Opposite effects of Ni\(^{2+}\) on *Xenopus* and rat ENaCs expressed in *Xenopus* oocytes

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Cucu, Dana, Jeannine Simaels, Jan Eggermont, Willy Van Driessche, and Wolfgang Zeiske. Opposite effects of Ni\(^{2+}\) on *Xenopus* and rat ENaCs expressed in *Xenopus* oocytes. Am J Physiol Cell Physiol 289: C946–C958, 2005. First published June 8, 2005; doi:10.1152/ajpcell.00419.2004.—The epithelial Na\(^+\) channel (ENaC) is modulated by various extracellular factors, including Na\(^+\), organic or inorganic cations, and serine proteases. To identify the effect of the divalent Ni\(^{2+}\) cation on ENaCs, we compared the Na\(^+\) permeability and amiloride kinetics of *Xenopus* ENaCs (xENaCs) and rat ENaCs (rENaCs) heterologously expressed in *Xenopus* oocytes. We found that the channel cloned from the kidney of the clawed toad *Xenopus laevis* [wild-type (WT) xENaC] was stimulated by external Ni\(^{2+}\), whereas the divalent cation inhibited the channel cloned from the rat colon (WT rENaC). The kinetics of amiloride binding were determined using noise analysis of blocker-induced fluctuation in current adapted for the transoocyte voltage-clamp method, and Na\(^+\) conductance was assessed using the dual electrode voltage-clamp (TEVC) technique. The inhibitory effect of Ni\(^{2+}\) on amiloride binding is not species dependent, because Ni\(^{2+}\) decreased the affinity (mainly reducing the association rate constant) of the blocker in both species in competition with Na\(^+\). Importantly, using the TEVC method, we found a prominent difference in channel conductance at hyperpolarizing voltage pulses. In WT xENaCs, the initial ohmic current response was stimulated by Ni\(^{2+}\), whereas the secondary voltage-activated current component remained unaffected. In WT rENaCs, only a voltage-dependent block by Ni\(^{2+}\) was obtained. To further study the origin of the xENaC stimulation by Ni\(^{2+}\), and based on the rationale of the well-known high affinity of Ni\(^{2+}\) for histidine residues, we designed α-subunit mutants of xENaCs by substituting histidines that were expressed in oocytes, together with WT β- and γ-subunits. Changing His\(^{245}\) to Asp in one putative amiloride-binding domain (WYRFHY) in the extracellular loop between Na\(^+\) channel membrane segments M1 and M2 had no influence on the stimulatory effect of Ni\(^{2+}\), and neither did complete deletion of this segment. Next, we mutated His\(^{351}\) to Arg in one putative amiloride-binding site (WYRWFY) in the intracellular loop between Na\(^+\) channel membrane segments M1 and M2, which increased the stimulatory effect of Ni\(^{2+}\). These findings support the idea that the channel structure is involved in the stimulatory effect of Ni\(^{2+}\).

Additional evidence of the stimulatory Ni\(^{2+}\) mechanism comes from the results of a scan forward and reverse analysis, which identified the segment 110-118 as the most important segment in the stimulatory Ni\(^{2+}\) mechanism. The stimulatory effect of Ni\(^{2+}\) is specific for the xENaC or whether stimulation is a general mechanism by which the activity of ENaCs is regulated in native Na\(^+\)-transporting epithelia is called self-inhibition, which can be observed as a peak current that relaxes to a lower value with a time course of a few seconds after a sudden increase in the extracellular Na\(^+\) concentration ([Na\(^+\)]\(_{o}\)) (6, 29, 36). Polyvalent cations such as Cd\(^{2+}\), Ni\(^{2+}\), Zn\(^{2+}\), and La\(^{3+}\) were shown to increase Na\(^+\) uptake in frog skin (35) by interfering with the self-inhibition process, whereas in oocytes injected with the cRNA of the homologous rodent ENaC subunits, Ni\(^{2+}\) exerted opposite effects compared with those in the native tissues, and some heavy metal ions had no effect at all (25). However, Zn\(^{2+}\) turned out to be a self-inhibition blocker in the mouse ENaC (mENaC) (29). Most intriguingly, in frog skin but also in a cell line derived from the *Xenopus* kidney (A6), Ni\(^{2+}\) and other divalent cations stimulated active Na\(^+\) transport (8, 10), whereas it had inhibitory effects in *Xenopus* oocytes expressing mENaCs (28) and rat ENaCs (rENaCs) (27).

The use of Ni\(^{2+}\) as an inorganic Na\(^+\) channel probe is well suited to the detection of strategic cysteine and histidine residues in the Na\(^+\) channel structure. Moreover, the study of toxic effects of this heavy metal, well known to be a very harmful pathogen in technology, will allow for the assessment of risk factors arising from interactions with accessible apical membrane transporters such as the ENaC. In a previous study (8), we used A6 epithelia, an immortalized cell line derived from the distal nephron of the clawed toad *Xenopus laevis*, to explore the effects of external Ni\(^{2+}\) on Na\(^+\) transport. Ni\(^{2+}\) stimulates Na\(^+\) transport in A6 epithelia. Amiloride-induced current fluctuation analysis demonstrated competition between Na\(^+\) and amiloride on the one hand and between Ni\(^{2+}\) and Na\(^+\) as well as amiloride on the other hand. In the present study, to better understand the stimulatory mechanism of Ni\(^{2+}\) on ENaCs and the competition with amiloride and Na\(^+\), we investigated the effects of Ni\(^{2+}\) on the channel cloned from *Xenopus* ENaC (xENaC) A6 cells expressed in *Xenopus* oocytes.

Moreover, we have investigated whether the effect of Ni\(^{2+}\) is specific for the xENaC or whether stimulation is a general characteristic of ENaCs cloned from other organs and/or species. Therefore, we compared the effect of Ni\(^{2+}\) on rENaCs transport epithelia such as those in the distal nephron, the distal colon, lung epithelia, and duct cells of exocrine glands. The activity of ENaCs is highly regulated by hormones such as aldosterone and vasopressin but also by extracellular factors via mechanisms that are not completely understood. One remarkable mechanism by which the activity of ENaCs is regulated in native Na\(^+\)-transporting epithelia is called self-inhibition, which can be observed as a peak current that relaxes to a lower value with a time course of a few seconds after a sudden increase in the extracellular Na\(^+\) concentration ([Na\(^+\)]\(_{o}\)) (6, 29, 36). Polyvalent cations such as Cd\(^{2+}\), Ni\(^{2+}\), Zn\(^{2+}\), and La\(^{3+}\) were shown to increase Na\(^+\) uptake in frog skin (35) by interfering with the self-inhibition process, whereas in oocytes injected with the cRNA of the homologous rodent ENaC subunits, Ni\(^{2+}\) exerted opposite effects compared with those in the native tissues, and heavy metal ions had no effect at all (25). However, Zn\(^{2+}\) turned out to be a self-inhibition blocker in the mouse ENaC (mENaC) (29). Most intriguingly, in frog skin but also in a cell line derived from the *Xenopus* kidney (A6), Ni\(^{2+}\) and other divalent cations stimulated active Na\(^+\) transport (8, 10), whereas it had inhibitory effects in *Xenopus* oocytes expressing mENaCs (28) and rat ENaCs (rENaCs) (27).

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cloned from rat colon and expressed in *Xenopus* oocytes. We found that Ni\(^{2+}\) stimulates Na\(^+\) current and conductance in xENaCs and has an inhibitory effect in rENaCs (27). The results obtained in rENaCs are also in agreement with previous observations made regarding mENaCs (28). Most interestingly, a recent report by Sheng et al. (29) may have shown that Zn\(^{2+}\), in contrast to Ni\(^{2+}\), was able to stimulate the current through mENaCs and that a cysteine was pinned down as a possible reaction partner. In their former study of Ni\(^{2+}\), these authors identified key extracellular histidine residues (α-His\(^{282}\) and γ-His\(^{239}\)) within conserved regions of the mENaC α- and γ-subunits that were required for channel block. This segment (WYRFHY) represents a putative binding site for amiloride.

To extend our previous experience with rENaCs and xENaCs, we compared Na\(^+\) channel characteristics and amiloride binding for the wild-type (WT) channels of both species after expression in *Xenopus* oocytes. Moreover, in an effort to localize the site (presumably histidine) at which Ni\(^{2+}\) binds in the xENaC to exert its stimulatory effect, we performed mutagenesis studies of the extracellular segment of the α-subunit of xENaC. We investigated xENaCs (WT and mutated) and WT rENaCs by means of two electrophysiological methods. One method was the classic two-microelectrode voltage-clamp (TEVC) technique. The other approach used was the transoocyte voltage-clamp (TOVC) method recently developed in our laboratory (7). The TOVC method was used for the investigation of transoocyte current (I\(_{TO}\)), transoocyte conductance (G\(_{TO}\)), and amiloride-induced fluctuation in current. The TOVC method is an excellent technique with which to record current fluctuation spectra, to make accurate estimates of the amiloride association rate constant (k\(_a\)), and to compare this parameter obtained from the xENaC with that from the rENaC in the WT. Furthermore, we assessed the channel characteristics [i.e., current-voltage (I-V) relationships] as well as the amiloride kinetics before and after Ni\(^{2+}\) treatment and/or histidine mutation.

**MATERIALS AND METHODS**

The research and experiments described the present report were performed with the support of the Belgian Science Foundation (Fonds voor Wetenschappelijk Onderzoek Vlaanderen). The use of *Xenopus laevis* in the Laboratory of Physiology in our study was approved by the Ministry of Agriculture (approval no. LA1210202).

Preparation of ENaC mRNA and ENaC mutants. *Xenopus* and rat ENaC cDNA encoding the α-, β-, and γ-subunits (gift of B. Rossier and J. D. Horisberger, University of Lausanne, Lausanne, Switzerland) were cloned into the pSDEasy and pSport 1 vectors, respectively. Point mutations were generated in α-xENaC cDNA with the sequential polymerase chain reaction (PCR) method using Pfu DNA polymerase (QuickChange site-directed mutagenesis kit; Stratagene, La Jolla, CA).

Expression of ENaCs in *Xenopus* oocytes. *Xenopus* females were purchased from the African *Xenopus* Facility (Knysna, South Africa). They were anesthetized by inducing hypothermia, and the ovarian lobes containing oocytes were removed. Oocytes were defolliculated by incubation in collagenase (1 mg/ml; Serva, Mannheim, Germany) for 2 h and subsequently washed with Ca\(^{2+}\)-free Ringer solution for 10 min (see below for composition). cRNA of each of the α-, β-, and γ-subunits were synthesized, and equal amounts of subunit cRNA (5 ng of total cRNA) were injected into oocytes.

**Solutions and chemicals.** Native noninjected oocytes were incubated in Ringer solution containing (in mM) 90 NaCl, 2 CaCl\(_2\), 3 KCl, and 5 HEPES, pH 7.6. For the storage of ENaC-injected oocytes, we used a low-Na\(^+\) Ringer solution that contained (in mM) 5 NaCl, 85 N-methyl-d-glucammonium Cl\(^-\) (NMDG-Cl), 2 CaCl\(_2\), 3 KCl, and 5 HEPES, pH 7.6. The experiments were performed in solutions with the following composition (in mM): 102 NaCl, 2.5 KH\(_2\)PO\(_4\), and 1 CaCl\(_2\), pH 8. NiCl\(_2\) (2 mM) was added to the solutions without osmolality adjustment.

**Transoocyte voltage clamp.** Transoocyte current and conductance from ENaC-expressing oocytes were measured using the TOVC technique as described previously (7). Briefly, the oocyte is mounted in a container designed to fit in an Ussing-type chamber. Figure 1A shows that one side of the oocyte was exposed to 102 mM NaCl-Ringer solution; this is referred to as the high-Na\(^+\) (HN) side, whereas
the other side was exposed to Na\textsuperscript{+}-free solution and is referred to as the zero-Na\textsuperscript{+} (ZN) side (NMDG-Cl-Ringer, no Na\textsuperscript{+}). In the TOVC arrangement, the positive current indicates cation movement from the HN side to the ZN side.

Transoocyte current and voltage were recorded with a digital signal processor (DSP) board (model 310B; Dalanco Spry, Rochester, NY) equipped with two high-speed analog-to-digital converters (14 bit) and two digital-to-analog converters (12 bit). A second DSP board was used to record the transoocyte resistance (r\text{TO}) simultaneously by continuously imposing low-frequency sine wave voltage (1 Hz) to the oocyte. r\text{TO} was calculated as the ratio of amplitude of the voltage and current response. Transoocyte conductance was G\text{TO} = 1/r\text{TO}.

This technique allows measurements of transoocyte current and conductance as well as current fluctuation analysis (7). For current fluctuation analysis, the HN side of the oocyte was exposed to different amiloride concentrations for short periods. Current noise was amplified, digitized, and Fourier-transformed to yield power density spectra. We recorded noise spectra as the mean of 50 sweeps of 2-s duration, resulting in a fundamental frequency of 0.5 Hz. The interaction of amiloride with the Na\textsuperscript{+} channel induced a Lorentzian component in the power density spectra. The Lorentzian parameters, the low-frequency plateau (S\text{c}), and the corner frequency (f\text{c}) were determined using nonlinear curve fitting of the spectra (see Fig. 2 and Ref. 32). The on (k\text{on}) and off (k\text{off}) rate constants of the blocking reaction were calculated from the fit of the following relationship:

\[
2\pi f_c = k_{\text{on}} [B] + k_{\text{off}}
\]

where [B] is the blocker concentration (32). However, statistical weight was assigned only to the easily calculable association rate. Numerous previous investigations (summarized in Ref. 32) showed that the fitted amiloride off rate is too close to the origin of the 2\pi f\text{c}-amiloride concentration relationship, so there is no reliability in the values (cf. Fig. 5A) that may exhibit a small shift after treatment with, e.g., Ni\textsuperscript{2+}, which makes determinations of K\text{b} (k\text{off}/k\text{on}) from noise analysis critical.

Amiloride-sensitive currents in ENaC-expressing oocytes were obtained by subtracting the currents remaining after 100 \mu M amiloride addition at the end of each experiment. To analyze titration curves for inhibition of macroscopic Na\textsuperscript{+} conductance (G\text{Na}) to that in the absence of the blocker (G\text{control}) is described by a Langmuir inhibition isotherm (22, 25):

\[
\frac{G_{\text{Na}}}{G_{\text{control}}} = \frac{K_i}{[B] + K_i}
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\[
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\]
animal donors, and

\[
\frac{G_{Na}}{G_0} = \frac{K_r'}{K_r' + [B]^n}
\]

(2)

where [B] is the concentration of amiloride, \(K_r\) is the inhibitory constant of the blocker, and \(n\) is a pseudo-Hill coefficient.

Two-microelectrode voltage-clamp technique. The measurements were performed using the standard TEVC technique (30). The oocytes were placed in a small Plexiglas chamber and continuously superfused with solutions. Microelectrodes were pulled from borosilicate glass capillaries with a thin filament (Clark Electromedical Instruments, Reading, UK) equipped with a Ag-AgCl wire. Both electrodes were filled with 3 M KCl. Using micromanipulators, the oocyte was impaled with the microelectrodes under a low-magnification stereomicroscope. We used a voltage-clamp amplifier manufactured by Warner Instruments (Hamden, CT). All transmembrane ion currents were measured as a deflection from the baseline current. The ground electrodes in the bath were also made of Ag-AgCl wires. The flow of positive charge (i.e., Na\(^+\)) from the bathing solution to the oocyte cytosol is termed inward current and is conventionally expressed with a negative sign. \(I-V\) relationships were studied with voltage pulses ranging from \(-140\) to +40 mV in increments of 20 mV. Between voltage pulses, the oocyte potential was held at 0 mV. The duration of a pulse was 400 ms, and the interval between pulses was 1,500 ms. The ENaC conductance was calculated by performing linear regression analysis of the data points between \(-140\) and +80 mV.

Analysis of voltage-activated currents. When a voltage pulse was applied (see above), the difference between the baseline current (zero voltage) and the current magnitude reached after 5 ms was termed instantaneous current (\(I_{inst}\)), representing a prompt reaction to the pulse (see Fig. 1B). Starting from this point, the subsequent slow voltage activation in the xENaC current was fitted with an exponential function whose amplitude was termed the voltage-activated current (\(\Delta I_{V}\)).

Statistics. Results are expressed as means ± SE. \(N\) is the number of animal donors, and \(n\) represents the number of experiments (oocytes).

RESULTS

Bioelectrical properties of ENaC-expressing oocytes and analysis of amiloride-induced current fluctuation (TOVC method). Xenopus oocytes have become the most frequently used expression system for studying the structure, function, and regulation of ENaC. Until now, experiments with oocytes expressing ENaCs have used the classic TEVC method. This method is very reliable for measurements of whole cell currents and conductance and for studies of \(I-V\) relationships. However, one major disadvantage in studying ENaC-expressing oocytes using the TEVC method is the potential intracellular Na\(^+\) loading during long-term exposure. Loading the oocytes with Na\(^+\) is certainly less pronounced when using the TOVC method, in which the oocytes are exposed to high Na\(^+\) on one side only as described in MATERIALS AND METHODS. The Na\(^+\) flux from the HN side to the ZN side was further enhanced by the application of a transoocyte potential of +40 mV, positive on the HN side. Under these conditions, \(I_{TO}\) for xENaCs was in the range of 2.1–5.2 \(\mu\)A, and \(G_{TO}\) ranged from 20 to 200 \(\mu\)S. Amiloride added to the HN side blocked the current and conductance in a dose-dependent manner. A typical example of an experiment in which the inhibitory effect of amiloride on \(G_{TO}\) and \(I_{TO}\) was studied is shown in Fig. 2A with \([Na^+]_{HN}\) of 102 mM. In the presence of an electrochemical gradient across the oocyte, a leak current around the oocyte cannot be excluded. Indeed, 100 \(\mu\)M amiloride in the HN bath did not block \(I_{TO}\) completely but reduced \(I_{TO}\) from 3.04 ± 0.94 to 0.48 ± 0.01 \(\mu\)A and \(G_{TO}\) from 181.0 ± 38.2 to 25.6 ± 1.5 \(\mu\)S (\(N = 3, n = 5\)). Under the assumption that the amiloride-insensitive current passes through a paracellular leak, we estimated the leak conductance as \(\sim 15\%\). The amiloride-sensitive components of \(I_{TO}\) and \(G_{TO}\) are referred to as \(I_{SN}\) and \(G_{SN}\) and were used for further calculations. We decided to quantify the inhibitory effect of different doses of amiloride by studying the effect on \(G_{SN}\), because the results obtained from the inhibition of \(I_{SN}\) were more scattered. Figure 2B shows the effect of amiloride on mean values of \(G_{relative}\) that represent the ratio of \(G_{SN}\) in the presence of amiloride to \(G_{SN}\) in control. We evaluated the dependence of the macroscopic \(K_i\) on [Na\(^+\)] with 102 or 30 mM [Na\(^+\)] on the HN side. \(K_i\) was determined by fitting the Langmuir isotherm equation (Eq. 2) to the data. Figure 2B shows that \(K_i\) was strongly [Na\(^+\)] dependent. \(K_i\) values were 185 ± 20 and 458 ± 10 nM at 30 and 102 mM [Na\(^+\)], respectively. This relationship suggests competition between Na\(^+\) and amiloride. Similarly, in our previous study (8), we found competition between Na\(^+\) and amiloride for xENaCs in A6 epithelia, with \(K_i\) = 126 ± 7 and 204 ± 2 nM for 30 and 102 mM apical Na\(^+\) ([Na\(^+\)]\(_{ap}\)), respectively.

The amiloride-induced fluctuation in \(I_{TO}\) was analyzed in oocytes expressing xENaCs and rENaCs under conditions in which transoocyte currents were partly inhibited with the diuretic on the HN side. Power density spectra could be recorded with amiloride concentrations ranging from 1 to 20 \(\mu\)M. In this concentration range, Lorentzian noise was markedly greater than instrumentation noise. Figure 2C shows power density spectra recorded from xENaCs for two amiloride concentrations. The averaged values of \(f_c\) were 13.0 ± 1.5 Hz at 3 \(\mu\)M and 30.7 ± 0.7 Hz at 8 \(\mu\)M amiloride concentration (\(N = 3, n = 10\)). The association (\(k_{on}\)) and dissociation (\(k_{off}\)) rate constants were determined by performing linear regression analysis of the \(2\pi f_c\) amiloride concentration data (Fig. 2D and Eq. 1). The strong reduction of \(k_{on}\) at higher [Na\(^+\)] confirms competition between Na\(^+\) and amiloride as suggested in Fig. 2B and as shown in Fig. 2D.

Obviously, Na\(^+\) entry at the HN side occurs through the ENaC and is amiloride sensitive. On the other hand, Na\(^+\) exit from the cell side to the ZN side could occur not only through the ENaC but also via the endogenous Na\(^+\)-K\(^+\)-ATPase (20). The effect of amiloride on outward Na\(^+\) currents has been studied in frog skin (31), and the amiloride inhibition constant was calculated from noise analysis data as 0.31 \(\mu\)M for outward Na\(^+\) currents compared with 0.19 \(\mu\)M for inward Na\(^+\) currents. Outward Na\(^+\) currents were also measured using the TEVC method in human ENaC (hENaC)-expressing oocytes (6). These currents could be inhibited by large (50 \(\mu\)M) doses of amiloride, but \(K_i\) for amiloride was not determined. We tried using amiloride from the ZN side, but we could measure amiloride-sensitive currents at zero potential in only 4 of 20 experiments. Figure 3A shows an experiment in xENaCs, in which, upon the addition of 100 \(\mu\)M amiloride to the ZN side, \(I_{TO}\) was blocked by \(\sim 50\%\). The inability of amiloride to block Na\(^+\) exit observed in 16 of 20 experiments may have been caused by different factors. A first possibility is that the exit of Na\(^+\) occurs only via the Na\(^+\)-K\(^+\)-ATPases on the ZN side. To verify this hypothesis, we added 100 \(\mu\)M ouabain to the ZN side. To avoid Na\(^+\) loading, the oocytes were incubated in Na\(^+\)-free solutions on both sides and were exposed for only brief periods to high [Na\(^+\)]\(_{HN}\) (Fig. 3B). The \(I_{TO}\) remained

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unaffected despite treatment with 100 μM ouabain for ~30 min. This result suggests that the exit of Na⁺ to the ZN side was not via Na⁺/K⁺ pumps. Another explanation could be that the exit of Na⁺ on the ZN membrane is mediated through other native Na⁺ transporters in the oocyte membrane. A voltage-dependent and amiloride-insensitive Na⁺ channel activated by long depolarization was found in the Xenopus oocyte membrane (4). Because we expected cell depolarization during exposure to high [Na⁺] on the HN side, it is likely that in our experimental arrangement, this Na⁺ channel was opened and mediated the exit of Na⁺. Of course, other transporters, if Na⁺ coupled, could mediate the efflux of Na⁺ at the ZN side as well.

**Ni²⁺ effects on ENaC-expressing oocytes: wild-type xENaCs vs. rENaCs.** We first studied the Ni²⁺ dose-response relationship of the oocyte conductance after expression of xENaCs or rENaCs. It turned out (data not shown) that Ni²⁺ stimulated xENaCs as shown previously for A6 cells (8) and blocked rENaCs as reported previously (27). xENaC stimulation occurred with Michaelis-Menten kinetics (Hill coefficient of 0.9, n = 12, N = 3) and a half-maximal Ni²⁺ concentration ([Ni²⁺]) of 210 ± 30 μM. Unfortunately, simple saturation kinetics with respect to the inhibitory effect on rENaCs were not that obvious when the data were tentatively fitted with the Langmuir equation (see MATERIALS AND METHODS). The apparent Hill coefficient was 0.6 (n = 9, N = 3), and a half-maximal [Ni²⁺] could be estimated only with low confidence as 1.3 ± 0.5 mM. It is likely that the maximal dose concentration for inhibition is of the order of 10 mM. However, [Ni²⁺] > 5 mM could not be tested, owing to solubility problems with the saline used.

Figure 4 shows the effects of a saturating dose of Ni²⁺ (2 mM) on current and conductance measured using the TOVC method for xENaCs and for a submaximal concentration of 2 mM [Ni²⁺] in the case of rENaCs. Here, too, the HN side contained 102 mM Na⁺, whereas on the ZN side, the oocyte was bathed in 102 mM NMDG-Cl solution. Figure 4, A and B, shows I_TO and G_TO in response to a voltage step from 0 to +40 mV (referenced to the ZN side) and the effect of amiloride. In xENaC-expressing oocytes, addition of 2 mM [Ni²⁺] to the HN side solutions augmented the amiloride-sensitive conductance by ~50 ± 1% (n = 6, N = 4). The striking similarity of
the degree of stimulation in xENaC-expressing oocytes and A6 cells (8) suggests that Ni²⁺ acts on the channel itself and not via cellular signaling mechanisms that most likely differ in these cellular systems. Therefore, the interaction of Ni²⁺ with other native proteins in A6 does not seem to cause the activation of the current.

Contrary to the effect on xENaCs, 2 mM [Ni²⁺] inhibited the current and conductance in rENaCs by 30 ± 2% (N = 3, n = 5). In xENaC- as well as in rENaC-expressing oocytes, the current recorded after application of Ni²⁺ is completely inhibited by large doses of amiloride applied on the HN side, which demonstrates that in both cases, Iₜₒ is carried by Na⁺ through the ENAC. The rapid response of both xENaCs and rENaCs to Ni²⁺ may indicate a site of action located on the extracellular side of the ENAC and thus may exclude any interference with intracellular targets. A previous study (28) showed that mENaCs expressed in oocytes were blocked by external Ni²⁺, just like in rENaCs. Sheng et al. identified extracellular histidine residues (α-His382 and γ-His399) within conserved regions of the α- and γ-subunits that were required for channel block by Ni²⁺. These residues are located in the extracellular loop of mouse α- and γ-ENaC subunits within a segment that represents a putative binding site for amiloride (14). However, the authors mentioned that the inhibitory channel interaction with Ni²⁺ is not competitive with amiloride, contrary to our findings regarding stimulatory Ni²⁺ for xENaCs in A6 cells (8). To clarify this point and to elucidate the opposite effect of Ni²⁺ in Xenopus vs. mammalian ENaCs, we performed noise analysis studies of amiloride-induced fluctuations in current at different Na⁺ concentrations.

The amiloride-induced Lorentzian components in the Iₜₒ noise spectra were recorded in the presence and in the absence of Ni²⁺. The addition of Ni²⁺ to the amiloride-containing solutions in 102 mM [Na⁺]ₜₒ on the HN side caused a marked diminution of kₜₒ in both xENaCs and rENaCs. Figure 5A demonstrates the influence of 2 mM [Ni²⁺] on the amiloride kₜₒ for xENaCs and rENaCs. For xENaCs, the control kₜₒ was 17.8 ± 0.4 μM⁻¹·s⁻¹ and is comparable to the value previously recorded in A6 cells as 20.2 μM⁻¹·s⁻¹ (8). In rENaC-expressing oocytes, kₜₒ was 21.6 ± 1.2 μM⁻¹·s⁻¹ and thus compares well with both the kₜₒ value obtained from A6 cells and that obtained from xENaC-expressing oocytes. Although Ni²⁺ exerts an opposite effect on the conductance of rENaCs vs. xENaCs, we thus found a similar, competition-like effect on the binding of amiloride. A comparable reduction of kₜₒ was obtained for both species, amounting to 59.7 ± 10.2% (n = 5, N = 2) and 59.2 ± 19.5% (n = 6, N = 3) for rENaCs and xENaCs, respectively. The reduction of kₜₒ in xENaCs as well as in rENaCs suggests direct competition between Ni²⁺ and amiloride for a common binding site. Alternatively, it is conceivable that a steric modification of the channel by Ni²⁺ hinders amiloride to reach its site for specific blocking farther away. Because both external Ni²⁺ (Fig. 5A) and high [Na⁺] (Fig. 2D) diminished amiloride kₜₒ, we hypothesized that the effect of both cations on amiloride binding occurs through an interaction at an analogous, if not identical, site in both channel species, thus suggesting competition between amiloride, Ni²⁺, and Na⁺.

This prompted us to examine the effect of 2 mM [Ni²⁺] at different [Na⁺]ₜₒ on the rate of amiloride binding. Current noise induced by 8 μM amiloride was recorded from xENaC- and rENaC-expressing oocytes in the [Na⁺]ₜₒ range between 30 and 102 mM. Figure 5B shows the effect of Ni²⁺ on the [Na⁺]ₜₒ dependence of the amiloride chemical binding rate. In control conditions, with xENaCs as well as with rENaCs, the amiloride reaction rate recorded with 8 μM diuretic decreased when [Na⁺] was elevated. Conspicuously, in the presence of 2 mM [Ni²⁺] (Fig. 5B), the corner frequency became independent of the [Na⁺] in both xENaC- and rENaC-expressing oocytes (circles) as well as in rENaC-expressing oocytes (squares).
the channel pore by Ni$^{2+}$ in rENaCs is conceivable if it impedes amiloride to bind to its high-affinity site located in the channel pore and thus blocks the entry of Na$^+$. To further explore the process of the interaction of Ni$^{2+}$ with both xENaCs and rENaCs, we recorded $I$-$V$ curves using the TEVC method.

Oocytes were clamped at 0 mV, and step changes in the membrane voltage were applied as described in MATERIALS AND METHODS. Generally, the oocytes were incubated in low-[Na$^+$/H$^+$] solution (5 mM) to avoid cell loading with Na$^+$, and they were exposed to high (102 mM [Na$^+$/H$^+$]) solutions only for brief periods. The time dependence of the current response to the voltage pulses, as well as $I$-$V$ relationships could be studied. The superimposed traces of the currents recorded during the voltage pulse protocol are shown in Fig. 6. From these records (Fig. 6A), it is clear that the membrane current in the case of xENaCs consists of two components: 1) an instantaneous current jump that reflects the conductance of the membrane at 0 mV and 2) a voltage-activated current that becomes particularly apparent at high, hyperpolarizing voltages. The instantaneous and the activated currents are completely abolished by amiloride (data not shown), demonstrating that both components represent an increase in the current flowing through ENaCs. To quantify the activation process, the amiloride-sensitive currents were fitted with a single exponent (see Fig. 1B). From this analysis, we obtained the instantaneous current ($I_{\text{inst}}$), which represents the current jump at the beginning of the voltage pulse and the voltage-activated current ($\Delta I_V$), determined as the amplitude of the exponential function. These activated Na$^+$ currents may be attributed to a slow (in the range of tenths of a second) process that is caused by the effect of voltage on channel gating. A similar activation of the ENaC by hyperpolarizing voltages was previously reported for both ENaCs from A6 cells (24) and of human origin (3).

Figure 6B shows the effect of Ni$^{2+}$ on $I_{\text{inst}}$ and on $\Delta I_V$ at different voltages in xENaCs. The addition of 2 mM [Ni$^{2+}$/H$^+$] stimulated $I_{\text{inst}}$. Interestingly, $\Delta I_V$ was apparently unaffected by the divalent cation. Like xENaCs, rENaCs exhibited large, inwardly rectifying Na$^+$ currents (Fig. 6C) that were amiloride sensitive but inhibited by Ni$^{2+}$. The process of current activation as shown with xENaC currents at hyperpolarizing voltages was not observed in oocytes expressing the rat homolog (Fig. 6C). The absence of current activation by voltage indicates that this process is dependent on the ENaC species as described in previous studies with regard to hENaCs (2) and herein regarding xENaCs, whereas it was less evident in rENaCs (2). The observation made by Awayda (2) was confirmed in our present experiments with rENaCs. The reason why the rENaC is not activated by strong hyperpolarization is not clear. It should be related to some differences in structure among ENaC species.
(xENaCs and rENaCs), resulting in different gating sensitivity to voltage. As shown in Fig. 6D, 2 mM [Ni2+] caused not only the blockade of the instantaneous current response to the voltage pulse in the case of the rENaC but also a further time- and voltage-dependent (and slower) inhibition within ~100 ms.

Next, we analyzed in more detail the effect of Ni2+ on \( I_{\text{inst}} \) and \( \Delta I/V \) for xENaCs by constructing the \( I-V \) relationships in the presence and absence of the divalent cation. Figure 7, A and B, shows that Ni2+ stimulated practically only \( I_{\text{inst}} \) in xENaCs. Furthermore, Ni2+ stimulated \( I_{\text{inst}} \) independent of voltage, because the ratio of the current in the presence of Ni2+ over control \( (I_{\text{inst}}^{\text{Ni}^{2+}}/I_{\text{inst}}^0) \) is ~2 at any voltage between ~140 and ~40 mV, which is shown in Fig. 7A. Together, these data are consistent with the idea of Na+ transport stimulation at a site located outside the voltage-sensing channel pore. Membrane Na+ conductance \( (G_{\text{Na}}) \) was calculated from the slope of the linear regression analysis of \( I_{\text{inst}} \) between ~140 and ~80 mV (see Fig. 7A). After Ni2+ addition, \( G_{\text{Na}} \) increased from 40.4 ± 2.3 to 83.8 ± 7 \( \mu \)S. These data confirm the results obtained from measurements in A6 cells (8). The current stimulation by Ni2+ is fast (Fig. 4A), which indicates that this voltage-insensitive site for the stimulatory action of Ni2+ must be easily accessible. Moreover, the process of stimulation of \( I_{\text{inst}} \) is not related to the secondary voltage activation of the channel (Fig. 7B).

For further comparison of the effect of Ni2+ on xENaCs and rENaCs, we analyzed the instantaneous current in rENaCs at the beginning of the voltage pulse. Ni2+ diminished \( I_{\text{inst}} \) at any voltage as shown in Fig. 7C. From the slope of the linear regression analysis between ~140 and ~80 mV, we calculated \( G_{\text{Na}} = 104.4 ± 2.8 \mu \)S in the absence of Ni2+ and 62 ± 0.4 \( \mu \)S in its presence.

Histidine mutations in the extracellular loop of the \( \alpha \)-xENaC in the domains WYRFH\( ^{215 \text{Y}} \) and HKSWG\( ^{310 \text{C}} \): stimulation by Ni2+ is unaffected, although amiloride and Na+ interact differently with xENaCs. All of the above results demonstrate that Ni2+ exerts opposite effects on Na+ current in xENaC- and rENaC-expressing oocytes, although the channels have strikingly similar biophysical properties. Moreover, we have demonstrated that in xENaCs (but also in rENaCs), amiloride binding is impaired by Ni2+ and interferes with Na+.

This result is most likely related to the fact that the binding of the diuretic and the divalent cations take place in areas that are in close proximity to each other, if not in identical locations. Therefore, our attempts to localize the binding site of Ni2+ were based on data available for the binding site of amiloride. A mutagenesis screen of amino acids preceding the second transmembrane segment of \( \alpha \)-, \( \beta \)-, and \( \gamma \)-ENaC identified the residues \( \alpha \)-Ser\( ^{583} \)\( \beta \)-Gly\( ^{525} \), and \( \gamma \)-Gly\( ^{537} \) that, when mutated, reduced 1,000-fold the channel affinity to amiloride (18). It has been shown that all ENaCs cloned to date share this site for amiloride inhibition. Before as well as after the identification of the critical role of the homologous \( \alpha \)-Ser\( ^{583} \)\( \beta \)-Gly\( ^{525} \), and \( \gamma \)-Gly\( ^{537} \) in amiloride binding, other mutations were made in search of other residues that contribute to amiloride block. Thus the short amino acid segment WYRFHY in the mouse and rat \( \alpha \)-ENaC domain was also proposed to participate in amiloride binding (14, 19). Deletion of this region resulted in a loss of amiloride binding to the channel. Clearly, the interaction between amiloride and the ENaC is complex and may involve other, as yet unidentified residues in the extracellular loop of the ENaC family. Within the WYRFHY segment, from \( \alpha \)- and \( \gamma \)-subunits of mENaCs, the mutation \( \alpha \)-His\( ^{582} \) to aspartate or double mutations to arginine \( \alpha \)-His\( ^{582} \)Arg/\( \gamma \)-His\( ^{230} \)Arg eliminated the Ni2+ block in oocytes expressing mENaCs (28). Finally, extracellular loop folding might bring the WYRFHY segment close to the \( \alpha \)-Ser\( ^{583} \)\( \beta \)-Gly\( ^{525} \)\( \gamma \)-Gly\( ^{537} \) amiloride-
binding motif at the pore entrance and thus form a complex drug-binding pocket.

To examine whether Ni\(^{2+}\) would bind to the homologous segment in xENaCs and thus contribute to Ni\(^{2+}\) stimulation, we substituted the corresponding histidine residue α-His\(^{215}\) within the WYRFHY segment of the Xenopus α-subunit with aspartate and coexpressed an α-His\(^{215}\)Asp ENaC with WT β- and γ-ENaCs. We did not observe any significant changes regarding Ni\(^{2+}\) action on amiloride-binding rates in xENaCs in two experiments (data not shown). Not unexpectedly, 2 mM Ni\(^{2+}\) stimulated the amiloride-sensitive current and conductance by about \(\sim 40\%\). Assuming that histidine is important for Ni\(^{2+}\) binding, this suggests that the site for Ni\(^{2+}\) binding and stimulation of xENaCs does not involve the histidine residue from WYRFHY, which, in contrast, participates in the inhibitory Ni\(^{2+}\) binding in mENaCs. Even more so, deleting the entire WYRFHY stretch led to the same result (data not shown), indicating that neither His\(^{215}\) nor its immediate surroundings are related to the xENaC stimulation by Ni\(^{2+}\).

In our previous report on A6 cells (8), we attempted to chemically characterize the residues involved in xENaC stimulation by Ni\(^{2+}\). We found that p-chloromercuribenzoate (PCMB), a reagent that binds to cysteine but not the histidine-reactive diethyl pyrocarbonate (DEPC) mimicked the stimulatory effect of Ni\(^{2+}\). However, from the chemical point of view, histidine rather than cysteine may be the most preferred partner with which to form complexes with Ni\(^{2+}\), e.g., in enzymes such as urease (34), and it has also become an important Ni\(^{2+}\)-binding site in the high-affinity site for the Ni\(^{2+}\) channel. Results are from oocytes bathed in Ni\(^{2+}\)-free solutions (Ctrl) compared with those exposed to 2 mM Ni\(^{2+}\).

Suggested a cysteine as one possible Ni\(^{2+}\)-binding site in the A6 ENaC (8). Figure 8 also shows that the corresponding segment in the rENaC contains no histidines, but we note that it still shares the cysteine position with Xenopus.

In the following section of the text, only α-subunit mutations of xENaCs are described, and they were expressed in oocytes together with WT β- and γ-subunits. We generated different point mutations by substitution of the α-histidine residue His\(^{416}\) with the amino acids alanine (A), arginine (R), tyrosine (Y), and aspartic acid (D). However, these mutations did not abolish the stimulating effect of Ni\(^{2+}\) or reverse it to an inhibitory type. Nevertheless, each mutation resulted in channels with different sensitivities to Ni\(^{2+}\) (concerning only the impact on the amiloride rate constant) and different \(I-V\) behavior. As shown with the use of the TOVC method, all substitutions of α-His\(^{416}\) diminished amiloride rate in control conditions (Ctrl); Table 1 in the absence of Ni\(^{2+}\) compared with the WT channels. Interestingly, \(k_{\text{on}}\) of all mutations becomes roughly two-thirds that of the WT. Also as in the WT, the addition of 2 mM [Ni\(^{2+}\)] further diminished \(k_{\text{on}}\), but the percentage of this effect was dependent on the amino acid substituted as shown in Table 1. For instance, in oocytes expressing α-His\(^{416}\) Tyr, β\(^{-}\)-WTγ\(^{-}\)-WT channels, Ni\(^{2+}\) decreased \(k_{\text{on}}\) by 27%, which is only half the effect caused by the divalent in WT channels. The contrary, in channels with α-His\(^{416}\) Ala, Ni\(^{2+}\) decreased the amiloride on rate by 77%. This reduction of \(k_{\text{on}}\) is not more than 30% more than in WT ENaCs. Nevertheless, the final \(k_{\text{on}}\) values in the presence of Ni\(^{2+}\) seem not to differ too much from the WT case. These observations indicate that the mutation-independent effect of Ni\(^{2+}\) on the amiloride association rate constant excludes any hypothetical Ni\(^{2+}\) interaction with the Hist\(^{416}\) residue. Moreover, because all mutations of α-His\(^{416}\) clearly affected the amiloride binding even before Ni\(^{2+}\) was added (see Table 1), an allosteric rather than direct histidine involvement in the observed effects may appear likely. Therefore, the question whether those just-described mutations would have an impact on control Na\(^{+}\) conductance (absence of blocker and stimulator) is most interesting. We addressed this question using the TEVC technique also on mutated channels before and after addition of 2 mM Ni\(^{2+}\) (cf. MATERIALS AND METHODS and Fig. 7 legend). Figure 9 shows the voltage dependence of the Na\(^{+}\)-specific \(I_{\text{inst}}\) in the presence and absence of Ni\(^{2+}\) for WT and channels with substitutions of α-His\(^{416}\). Oocytes expressing mutated channels exhibited an instantaneous voltage activation of current at hyperpolarizing pulses such as those

<table>
<thead>
<tr>
<th>Channel Type</th>
<th>(n)</th>
<th>Ctrl</th>
<th>Ni(^{2+})</th>
<th>(\Delta k_{\text{on}}) Induced by Ni(^{2+}), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>α(^{-})WTβ(^{-})γ(^{-})WT</td>
<td>12</td>
<td>17.9 ± 0.4</td>
<td>7.2 ± 0.3</td>
<td>59.2</td>
</tr>
<tr>
<td>α(^{-})His(^{416})Arg(^{-})β(^{-})γ(^{-})WT</td>
<td>10.5 ± 0.5</td>
<td>6.4 ± 0.2</td>
<td>39.0</td>
<td></td>
</tr>
<tr>
<td>α(^{-})His(^{416})Asp(^{-})β(^{-})γ(^{-})WT</td>
<td>13.1 ± 0.7</td>
<td>4.6 ± 0.1</td>
<td>64.9</td>
<td></td>
</tr>
<tr>
<td>α(^{-})His(^{416})Tyr(^{-})β(^{-})γ(^{-})WT</td>
<td>13.3 ± 0.4</td>
<td>9.7 ± 0.2</td>
<td>27.1</td>
<td></td>
</tr>
<tr>
<td>α(^{-})His(^{416})Ala(^{-})β(^{-})γ(^{-})WT</td>
<td>13.0 ± 0.5</td>
<td>3.0 ± 0.07</td>
<td>76.9</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\), no. of experiments. \(\Delta k_{\text{on}}\), shift in amiloride association rate constant; Ctrl, control; xENaCs, Xenopus laevis epithelial Na\(^{+}\) channels. Results are from oocytes bathed in Ni\(^{2+}\)-free solutions (Ctrl).
expressing the WT channel, although the magnitude of the current activation by voltage was sometimes too small for an accurate fit as, for instance, in the case of H9251-His416Tyr-WT. After addition of Ni²⁺, I_inst from oocytes expressing mutated channels was always significantly higher. This increase, known from WT channels, again shows that the site at which Ni²⁺ binds cannot be His416; thus other residues must participate in Ni²⁺ coordination. In light of the finding that the degree of stimulation is nevertheless dependent on the type of amino acid substituted for His416, we assume that His416 might influence the I-V relationships not directly but rather allosterically. We noticed that in oocytes expressing, for instance, channels with aspartate instead of His416, the control currents were much smaller in the absence of Ni²⁺ compared with the WT. Nevertheless, Ni²⁺ caused a remarkable increase in this current (Fig. 9A). In contrast, substitution of histidine with tyrosine resulted in currents even smaller than those observed with aspartate and was correlated with a low but still clear stimulation of I_inst by Ni²⁺ (Fig. 9C). To better analyze this process, we calculated the ratio of current stimulated by Ni²⁺ to the current in control conditions (I_inst). For oocytes expressing the WT channels, this ratio was ~2 at any voltage between -140 and -40 mV. Substitution of histidine with aspartate (Fig. 9A) caused a sixfold increase of I_inst/Ni²⁺ at all voltages, although starting from a much lower level. One simple explanation for this result may be that there is now a strong electrostatic interaction between the divalent Ni²⁺ and aspartate (negatively charged). Why the currents in the absence of Ni²⁺ are also smaller than in WT remains obscure at present but may reflect a similar strong interaction with the monovalent Na⁺ and therefore lead to a reduced conductance. For the channels containing as substitutes the small neutral alanine (Fig. 9D) but also the long and mobile, positively charged arginine (Fig. 9B) for histidine, the currents in control conditions as well as in Ni²⁺-containing solutions are comparable to those observed in the WT (Fig. 9A), suggesting no hindrance by these mutations. With respect to the drastically reduced currents with the histidine-to-tyrosine mutation (Fig. 9C), the bulkiness and thus presumably the immobility of tyrosine as opposed to the mobile aspartate results perhaps in a much-reduced channel accessibility to Na⁺ and may be the cause of these observations.

Thus far, we may conclude that our mutation assay data in Table 1 and Fig. 9 do not support a role of His⁴¹⁶ as a putative ligand of Ni²⁺ but might rather point to an allosteric influence on the mechanisms underlying Na⁺ conductance and amiloride block. This makes sense because the rENaC, in which the corresponding segment (see Fig. 8) is devoid of histidines, exhibits the very same features in the Na⁺-amiloride-Ni²⁺

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Fig. 9. Activation by Ni²⁺ of I_inst measured in oocytes expressing α-xENaC-mutated subunits together with β- and γ-WT-xENaC. The data obtained with WT-α-, β-, and γ-ENaC are indicated only in A (○, no Ni²⁺; ●, 2 mM Ni²⁺). For all mutations, Ni²⁺-free solution is denoted ○, and Ni²⁺-containing salines are indicated by ▲. Data represent means ± SE. A: results from oocytes containing the α-His⁴¹⁶Asp mutation (N = 2, n = 4). B: α-His⁴¹⁶Arg mutation (N = 3, n = 5). C: α-His⁴¹⁶Tyr mutation (N = 2, n = 3). D: α-His⁴¹⁶Ala-mutated channels (N = 4, n = 10).
interaction as does the xENaC (see Fig. 3) although Ni\(^{2+}\) blocks the former whereas it stimulates the latter channel type (see Fig. 4). While Sheng et al. (28) allocated the mENaC block by Ni\(^{2+}\) to the WYRFHY segment, we could not find a role of the corresponding xHis\(^{2+}\) (see above). Whether in the His\(^{411}\)KSWGHis\(^{448}\)Cys\(^{417}\) segment (cf. Fig. 8) His\(^{411}\) or Cys\(^{417}\)—one of the many extracellular cysteines—plays a role in Ni\(^{2+}\) stimulation of xENaCs remains to be elucidated.

We also analyzed the effect of α-His\(^{416}\) substitution on the process of voltage-activated currents. As depicted in Figs. 6B and 7B for control (His\(^{416}\)), Ni\(^{2+}\) had no consistent effect on Δν, and this was also the case for all mutations (data not shown). Therefore we shall not deal with this issue further.

DISCUSSION

The ENaC is an important component of the transepithelial Na\(^{+}\) transport route. The ENaC is responsible for Na\(^{+}\) balance and thus controls extracellular fluid volume, arterial blood pressure, and the regulation of airway surface fluid. While the regulation of channel synthesis and surface density by hormones has been well explored, the control by extracellular factors involved in the regulation of channel kinetics is poorly understood. Regulation of ENaC activity is possible, e.g., by extracellular Na\(^{+}\) (through a phenomenon called self-inhibition) but also by several organic or inorganic cations that seem to interfere with self-inhibition. Each of the three channel subunits has a large, cysteine-rich extracellular loop of as yet unknown function. Although a direct interaction with this extracellular domain of ENaC has not yet been demonstrated for the above-mentioned extracellular modulators, the available data strongly suggest that ENaC behaves as a ligand-gated channel similar to several other members of the ENaC-degenerin family (13).

In the present study, we have provided the first direct comparison of the functional properties and the effect of Ni\(^{2+}\) in heterologously expressed xENaCs and rENaCs. We report significant differences between these channels with respect to Ni\(^{2+}\) sensitivity and the process of voltage activation. Ni\(^{2+}\) ions may become important tools for molecular identification of regulatory sites in ENaCs from various cell types and tissues. Ni\(^{2+}\) exerts opposite effects on Na\(^{+}\) current in WT xENaCs and rENaCs. We found in this study that Ni\(^{2+}\) stimulated xENaCs. The stimulatory effect of Ni\(^{2+}\) is in agreement with the findings of previous studies that showed that Ni\(^{2+}\) as well as other polyvalent cations, such as La\(^{3+}\), Zn\(^{2+}\) (36), and Cu\(^{2+}\) (9), stimulated Na\(^{+}\) uptake in frog skin. To the contrary, in toad urinary bladder, divalent cations such as Ca\(^{2+}\), Mg\(^{2+}\), or Ba\(^{2+}\) blocked the ENaC from the external side in a voltage-dependent manner with an estimated K\(_i\) of ~200 mM (23). Because all of the above results were obtained from measurements of Na\(^{+}\) transport in amphibian tissues, it is clear that the reported effects might be species dependent. In view of the fact that Ni\(^{2+}\) is only one of many cationic agents that stimulate ENaCs, its action may occur at a site that is not very selective. However, studies conducted at our laboratory, have shown that divalent and polyvalent cations exert different influences on Na\(^{+}\) transport in the very same epithelial tissue. For instance, in A6 epithelia, Zn\(^{2+}\) inhibits Na\(^{+}\) transport with a K\(_i\) of 45 μM (1), whereas Ni\(^{2+}\) stimulated I_{Na} with a K\(_m\) of 0.5 mM (8) and Mg\(^{2+}\) has no effect when added on the apical side (15). In rENaCs (and also mENaCs), Ni\(^{2+}\) is a blocker whereas Zn\(^{2+}\) turned out to be a stimulator in mENaCs, obviously counteracting self-inhibition (29). Clearly, the action of heavy metal ions such as Ni\(^{2+}\) must occur at selective binding sites. On the other hand, the action of divalent cations may be influenced by other regulatory proteins specific to each of these tissues. To circumvent this problem, we analyzed the influence of Ni\(^{2+}\) on oocytes expressing ENaCs. The Xenopus oocyte expression system enabled us to study the influence of Ni\(^{2+}\) on ENaCs cloned from A6 cells and from rat colon. In this way, we were able to compare the results obtained from xENaC- and rENaC-expressing oocytes with those from epithelial cells.

Comparison of amiloride noise (TOVC) and whole cell Na\(^{+}\) current (TEVC). Previous studies of xENaCs and mENaCs have produced conflicting results regarding the relationship between extracellular divalent cations and amiloride. In the case of A6 cells, xENaC direct activation by Ni\(^{2+}\) and competition with amiloride was reported (8). Because the effect of Ni\(^{2+}\) on A6 cells was investigated using the noise analysis technique, we aimed to implement the same method to study amiloride-induced current fluctuation in intact oocytes. In early studies, noise analysis was widely used for the estimation of the amiloride rate constants in Na\(^{+}\)-transporting epithelia. More recently, studies of oocytes expressing ENaCs revealed limits for the accurate calculation of the amiloride inhibition constant and the analysis of I_{Na} block. The reason why k_{on} and k_{off} rate constants of amiloride are not reported for patch-clamp experiments in ENaC-expressing oocytes may be due to the slow gating mode of ENaCs that complicates the kinetic analysis (17). The blocking events by amiloride are indistinguishable from the channel-closed state because they have about the same duration. Only one group of researchers (17) has reported amiloride rate constants calculated from blockade of rENaCs expressed in oocytes. To determine the association rate of the blocker, these authors used ENaCs formed by α- and β-subunits only, because this channel is almost constantly open. Therefore, blocking events could be readily distinguished from the rare spontaneous channel closing. However, a precise estimate of the amiloride rate constants in α-, β-, and γ-ENaC-expressing oocytes measured using the patch-clamp technique has not been reported to date.

We implemented the noise analysis method adapted for the TEVC method as reported by Segal et al. (26). The disadvantage of this technique is that the amplifier noise level is elevated and produces a limited bandwidth. With the development of the TOVC technique (7), we are now able to compare the amiloride rate constants obtained from A6 cells with those obtained from oocytes expressing xENaCs. We calculated k_{on} as 17.9 ± 0.4 μM\(^{-1}\)s\(^{-1}\) and k_{off} as 9.1 ± 3.3 s\(^{-1}\), which is in excellent agreement with the values obtained from A6 cells. Our results obtained with oocytes indicated that Ni\(^{2+}\) diminishes the amiloride K\(_i\) by decreasing the on rate to 7.2 ± 0.3 μM\(^{-1}\)s\(^{-1}\) and increasing the off rate constant to 11.6 ± 2.7 s\(^{-1}\). It is clear that the determination of the off rate constant is relatively inaccurate (see MATERIALS AND METHODS), so more weight should be given to k_{on}, and the conclusions derived from this analysis are fully consistent with those from the study of macroscopic K\(_i\) behavior; that is, the amiloride on rate is the parameter that is influenced by Ni\(^{2+}\) and/or Na\(^{+}\). One simple way to interpret the data shown in Fig. 5 (amiloride-Na\(^{+}\)-Ni\(^{2+}\)
interaction) would be that Ni\textsuperscript{2+} competes with amiloride and Na\textsuperscript{+} at the very same site on the channel protein and that this is true for xENaCs and rENaCs. However, we would then expect Ni\textsuperscript{2+} not to stimulate but rather to block xENaCs as observed with rENaCs. Alternatively, at a site outside the channel pore, Ni\textsuperscript{2+} might exert an allosteric effect on Na\textsuperscript{+}-amiloride binding. This mechanism could then stimulate Na\textsuperscript{+} entry by affecting the channel in a way that hinders amiloride from reaching its site, i.e., by establishing an “apparent competition” due to mutual exclusion effects on different binding sites for Na\textsuperscript{+}-amiloride and Ni\textsuperscript{2+}, respectively.

Another important finding of an earlier report (27) and of our present study is that Ni\textsuperscript{2+} inhibits the amiloride-sensitive current in oocytes expressing rENaC. The blocking effect of Ni\textsuperscript{2+} is in good agreement with the observations of Sheng et al. (28), who reported that Ni\textsuperscript{2+} inhibited mENaC current, but with a \( K_i \) of \(-0.5 \) mM, or \(-1 \) order of magnitude smaller than that in rENaCs, which might suggest molecularly different blocking sites and/or Ni\textsuperscript{2+} accessibility in the two species. This is supported by the findings of Sheng et al. (28), who identified histidine in the WYRFHY section as a ligand for the inhibitory Ni\textsuperscript{2+} in mENaCs. Our data regarding xENaC-WYRFHY could exclude His\textsuperscript{215} as ligand of the stimulatory Ni\textsuperscript{2+}. Contrary to the Sheng et al. report on mENaCs, our findings regarding both xENaCs and rENaCs indicate that Ni\textsuperscript{2+} diminishes amiloride \( k_{oa} \) by 59.7 \pm 10.2\%. Again suggesting possibly allosteric competition between the blocker and the divalent cation. To gain insight into why the effect of Ni\textsuperscript{2+} on \( I_{Na} \) is different in xENaCs and rENaCs, we studied the influence of Ni\textsuperscript{2+} on \( I_{Na} \) in the absence of amiloride at rapid step voltages between \(-140 \) and \(+40 \) mV.

An interesting finding from our TEVC study is that in the xENaC, which is sensitive to voltage, only \( I_{Na} \) is stimulated by Ni\textsuperscript{2+} rather than the voltage-activated part, \( \Delta I \). The rENaC, however, behaves in a fully ohmic manner and is inhibited by Ni\textsuperscript{2+} not only in the instantaneous current jump phase but in a secondary, slow Ni\textsuperscript{2+}-dependent inhibition of the current that becomes apparent and is even more effective at more negative voltages (Fig. 6). A voltage-dependent blockade by Ni\textsuperscript{2+} of rENaC can be the result of two different mechanisms. 1) Ni\textsuperscript{2+} blocks the rENaC pore by occupying a site located within the permeation pathway, where membrane voltage drops. 2) Ni\textsuperscript{2+}, rather than obstructing the permeation pathway, stabilizes the closed state of the channel through a mechanism that would be independent of the blocking itself but nevertheless would be dependent on voltage. For instance, in studies of high-voltage-activated Ca\textsuperscript{2+} channels, investigators have proposed the existence of two binding sites for Ni\textsuperscript{2+}: one accounting for the direct blocking and an additional one that stabilizes the Ca\textsuperscript{2+} channel-closed state (21).

The species difference in the Ni\textsuperscript{2+} effect, stimulating the xENaC but inhibiting the rENaC, could also be only an apparent difference; for instance, it might be the result of a different time lag of the processes involved. In xENaC, voltage activation is slow and thus visible in the recordings, whereas in rENaC the process could be much faster and might be terminated already during the settling time of the voltage clamp, within the first few milliseconds after the voltage step is initiated. Because of the lack of evidence, such an idea seems far fetched. We therefore propose a working model in which Ni\textsuperscript{2+} binds in the extracellular, voltage-insensitive domain of the xENaC, thereby changing the channel allosterically in such a way that amiloride and its competitor Na\textsuperscript{+} are impaired in binding to their common site. Such a site should also be available in the rENaC to allow the observed interaction of Na\textsuperscript{+}, Ni\textsuperscript{2+}, and amiloride (Fig. 5). At present, we cannot tell whether the sites in question in xENaCs and rENaCs share analogous chemical features. Previous studies showed that Ni\textsuperscript{2+} affects a variety of ion channels, such as voltage-gated Ca\textsuperscript{2+} channels (11), voltage-gated Na\textsuperscript{+} channels (33), P2X receptors (33), and glutamate receptors (33). The mechanism of Ni\textsuperscript{2+} effects on these membrane proteins is not always clear in detail, but both direct blocking and allosteric processes have been suggested (12, 21).

As demonstrated herein for Ni\textsuperscript{2+}-induced xENaC stimulation but inhibition of rENaC and mENaC stimulation (28), other transporters are affected by Ni\textsuperscript{2+} as well, e.g., the human aquaporin 3 (37). High doses of Ni\textsuperscript{2+} predispose to asthma, lung fibrosis, lung cancer, and kidney cancer. Taken together, these observations suggest that Ni\textsuperscript{2+}-related diseases may originate from epithelial effects in the first place.

**Lessons from mutation experiments.** More recently, Sheng et al. (28) suggested that a histidine residue from the extracellular WYRFHY domain of the \( \alpha \)-subunit and one from the extracellular domain of the \( \gamma \)-subunit form the inhibitory Ni\textsuperscript{2+}-binding site in mENaCs. The residue from the \( \alpha \)-subunit is highly conserved among the species mouse, rat, and Xenopus, and substitution of the homologous histidine residue from mENaCs with aspartic acid abolished the Ni\textsuperscript{2+} effect on amiloride-sensitive Na\textsuperscript{+} currents. We identified this histidine residue in the \( \alpha \)-subunit of the xENaC as His\textsuperscript{215}, and we generated the point mutation His\textsuperscript{215} to aspartate. As in the WT, in these channels, Ni\textsuperscript{2+} caused stimulation of \( I_{TO} \) and diminution of the amiloride \( k_{oa} \) by \(-60\% \) (experiments not shown). This result indicates that the mutation \( \alpha \)-His\textsuperscript{215}Asp is not involved as Ni\textsuperscript{2+}-binding site in xENaCs. Moreover, complete removal of the \( \alpha \)-WYRFHY sequence likewise did not impede stimulation by Ni\textsuperscript{2+}, suggesting a different binding site for this divalent cation. Finally, we again want to shed light on a recent finding regarding A6 cells (8), in which the thiol reagent PCMB, but not the histidine tracer DEPC, stimulated xENaC-like Ni\textsuperscript{2+} ions. It therefore is unlikely that Cys\textsuperscript{417}, the neighbor of the mutated His\textsuperscript{416}, may be involved in Ni\textsuperscript{2+} binding. On the other hand, there are plenty of cysteines (almost diagnostic for ENaCs) that might be possible Ni\textsuperscript{2+} targets in the extracellular loop. The world of Na\textsuperscript{+} channels and heavy metals seems much more complicated than expected.

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