Effect of divalent heavy metals on epithelial Na\(^+\) channels in A6 cells

Ling Yu,\(^1\) Douglas C. Eaton,\(^{1,2,3}\) and My N. Helms\(^3,3\)

Departments of \(^1\)Physiology and \(^2\)Pediatrics and \(^3\)The Center for Cell and Molecular Signaling, Emory University School of Medicine, Atlanta, Georgia

Submitted 2 January 2007; accepted in final form 6 April 2007

Yu L, Eaton DC, Helms MN. Effect of divalent heavy metals on epithelial Na\(^+\) channels in A6 cells. Am J Physiol Renal Physiol 293: F236–F244, 2007. First published April 11, 2007; doi:10.1152/ajprenal.00002.2007.—To better understand how renal function is modified histidine residues, affected neither channel activity [measured as the number of channels (N) \(\times\) open probability (P\(o\))] was decreased by Cd\(^2+\) and Hg\(^2+\) and increased by Cu\(^2+\), Zn\(^2+\), and Ni\(^2+\) but was not changed by Pb\(^2+\). Of the cations that induced an increase in Na\(^+\) channel function, Zn\(^2+\) increased N, Ni\(^2+\) increased P\(o\), and Cu\(^2+\) increased both. The cysteine modification reagent [2-(trimethylammoniomethyl)ethyl]methanethiosulfonate bromide also increased N, whereas diethylpyrocarbonate, which covalently modifies histidine residues, affected neither P\(o\) nor N. Cu\(^2+\) increased N and stimulated P\(o\), by reducing Na\(^+\) self-inhibition. Furthermore, we observed that ENaC activity is slightly voltage dependent and that the voltage dependence of ENaC is insensitive to extracellular Na\(^+\) concentration; however, apical application of Ni\(^2+\) or diethylpyrocarbonate reduced the channel voltage dependence. Thus the voltage sensor of Xenopus ENaC is different from that of typical voltage-gated channels, since voltage appears to be sensed by histidine residues in the extracellular loops of ENaC, rather than by charged amino acids in a transmembrane domain.

divalent cations; single-channel recording; sodium self-inhibition

HEAVY METALS ARE MAJOR ENVIRONMENTAL pollutants that result in globally important health problems. Heavy metals tend to accumulate in specific tissues in the human body, such as liver, bones, and kidneys. Heavy metal toxicity is most commonly associated with cognitive deficits and renal pathology. The renal pathology is not surprising, since, except for some excretion via the gastrointestinal tract, most heavy metals are concentrated and excreted by the kidneys (11). In the central nervous system, heavy metals often target ion channels (18, 32), but whether the renal effects of heavy metals also involve ion channels is less clear. In particular, epithelial Na\(^+\) channels (ENaC) in the distal nephron are exposed to high levels of heavy metal because of tubular water reabsorption in earlier parts of the nephron.

ENaC are responsible for the kidney’s ability to regulate total body Na\(^+\) balance and, thereby, mean blood pressure. ENaC consists of three conserved subunits, \(\alpha\), \(\beta\), and \(\gamma\). Each subunit has two transmembrane-spanning domains (TM1 and TM2), a large extracellular domain between TM1 and TM2, and NH\(_2\) and COOH termini within the cytoplasm (3). Inappropriate alteration of ENaC activity is linked to several human genetic diseases: a gain-of-function mutation is associated with Liddle’s syndrome (31), and a loss-of-function mutation leads to pseudohypoaldosteronism type 1 (23, 34). ENaC is also well conserved among different species: identity between Xenopus and human \(\alpha\), \(\beta\), and \(\gamma\)-subunit ENaC proteins is 54%, 59%, and 55%, respectively.

Because A6 cells are derived from distal tubules of Xenopus laevis and express ENaC protein, they are a useful model for characterization of ENaC regulation and function. Examination of the effect of heavy metals on ENaC activity in A6 cells should provide useful insight into the mechanisms of kidney cell injury caused by divalent metal cations and, thus, the role of heavy metals in renal pathology.

From previous research on transport in frog skin, it is known that heavy metals affect epithelial Na\(^+\) transport (10). The effects of heavy metals were reported as changes of transepithelial short-circuit current (SCC), but the precise characteristics of the channels in the frog skin carrying this current were not clearly defined. More recently, the effects of heavy metals on ENaC have been examined in cultured epithelial cells (6) and ENaC expressed in Xenopus oocytes (1, 29, 30). In these experiments, ENaC were defined as amiloride-sensitive channels, but the currents were measured as transepithelial SCC or as whole cell current. The properties of amiloride-sensitive channels can vary widely (7), but only the channel consisting of \(\alpha\)-, \(\beta\)-, and \(\gamma\)-subunits with a single-channel conductance of \(\sim 5\) pS is characteristic of ENaC in principal cells of the distal nephron (7). Single-channel recording unequivocally determines channel characteristics, including unit conductance and channel activity [measured as \(NP-o\)], i.e., the number of channels (N) \(\times\) open probability (P\(o\)). Single-channel measurements can also provide information about N and P\(o\).

In this study, we have examined the effects of apical application (within the patch pipette) of Zn\(^2+\), Ni\(^2+\), Cu\(^2+\), Pb\(^2+\), Cd\(^2+\), and Hg\(^2+\) on ENaC activity. Zn\(^2+\), Ni\(^2+\), and Cu\(^2+\) increased channel activity, Cd\(^2+\) and Hg\(^2+\) strongly inhibited activity, and Pb\(^2+\) had a marginal effect on ENaC function. Our experimental evidence suggests that each metal has its own coordination site in the extracellular loops (ECLs) of Xenopus ENaC. We also found that ENaC in A6 cells is voltage dependent and that the voltage is likely sensed by His residues in the ECLs.

MATERIALS AND METHODS

A6 cell culture preparation. A6 cells (subclone 2F3; obtained from Drs. Kranenbuhl and Rossier) were maintained in plastic tissue culture flasks, as described previously (2). For single-channel patch-clamp experiments, A6 cells were seeded onto collagen-coated permeable

Address for reprint requests and other correspondence: L. Yu, Emory Univ. School of Medicine, Dept. of Physiology, Whitehead Biomedical Research Bldg., 615 Michael St., Atlanta, GA 30322 (e-mail: lyu@physio.emory.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

0363-6127/07 $8.00 Copyright © 2007 the American Physiological Society

http://www.ajprenal.org

F236
support inserts until they reached confluency. A6 cell culture medium, which consists of three parts Coon’s medium F-12 and seven parts Leibovitz’s medium L-15 modified for amphibian cells with 104 mM NaCl, 25 mM NaHCO3, 10% fetal bovine serum (GIBCO, Grand Island, NY), 1% streptomycin, and 0.6% penicillin (Irvine Scientific, Santa Ana, CA) with pH 7.4, was replaced three times per week.

Patch-clamp experiments were carried out in A6 cells between passages 97 and 104.

Single-channel recordings. The cell-attached configuration was used in all patch-clamp studies. Micropipettes were pulled from filamented borosilicate glass capillaries (TW-150, World Precision Instruments) with a two-stage vertical puller (Narishige, Tokyo, Japan). Resistance of the pipettes was 7–10 MΩ. MTSET, DEPC, and Pb(acetate)2 solutions were made fresh with DEPC. MTSET, DEPC, and Pb(acetate)2 solutions were made fresh with DEPC.

Data analysis. After formation of a high-resistance (–5 GΩ) seal, the channel currents were recorded at 1 kHz with an Axopatch 1-D amplifier (Molecular Devices) with a low-pass 100-Hz Bessel filter. Channel activity (NPo) was calculated from pClampfit 9.2 data analysis software (Molecular Devices), N was determined from the maximal number of transitions during 20–25 min of recording, and Pn was calculated as the ratio of NPo to N. Values are means ± SE. Differences between groups were evaluated with one-way ANOVA, and differences of properties of the same cell at different membrane potentials were tested by paired t-test. Parameter estimation from curve fitting was done with Sigmaplot and Sigmasstat (San Rafael, CA).

RESULTS

The conductance and selectivity of ENaC in cultured renal cells are affected by culture conditions (9). In our work, A6 cells were grown on permeable supports in the presence of aldosterone; under this condition, the only cation channel that was observed was a channel with 4- to 5-pS conductance and slow gating kinetics, which we previously identified as ENaC. These properties are characteristic of Na+ channels found in many tight epithelia. All the results reported here are from this type of channel.

Different heavy metals affect ENaC activity differently. We used single-channel, cell-attached patch-clamp analysis to test the effects of six divalent metals on ENaC activity. These metals were individually applied to the apical surface via the pipette solution. To eliminate variability, which might arise between cells from different passages and even different wells from the same passage, we formed patches and recorded separately from nearby cells in the same culture well, first with a pipette without heavy metal and then with a pipette containing one of the heavy metals. None of the metals produced a statistically significant change in single-channel conductance (Table 1), but all the metals, except Pb2+, produced an easily discernible change in ENaC activity (NPo; Fig. 1). NPo from cells exposed to heavy metals, as well as their respective controls, are summarized in Fig. 2. Figure 2A shows a significant increase of NPo in cells treated with 2 mM Ni2+ compared with control cells (0.95 ± 0.16 vs. 0.40 ± 0.12, P = 0.029). We also tested the effect of 200 μM Ni2+ on ENaC activity and found no significant effect at this lower concentration (data not shown.) Figure 2A also shows that 100 μM Zn2+ (0.87 ± 0.39) failed to significantly alter ENaC activity when the pipette holding potential was 0 mV. (Zn2+ and Ni2+ treatments shared a common group of control recordings.) In a separate study (Fig. 2B), 100 μM Cu2+ significantly increased NPo almost fivefold compared with control values (from 0.16 ± 0.06 to 0.83 ± 0.22, P = 0.01). Interestingly, the effect of 100 μM Cd2+ and 20 μM Hg2+ (with the same outer shell electronic structure as Zn2+) differed from the effects of Zn2+. In cells exposed to Cd2+, NPo decreased to <15% of its control value (from 2.85 ± 0.55 to 0.34 ± 0.19, P < 0.01). The inhibitory effect of Hg2+ can be seen at concentrations as low as 2 μM (Fig. 2D), and 20 μM Hg2+ decreased the channel activity from the control value of 0.79 ± 0.34 to 0.02 ± 0.004 (P < 0.01). The results presented in Fig. 2D were recorded from cells with a holding potential of −60 mV to increase overall channel activity (see below). The effect of 100 μM Pb2+ was also tested; compared with its control, there was no significant effect (see NPo during initial recording period at 0 mV in Fig. 3D). All results and statistical evaluation are calculated on the basis of patches with active channels. However, the statistical significance would not change if silent patches were included.

ENaC voltage dependence is altered by heavy metals. ENaC is typically regarded as a constitutively active channel, the activity of which is modulated by hormonal agents. However, when all three subunits are expressed in Xenopus oocytes, Xenopus ENaC is slightly voltage dependent (5), and native ENaC in rat cortical collecting tubule principal cells is also voltage dependent (21). To test the voltage dependence of Xenopus ENaC in A6 cells, the channel activity was first recorded at a holding potential of 0 mV immediately after formation of a tight seal. Then activity from the same patch was recorded at a more hyperpolarizing membrane potential, from 0 to −100 mV in −20-mV steps. To check whether the channel activity remained stable after the voltage steps, we returned each patch to a holding potential of 0 mV for a final recording period. Because NPo from most of the patch record-

Table 1. Unit conductance of ENaC channels in metal-treated and untreated patches

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conductance, pS</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn2+</td>
<td>3.55 ± 0.27</td>
<td>12</td>
</tr>
<tr>
<td>Ni2+</td>
<td>4.29 ± 0.17</td>
<td>12</td>
</tr>
<tr>
<td>Untreated</td>
<td>4.10 ± 0.19</td>
<td>12</td>
</tr>
<tr>
<td>Cu2+</td>
<td>4.65 ± 0.19</td>
<td>13</td>
</tr>
<tr>
<td>Untreated</td>
<td>4.29 ± 0.4</td>
<td>13</td>
</tr>
<tr>
<td>Cd2+</td>
<td>3.76 ± 0.31</td>
<td>7</td>
</tr>
<tr>
<td>Untreated</td>
<td>3.65 ± 0.36</td>
<td>8</td>
</tr>
<tr>
<td>Hg2+</td>
<td>4.07 ± 0.45</td>
<td>7</td>
</tr>
<tr>
<td>Untreated</td>
<td>3.99 ± 0.37</td>
<td>10</td>
</tr>
<tr>
<td>Pb2+</td>
<td>3.84 ± 0.22</td>
<td>13</td>
</tr>
<tr>
<td>Untreated</td>
<td>3.72 ± 0.18</td>
<td>12</td>
</tr>
</tbody>
</table>

Values are means ± SE. ENaC, epithelial Na+ channel. Conductance was measured as the slope of the current-voltage relationship between 0 and +60 mV applied pipette potential. No mean value is significantly different from any other mean (ANOVA) for all treatments, and no mean for an untreated group from the same passage number is different from the mean conductance for metal-treated patches (ANOVA or t-test). Because power of the tests is low, a small effect for some of the metals cannot be ruled out.
ings remained stable for \(\sim 30\) min, for completion of all the recordings at different membrane potentials before loss of channel activity, the duration of the first 0-mV recording period was \(7–10\) min and the duration of all subsequent recordings at different holding potentials was 2–3 min. From the results of Fig. 3 (as well as Fig. 7 A), where \(N_{Po}\) is plotted vs. the different pipette holding potentials \((-V_{pip})\), it is clear that ENaC activity in A6 cells is enhanced at hyperpolarized membrane potentials. In Fig. 3D, however, the channel activity did not respond strongly to voltage changes. This is likely due to rundown of channel activity. Nevertheless, the increase in \(N_{Po}\) at hyperpolarized membrane potentials is clear. The reduced voltage sensitivity was also observed in cells from the same passage recorded in pairs exposed to 100 \(\mu\)M \(\text{Pb}^{2+}\) (Fig. 3D).

The comparison of channel activity at \(-100\) and 0 mV recorded immediately thereafter would be the strongest measure of the channel voltage dependence, but the recording duration at these two voltage steps is too short to provide consistently statistically different values for \(N_{Po}\). Therefore, \(N_{Po}\) of each cell at \(-60\) to \(-100\) mV \((6–9\) min total duration) were averaged and compared with \(N_{Po}\) of the same cell during the initial recording period at 0 mV \((\sim 7–10\) min total dura-

---

**Fig. 1.** Typical single-channel recordings from epithelial Na\(^+\) channels (ENaC) in A6 cells exposed to heavy metals. A6 cells were grown on Nunc filters for 8–12 days to form tight confluent monolayers; then the patch-clamp technique was used to record single-channel activity in the cell-attached configuration. Inward currents are downward deflections. Downward deflections from the closed state are individual channel openings, O. C, closed level. Controls (Ctrl) and treatments were always recorded in pairs from cells growing in the same dish. All traces were recorded at a holding potential of 0 mV.

**Fig. 2.** Effect of divalent heavy metals on ENaC activity. Channel activity is presented as channel density \(N_{Po}\) (i.e., number of channels per patch \((N)\) \(\times\) open probability \((P_o)\)). Numbers above columns represent number of patches with active channels; numbers in parentheses represent number of cells on which seals were formed but in which the patches had no active channels (“silent” patches). Average \(N_{Po}\) was recorded from cells with active channels. Values are means \(\pm\) SE. \(*P < 0.02\). A: 100 \(\mu\)M \(\text{Zn}^{2+}\) or 2 mM \(\text{Ni}^{2+}\) in pipette solution. \(\text{Zn}^{2+}\) and \(\text{Ni}^{2+}\) share a common control group. \(N_{Po}\) was recorded at pipette holding potential of 0 mV. B and C: 100 \(\mu\)M \(\text{Cu}^{2+}\) or \(\text{Cd}^{2+}\) in pipette solution. Data were compared with their own control groups at 0 mV. D: 20 \(\mu\)M \(\text{Hg}^{2+}\) and 2 \(\mu\)M \(\text{Hg}^{2+}\) in pipette solution. Data were recorded at -60 mV and compared with control cells.

**Fig. 3.** Activity of ENaC is enhanced by hyperpolarizing membrane potentials. \(N_{Po}\) was recorded at pipette holding potential \((-V_{pip})\) of 0 to \(-100\) mV in 20-mV steps and again at 0 mV. Duration of the first 0-mV recording period was \(7–10\) min; remaining potentials were recorded for 2–3 min. \(N_{Po}\) for control and treated cells are plotted in pairs vs. pipette holding potential in A, B, C, and D. Results in A, B, and C are recorded from cells in Fig. 2. A, B, and C, which had active channels. In D, there were 13 patches on \(\text{Pb}^{2+}\)-treated cells and 12 patches on control cells.
tials with pipettes filled with 100 nM of the three ENaC subunits. To explore whether the ECLs of there is no such cluster in the transmembrane domains of any residues of each other in the transmembrane region; however, a cluster of charged amino acids spaced within three to four

\[
p \alpha \propto \exp \left( -\frac{\alpha VF}{RT} \right)
\]

where \(P_o(V)\) is \(P_o\) at a voltage \(V\), \(P_o(0)\) is \(P_o\) at 0 mV, \(\alpha\) is voltage dependence of the channel, and \(R\), \(T\), and \(F\) are constants.

One way to interpret \(\alpha\) is as the fraction of the membrane field detected by charged sites on the channel. The value for \(\alpha\) can be obtained by determining the slope of the relationship between log \(P_o\) and voltage (or log \(NP_o\) and voltage, since \(N\) only represents a scaling factor). Figure 4 shows just such plots for Ni\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), and Pb\(^{2+}\): Ni\(^{2+}\) decreases, Cu\(^{2+}\) and Zn\(^{2+}\) increase, and Pb\(^{2+}\) does not change the voltage dependence of the channel (measured as slopes of the lines). Figure 5 shows the actual values of \(\alpha\) determined from the slopes for different metals and their respective controls.

Effects of Zn\(^{2+}\), Ni\(^{2+}\), and Cu\(^{2+}\) suggest different binding sites. The channel activity we have presented has been reported as \(NP_o\), which combines \(N\) and \(P_o\). We have shown that \(N\) can be determined with a high degree of confidence if the duration of recording is long enough and \(P_o\) is close enough to 0.5, when \(P_o\) can be calculated by dividing \(NP_o\) by \(N\) (17). All results summarized in Fig. 3 are from cell recordings of ~20- to 25-min duration, which is sufficient to allow a good estimate of \(N\) for each patch. This estimate of \(N\) is more accurate when it is determined from recordings held at hyperpolarizing potentials (since \(P_o\) is closer to 0.5 than at more depolarized potentials). The average \(N\) from patches exposed to Zn\(^{2+}\) and Ni\(^{2+}\) were 5.17 ± 0.64 (n = 12) and 3.67 ± 0.90 (n = 12), respectively, compared with the control value of 3.27 ± 0.45 (n = 12; Fig. 6A). In patches exposed to Cu\(^{2+}\), average \(N\) was 4.69 ± 0.63 (n = 13), and average \(N\) of its paired control cell recordings was 2.85 ± 0.37 (n = 13; Fig. 6C). Since \(NP_o\) is voltage dependent, \(P_o\) was calculated from \(NP_o\) at hyperpolarizing membrane potentials. The average \(P_o\) from patches exposed to Zn\(^{2+}\) or Ni\(^{2+}\) was 0.306 ± 0.032 (n = 12) or 0.475 ± 0.049 (n = 12), respectively, and average \(P_o\) of their control recordings was 0.222 ± 0.027 (n = 12; Fig. 6B). For patches exposed to Cu\(^{2+}\), average \(P_o\) was 0.406 ± 0.033 (n = 13) compared with average control \(P_o\) of 0.140 ± 0.022 (n = 13; Fig. 6D). When the same calculation was performed for Pb\(^{2+}\)-exposed and paired control cells, average \(N\) and \(P_o\) were 3.57 ± 0.77 and 0.19 ± 0.03 (n = 14), respectively, compared with control values of 1.92 ± 0.42 and 0.15 ± 0.3 (n = 12), respectively. In summary, we found that Zn\(^{2+}\) significantly increased \(N\) (\(P = 0.026\)), Ni\(^{2+}\) significantly increased \(P_o\) at hyperpolarizing potentials \(P = 0.01\), and Cu\(^{2+}\) significantly increased \(N\) and \(P_o\) (\(P = 0.019\) and \(P = 8.4 \times 10^{-5}\)); however, Pb\(^{2+}\) had no significant effect on \(N\) or \(P_o\). All four metals affect

![Fig. 4. Effects of heavy metal on ENaC voltage dependence. Mean \(NP_o\) values were calculated for individual cells, log \(NP_o\) vs. voltage was plotted (means ± SE), and values were fitted to the following equation: \(NP_o(V) = NP_o(0) \exp \left( -\frac{\alpha VF}{RT} \right)\), where \(NP_o(V)\) is \(NP_o\) at voltage \(V\), \(NP_o(0)\) is \(NP_o\) at 0 mV, \(\alpha\) is voltage dependence, and \(R\), \(T\), and \(F\) are constants. Solid lines are best-fit values for \(NP_o\) based on mean \(\alpha\). Determination of \(\alpha\) has the advantage that it is independent of \(N\). A value of \(\alpha\) of 0.30 implies that the voltage-sensing residues in the channel sense ~30% of the membrane field and that there is an e-fold change in \(P_o\) for an 83-mV change in membrane potential. For concentrations of heavy metals, see Fig. 2 legend.](http://ajprenal.physiology.org/)

![Fig. 5. Effects of heavy metals on voltage dependence of ENaC. Zn\(^{2+}\) and Cu\(^{2+}\) increase voltage dependence, Ni\(^{2+}\) decreases voltage dependence, and Pb\(^{2+}\) does not affect voltage dependence. For concentrations of heavy metals, see Fig. 2 legend.](http://ajprenal.physiology.org/)
channel activity, albeit in different ways, suggesting that each metal may have different specific binding sites in the ECLs.

MTSET mimics effects of Zn$^{2+}$ and Cu$^{2+}$, and DEPC mimics the effect of Ni$^{2+}$. Divalent metals preferentially bind His and Cys residues (8); indeed, it has been shown that His and Cys residues in ECLs of mouse ENaC are important ligand binding sites for Ni$^{2+}$ and Zn$^{2+}$ (29). The effect of heavy metals on ENaC $P_o$ has not been specifically studied. Our present study provides information regarding ligand specificity of the metals. To distinguish the metals’ binding sites on ENaC, we covalently modified extracellular Cys or His residues, respectively, by adding MTSET or DEPC to the pipette solution before recording the channel activity at different membrane potentials. The protocol for data collection was the same as that described above, with a common control from cells on the same plates treated with MTSET or DEPC. In Fig. 7A, it is clear that the channel activity is higher at hyperpolarized membrane potentials in control and MTSET-treated, but not DEPC-treated, cells. $P_o$ at hyperpolarized membrane potential was averaged and presented together with $N_p$ at 0 mV in Fig. 7B. Specifically, at 0 mV, $N_p$ of cells exposed to MTSET, DEPC, and the untreated controls were 0.60 ± 0.14 ($n$ = 24), 0.49 ± 0.13 ($n$ = 19), and 0.32 ± 0.08 ($n$ = 19), respectively. Compared with control values, neither MTSET nor DEPC significantly stimulated ENaC activity at 0-mV membrane potential. On the other hand, the average $N_p$ at hyperpolarizing membrane potentials was 1.33 ± 0.24 for cells exposed to MTSET ($n$ = 24) and 0.74 ± 0.21 for cells exposed to DEPC ($n$ = 19), and their control value was 0.923 ± 0.220 ($n$ = 19). Thus the Na$^+$ channels in control and MTSET-treated cells are voltage dependent ($P$ = 0.005 and $P$ = 8.8 × 10$^{-5}$), whereas the Na$^+$ channels in DEPC-treated cells resemble the ENaC of Ni$^{2+}$-exposed cells. DEPC (and Ni$^{2+}$) eliminated the channel voltage dependence measured as the slope of log $N_p$ vs. voltage curves (Fig. 8). No significant difference in $N_p$ at hyperpolarizing membrane potentials was apparent among control and the two reagent treatments in Fig. 7B.

However, because the power of the test was low, it is possible that we failed to detect a difference that was actually present. Since $N_p$ consists of two independent variables, $N$ and $P_o$, both of which contribute to the overall variability, we separated the variables on the basis of our best estimates of $N$. When we examined the effect of MTSET and DEPC on $N$ and $P_o$ separately, there was a difference. The average $N$ of control cells was 3.26 ± 0.26 ($n$ = 19). In cells exposed to MTSET, average $N$ was 4.19 ± 0.24 ($n$ = 24), which was significantly different from control ($P$ = 0.038); in cells exposed to DEPC, average $N$ was 2.81 ± 0.19, which was not significantly different from control ($n$ = 19; Fig. 7C). These effects of Cys modification by MTSET are not unlike those observed in Zn$^{2+}$- and Cu$^{2+}$-treated cells. Cys modification significantly increased average $N$, whereas His modification had no effect on $N$. However, neither MTSET nor DEPC altered ENaC $P_o$ at hyperpolarizing membrane potentials (Fig. 7D).

Heavy metals apparently do not alter $P_o$ by interaction with His residues. Our results show that Cu$^{2+}$ and Ni$^{2+}$ increased $P_o$, but DEPC had no effect on $P_o$, despite the fact that His residues are known to bind Ni$^{2+}$. To further demonstrate that extracellular His is not involved in the heavy metal effects on $P_o$, we performed another set of experiments in which cells were challenged with DEPC and Cu$^{2+}$ simultaneously. We used Cu$^{2+}$, instead of Ni$^{2+}$, because Cu$^{2+}$ produced a stronger

Fig. 6. Stimulatory effects of Zn$^{2+}$, Ni$^{2+}$, and Cu$^{2+}$ on ENaC activity. A and C: $N$ after treatment vs. control. B and D: $P_o$ after treatment vs. control. For concentrations of heavy metals, see Fig. 2 legend. Values are means ± SE. **$P$ < 0.01; ***$P$ < 0.001.

Fig. 7. Effects of [2-(trimethylammonium)ethyl]methanethiosulfonate bromide (MTSET) and diethylpyrocarbonate (DEPC) on ENaC activity. A: ENaC activity recorded at different membrane potentials from cells exposed to 2 mM MTSET ($n$ = 24), 2 mM DEPC ($n$ = 19), and their control ($n$ = 19). B: verification of voltage dependence of channel. First recording period shows channel activity as $N_p$ at 0 mV; hyperpolarized value is average $N_p$ of channel activity at ~60, ~80, and ~100 mV. C and D: averaged $N_p$ differentiated into $N$ and average $P_o$. Numbers above columns represent number of cells with active channels. Values are means ± SE. **$P$ < 0.01; ***$P$ < 0.001.

Fig. 8. Effects of 2 mM MTSET and 2 mM DEPC on voltage dependence of ENaC. Voltage dependence was increased by MTSET and decreased by DEPC.
stimulatory effect on ENaC than Ni\(^{2+}\). Figure 9A shows that channels maintained their voltage dependence after exposure to DEPC + Cu\(^{2+}\). ENaC in cells treated only with DEPC lost their voltage dependence (Figs. 7A and 8). \(N_{\text{Po}}\) was significantly higher in cells treated with DEPC + Cu\(^{2+}\) than in cells treated with DEPC only (\(P < 0.05\)) at all holding potentials except \(-40\) mV. Furthermore, in cells treated with DEPC + Cu\(^{2+}\), hyperpolarizing membrane potentials substantially increased \(N_{\text{Po}}\). Figure 9B summarizes \(N_{\text{Po}}\) at 0 mV and at hyperpolarized potentials. \(N_{\text{Po}}\) for hyperpolarized cells treated with DEPC + Cu\(^{2+}\) was 1.801 ± 0.257, which is significantly higher than the initial \(N_{\text{Po}}\) of 1.02 ± 0.23 (\(n = 14, P = 0.025\)) at 0 mV in the same cells. The average \(N_{\text{Po}}\) of hyperpolarized DEPC-treated cells was 0.667 ± 0.181, which is not significantly different from the initial \(N_{\text{Po}}\) of 0.34 ± 0.12 (\(n = 11\)) at 0 mV. As shown in Fig. 9, C and D, cells treated with DEPC + Cu\(^{2+}\) showed an increase in \(N\) and \(P_{\text{o}}\) compared with cells treated with DEPC only: \(N\) was 2.06 ± 0.53 for DEPC-treated cells and 4.00 ± 0.73 for cells treated with DEPC + Cu\(^{2+}\) (\(P = 0.039\)), and \(P_{\text{o}}\) was 0.17 ± 0.02 for DEPC-treated cells, which was significantly lower than that for cells treated with DEPC + Cu\(^{2+}\) (0.30 ± 0.03, \(P = 0.045\)). Together, these results suggest that DEPC-accessible His residues in the ECLs of ENaC are not the binding site for metal-induced effects on \(P_{\text{o}}\).

Stimulatory effects of heavy metals on ENaC activities are due to a reduction of Na\(^{+}\) self-inhibition. Our data show that Zn\(^{2+}\), Ni\(^{2+}\), and Cu\(^{2+}\) stimulate Na\(^{+}\) channel activity. Interestingly, Zn\(^{2+}\) has been reported to stimulate mouse ENaC activity by reducing Na\(^{+}\) self-inhibition (30). Na\(^{+}\) self-inhibition has been observed when external Na\(^{+}\) concentration was >30 mM on oocytes (26). Unfortunately, single-channel activity cannot be resolved with a pipette solution containing only 30 mM Na\(^{+}\).

Therefore, to test metal-induced reduction of Na\(^{+}\) self-inhibition, we recorded single-channel activity with a pipette solution containing 40 mM Na\(^{+}\). At this external Na\(^{+}\) concentration, Na\(^{+}\) channel self-inhibition was negligible, whereas the single-channel activity could be resolved clearly (Fig. 10A). We recorded channel activity at holding potentials (\(-V_{\text{pip}}\)) from −20 to −100 mV in 20-mV steps with or without 100 \(\mu\)M Cu\(^{2+}\) in the pipette solution. The duration was 8–10 min for the first recording period at −20 mV, 4–5 min at −80 and −100 mV, and 2–3 min at the other potentials (Fig. 10B). At lower extracellular Na\(^{+}\) concentration, Cu\(^{2+}\) no longer stimulated N [4.49 ± 0.29 (\(n = 12\)) vs. 4.63 ± 0.56 (\(n = 11\)) for treatment vs. control] or \(P_{\text{o}}\) (0.34 ± 0.03 vs. 0.34 ± 0.04 for treatment vs. control). ENaC remained voltage dependent in control and treated cells. \(N_{\text{Po}}\) at −20 mV was 0.65 ± 0.14 (control); 0.77 ± 0.16 (treated) and average \(N_{\text{Po}}\), at −80 and −100 mV was 1.71 ± 0.30 and 1.77 ± 0.23, respectively. Hence, the mechanism of ENaC voltage dependence is independent of Na\(^{+}\) self-inhibition.

**DISCUSSION**

Properties and summary of effects of heavy metals on properties of ENaC. Because the coordination chemistry of the heavy metals is complex, it is not surprising that the metal ions used in this study produce substantially different effects on ENaC. The three effects, change of \(N\), change of \(P_{\text{o}}\), and change of voltage dependence of the channel, are summarized in Table 2, along with some of the physical chemical properties of the divalent cations. It is also important to recognize that, because of the application and measurement methods, the effects we describe are acute effects that alter only the external surface of ENaC.

There are multiple heavy metal interaction sites in ENaC ECLs. Using single-channel recording, we have reported, for the first time, the effects of heavy metals on ENaC activity. After separating the single-channel \(N_{\text{Po}}\) into \(N\) and \(P_{\text{o}}\), we found that the stimulatory effects of Zn\(^{2+}\), Ni\(^{2+}\), and Cu\(^{2+}\) on ENaC activity were different, which suggests that these metals coordinate with different binding sites in ENaC ECLs. The effect of Ni\(^{2+}\) on ENaC seems species dependent, since Ni\(^{2+}\) increases instantaneous current of Xenopus ENaC expressed in Xenopus oocytes at a holding potential of 0 mV (5), which is consistent with our observation that Ni\(^{2+}\) increases channel \(N_{\text{Po}}\) at depolarizing membrane potentials. Mouse and rat ENaC, however, were inhibited by external Ni\(^{2+}\) when the respective cRNAs were expressed in Xenopus oocytes (24, 29). Sheng et al. (29) reported that, in the ECLs of mouse ENaC,
The radius of Zn$^{2+}$ bind extracellular His residues, they stimulate most profound effects of Zn$^{2+}$.

The radius of Cd$^{2+}$ and Hg$^{2+}$ are similar to but different from those of Zn$^{2+}$, implying that these ions are almost as strong as covalent interactions (8). However, the radius of Cd$^{2+}$ is larger than that of Zn$^{2+}$, which means that their hydrated radius is smaller (Table 2). In addition, the electronic configuration of Cd$^{2+}$ and Hg$^{2+}$ is similar to that of Zn$^{2+}$, suggesting that this Cys residue is facing away from the Na$^+$ conductivity pathway (28). However, Cd$^{2+}$, an ion similar in size to Cd$^{2+}$ but much more polarizable, inhibited mouse ENaC activity in the same oocyte expression system. Human ENaC expressed in Xenopus oocytes is inhibited by a small thiol-modifying reagent, methylthiosulfonate ethylammonium, but not by the large MTSET reagent. Furthermore, this inhibition is due to methylthiosulfonate ethylammonium binding to the Cys residue within the tract (33). Hence, the strong inhibitory effect of Hg$^{2+}$ and Cd$^{2+}$ on A6 ENaC is quite possibly due to binding of these two ions to the same Cys residue in the selectivity filter of the $\gamma$-subunit, with Hg$^{2+}$ having easier access and stronger binding. These metal cations, therefore, may interact with the same sites as Zn$^{2+}$, but in addition, they also interact with sites in the channel pore inaccessible to the larger Zn$^{2+}$. When Hg$^{2+}$ and Cd$^{2+}$ interact with the sites in the pore, they destabilize the open state and, thereby, reduce $P_o$. In a previous study, 100 $\mu$M Cd$^{2+}$ was reported to reduce frog skin transepithelial resistance but increase SCC (10); in this study, the effect of Cd$^{2+}$ on the frog skin was imitated by a Ca$^{2+}$ chelator, EDTA. In another report, Cd$^{2+}$ disrupted cadherin structure by replacing its binding Ca$^{2+}$ (22). Hence, the stimulatory effect of Cd$^{2+}$ on the whole frog skin may be due to disruption of the tight epithelial connection or, perhaps, might even affect a channel type other than ENaC, since there are different types of channels in frog skin.

Table 2. Properties of heavy metals and a summary of their effects on ENaC

<table>
<thead>
<tr>
<th>Ion</th>
<th>Electronic Configuration</th>
<th>Crystal Radius, nm</th>
<th>Hydrated Radius, nm</th>
<th>Effect on $N$</th>
<th>Effect on $P_o$</th>
<th>Effect on Voltage Dependence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni$^{2+}$</td>
<td>$-8-16-2$</td>
<td>0.070</td>
<td>0.404</td>
<td>None</td>
<td>Increase</td>
<td>None</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>$-8-18-1$</td>
<td>0.072</td>
<td>0.419</td>
<td>Increase</td>
<td>Increase</td>
<td>Increase</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>$-8-18-2$</td>
<td>0.074</td>
<td>0.430</td>
<td>Increase</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cd$^{2+}$</td>
<td>$-18-18-2$</td>
<td>0.097</td>
<td>0.426</td>
<td>Decrease</td>
<td>Decrease</td>
<td>NA</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>$-32-18-2$</td>
<td>0.11</td>
<td>0.411</td>
<td>Decrease</td>
<td>Decrease</td>
<td>NA</td>
</tr>
<tr>
<td>Pb$^{2+}$</td>
<td>$-32-18-4$</td>
<td>0.12</td>
<td>0.401</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Data are from Refs. 19 and 35. $N$, channel density (i.e., number of channels per patch); $P_o$, open channel probability; NA, not available.
HEAVY METAL EFFECTS ON ENaC

is no such cluster within ENaC transmembrane domains of all three subunits. ENaC voltage sensitivity is abolished when the cell’s apical membrane is exposed to DEPC. Because DEPC is slightly membrane permeable, His residues in ENaC transmembrane domains, as well as in ECLs, may act as the voltage “sensor.” A comparison of all the amino acids in the transmembrane domains of Xenopus, rat, and mouse ENaC revealed the existence of a His residue conserved in the second transmembrane domain of the α-subunit of Xenopus and mouse ENαC. However, Xenopus, but not mouse, ENαC is voltage dependent (5). Thus the conserved His in the transmembrane region is not likely to be the source of the ENαC voltage sensor. Additionally, since Ni2+ and Cu2+ are membrane impermeable, yet they could still affect voltage sensitivity (Fig. 3B), it is unlikely that the voltage sensor is in the transmembrane domains. On the basis of our studies, the voltage sensor is most likely in the ECLs. This implies that the external vestibule of the channel formed by the ECLs must be sufficiently resistive to allow the potential field of the membrane to extend beyond the lipid head group surface of the apical membrane.

His is negatively charged at neutral pH. The values for channel voltage dependence, α, imply that the voltage-sensing charged residue senses very little of the overall membrane field (Fig. 5). If there is only one charge, it would need to sense only ~30% of the membrane field, and two or more charges would sense proportionately less, so it is reasonable to suggest that some portion of the field could extend into the external vestibule (considering the highly resistive, low unit conductance nature of the channel).

Heavy metal stimulation of ENaC activity is due to reduction of Na+ self-inhibition. Na+ self-inhibition refers to ENαC activity that is inhibited by high extracellular Na+ concentration. All cloned ENαCs from species that have been tested display Na+ self-inhibition. Heavy metal-stimulated ENαC activity was examined after Na+ self-inhibition was reduced. Zn2+ can only stimulate mouse ENαC activity (in an oocyte expression system) with high external Na+ concentrations (30). As indicated by transepithelial SCC, Ni2+ increases native ENαC in A6 cells by releasing Na+ self-inhibition (6). By recording single-channel activity in A6 cells using similar procedures, we found that Zn2+ increases N, Ni2+ increases P, and Cu2+ stimulates N and P. These results imply that there are at least two Na+ self-inhibition sites in the ECLs: one is involved in determining P, and the other is involved in switching channels on or off. This suggestion is further supported by another set of experiments (Fig. 8B) in which, at low extracellular Na+ concentration, Cu2+ no longer was capable of altering N or P.

Na+ self-inhibition has been proven to be an intrinsic characteristic of the channel. Two His residues in ECLs of mouse ENαC α- and γ-subunits are considered to play opposite roles in the Na+ self-inhibition process: mutation of the α-subunit His285 enhances, whereas mutation of the γ-subunit His299 eliminates, Na+ self-inhibition (26). Recent studies demonstrate that Na+ self-inhibition is related in some way to proteolytic maturation of ENαC. According to a biochemical analysis of ENαC α-, β-, and γ-subunits, two pools of ENαC coexist in the plasma membrane: one is completely posttranslationally processed, with high P, and the other is incompletely processed, with low or very low P (12). Proteolytic cleavage of ENαC α- and γ-subunit ECLs is a critical process in conversion of channels with low activity to channels with high activity (13). Chraibi and Horisberger (4) observed that Na+ self-inhibition was markedly diminished by extracellular application of proteases. Furthermore, Sheng et al. (25) reported that expression of mouse ENαC in Xenopus oocytes with furin cleavage sites on the α- or γ-subunit eliminated by mutation greatly enhanced Na+ self-inhibition. Na+ self-inhibition appears to be a primary response of noncleaved channels in the plasma membrane. The noncleaved channel has higher affinity to Na+ than cleaved channels. At low extracellular Na+ concentrations, both channel types have similar activity, but at high external Na+ concentration the noncleaved channels may bind Na+ and allosterically change the ECLs of ENαC and, thus, change the channel gating (25). When a furin cleavage site on the γ-subunit was eliminated, P of mouse ENαC was significantly reduced at high external Na+ concentration, which is consistent with our observation that Cu2+ stimulates P only at high external Na+ concentration. It is quite possible that binding of Cu2+ (and, probably, Ni2+) to ECLs prevents Na+ from binding to the self-inhibition site and, thus, reduces Na+ self-inhibition. If this is true, then the furin cleavage site, the Na+ self-inhibition site, and the Cu2+-binding site are likely physically close to one another.

Interpreting how heavy metals affect human health. In summary, all the metals we examined, except Pb2+, exerted significant effects on ENαC in A6 cells. Hg2+ tends to inhibit the channel activity at concentrations as low as 2 μM, whereas Cu2+ increases ENαC activity by several fold. The effect of these metals on ENαC activity is mainly through Cys and His residues of ECLs of ENαC; the conservation of Cys residues between Xenopus and human ENαC is 100% in α- and γ-subunits, whereas in the β-subunit it is ~90%. The heavy metal concentrations we used in our experiments (except Ni2+) are approximately within the physiological range of those found in human kidney cortex tissue in cases of heavy metal poisonings (16). Therefore, the effects of these metals on ENαC in A6 cells are likely to be similar to their effects on ENαC in human kidneys. Since the stimulatory functions of heavy metals on ENαC require high external Na+ concentrations, in those at risk of heavy metal toxicity, a low-Na+ diet may help prevent heavy metal-induced hypertension.

REFERENCES

HEAVY METAL EFFECTS ON ENaC