²⁺] (i.e. current in the presence of Zn²⁺ normalized to current in the absence of Zn²⁺); I_{max} and I_{min} are the maximal and minimal normalized currents, respectively; C is the Zn²⁺ concentration; and EC_{50} and IC_{50} are the concentrations with 50% effects for the activating and inhibitory phases, respectively.

**Statistical Analysis**—Data are presented as means ± S.E. To minimize bias from batch-to-batch variation in oocytes, the significance in the differences between mutant and WT channels was always analyzed from data obtained in the same batches of oocytes. Student’s t tests were used for comparison, and the significance levels are specified below and in the figure legends. Curve fittings were performed with OriginPro 8.5 (OriginLab, Northampton, MA).

**RESULTS**

**External Zn²⁺ Regulates mENaC by Interacting at Distinct Sites**—Because extracellular Zn²⁺ activates ENaC by relieving Na⁺ self-inhibition (25), the Na⁺ self-inhibition response and the effect of Zn²⁺ were examined sequentially in each oocyte.
The Na\(^+\) self-inhibition response reflects a decrease in current from a peak (\(I_{\text{peak}}\)) to a steady state (\(I_{\text{ss}}\)), which is elicited by a rapid increase in the extracellular Na\(^+\) concentration from 1 to 110 mM (Fig. 1). As currents approached a steady-state level, increasing concentrations of Zn\(^{2+}\) were applied, and changes in current were measured. Increasing extracellular [Zn\(^{2+}\)] in the range of ~0.1–100 \(\mu\)M led to a dose-dependent increase in current. The current fell when extracellular [Zn\(^{2+}\)] was increased above 100 \(\mu\)M. The maximal current in the presence of Zn\(^{2+}\) (\(I_{\text{znm}}\)) was essentially identical to the \(I_{\text{peak}}\) observed following the increase in extracellular [Na\(^+\)] (\(I_{\text{znm}}/I_{\text{peak}} = 1.00 \pm 0.01, n = 32\)), consistent with the notion that Zn\(^{2+}\) relieves ENaC inhibition from external Na\(^+\).

Fig. 1B illustrates the Zn\(^{2+}\) dose-response relationship. Mean amiloride-sensitive currents in the presence of increasing extracellular [Zn\(^{2+}\)] were normalized to the base-line current. Similar to our previous report (25), we observed a bell-shaped dose response to Zn\(^{2+}\). This relationship fits a model of ENaC possessing a high-affinity Zn\(^{2+}\) activating site and a low-affinity Zn\(^{2+}\) inhibitory site \((R^2 = 0.997, \text{Equation 1, see "Experimental Procedures"})\) with an estimated EC\(_{50}\) and IC\(_{50}\) of 2.1 \(\pm 0.1\) \(\mu\)M (\(n = 6\)) and 2.1 \(\pm 0.1\) mM (\(n = 6\)), respectively. The maximal relative current observed at 110 \(\mu\)M Zn\(^{2+}\) was 1.68 \(\pm 0.07\) (\(n = 6\)).

Ala Substitutions of Selected His Residues in the Extracellular Region of \(\gamma\)mENaC Alter the Effect of Zn\(^{2+}\) —There are a total of 10 His residues within the ECD of the \(\gamma\)-subunit. Four units are located within the finger subdomain, three in the \(\beta\)-ball subdomain, two in the palm subdomain, and one in the thumb subdomain (Fig. 2). We individually mutated these His residues to Ala and expressed the mutant \(\gamma\)-subunits together with WT \(\alpha\)- and \(\beta\)-subunits in oocytes. We sequentially examined Na\(^+\) self-inhibition and the response to Zn\(^{2+}\) (Figs. 3 and 4) to determine whether a mutation selectively disrupted the effect of Zn\(^{2+}\) on channel activity or secondarily altered the Zn\(^{2+}\) effect by modifying the Na\(^+\) self-inhibition response.

Ala substitutions at 4 of the 10 extracellular \(\gamma\)-subunit His residues (\(\gamma\)H193A, \(\gamma\)H200A, \(\gamma\)H202A, and \(\gamma\)H239A) significantly reduced maximal ENaC activation by Zn\(^{2+}\) (Figs. 3 and 4). Interestingly, all four of the extracellular His residues whose mutations reduced Zn\(^{2+}\) activation are within the predicted finger subdomain of the channel. In addition to suppressing Zn\(^{2+}\) activation, \(\gamma\)H239A also diminished Na\(^+\) self-inhibition, similar to what was previously observed when \(\gamma\)His\(^{239}\) was substituted with other residues (37, 38). As the \(\gamma\)H239A mutant was relieved of Na\(^+\) self-inhibition prior to the addition of Zn\(^{2+}\), it is not possible to determine whether this residue is involved in Zn\(^{2+}\) binding to the channel. On the other hand, \(\gamma\)H193A, \(\gamma\)H200A, and \(\gamma\)H202A did not significantly alter the Na\(^+\) self-inhibition response, suggesting that these three His residues in the finger subdomain have specific roles in Zn\(^{2+}\) activation of ENaC.

Although \(\gamma\)H88A did not affect the maximal Zn\(^{2+}\) activation (\(p > 0.05\) versus WT) or the Na\(^+\) self-inhibition response, it converted the Zn\(^{2+}\) dose response from bell-shaped to monophasic by selectively eliminating the inhibitory effect of high Zn\(^{2+}\) (Figs. 3 and 4). These results are consistent with a mutation-induced loss of a low-affinity Zn\(^{2+}\)-binding site that mediates channel inhibition at high Zn\(^{2+}\). One mutation (\(\gamma\)H283A) moderately increased the maximal Zn\(^{2+}\) activation (\(p < 0.01\) versus WT) (Fig. 4). Collectively, these observations suggest that \(\gamma\)His\(^{88}\) within the palm subdomain and \(\gamma\)His\(^{193}\),
His200, and His202 within the finger subdomain are involved in Zn2⁺-dependent regulation of ENaC.

Mutation of His193, His200, or His202 within the Finger Subdomain Reduces the Apparent Zn2⁺ Binding Affinity of the Activating Site—We analyzed the responses of WT and mutant channels to increasing [Zn2⁺] using a two-site model. Representative traces for current changes in responses to [Na⁺] increases and Zn2⁺ applications are from oocytes expressing αβγyENaC (WT), αβγH88A, αβγH193A, αβγH200A, αβγH202A, and αβγH239A mENaCs. A simplified protocol for examining the effects of Zn2⁺ at only six concentrations compared with the one in Fig. 1 was used for both WT and mutant channels. The recording portions with 10 μM amiloride at the end of the experiments were omitted for clarity. These recordings represent at least six observations.

![Figure 3](https://example.com/figure3.png)

**FIGURE 3.** Mutations of five γHis residues differentially alter the effect of Zn2⁺. Representative traces for current changes in responses to [Na⁺] increases and Zn2⁺ applications are from oocytes expressing αβγmENaC (WT), αβγH88A, αβγH193A, αβγH200A, αβγH202A, and αβγH239A mENaCs. A simplified protocol for examining the effects of Zn2⁺ at only six concentrations compared with the one in Fig. 1 was used for both WT and mutant channels. The recording portions with 10 μM amiloride at the end of the experiments were omitted for clarity. These recordings represent at least six observations.

![Figure 4](https://example.com/figure4.png)

**FIGURE 4.** Mutation-induced changes in the magnitude of Na⁺ self-inhibition and the maximal Zn2⁺ activation of mENaC. I\textsubscript{ss}/I\textsubscript{peak} values represent the maximal relative currents in the presence of Zn2⁺. They were typically observed with 100 μM Zn2⁺ except for the γH88A mutant, which had I\textsubscript{ss} at 1 mM. Note that I\textsubscript{ss} is the observed value and different from the I\textsubscript{max} (Table 1) that was derived from a best fit of the dose-response data. Black bars indicate the values of mutants were significantly different (p < 0.05) from those of WT (n = 5–10), obtained in the same batch of oocytes. The dashed lines show the mean values pooled from all oocytes expressing WT mENaC obtained in the analyses of these mutants (I\textsubscript{ss}/I\textsubscript{peak} = 0.60 ± 0.01, I\textsubscript{ss} = 1.73 ± 0.03, n = 32).

γHis\textsuperscript{200}, and γHis\textsuperscript{202} within the finger subdomain are involved in Zn2⁺-dependent regulation of ENaC.

Mutation of γHis\textsuperscript{193}, γHis\textsuperscript{200}, or γHis\textsuperscript{202} within the Finger Subdomain Reduces the Apparent Zn2⁺ Binding Affinity of the Activating Site—We analyzed the responses of WT and mutant channels to increasing [Zn2⁺] using a two-site model. Representative dose-response curves are shown in Fig. 5, and derived values for EC\textsubscript{50} and IC\textsubscript{50} are shown in Table 1. Our results show that γH88A did not affect the EC\textsubscript{50} for the activating site,
although it essentially eliminated the decrease in channel activity observed with higher [Zn\(^{2+}\)]. In contrast, γH193A, γH200A, and γH202A reduced the sensitivity of mENaC to activation by extracellular Zn\(^{2+}\) and did not affect the inhibitory effect of high [Zn\(^{2+}\)], leading to increased EC\(_{50}\) values and no change in the IC\(_{50}\) relative to WT. The results imply that γH193A, γH200A, and γH202A selectively reduce the apparent affinity of the potentiating site for Zn\(^{2+}\) while not interfering with the low-affinity Zn\(^{2+}\) inhibitory site.

We also examined whether substitutions at multiple His residues led to further increases in the EC\(_{50}\) for Zn\(^{2+}\). The Zn\(^{2+}\) dose-response relationships of γH200A/γH202A and γH193A/γH200A/γH202A were similar to those found in single mutants (Fig. 6). Thus, all three His residues within the finger subdomain are required for Zn\(^{2+}\)-dependent channel activation. The lack of an additive effect of multiple His substitutions suggests that these His residues participate in a common Zn\(^{2+}\) binding site.

In addition to increasing the EC\(_{50}\) values of Zn\(^{2+}\) activation, γH193A, γH200A, γH202A, γH200A/γH202A, and γH193A/γH200A/γH202A also reduced the magnitude of the increase in current in response to Zn\(^{2+}\) (I\(_{\text{max}}\)) (Table 1). The reduced I\(_{\text{max}}\) observed in the mutants could be explained either by a reduction in the efficacy of channel activation by Zn\(^{2+}\) or by the presence of an inhibitory effect that limits the extent of channel activation. To distinguish these two possibilities, we examined channels with His substitutions at Zn\(^{2+}\)-activating sites (γH200A/γH202A) and the Zn\(^{2+}\) inhibitory site (γH88A). As shown in Fig. 6E, γH88A/γH200A/γH202A reduced Zn\(^{2+}\) sensitivity by 8-fold compared with γH88A channels (EC\(_{50}\) = 20.4 ± 3.0 μM (n = 6) versus 2.6 ± 0.3 μM (n = 10); p < 0.001). However, both mutants had similar I\(_{\text{max}}\) values (1.81 ± 0.11 (n = 6) versus 1.79 ± 0.09 (n = 10); p > 0.05), demonstrating that mutations of the Zn\(^{2+}\) activating sites within the finger subdomain do not alter the magnitude of the maximal Zn\(^{2+}\) activation when the low-affinity Zn\(^{2+}\) inhibitory site is not

### TABLE 1

| Oocytes       | I\(_{\text{max}}\) | EC\(_{50}\) | IC\(_{50}\) |
|---------------|-------------------|------------|------------|
| WT            |                   | 1.76 ± 0.03| 2.10 ± 0.12| 2.13 ± 0.14|
| γH193A        | 10                | 1.79 ± 0.09| 2.58 ± 0.32| >1000       |
| γH200A        | 6                 | 1.54 ± 0.03| 4.59 ± 0.46| 1.97 ± 0.28|
| γH202A        | 14                | 1.59 ± 0.03| 5.94 ± 0.27| 3.69 ± 0.02|
| γH200A/γH202A | 15                | 1.50 ± 0.04| 6.29 ± 0.56| 3.22 ± 0.36|
| γH202A        | 9                 | 1.18 ± 0.02| 3.03 ± 0.77| 1.09 ± 0.24|
| γH282A        | 10                | 1.69 ± 0.09| 1.79 ± 0.31| 1.61 ± 0.21|
| γH282A        | 5                 | 2.20 ± 0.09| 2.11 ± 0.31| 1.31 ± 0.10|
| γH338A        | 6                 | 1.88 ± 0.06| 2.02 ± 0.37| 3.10 ± 0.63|
| γH364A        | 6                 | 1.74 ± 0.07| 2.44 ± 0.25| 1.68 ± 0.28|
| γH434A        | 12                | 1.61 ± 0.07| 1.91 ± 0.24| 1.71 ± 0.18|
| γH200A/γH202A | 9                 | 1.45 ± 0.03| 6.74 ± 0.52| 2.46 ± 0.36|
| γH193A/γH200A/γH202A | 6 | 1.48 ± 0.03| 6.41 ± 0.48| 1.34 ± 0.23|
| γH88A/γH200A/γH202A | 6 | 1.81 ± 0.11| 20.4 ± 3.00| >1000       |

*The values were significantly different from the WT values in the same batch of oocytes (p < 0.01).*

**FIGURE 6. Effects of Zn\(^{2+}\) on double and triple γHis mutations.** Representative traces are from oocytes expressing αβγH200A/γH202A (A; n = 9), αγH193A/γH200A/γH202A (B; n = 8), and αβγH88A/γH200A/γH202A (C; n = 6) mENaCs. Experiments were performed as described in the legend to Fig. 5. Curve fittings in D and E were carried out as described in the legend to Fig. 5. Derived parameters in D were as follows: I\(_{\text{max}}\) = 1.82, EC\(_{50}\) = 2.1 μM, and IC\(_{50}\) = 2.7 mm for WT; I\(_{\text{max}}\) = 1.45, EC\(_{50}\) = 6.5 μM, and IC\(_{50}\) = 2.3 mm for γH200A/γH202A; and I\(_{\text{max}}\) = 1.48, EC\(_{50}\) = 6.5 μM, and IC\(_{50}\) = 1.2 mm for γH193A/γH200A/γH202A. Parameters in E were as follows: I\(_{\text{max}}\) = 1.79, EC\(_{50}\) = 2.4 μM, and IC\(_{50}\) > 1 m for γH88A and I\(_{\text{max}}\) = 1.72, EC\(_{50}\) = 18.0 μM, and IC\(_{50}\) > 1 m for γH88A/γH200A/γH202A. Parameters from individual oocytes are shown in Table 1.
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FIGURE 7. Na\(^+\) self-inhibition-enhancing mutation \(\alpha G481A\) “restores” Zn\(^{2+}\) activation that is diminished by \(\gamma H239A\). A, representative recording in oocytes expressing \(\alpha G481A\)/\(\gamma mENaC\) \((n = 6)\). B, representative recording of \(\alpha G481A\!/\(\beta,\gamma \gamma h239A\) mENaC \((n = 6)\). C, dose-response curves. Fitting parameters were as follows: \(I_{\text{max}} = 1.80\), EC\(_{50} = 2.9\ \mu \text{A}\), and IC\(_{50} = 1.7\ \mu \text{mM}\) for WT; \(I_{\text{max}} = 3.4,\ EC_{50} = 9.6\ \mu \text{mM}\), and IC\(_{50} = 1.8\ \mu \text{mM}\) for \(\alpha G481A\beta\gamma\gamma h239A\); and \(I_{\text{max}} = 3.7,\ EC_{50} = 4.2\ \mu \text{mM}\), and IC\(_{50} = 1.2\ \mu \text{mM}\) for \(\alpha G481A\beta\gamma\h239A\).

present. Considered together, these results are consistent with the idea that \(\gamma H200A/\gamma H202A\) reduces the apparent Zn\(^{2+}\) binding affinity for the activating site without altering its efficacy. The inhibitory site containing \(\gamma H225\) limits the efficacy of channel activation by Zn\(^{2+}\).

\(\gamma H225\) Does Not participate in Zn\(^{2+}\) Binding—The \(\gamma H225\) mutation eliminated both Na\(^+\) self-inhibition and Zn\(^{2+}\) activation of ENaC (Figs. 3–5). As Zn\(^{2+}\) activates ENaC through relief of Na\(^+\) self-inhibition, it is unclear whether \(\gamma H225\) is a Zn\(^{2+}\)-binding site similar to the other finger subdomain His residues or whether the \(\gamma H225\) mutation simply prevents channel inhibition by external Na\(^+\). To distinguish between these two possibilities, additional mutations were added to restore Na\(^+\) self-inhibition in the setting of the \(\gamma H225\) mutant. Previously, we found that the G481A mutation within the \(\alpha\) subunit greatly enhanced the Na\(^+\) self-inhibition response (39). Channels bearing both the \(\alpha G481A\) and \(\gamma H225\) mutations exhibited a robust Na\(^+\) self-inhibition response and a biphatic response to Zn\(^{2+}\) (Fig. 7). Both \(\alpha G481A\) and \(\alpha G481A/\gamma H225\) channels had \(I_{\text{max}}\) values that were significantly greater than the WT values (3.4 \pm 0.2 \((n = 6)\) and 3.7 \pm 0.2 \((n = 6)\), respectively), presumably as a result of the enhanced Na\(^+\) self-inhibition. The EC\(_{50}\) for Zn\(^{2+}\) activation of \(\alpha G481A/\gamma H225\) was similar to that of WT (4.2 \pm 0.5 \mu M \((n = 6)\) versus WT; \(p > 0.05\)). Thus, \(\gamma H225\) did not weaken the apparent Zn\(^{2+}\) affinity of either the high- or low-affinity site.

Region Surrounding \(\gamma H225\) Is a Determinant of the Low-affinity Inhibitory Site for Zn\(^{2+}\).—Next, the importance of the palm subdomain \(\gamma H225\) and surrounding region was examined to explore the nature of the low-affinity Zn\(^{2+}\) inhibitory site. As shown in Figs. 3 and 5, mutation of \(\gamma H225\) to Ala prevented ENaC inhibition by high concentrations of Zn\(^{2+}\). Mutation of \(\gamma H225\) to either Arg or Asp also rendered the Zn\(^{2+}\) dose responses monophasic and did not significantly alter the Na\(^+\) self-inhibition response (Fig. 8, A and B). These results suggest that a His residue at this position is an essential determinant of the Zn\(^{2+}\) inhibitory site.

Zn\(^{2+}\) is often coordinated by multiple neighboring amino acid residues in metal-binding proteins (32). To define additional residues that contribute to the low-affinity Zn\(^{2+}\) inhibitory site, we searched for additional potential Zn\(^{2+}\)-binding residues using sequence alignments of ENaC and ASIC and the resolved cASIC1 structure (40). \(\gamma H225\) aligns with Ala\(^{82}\) in cASIC1, which is located immediately after the first \(\beta\) strand of ECD (\(\beta\)1) within the palm subdomain (Fig. 8C). Multiple regions within the same subunit (strand \(\beta\)1 and loops \(\beta\)1-\(\beta\)2, \(\beta\)11-\(\beta\)12, and \(\beta\)5-\(\beta\)6) and an adjacent subunit (N-terminal end of helix \(\alpha\)4 and loop \(\alpha\)5-\(\beta\)10) are in close proximity to Ala\(^{82}\) in cASIC1. Eleven residues in these regions are within 6 Å of the Ala\(^{82}\) (Fig. 8C). Among these residues, only Ala\(^{81}\) and Met\(^{364}\) have homologous residues in mENaC that are often found within a Zn\(^{2+}\)-coordination shell (\(\gamma A\)365 and \(\beta G\)444, respectively). We examined whether these sites are involved in ENaC regulation by Zn\(^{2+}\). As shown in Fig. 8 (D and E), \(\gamma D516A\) and \(\gamma D516R\) largely prevented ENaC inhibition by high [Zn\(^{2+}\)], similar to what we observed with the \(\gamma H225\) mutant (Fig. 5). Mutation of the other residue that was predicted to be in close proximity to \(\gamma H225\) (\(\beta E444A\)) did not significantly alter the Zn\(^{2+}\) effects on ENaC (data not shown). The proximity of \(\gamma A\)365 to \(\gamma H225\) and their similar mutation-induced changes in the Zn\(^{2+}\) response suggest that the two residues share a functional role in constituting the low-affinity Zn\(^{2+}\) inhibitory site.

The residues corresponding to \(\gamma H225\) and \(\gamma A\)365 in cASIC1 (Ala\(^{82}\) and Ala\(^{413}\)) are located at a subunit interface (Fig. 8C). According to the suggested counterclockwise arrangement (viewed from above) of the ENaC \(\alpha\), \(\beta\), and \(\gamma\) subunits (14, 28), \(\gamma H225\) (Ala\(^{82}\) in cASIC1) and \(\gamma A\)365 (Ala\(^{413}\) in cASIC1) are expected to interact with residues within the thumb subdomain of \(\beta\)ENaC. There are five negatively charged \(\beta\)ENaC residues within loops \(\beta\)9-\(\alpha\)4 and \(\alpha\)5-\(\beta\)10 of the thumb subdomain: \(\beta G\)454, \(\beta A\)365, \(\beta G\)444, \(\beta D\)446, and \(\beta D\)446. Given their locations within flexible loops, they might be in close proximity to \(\gamma H225\) and \(\gamma A\)365 and contribute to the low-affinity inhibitory site. Mutations of these \(\beta\) subunit residues to Ala did not alter the channel’s response to Zn\(^{2+}\) (data not shown). Thus, it is unlikely that these residues within the thumb domain of \(\beta\)ENaC are involved in Zn\(^{2+}\) regulation of the channel.
On the basis of these observations, we propose that residues in the activating site or the inhibitory site, respectively, be selectively eliminated by targeted mutations of specific residues. (i) Mutation of these residues increased the EC50 of Zn2+ activation and had no effect on the IC50 (Fig. 5 and Table 1), suggesting that these residues are specifically involved in Zn2+ binding to an activating site. (ii) Although mutations of γHis193, γHis200, and γHis202 reduced the maximal ENaC current elicited by Zn2+, maximal Zn2+ activation was not affected when the Zn2+ inhibitory site was also mutated (γH88A/γH200A/γH202A). Thus, γH200A/γH202A appears to primarily affect the affinity of the activating site and does not alter the efficacy of channel activation. (iii) γHis193, γHis200, and γHis202 appear to share a similar functional role in mediating Zn2+ effects on the regulation of ENaC by Zn2+.

**DISCUSSION**

The major goal of this study was to probe the structural basis of the regulation of ENaC by Zn2+. The biphasic dose response of Zn2+ on the purinergic receptor P2X is believed to originate from Zn2+ binding at two distinct sites within the receptor: a high-affinity activating site and a low-affinity inhibitory site (41). Because the ENaC/degenerin family is structurally similar to P2X (40), we hypothesized that the bell-shaped Zn2+ dose-response relationship with ENaC activity is due to the presence of a high-affinity activating site and a low-affinity inhibitory site within the ECD. We found that a minimal two-site model sufficiently describes the bell-shaped dose response of mouse ENaC to external Zn2+. The presence of a high-affinity activating site and a low-affinity inhibitory site is supported by our observations that Zn2+-mediated activation or inhibition can be selectively eliminated by targeted mutations of specific residues in the activating site or the inhibitory site, respectively. On the basis of these observations, we propose that γHi 193, γH88, and γH202 within the finger subdomain contribute to a high-affinity Zn2+ binding site and that γHi 193 and γAsp516 in the palm subdomain are key determinants of a low-affinity Zn2+ binding site (Fig. 9).

The following observations suggest that γHi 193, γHi 200, and γHi 202, presumably within the finger subdomain, contribute to a high-affinity Zn2+ activating site. (i) Mutation of these residues increased the EC50 of Zn2+ activation and had no effect on the IC50 (Fig. 5 and Table 1), suggesting that these residues are specifically involved in Zn2+ binding to an activating site. (ii) Although mutations of γHi 193, γHi 200, and γHi 202 reduced the maximal ENaC current elicited by Zn2+, maximal Zn2+ activation was not affected when the Zn2+ inhibitory site was also mutated (γH88A/γH200A/γH202A). Thus, γH200A/γH202A appears to primarily affect the apparent affinity of the activating site and does not alter the efficacy of channel activation. (iii) γHi 193, γHi 200, and γHi 202 appear to share a similar functional role in mediating Zn2+ effects on the regulation of ENaC by Zn2+.
the channel. Individual mutation of any of the three His residues induced a similar phenotype, including an increase in EC_{50} and a lack of effect on Na^+ self-inhibition. Moreover, mutation-induced changes in EC_{50} were not additive in γHiS193, γHiS200, and γHiS202 (Fig. 9). Further studies are needed to identify additional sites involved in Zn^{2+} activation of ENaC.

Our results demonstrate that γHiS^{88} within the palm subdomain is a key determinant for the low-affinity inhibitory Zn^{2+}-binding site. Interestingly, like the three finger subdomain His residues, γHiS^{88} is also not conserved among ENaC subunits (Fig. 9). Taking advantage of the structural information of ASIC1, a negatively charged residue, γAsp^{516}, was identified that lies in close proximity to γHiS^{88} and is likely involved in the inhibitory effect of Zn^{2+} as well (Fig. 9). However, it is also possible that mutations of γHiS^{88} and γAsp^{516} may diminish the inhibitory component in the biphasic Zn^{2+} dose response by disrupting conformational changes induced by Zn^{2+} binding, instead of abolishing Zn^{2+} binding. Nevertheless, our observations suggest that this region (loops β1-β2 and β11-β12), located at the middle of the palm subdomain, contributes to a general inhibitory site for different channel regulators (Fig. 9). Indeed, a putative β1-β2-binding site and a Cu^{2+}-binding site have been identified near this region in human ENaCs (14, 28). The corresponding region in ASIC1 has been implicated in H^+ sensing and desensitization (27, 45–47). This solvent-accessible region appears to be an excellent target for the development of therapeutic agents that modulate activities of these related channels.

The activating effect of Zn^{2+} on ENaC depends on the presence of Na^+ self-inhibition (Figs. 3–5) (25). External Zn^{2+} may disrupt either Na^+ binding to its receptor site or Na^{2+}-induced conformational changes. Our observations that mutations at the implicated Zn^{2+}-binding sites (γHiH9251, γHiH9252, and γHiH9253) did not alter the Na^+ self-inhibition response suggest that the binding sites for Zn^{2+} and Na^+ are distinct. The lack of effect on the apparent affinity for Zn^{2+} activation of a mutation of a critical site for Na^+ self-inhibition (γHiH9239A) also supports the notion of distinct Zn^{2+}- and Na^{2+}-binding sites. Therefore, it is likely that Zn^{2+} binding interferes with Na^+ induced motions within the ECD. Similar to Zn^{2+}, several extracellular factors such as protons, Cl^-, H^+, and small molecules are thought to regulate ENaC gating in part by altering the Na^+ self-inhibition response (13, 15, 28, 36, 48). It would be of great interest to determine whether other extracellular regulators affect Na^+ self-inhibition in a similar manner to Zn^{2+}.

Recent studies suggest that ENaCs may belong to ligand-gated channels, like other members of the ENaC/degenerin family (25, 26, 49). Multiple external regulators of ENaC, including transition metals, Cl^-, H^+, and other small molecules, exert their effects on ENaC gating by binding to regions within the ECD that are structurally distant from the channel pore (13–15, 28). These gating regulators are expected to remotely influence the channel gate through a series of conformational changes. The identification of these allosteric regulatory sites will advance our understanding regarding mechanisms by which the binding of allosteric regulators alters channel gating.

In summary, this study provides novel insights into the structural basis for Zn^{2+} regulation of ENaC. Our results suggest that external Zn^{2+} regulates ENaC activity by binding to mul-
multiple extracellular sites, including a high-affinity activating site in the finger subdomain and a low-affinity inhibitory site in the palm subdomain of the γ-subunit. These findings advance our understanding of the regulation of ENaC gating by extracellular factors.

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