Extracellular Zn$^{2+}$ Activates Epithelial Na$^+$ Channels by Eliminating Na$^+$ Self-inhibition*  

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Inhibition of epithelial Na$^+$ channel (ENaC) activity by high concentrations of extracellular Na$^+$ is referred to as Na$^+$ self-inhibition. We investigated the effects of external Zn$^{2+}$ on whole cell Na$^+$ currents and on the Na$^+$ self-inhibition response in Xenopus oocytes expressing mouse αβγ ENaC. Na$^+$ self-inhibition was examined by analyzing inward current decay from a peak current to a steady-state current following a fast switching of a low Na$^+$ (1 mM) bath solution to a high Na$^+$ (110 mM) solution. Our results indicate that external Zn$^{2+}$ rapidly and reversibly activates ENaC in a dose-dependent manner with an estimated EC$_{50}$ of 2 μM. External Zn$^{2+}$ in the high Na$^+$ bath also prevents or reverses Na$^+$ self-inhibition with similar affinity. Zn$^{2+}$ activation is dependent on extracellular Na$^+$ concentration and is absent in ENaCs containing αH239 mutations that eliminate Na$^+$ self-inhibition and in oocytes expressing αS880Cβγ following covalent modification by a sulfhydryl-reactive reagent that locks the channels in a fully open state. In contrast, external Ni$^{2+}$ inhibition of ENaC currents appears to be additive to Na$^+$ self-inhibition when Ni$^{2+}$ is present in the high Na$^+$ bath. Pretreatment of oocytes with Ni$^{2+}$ in a low Na$^+$ bath also prevents the current decay following a switch to a high Na$^+$ bath but rendered the currents below the control steady-state level measured in the absence of Ni$^{2+}$ pretreatment. Our results suggest that external Zn$^{2+}$ activates ENaC by relieving the channel from Na$^+$ self-inhibition, and that external Ni$^{2+}$ mimics or masks Na$^+$ self-inhibition.

ENTaC activity is regulated by a variety of both intracellular and extracellular factors, including selected hormones, cations, enzymes, and other channel proteins (3, 4). Na$^+$ exhibits two types of inhibitory effects on ENaC activity: self-inhibition and feedback inhibition that are due to increases in either extracellular or intracellular Na$^+$ concentration, respectively. These regulatory phenomena have been proposed to provide a mechanism to prevent sudden or excessive increases in intracellular Na$^+$ concentration (5, 6).

Although most studies on Na$^+$ self-inhibition have utilized native Na$^+$-transporting tissues, including frog skin, toad urinary bladder, and kidney collecting tubule, Na$^+$ self-inhibition has also been observed in Xenopus oocytes expressing ENaCs (7–9). Extracellular cations have been reported to affect ENaC activity in native tissues that may reflect changes in Na$^+$ self-inhibition (3, 5). We and others previously reported that external Ni$^{2+}$ blocks whole cell currents of αβγ ENaCs expressed in oocytes (10, 11). In contrast, external Ni$^{2+}$ is reported to stimulate Na$^+$ currents in A6 cells by relieving ENaC from Na$^+$ self-inhibition (12). Recent work suggested that external Zn$^{2+}$ is a voltage-dependent blocker of ENaC (13). In this report, we examined the effects of external Zn$^{2+}$ on amiloride-sensitive Na$^+$ currents in oocytes expressing αβγ mouse ENaC (mENaC), and the effects of external Zn$^{2+}$ and Ni$^{2+}$ on Na$^+$ self-inhibition of αβγ mENaC. We report that external Zn$^{2+}$ reversibly activates ENaC by eliminating Na$^+$ self-inhibition, whereas external Ni$^{2+}$ appears to mimic Na$^+$ self-inhibition.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—All ENaC clones used in this study are mouse ENaC subunits whose cDNAs were inserted into pBluescript SK- (Stratagene, La Jolla, CA) (14). Point mutations were generated previously by using a PCR-based method (11). ENaC Expression and Two-electrode Voltage Clamp—ENaC expression in Xenopus oocytes and two-electrode voltage clamp were performed as previously reported (11). Stage V and VI oocytes free of follicle cell layers were injected with 1–4 ng of cRNA for each mENaC subunit per oocyte and incubated at 18 °C in modified Barth's saline (MBS, 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO$_3$, 15 mM HEPES, 0.3 mM Ca(NO$_3$_)$_2$, 0.41 mM CaCl$_2$, 0.82 mM MgSO$_4$, 10 μM/ml sodium penicillin, 10 μg/ml streptomycin sulfate, 100 μg/ml gentamycin sulfate, pH 7.4). All experiments were performed at room temperature (20–24 °C). Oocytes were continuously clamped at −60 or −100 mV in most experiments. Current-voltage relationships were determined by clamping oocytes at holding potentials in the range of −140 to 60 mV in 20-mV increments.

The responses of Na$^+$ self-inhibition were examined as previously reported (7, 9). A current decay from a peak current to a relatively steady-state current was considered the response for Na$^+$ self-inhibition. The current decay was initiated by rapidly replacing a low Na$^+$ bath solution (NaCl-I: containing 1 mM NaCl, 109 mM N-methyl-D-glucamine, 2 mM KCl, 2 mM CaCl$_2$, 10 mM HEPES, pH 7.4) with a high Na$^+$ bath solution (NaCl-II: containing 110 mM NaCl, 2 mM KCl, 2 mM CaCl$_2$, 10 mM HEPES, pH 7.4). Rapid solution exchange was performed with a 6-channel Teflon valve perfusion system from Warner Instruments (Hamden, CT). At the end of an experiment, 10 μM amiloride was added.
added to the bath to obtain the amiloride-insensitive current. Whole cell currents in the presence of 10 μM amiloride were generally less than 200 nA at −60 or −100 mV. Results from oocytes that showed unusually large amiloride-insensitive currents (>5% of total currents) were discarded to minimize current contamination from endogenous channels and membrane leak. The Na⁺ self-inhibition response was described with two parameters, a time constant (τ) and the ratio of the steady-state current (I_{ss}) and the peak current (I_{peak}) (see Ref. 9).

The time course of Na⁺ self-inhibition and the effects of Zn²⁺ on Na⁺ currents on this process were analyzed as previously described (9). Briefly, the first 40 s of current decay (or increase) were fitted with an exponential equation by Clampfit 9.0 (Axon Instruments Inc.). The concentration at which half-maximal effects were observed (EC_{50}) was estimated by non-linear least square curve-fitting of the dose response data with the Hill equation: \( R = C + (C - C_{\text{MAX}})/\left(1 + (I/I_{\text{E}0})^n\right) \), in which I is the relative I_{peak} or I_{ss} and C refers to the Na⁺ concentration used to initiate self-inhibition. The apparent inhibitory constant (K_I) of Na⁺ self-inhibition was estimated from a best fitting of the data with the equation: \( J/J_{\text{max}} - K_I/C + K_I/C_{\text{MAX}} \), in which C and n are the concentration of Na⁺ and Hill coefficient, respectively.

Superpure NiCl₂ and ZnCl₂ (>99.99%) were purchased from Sigma-Aldrich and dissolved at 1 M in water and diluted to the desired concentrations in bath solutions. The addition of NiCl₂ or ZnCl₂ to the bath solutions at the highest concentrations (1 mM for NiCl₂, 5 mM for ZnCl₂) used in this study did not alter the pH of the solutions or form precipitates.

Statistical Analysis—Data are presented as mean ± S.E. Significance comparisons between groups were performed using Student's t test. Curve fittings were performed with Clampfit 9.0 (Axon Instruments Inc., Union City, CA) and Origin Pro 7.0 (OriginLab Corp., Northampton, MA).

RESULTS

External Zn²⁺ Activates αβγ mENaC Expressed in Xenopus Oocytes—The effect of extracellular Zn²⁺ on αβγ mENaC expressed in Xenopus oocytes was examined by comparing amiloride-sensitive Na⁺ currents prior to and following the addition of ZnCl₂ into the bath solution. Oocytes expressing wild type (WT) αβγ mENaC were clamped from a holding potential (V_{HOLD}) to −60 mV in NaCl-110 NaCl-110 with 100 mM ZnCl₂, and NaCl-110 with 100 μM amiloride were blocked completely by 10 μM amiloride. Similar magnitude of the stimulation of the current was observed with repetitive applications of Zn²⁺ without an obvious decline in its effect.

Xenopus oocytes express several types of endogenous channels that may conduct Na⁺ (15–18). To exclude the possibility that the observed increase in whole cell currents by extracellular Zn²⁺ was due to activation of an endogenous channel, we examined the effect of 100 μM ZnCl₂ on the whole cell currents in H₂O-injected oocytes. The currents measured from six oocytes at −100 mV in NaCl-110 NaCl-110 with 100 μM ZnCl₂, and NaCl-110 with 10 μM amiloride were −66.7 ± 27.9, −50.0 ± 12.9, and −83.2 ± 16.7 nA (p > 0.05), respectively, suggesting that 100 μM external Zn²⁺ does not activate endogenous currents. Furthermore, no changes in whole cell currents were observed in oocytes expressing αβγ mENaC when 10 μM ZnCl₂ was externally applied in the presence of 10 μM amiloride. The relative currents (i.e. currents normalized to values in the absence of amiloride) in the presence of amiloride alone and amiloride with Zn²⁺ were 1.9 ± 0.6 and 1.7 ± 0.3% (n = 6), respectively (p > 0.05). The results suggest that the stimulatory effect of external Zn²⁺ on currents in oocytes expressing ENaC results from activation of ENaC. These findings differ from the reported blocking effect of external Zn²⁺ in oocytes expressing αβγ rat ENaCs and in A6 cells that express endogenous ENaC (13).

External Zn²⁺ Eliminates Na⁺ Self-inhibition—The stimulation of the amiloride-sensitive whole cell currents by extracellular Zn²⁺ may result from an increase in unitary current, open probability, or number of active channels in oocyte membranes. Because the extracellular Na⁺ concentration was constant and Zn²⁺ did not change the reversal potentials (Fig. 1B), it is unlikely that Zn²⁺ had a significant effect on the driving forces for generating the whole cell currents. The rapid time course of Zn²⁺ activation and recovery following washout of Zn²⁺ suggest a direct effect of Zn²⁺ on single channel properties rather than a change in surface channel density. Several extracellular divalent cations are known to stimulate Na⁺ transport in model epithelia such as frog skin and toad bladder, possibly through interfering with Na⁺ self-inhibition (5). To investigate the mechanism of ENaC activation by external Zn²⁺, we examined whether extracellular Zn²⁺ altered the Na⁺ self-inhibition response of αβγ mENaC expressed in Xenopus oocytes. Na⁺ self-inhibition was studied by monitoring changes in whole cell Na⁺ currents measured at either −60 or −100 mV by two-electrode voltage clamp during a rapid increase in extracellular Na⁺ concentration. We previously reported that αβγ mENaCs exhibit a Na⁺ self-inhibition response that is similar to that of rat, human, and Xenopus αβγ ENaC (7–9). The effects of external Zn²⁺ on Na⁺ self-inhibition were studied with two sets of experiments. First, we examined the effect of the presence of Zn²⁺ in the high Na⁺ bath solution (NaCl-110). A typical response for Na⁺ self-inhibition of αβγ mENaC is shown in Fig. 2A. The current reached a maximal level (termed as I_{peak}) and declined to a relatively steady level (termed as I_{ss}).
following a fast solution exchange that increased the extracellular Na\textsuperscript{+} concentration from 1 to 110 mM. The current decay reflects the Na\textsuperscript{+} self-inhibition response. After successful observation of a typical response of Na\textsuperscript{+} currents expressed αβγ mENaC, we examined the self-inhibition response when the low Na\textsuperscript{+} solution (NaCl-I) was replaced by the NaCl-110 solution containing 100 μM ZnCl\textsubscript{2}. No current decay was observed, and the current remained at a level slightly higher than the I\textsubscript{peak} observed in the previous test, as shown in Fig. 2A. The Zn\textsuperscript{2+}-activated currents were completely blocked by 10 μM amiloride or by reducing bath Na\textsuperscript{+} to 1 mM (Fig. 2, A and B), indicating that all the currents in the presence of Zn\textsuperscript{2+} were conducted through ENaC. These results indicate that external Zn\textsuperscript{2+} at 100 μM completely prevented Na\textsuperscript{+} self-inhibition. This Zn\textsuperscript{2+} effect was also reversible, because washout of Zn\textsuperscript{2+} quickly restored the typical self-inhibition response (Fig. 2A). We also examined whether the addition of Zn\textsuperscript{2+} was able to reverse current inhibition by extracellular Na\textsuperscript{+} by adding 100 μM Zn\textsuperscript{2+} to NaCl-110 when the currents reached a steady-state level following the Na\textsuperscript{+} self-inhibition response. As seen in Fig. 2C, Zn\textsuperscript{2+} increased the current to a level near the I\textsubscript{peak} observed in the absence of Zn\textsuperscript{2+}, and washout of Zn\textsuperscript{2+} returned the current to the inhibited level. Therefore, external Zn\textsuperscript{2+} not only prevents Na\textsuperscript{+} self-inhibition, it also reverses Na\textsuperscript{+} self-inhibition.

In the second set of experiments, we examined whether pretreatment of oocytes expressing αβγ mENaC with Zn\textsuperscript{2+} affected Na\textsuperscript{+} self-inhibition. Oocytes were perfused with NaCl-I containing 100 μM ZnCl\textsubscript{2} and then perfused with NaCl-110 without ZnCl\textsubscript{2}. A current decay was observed with an elevated I\textsubscript{peak} and an unchanged I\textsubscript{leak} compared with the values in the control test (Fig. 2D). Although Zn\textsuperscript{2+} added at the same time as high concentration of Na\textsuperscript{+} was sufficient to prevent Na\textsuperscript{+} from causing the self-inhibition, Zn\textsuperscript{2+} pretreatment was unable to prevent Na\textsuperscript{+} self-inhibition (Fig. 2). To estimate the apparent affinity for Zn\textsuperscript{2+} elimination of

**Fig. 1. External Zn\textsuperscript{2+} activates αβγ mENaC.** A, representative recordings of whole cell currents in the absence, presence, and after washout of 100 μM ZnCl\textsubscript{2} in NaCl-110. The oocyte expressing αβγ mENaC was clamped from −140 to 60 mV in 20-mV increments. The currents recorded in the presence of 10 μM amiloride in bath solution NaCl-110 are shown on the right. The recording is representative of 15 experiments. B, current-voltage (I-V) relationship curves were obtained by plotting the currents measured at 0.4 s following the initiation of the voltage step from recordings in (A) against clamping voltages. C, dose-response curve of the Zn\textsuperscript{2+} effect. Relative currents are the ratios of amiloride-sensitive Na\textsuperscript{+} currents measured at −60 mV in the presence of increasing concentrations of ZnCl\textsubscript{2} in NaCl-110, relative to the amiloride-sensitive currents measured prior to addition of ZnCl\textsubscript{2}. D, re-plot of the Zn\textsuperscript{2+} dose-response data in C to determine the half-maximal effective concentration (EC\textsubscript{50}). The relative responses represent the increases in current in response to 10\textsuperscript{-8}, 10\textsuperscript{-7}, 10\textsuperscript{-6}, 10\textsuperscript{-5}, or 10\textsuperscript{-4} M Zn\textsuperscript{2+}, normalized to the current increase observed with 10\textsuperscript{-4} M Zn\textsuperscript{2+} at which maximal Zn\textsuperscript{2+} activation was observed. The curve is a best fit of the data by non-linear least-square fitting with a Hill equation: Response = C\textsuperscript{n} / (C\textsuperscript{n} + EC\textsubscript{50}\textsuperscript{n}), where “Response” represents the relative response and “n” is the Hill coefficient. The fitting parameters are: EC\textsubscript{50} 1.74 μM; Hill coefficient, 0.77; and correlation coefficient (R\textsuperscript{2}), 0.9954. E, time course of Zn\textsuperscript{2+} activation. An αβγ mENaC-expressing oocyte was bathed in NaCl-110 solution containing 100 μM ZnCl\textsubscript{2}. The arrow indicates the period of time when the oocyte was perfused with NaCl-110 containing 100 μM ZnCl\textsubscript{2}. The gray bar indicates the period of time when the oocyte was perfused with NaCl-110 containing 100 μM ZnCl\textsubscript{2}. The trace is representative of six experiments.
Na\(^{+}\) self-inhibition, we examined Na\(^{+}\) self-inhibition responses in the presence of increasing concentrations of ZnCl\(_2\) in the NaCl-110 solution (Table I). A typical experiment is shown in Fig. 3A. The effect of external Zn\(^{2+}\) on preventing Na\(^{+}\) self-inhibition was dose-dependent (Fig. 3B and Table I). The estimated EC\(_{50}\) for Zn\(^{2+}\) was 1.3 \(\mu\)M by a best fitting of the dose-response relationship (Fig. 3B, inset). The EC\(_{50}\) value was almost identical to that obtained from analysis of the Zn\(^{2+}\) dose-response curve for activation of ENaC currents (Fig. 1D), suggesting a link between ENaC activation and loss of Na\(^{+}\) self-inhibition by external Zn\(^{2+}\).

The Effect of External Zn\(^{2+}\) on ENaC Is Dependent on the Extracellular Na\(^{+}\) Concentration—External Zn\(^{2+}\) may eliminate Na\(^{+}\) self-inhibition by three possible mechanisms: (i) interfering with Na\(^{+}\) binding to a “receptor,” (ii) preventing conformational changes induced by Na\(^{+}\) binding, or (iii) locking ENaC in a fully open state that is insensitive to regulation by extracellular Na\(^{+}\). Na\(^{+}\) self-inhibition is considered a low affinity event with an estimated inhibitory constant of \(>100\) mM (7, 9). To determine whether Zn\(^{2+}\) and Na\(^{+}\) are binding to a common site, we analyzed the changes in the Na\(^{+}\) concentration-current relationship by low concentrations of Zn\(^{2+}\). The Na\(^{+}\) concentration-current relationships for the peak and steady-state currents were examined in the presence of 1 and 10 \(\mu\)M of ZnCl\(_2\). In the presence of 1 and 10 \(\mu\)M Zn\(^{2+}\), the estimated \(K_m\) values for \(I_p\) were significantly higher than the values obtained in the absence of Zn\(^{2+}\) (\(p < 0.01\) or 0.001), whereas the \(K_m\) values for \(I_{\text{peak}}\) were not significantly different from the control value (Table I). The apparent inhibitory constants (\(K_i\)) for Na\(^{+}\) self-inhibition in the presence of 1 or 10 \(\mu\)M Zn\(^{2+}\) were also significantly higher than the apparent \(K_i\) in the absence of Zn\(^{2+}\). As shown in Fig. 4A, Zn\(^{2+}\) shifted the relationship between \(I_{\text{peak}}/I_p\) and Na\(^{+}\) concentrations to the right. A complete analysis of the shift is precluded due to limitations of further increases of the extracellular Na\(^{+}\) concentration. These data suggest that Zn\(^{2+}\) and Na\(^{+}\) may compete for a common binding site.

Our results indicate that external Zn\(^{2+}\) activates ENaC by eliminating Na\(^{+}\) self-inhibition, a process that is dependent on the extracellular Na\(^{+}\) concentration. If extracellular Na\(^{+}\) self-inhibition is below the minimal concentration causing Na\(^{+}\) self-inhibition, Zn\(^{2+}\) activation should be abolished. We examined the effect of Zn\(^{2+}\) on ENaC currents in oocytes that were expressing \(\alpha \beta \gamma\) mENaCs and bathed in a low Na\(^{+}\) concentration solution (10 mM). External Zn\(^{2+}\) did not significantly alter amiloride-sensitive Na\(^{+}\) currents in oocytes that had been incubated in either regular Na\(^{+}\) MBS (88 mM) or low Na\(^{+}\) MBS (10 mM) following cRNA injections (Fig. 4B). The MBS solution with a low Na\(^{+}\) concentration was used to prevent Na\(^{+}\) loading of oocytes in the period following the injection of ENaC cRNAs and preceding the voltage clamp experiments, as Amuzescu et al. had reported Zn\(^{2+}\)-dependent block of ENaC in oocytes that were maintained in a low Na\(^{+}\) concentration bath prior to functional assays (13). We did not observe Zn\(^{2+}\)-dependent inhibition of ENaC currents. The effects of external Zn\(^{2+}\) were also examined in studies performed with the membrane voltage held at \(-100\) mV while continuously monitoring the whole cell current. Increasing bath Na\(^{+}\) concentration from 1 to 10 mM led to no obvious current decay, as we previously reported (9). The addition and washout of 10 \(\mu\)M Zn\(^{2+}\) did not significantly affect the currents following an increase of the bath Na\(^{+}\) concentration from 1 mM to 10 mM (Fig. 4, C and D).

Mutations at \(\gamma\)H239 That Disrupt Na\(^{+}\) Self-Inhibition Diminish Zn\(^{2+}\) Activation—We recently reported that mutations of a His residue within the extracellular loop (ECL) of \(\gamma\) mENaC (\(\gamma\)H239R, \(\gamma\)H239D, and \(\gamma\)H239C) eliminated Na\(^{+}\) self-inhibition (9). If the stimulatory effect of external Zn\(^{2+}\) depends on the presence of Na\(^{+}\) self-inhibition, Zn\(^{2+}\) should have no effect on channels with \(\gamma\)H239 mutations. No current decay was observed in oocytes expressing \(\alpha \beta \gamma\)H239R following a fast increase in bath Na\(^{+}\) concentration to 110 mM in the absence or...
Characterizations of Na⁺ self-inhibition in the absence and presence of external Zn²⁺ or Ni²⁺

The time constants (τ) for Na⁺ self-inhibition were obtained by exponential fitting of the current decay at the clamping voltage of −60 mV following a rapid increase of bath Na⁺ concentrations from 1 to 110 mM. Both Ipeak and Ias are amiloride-sensitive inward Na⁺ currents expressed as negative values by convention and are not compared due to different batches of oocytes used in the experiments. Fitting for 100 μM Zn²⁺ and 1 mM Ni²⁺ pretreatment failed to generate a time constant due to a absence of current decay. Zn²⁺ and Ni²⁺ were added to either the NaCl-110 solution or the NaCl-1 solution (pretreatment).

| Experiment | Oocytes | t | Ipeak | Ias | Ias/Ipeak |
|------------|---------|---|--------|-----|-----------|
| Control    | 25      | s | −7.2 ± 0.8 | −4.9 ± 0.6 | 0.66 ± 0.02 |
| 1 μM Zn²⁺  | 13      | 11 | −6.4 ± 1.0 | −5.1 ± 0.8 | 0.81 ± 0.01 |
| 10 μM Zn²⁺ | 11      | 19.0 ± 2.5 | −8.7 ± 1.1 | −7.9 ± 1.0 | 0.91 ± 0.02 |
| 100 μM Zn²⁺| 10      | ND | −4.9 ± 0.8 | −4.7 ± 0.7 | 0.97 ± 0.00 |
| 100 μM Zn²⁺pretreatment | 8 | 12.2 ± 1.0 | −5.6 ± 0.8 | −2.9 ± 0.6 | 0.49 ± 0.05 |
| 1 mM Ni²⁺  | 6       | 4.5 ± 0.5 | −3.2 ± 0.8 | −1.5 ± 0.4 | 0.43 ± 0.03 |
| 1 mM Ni²⁺pretreatment | 7 | ND | −1.1 ± 0.4 | −1.1 ± 0.3 | 1.03 ± 0.05 |

Table I

Values are p < 0.001 from Student’s t test between control and other groups.

Values are p < 0.01 from Student’s t test between control and other groups.

Values are p < 0.05 from Student’s t test between control and other groups.

Fig. 3. Dose-dependent effect of Zn²⁺ on Na⁺ self-inhibition. A, a typical recording shows Na⁺ self-inhibition responses in the presence of 1, 10, or 100 μM ZnCl₂ in NaCl-110. A control Na⁺ self-inhibition response was performed before the Zn²⁺ effect was examined. Na⁺ concentrations are indicated as open (1 μM) or black bars (110 μM). The trace is representative of experiments performed in six oocytes. B, dose-response curve of Zn²⁺ effect on Na⁺ self-inhibition. The maximal inward currents measured following quick increase of bath Na⁺ from 1 to 110 mM are referred to as the peak current (Ipeak), and the current measured at 40 s after the Ipeak measurement was referred to as the steady state current (Ias). The Ias/Ipeak was used as a parameter to describe the magnitude of Na⁺ self-inhibition. A value of 1 indicates a lack of self-inhibition. The inset shows a best fitting of the mean relative response versus Zn²⁺ concentrations with the Hill equation to estimate the EC₅₀. The relative response was the difference between the mean Ias/Ipeak values in the presence of 100 μM ZnCl₂ (which eliminated Na⁺ self-inhibition) and the absence of Zn²⁺. The fitting parameters are shown in the inset.

presence of 100 μM Zn²⁺ (Fig. 5A). In experiments using the same protocol as in Fig. 1C for WT ENaCs, whole cell amiloride-sensitive Na⁺ currents in oocytes expressing αβγH239R also remained constant in the presence of increasing concentrations of external Zn²⁺ (Fig. 5B). These data suggest that external Zn²⁺ activation of ENaC depends on the presence of Na⁺ self-inhibition. In contrast to the effect of γH239 mutations, we previously reported that αH282Dβγ channels show an enhanced Na⁺ self-inhibition response (9). Addition of Zn²⁺ to the NaCl-110 solution blunted, but did not eliminate Na⁺ self-inhibition in oocytes expressing αH282Dβγ mENaCs (Fig. 5C). The Ias was much higher than the Ias observed in the absence of Zn²⁺. Addition of 100 μM ZnCl₂ into high Na⁺ bath significantly increased the Ias following a self-inhibition response but did not fully restore the current to the Ipeak (Fig. 5D). These results suggest that external Zn²⁺ results in a "partial" activation of αH282Dβγ mENaCs, in contrast to a "full" activation of WT.

External Zn²⁺ Has No Effect on ENaCs with a High Open Probability—We previously reported that αS580Cβγ channels following modification by external MTSET have a high open probability and do not exhibit Na⁺ self-inhibition (9, 19). If the stimulatory effect of external Zn²⁺ on ENaC currents is due to an increase in channel open probability, the effect of Zn²⁺ on ENaC currents should be abolished in oocytes expressing αS580Cβγ following MTSET treatment. Fig. 6 shows a typical experiment we performed to test this possibility. Addition of 100 μM ZnCl₂ to the bath solution significantly increased whole cell currents in oocytes expressing αS580Cβγ. External application of 1 mM MTSET caused an irreversible increase in the inward current and removed the typical response for Na⁺ self-inhibition, as we previously reported (9). Subsequent application of 100 μM ZnCl₂ resulted in a very minimal change in the current. These results demonstrate that external Zn²⁺ has a minimal effect on channels with a high intrinsic open probability and suggest that Zn²⁺ activation of ENaC reflects an increase in Pₒ.

External Ni²⁺ and Na⁺ Self-Inhibition—We and others reported that external Ni²⁺ is a blocker of mouse and rat αβγ ENaC expressed in Xenopus oocytes (10, 11). In contrast, transepithelial Na⁺ transport in A6 cells was stimulated by external Ni²⁺, which was proposed to compete with extracellular Na⁺ and relieve ENaC from self-inhibition (12). We examined the effect of external Ni²⁺ on Na⁺ self-inhibition of αβγ mENaCs in oocytes. When 1 mM Ni²⁺ was added only to the high Na⁺ bath solution, the current declined was faster and deeper than the control response in the absence of Ni²⁺ (Fig. 7A and Table I). Furthermore, the peak and steady-state currents were lower than the currents observed in the absence of Ni²⁺. The enhanced current decay following the change to a high Na⁺ bath solution that contained Ni²⁺ may reflect additive effects of...
The current decays were examined in the same oocytes as external Na\(^{+}\) was increased rapidly from 1 mM to 110, 60, 30, 10, or 3 mM. The \(K_m\) and \(K_i\) values were obtained by fitting the \(I_{\text{peak}}\) or \(I_{\text{ss}}\) values against Na\(^{+}\) concentrations with equations described under ‘Experimental Procedures.’

| [Zn\(^{2+}\)] (µM) | Oocytes | \(I_{\text{peak}}\) (µA) | \(I_{\text{ss}}\) (µA) | \(K_m\) | \(K_i\) |
|----------------|--------|-----------------|-----------------|----------|--------|
| 0             | 7      | 42.3 ± 8.3      | 21.9 ± 3.5      | 206.5 ± 16.5 | 1.10 ± 0.04 |
| 1             | 8      | 51.2 ± 3.4      | 35.9 ± 2.2\(^a\) | 665.8 ± 109.6\(^a\) | 0.84 ± 0.05\(^a\) |
| 10            | 7      | 50.6 ± 2.7      | 41.7 ± 2.1\(^b\) | 1118.3 ± 157.8\(^b\) | 0.88 ± 0.06\(^b\) |

\(^a\) \(p < 0.01\) versus control group (0 Zn\(^{2+}\)).

\(^b\) \(p < 0.001\) versus control group (0 Zn\(^{2+}\)).

**Table II**

**Fig. 4. Dependence of the Zn\(^{2+}\) effect on the Na\(^{+}\) concentration.** Zn\(^{2+}\) effects were examined in oocytes expressing αβγ ENaCs. A, the effects of 1 and 10 µM ZnCl\(_2\) on the \(I_{\text{peak}}/I_{\text{ss}}\) ratio representing the amplitude of the Na\(^{+}\) self-inhibition responses initiated by different Na\(^{+}\) concentrations. Numbers of oocytes used in the experiments were 7, 8, and 7 for the 0 Zn\(^{2+}\), 1 µM Zn\(^{2+}\), and 10 µM Zn\(^{2+}\) groups, respectively. B, the dose response of Zn\(^{2+}\) on amiloride-sensitive Na\(^{+}\) currents measured with a 10 mM Na\(^{+}\) bath solution. Oocytes injected with WT αβγ mENaC cRNAs were kept in either regular MBS with 88 mM Na\(^{+}\) (open circle) or low Na\(^{+}\) MBS with 10 mM Na\(^{+}\) and 78 mM N-methyl-D-glucamine (open square). A clamping voltage of −100 mV was used in this experiment. Relative currents have the same meaning as in Fig. 1C. The Zn\(^{2+}\) effects were examined in a low Na\(^{+}\) bath solution (10 mM). Amiloride (100 µM) was used to define amiloride-insensitive currents. Numbers of observations were 5 for the MBS group and 14 for the 10 mM Na\(^{+}\) MBS group. C, a representative recording showing the effect of 10 µM Zn\(^{2+}\) on the steady-state current in a 10 mM Na\(^{+}\) bath solution. The oocyte was clamped to −100 mV. The trace is representative of five experiments. D, a recording showing the effect of 10 µM Zn\(^{2+}\) on Na\(^{+}\) self-inhibition initiated by 10 mM Na\(^{+}\). The oocyte was clamped to −100 mV. After a control self-inhibition response to 10 mM Na\(^{+}\), ZnCl\(_2\) (10 µM) was included in the 10 mM Na\(^{+}\) solution for the second test. During the third test, NaCl-1 was rapidly changed to NaCl-110 to verify that the channels exhibit a typical Na\(^{+}\) self-inhibition response. In C and D, open, dashed, and solid bars indicate 1, 10, or 110 mM Na\(^{+}\) concentrations in the bath, respectively. The gray bar indicates the period in the presence of 10 µM ZnCl\(_2\) in the 10 mM Na\(^{+}\) bath solution.

Na\(^{+}\) self-inhibition and Ni\(^{2+}\) inhibition. In contrast to Zn\(^{2+}\), external Ni\(^{2+}\) did not prevent or reverse Na\(^{+}\) self-inhibition. The \(I_{\text{ss}}\) in NaCl-110 was further blocked by 1 mM NiCl\(_2\) (Fig. 7B). Interestingly, the relative difference (dashed arrow in Fig. 7A) between the initial \(I_{\text{peak}}\) prior to Ni\(^{2+}\) application and the \(I_{\text{ss}}\) in the presence of Ni\(^{2+}\) was similar in magnitude to the relative difference (dashed arrow in Fig. 7B) between the \(I_{\text{peak}}\) prior to Ni\(^{2+}\) and the current following a sequential inhibition by high Na\(^{+}\) and Ni\(^{2+}\). Additional experiments were performed with oocytes expressing αβγ mENaC treated with 1 mM NiCl\(_2\) in the low (1 mM) Na\(^{+}\) bath for 1 min. The bath was then changed to NaCl-110 in the absence of NiCl\(_2\). Almost no current decay was observed, and the steady-state current was reduced to a level lower than the \(I_{\text{ss}}\) before the Ni\(^{2+}\) pretreatment. The typical Na\(^{+}\) self-inhibition response was gradually restored after washing out Ni\(^{2+}\) for a few minutes (Fig. 7C). It appears that Na\(^{+}\) self-inhibition is “masked” by Ni\(^{2+}\) inhibition, which reflects a decrease in ENaC \(P_v\) (10, 11).

We previously observed that αH282Dβγ was not blocked by external Ni\(^{2+}\) but showed enhanced Na\(^{+}\) self-inhibition (9, 11). Interestingly, Ni\(^{2+}\) added in NaCl-110 had no effect on the Na\(^{+}\) self-inhibition response of αH282Dβγ (Fig. 8A), suggesting that Ni\(^{2+}\) inhibition and Na\(^{+}\) self-inhibition may be separate events that share a final pathway leading to a reduced channel \(P_v\). When 1 mM Ni\(^{2+}\) was added only to the low Na\(^{+}\) bath, a slower (\(7.2 ± 1.0\) s, \(n = 5\)), compared with 3.4 ± 0.4 s without Ni\(^{2+}\) pretreatment, \(n = 5\), \(p < 0.05\)) and smaller (\(I_{\text{ss}}/I_{\text{peak}}\) 0.47 ± 0.03, \(n = 5\), compared with 0.27 ± 0.03 without Ni\(^{2+}\) pretreatment, \(n = 5\), \(p < 0.01\)) response of Na\(^{+}\) self-inhibition was observed (Fig. 8A). The smaller magnitude of self-inhibition...
FIG. 5. Zn$^{2+}$ activation is absent in oocytes expressing $\alpha\beta\gamma$ mENaCs with $\gamma$H239 mutations. Open, solid, and gray bars have the same meanings as in other figures. Application of 10 $\mu$M amiloride is indicated by an arrow. All experiments in this figure have been repeated at least six times, and the results are indistinguishable. A, lack of effect of 100 $\mu$M Zn$^{2+}$ on the Na$^+$ self-inhibition response in an oocyte expressing $\alpha\beta\gamma$H239R. B, dose-response curve for external Zn$^{2+}$ effect on amiloride-sensitive Na$^+$ currents measured at −80 mV and normalized to control values in oocytes expressing $\alpha\beta\gamma$H239R. Experiments were performed with the same protocol as for Fig. 1C. A recording with a protocol similar to A in an oocyte expressing $\alpha$H282D$\beta$ mENaC. C, a representative recording shows the effect of 100 $\mu$M Zn$^{2+}$ on the Na$^+$ current following a Na$^+$ self-inhibition response in an oocyte expressing $\alpha$H282D$\beta$.

Fig. 6. Effect of external Zn$^{2+}$ on ENaC is absent when channels are gated open. The tracing is representative of six experiments from oocytes expressing $\alpha$S580C$\beta$.$\gamma$. The oocyte was continuously clamped at −60 mV throughout the recording. Bath Na$^+$ concentrations are indicated by open (1 mM) or solid (110 mM) bars. External applica-
tions of Zn$^{2+}$ are indicated by gray bars, and 1 mM MTSET is indicated by a double-arrowed line.

was obviously due to a reduction of $I_{peak}$, because the $I_{sa}$ was not affected by Ni$^{2+}$ treatment. A reduction in the outward current in the low Na$^+$ bath (Fig. 8A) was also observed. These data suggest that Ni$^{2+}$ inhibits $\alpha$H282D$\beta$.$\gamma$ channels when the extracellular Na$^+$ concentration is low (no self-inhibition) but does not inhibit $\alpha$H282D$\beta$.$\gamma$ when the extracellular Na$^+$ concentration is high (strong self-inhibition). Mutations of $\gamma$H239 led to a loss of Na$^+$ self-inhibition and a partial loss of Ni$^{2+}$ inhibition of ENaC (Fig. 5 and Refs. 9 and 11). Although $\alpha$H282D$\beta$ does not exhibit Na$^+$ self-inhibition or Zn$^{2+}$ activation, the addition of 1 mM Ni$^{2+}$ to the NaCl-110 bath resulted in a modest current decay (Fig. 8B).

DISCUSSION

Our results demonstrate that external Zn$^{2+}$ stimulates amiloride-sensitive Na$^+$ currents in oocytes expressing $\alpha\beta\gamma$ mENaC in a dose-dependent manner in association with a loss of Na$^+$ self-inhibition. The stimulatory effect of Zn$^{2+}$ depends on the extracellular Na$^+$ concentration, a key factor in the Na$^+$ self-inhibition response. Extracellular divalent cations, including Zn$^{2+}$, Cd$^{2+}$, Cu$^{2+}$, and Ni$^{2+}$, have been previously shown to stimulate amiloride-sensitive Na$^+$ currents in Na$^+$-transporting epithelial tissues such as frog skin and toad urinary bladder (5, 20). Although external Ni$^{2+}$ is a blocker of mouse and rat ENaCs expressed in Xenopus oocytes (10, 11), extracellular Ni$^{2+}$ was found to stimulate amiloride-sensitive short-circuit current in A6 cells by competitively interfering with Na$^+$ self-

inhibition (12). The differences in the response to external Ni$^{2+}$ may reflect species differences in the structures that mediate Ni$^{2+}$ binding and Na$^+$ self-inhibition. We anticipate that future studies will address whether other members of ENaC/DEG family are also regulated by external transition metals. For example, extracellular Zn$^{2+}$ facilitates H$^+$ activation of the acid sensing ion channels that are homologous to ENaCs (21).

The stimulatory effect of external Zn$^{2+}$ on ENaC currents that we observed contrasts with the voltage-dependent block of the endogenous and expressed ENaCs by external Zn$^{2+}$ that was recently reported by Amuzescu and co-workers (13). It is not clear why opposite effects of Zn$^{2+}$ on ENaC activity were observed. The stimulatory effect of external Zn$^{2+}$ on ENaC is dependent on the magnitude of Na$^+$ self-inhibition and on the extracellular Na$^+$ concentration (Fig. 4). External Zn$^{2+}$ only stimulates ENaC currents measured in bath Na$^+$ con-
centrations greater than the minimal concentration required for Na$^+$ self-inhibition (−10 mM). The blocking effect of Zn$^{2+}$ on ENaC currents reported by Amuzescu et al. was observed in oocytes bathed in a solution containing 10 mM Na$^+$ (13) at Zn$^{2+}$ con-
centrations of 1−10 $\mu$M. We have not observed an inhibitory effect of extracellular Zn$^{2+}$ in the concentration range of 10 nM to 1 mM when oocytes were bathed in a solution containing 10 mM Na$^+$ (Fig. 4B). Amuzescu et al. also reported that 100 $\mu$M Zn$^{2+}$ stimulated $\alpha\beta\gamma$ rENaC, in agreement with our findings (13). Although ENaCs derived from different species were used in these two studies, it is unlikely that the conflicting results are due to species differences, because rat and mouse ENaCs have >95% amino acid identity (14). Furthermore, we observed similar activation of the whole cell Na$^+$ currents and elimina-
tion of Na$^+$ self-inhibition by external Zn$^{2+}$ in oocytes expressing human $\alpha\beta\gamma$ ENaCs. Our results are also consistent with a previous report (22) demonstrating a lack of an effect of external Zn$^{2+}$ at the concentrations up to 1 mM on inward Li$^+$ currents in oocytes expressing $\alpha\beta\gamma$ rENaC and bathed in a low Li$^+$ (20 mM) bath.

Our results suggest that external Zn$^{2+}$ activates ENaC by reversing or preventing Na$^+$ self-inhibition, although the mechanism by which Zn$^{2+}$ alters the Na$^+$ self-inhibition re-

$^a$ S. Sheng, C. J. Perry, and T. R. Kleyman, unpublished data.
response is unclear. Na⁺ self-inhibition appears to be dependent on an external Na⁺ sensor or receptor. Zn²⁺ may interfere with Na⁺ binding to its receptor by competing with Na⁺ for the same binding site or blocking Na⁺ access to the site. If so, the conformational changes in association with Na⁺ binding that result in a decrease in channel P₀ do not occur when Zn²⁺ is bound to this site. Alternatively, Zn²⁺ binding to the channel may interfere with the conformational changes that are induced by Na⁺ binding to an external site and are required for Na⁺ self-inhibition. It is also possible that Zn²⁺ increases ENaC P₀ through a mechanism that is distinct from Na⁺ self-inhibition. Several observations support the possibility that Zn²⁺ and Na⁺ interact with the channel at a common external site, including (i) the dependence of Zn²⁺ activation on the external Na⁺ concentration, (ii) the rapid activation of ENaC currents (τ of 5 s) by external Zn²⁺ and the rapid reversal of this effect following the removal of Zn²⁺, and (iii) a Zn²⁺-dependent rightward shift in Na⁺ concentration versus I��/I_peak curves.

Alkali metal ions such as Na⁺ favor oxygen atoms as a binding ligand, whereas Zn²⁺ is an intermediate divalent cation that prefers nitrogen atoms from imidazoles and sulfur atoms from Cys side chains as binding ligands. Because the coordination chemistries of Zn²⁺ and Na⁺ differ, the presence of a common (or overlapping) binding site for both Zn²⁺ and Na⁺ would impose certain constraints, such that some residues participate in the coordination of both Zn²⁺ and Na⁺, whereas other residues would participate in coordination of either Zn²⁺ or Na⁺.

We recently reported that a His residue within the extracellular loop of γENaC (H239) is required for Na⁺ self-inhibition, because substitutions of γH239 with Arg, Asp, or Cys eliminated Na⁺ self-inhibition (9). Our current study suggests that γH239 is also required for Zn²⁺ activation of ENaC. Based on these observations, we propose that γH239 may provide a binding ligand for both Na⁺ and Zn²⁺. A direct σ-interaction between Na⁺ and an imidazole nitrogen atom has been observed in resolved structures (23). A Na⁺ is coordinated by three imidazole nitrogen atoms and one water oxygen atom in the structure of a mannos-binding protein (PDB ID, 1BCH, see Fig. 9A). Given the fact that the most preferred coordination numbers of Na⁺ are 6 and 8, we speculate that Na⁺ may be coordinated by a number of different ligands, including the γH239 nitrogen, as well as oxygen atoms from other residues or from the solvent.

Based on our current observations and previous reports, we present two working models to illustrate two possible mechanisms for Na⁺ self-inhibition and the Zn²⁺ effect on self-inhibition (Fig. 9, B and C). In both models, Na⁺ binds to a site within the extracellular allosteric regulatory sites (EARS) of the ENaC ECLs that we previously proposed (9) and induces local conformational changes that are transmitted to a putative gate located at the outer pore of the channel (19, 24, 25). Working model 1 was generated to emphasize an overlapping binding site for Na⁺ and Zn²⁺ within the EARS. Residue γH239 is proposed to provide coordination for both Na⁺ and Zn²⁺ based on our observation of the loss of both Na⁺ self-inhibition and Zn²⁺ activation of channels with mutations of
The role of αH282 in Na\(^+\) and Zn\(^{2+}\) binding is not clear, because mutation of αH282 to an Arg, Asp, Cys, or Trp residue result in enhanced Na\(^+\) self-inhibition (9), and the magnitude of Zn\(^{2+}\) activation of αH282 mutants was similar to wild type ENaC. The Na\(^+\) binding site is shown to contain several oxygen atoms from unspecified residues or solvent, because Na\(^+\) coordination often involves multiple oxygen atoms. Other binding ligands for Zn\(^{2+}\) are also included in this model based on a favorable Zn\(^{2+}\) coordination pattern and on previous work on Na\(^+\) self-inhibition. Some Zn\(^{2+}\) coordination shells involve a number of different ligands, including nitrogen, sulfur, and perhaps oxygen atoms. In addition to the imidazole nitrogen of γH239, other moieties such as -SH groups from Cys residues that are abundant within the ECLs of ENaC subunits may participate in forming a high affinity binding site for Zn\(^{2+}\). Reagents that are capable of reacting with sulfhydryl groups, as well as extracellular cations that are often coordinated by Cys (Cd\(^{2+}\), Zn\(^{2+}\), and Cu\(^{2+}\)), have been shown to stimulate Na\(^+\) transport across model epithelia, a response that likely reflects Na\(^+\) self-inhibition (5). According to this model, Zn\(^{2+}\) binding would prevent or reverse Na\(^+\) binding and thus eliminate Na\(^+\)-induced conformational changes that ultimately lead to a \(P_e\) reduction. The second model (Fig. 9C) was generated to show an alternative mechanism of the Zn\(^{2+}\) effect on Na\(^+\) self-inhibition. Residue γH239 is proposed to reside in a putative gate, which swings in to close the pore during channel closing and not to interact directly with either Zn\(^{2+}\) or Na\(^+\). In this model, Na\(^+\) and Zn\(^{2+}\) bind to their sites within the EARS that may or may not overlap. The conformational changes (shown as magenta arrows) induced by Na\(^+\) binding are passed to the gate and promote the gate to close the outer pore. In contrast, Zn\(^{2+}\) binding induces the opposite conformational changes (shown as green arrows) within the EARS that hold the gate in a location to keep the pore open. Thus Zn\(^{2+}\) prevents or reverses Na\(^+\)-induced conformational changes that promote channel closing and eliminates Na\(^+\) self-inhibition. Consistent with this possibility, external Zn\(^{2+}\) did not activate currents in oocytes expressing αSS80Cβγ following MTSET treatment, which locks the channels in a fully open state.

We and others previously reported that external Ni\(^{2+}\) inhibits whole cell amiloride-sensitive Na\(^+\) currents due to a decrease in ENaC open probability (10, 11). Although Ni\(^{2+}\) and Zn\(^{2+}\) share several properties such as size, charge, and binding ligands, they exhibit opposing effects on Na\(^+\) self-inhibition. Pretreatment of oocytes expressing αβγ mENaC with 1 mM Ni\(^{2+}\) in the low Na\(^+\) bath solution prevented the typical current decay following an increase in bath Na\(^+\) concentration, and the steady-state currents were below the \(I_{ox}\) levels measured prior to Ni\(^{2+}\) pretreatment and following Ni\(^{2+}\) washout (Fig. 7C). A more rapid and deeper Na\(^+\) self-inhibition response was observed when Ni\(^{2+}\) was present only in the NaCl-110 solution. This response likely reflects additive inhibitory effects of Na\(^+\) and Ni\(^{2+}\). Although Zn\(^{2+}\) activation appears to be dependent on Na\(^+\) self-inhibition, the effect of Ni\(^{2+}\) on ENaC activity is independent of Na\(^+\) self-inhibition, because Ni\(^{2+}\) inhibits ENaC activity when added to either the NaCl-1 or NaCl-110 solution. We previously reported that αH282Dγβ is not blocked by Ni\(^{2+}\) (11). In agreement with our previous observations, the addition of Ni\(^{2+}\) to the NaCl-110 solution did not alter the Na\(^+\) self-inhibition response of αH282Dγβ (9). It is possible that Ni\(^{2+}\) binds to a site that overlaps with Zn\(^{2+}\) and Na\(^+\) binding sites. Other investigators have suggested that Ni\(^{2+}\) competes with Na\(^+\) for a common binding site (12). Our results suggest that Ni\(^{2+}\)-bound ENaC has a low open probability (11) regardless of the external Na\(^+\) concentration and that the Na\(^+\) self-inhibition response is “masked” by Ni\(^{2+}\). Na\(^+\) self-inhibition and Ni\(^{2+}\) inhibition of ENaC may share a common mechanism.

Epithelial Na\(^+\) channels are expressed in the distal nephron, distal colon, and within both the airway and alveoli. The concentrations of Na\(^+\) in the distal nephron where ENaCs are expressed are highly variable. For example, human urine Na\(^+\) concentrations vary in a wide range from <10 mM to >100 mM and are altered by changes in dietary Na\(^+\) intake as well as by changes in extracellular fluid volume (26). Luminal Na\(^+\) concentrations in rat late distal tubules are reported in the range of 24–48 mM (27). Colonic fluid Na\(^+\) concentrations are likely variable as well. Recent studies suggest that Na\(^+\) concentrations in distal airway surface liquid are high (>100 mM) (28). Na\(^+\) concentrations in the lumen of ENaC-expressing tissues are frequently above the minimal concentration (≈30 mM) at which Na\(^+\) self-inhibition is observed, suggesting that Na\(^+\) self-inhibition may participate in the physiological regulation of transepithelial Na\(^+\) transport (6). These observations, together with our data indicating that external Zn\(^{2+}\) at concentrations in the low micromolar range enhances Na\(^+\) influx via ENaC by relieving Na\(^+\) self-inhibition, suggest that extracellular Zn\(^{2+}\) may be a physiological regulator of ENaC. Urinary Zn\(^{2+}\) concentrations of ≈5 μM have been reported in humans (29, 30), and given the low concentration of proteins and amino acids in urine that could potentially bind Zn\(^{2+}\), free urinary Zn\(^{2+}\) concentrations likely reach levels that affect ENaC activity. Because increases in rates of urinary Zn\(^{2+}\) excretion may be associated with increases in ENaC activity in collecting ducts by relieving Na\(^+\) self-inhibition, we propose that increases in the urinary concentration of Zn\(^{2+}\) may increase the risk of developing salt-sensitive hypertension. Angiotensin-converting enzyme has a key role in blood pressure regulation.
Fig. 9. Working models for the mechanisms of Na\(^+\) self-inhibition and Zn\(^{2+}\) activation of ENaC. A, the Na\(^+\) coordination shell is displayed in ball-and-cylinder mode from coordinates of a structure of mannose-binding protein (PDB ID, 1BCH). The colors are cyan for carbon, red for oxygen, and blue for nitrogen. A Na\(^+\) is shown as a magenta sphere, and a water molecule is shown as a red sphere. The Na\(^+\) is coordinated by a water molecule, and three \(\delta\)-nitrogen atoms from histidine residues that are located in three helices from three identical peptide chains A, B, and C. B, working model 1 illustrating overlapping putative Na\(^+\) and Zn\(^{2+}\) binding sites. ENaC pore is shown in blue, and a bound Na\(^+\) is displayed as a magenta sphere with putative coordinating oxygen atoms in red. The yellow and green portions represent parts of the extracellular loops of ENaC subunits with an emphasis on the hypothesized extracellular allosteric regulatory site (EARS). A putative Zn\(^{2+}\) binding site depicted as a green dashed circle is formed by two imidazole nitrogen atoms from \(\Psi H239\) and an unidentified histidine, a sulfur atom from an unidentified cysteine residue, and an oxygen atom from the backbone or side chain of an unidentified residue. The Na\(^+\) binding site depicted as a magenta dashed circle is constituted by one imidazole nitrogen atom from \(\Psi H239\) and several oxygen atoms from solvent or unidentified residues. The blue arrows indicate potential movements of the outer pore in ENaC gating as suggested by us and other investigators (19, 24, 25). The green arrows indicate possible movements that transmit the local conformational changes induced by Na\(^+\) binding to its receptor onto a putative gate. C, working model 2 showing \(\Psi H239\) as a key residue in a putative gate that is in a location distinct from the Na\(^+\) and Zn\(^{2+}\) binding sites. The blue and yellow portions represent the pore and the EARS within ECL of ENaC, respectively. A putative gate within ENaC is shown as a black rod whose movement during channel closing is indicated as a black arrow. In the model, Zn\(^{2+}\) and Na\(^+\) binding sites are proposed within the EARS and may or may not overlap. The directions of conformational changes induced by Na\(^+\)and Zn\(^{2+}\) are shown as magenta and green arrows, respectively. Part of the EARS is proposed to interact with the putative gate during channel gating.

and is a Zn\(^{2+}\)-dependent protease (31). An association between increases in erythrocyte Zn\(^{2+}\) and essential hypertension has been reported (32). A relationship between urinary Zn\(^{2+}\) excretion and blood pressure has not been established, although studies with small numbers of patients have suggested an association between increases in urinary Zn\(^{2+}\) excretion and hypertension (33, 34).

In summary, our results demonstrate that extracellular Zn\(^{2+}\) activates mouse a\(\phi\)ly ENaCs expressed in oocytes bathed in high Na\(^+\) by eliminating Na\(^+\) self-inhibition. Our previous results and work from other investigators suggest that ENaC gating is regulated in an allosteric manner by extracellular cues (5, 9, 11). These observations raise the possibility that ENaC is a ligand-gated channel, similar to ATP-sensitive inward rectifier K\(^+\) channels (K\(_{\text{ATP}}\)) (35), cyclic nucleotide-gated channels (36), and glutamate receptor ion channels (37). Extracellular Na\(^+\) would serve as a ligand whose binding to ENaC reduces channel open probability, and Zn\(^{2+}\) prevents or reverses Na\(^+\) self-inhibition as a potential physiological regulator or ligand.

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