An extracellular acidic cleft confers profound H\(^+\)-sensitivity to epithelial sodium channels containing the \(\delta\)-subunit in *Xenopus laevis*

Received for publication, March 2, 2019, and in revised form, June 27, 2019. Published, Papers in Press, June 27, 2019, DOI 10.1074/jbc.RA119.008255

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Edited by Mike Shipston

The limited sodium availability of freshwater and terrestrial environments was a major physiological challenge during vertebrate evolution. The epithelial sodium channel (ENaC) is present in the apical membrane of sodium-absorbing vertebrate epithelia and evolved as part of a machinery for efficient sodium conservation. ENaC belongs to the degenerin/ENaC protein family and is the only member that opens without an external stimulus. We hypothesized that ENaC evolved from a proton-activated sodium channel present in ionocytes of freshwater vertebrates and therefore investigated whether such ancestral traits are present in ENaC isoforms of the aquatic pipid frog *Xenopus laevis*. Using whole-cell and single-channel electrophysiology of *Xenopus* oocytes expressing ENaC isoforms assembled from \(\alpha\beta\gamma\)- or \(\delta\beta\gamma\)-subunit combinations, we demonstrate that *Xenopus* \(\delta\beta\gamma\)-ENaC is profoundly activated by extracellular acidification within biologically relevant ranges (pH 8.0–6.0). This effect was not observed in *Xenopus* \(\alpha\beta\gamma\)-ENaC or human ENaC orthologs. We show that protons interfere with allosteric ENaC inhibition by extracellular sodium ions, thereby increasing the probability of channel opening.

Using homology modeling of ENaC structure and site-directed mutagenesis, we identified a cleft region within the extracellular loop of the \(\delta\)-subunit that contains several acidic amino acid residues that confer proton-sensitivity and enable allosteric inhibition by extracellular sodium ions. We propose that *Xenopus* \(\delta\beta\gamma\)-ENaC can serve as a model for investigating ENaC transformation from a proton-activated toward a constitutively-active ion channel. Such transformation might have occurred during the evolution of tetrapod vertebrates to enable bulk sodium absorption during the water–to–land transition.

Water–to–land transition was a key event in the evolution of tetrapod vertebrates. By transitioning into terrestrial habitats, tetrapod ancestors faced major physiological challenges, such as the need for breathing air, as well as a limited availability of water and sodium. Mechanisms that permitted sodium absorption from a sodium-scarce environment are likely to have evolved while vertebrate ancestors invaded freshwater habitats. This suggests that tetrapod ancestors were likely equipped with a molecular machinery allowing them to evolve efficient mechanisms for bulk sodium and, consequently, water conservation.

Although comparative physiological studies reveal evolutionary adaptations at an organ level (1), not much is known about functional adaptations of proteins required for sodium and water conservation. In tetrapod vertebrates, a key protein for conserving sodium is the epithelial sodium channel (ENaC). The presence of ENaC genes in modern jawless vertebrates (2) suggests that ENaC evolved early within the vertebrate lineage. In sodium-absorbing epithelia of tetrapod vertebrae, ENaC is located in the apical epithelial membrane and is rate-limiting for the uptake of sodium ions into epithelial cells. In conjunction with basolateral Na\(^+\)/K\(^+\)-ATPases, ENaC facilitates the vectorial absorption of sodium ions, which also generates osmotic forces that drive water absorption (3). In humans, an increased volume of airway surface liquid as well as urinary salt-loss in patients suffering from type 1 pseudohypoaldosteronism are clinical manifestations of ENaC loss-of-function mutations mirroring insufficient adaptation to terrestrial life (4). In severe cases, patients require a daily uptake of 18 g of sodium (2) as compared with a recommended daily value of a maximum of 1.5 g (5).

Canonical ENaCs assemble as heterotrimers consisting of three homologous subunits (\(\alpha\), \(\beta\), and \(\gamma\)). A fourth \(\delta\)-subunit can replace the \(\alpha\)-subunit and form heteromeric ENaCs with different biophysical and functional properties (6). A recent study resolving the structure of human \(\alpha\beta\gamma\)-ENaC revealed that each subunit contains short intracellular N and C termini that are connected by two transmembrane helices and a large extracellular domain (7). The topology of this ectodomain resembles a clenched hand comprising the “palm,” “knuckle,”

The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Table S1.

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2 The abbreviations used are: ENaC, epithelial sodium channel; ANOVA, analysis of variance; ASIC, acid-sensing ion channel; SSI, sodium self-inhibition; PDB, Protein Data Bank; TEVC, two-electrode voltage-clamp; NMDG, \(N\)-methyl-D-glucamine.

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“finger,” and “thumb” domains, surrounding a central “β-ball.” Aside from the ENaC-specific GRIP (gating relief of inhibition by proteolysis) domain, which confers channel sensitivity to proteolytic activation, this structural organization is similar to that of acid-sensing ion channels (ASICs) (7). However, despite a shared channel architecture, representatives of the degenerin/ENaC family are functionally distinct, as they open in response to various external stimuli (8). This includes mechanosensitive degenerin channel complexes in Caenorhabditis elegans (9), peptide-gated channels in molluscs (10), and proton-sensitive ASICs (11). ENaC emerged in vertebrates and is the only member of this protein family that opens without an external stimulus, representing an evolutionary adaptation for bulk sodium absorption. The molecular changes that enabled an evolutionary transition from a stimulus-activated ancestor to a constitutively-active ENaC are unknown. Many freshwater vertebrates absorb sodium ions from the environment by specialized gill epithelial cells (ionocytes) that secrete protons via apical vacuolar-type H⁺-ATPases (12). Protons are either used to co-transport sodium ions into ionocytes via proton-coupled sodium transporters or sodium-permeable ion channels that open in response to apical extracellular acidification (12).

We hypothesize that ENaC evolved from an ancestral ion channel that was originally opened in response to protons and then progressed to constitutive activity during water–to–land transition. At a molecular level, this hypothesis is supported by the close evolutionary relationship and the structural similarities between ENaC and proton-gated ASICs, which are evolutionarily older. ASICs recently have been suggested as possible candidates for proton-coupled sodium uptake in ionocytes of freshwater zebrafish (12) and rainbow trout (13). Furthermore, mammalian ENaCs are sensitive to extracellular pH in a species-specific manner, as exemplified by a 40% increase in human αβγ-ENaC activity due to extracellular acidification (pH 6.0) (14). Precedents for evolutionary gain or loss of proton-sensitivity within vertebrate degenerin/ENaC proteins have recently been demonstrated for ASIC1, which gained proton-sensitivity during the emergence of bony fishes (15), and ASIC4, which lost proton-sensitivity in the mammalian lineage (16).

We and others (17) have recently shown that δβγ-ENaC in the South African clawed frog Xenopus laevis generates sodium currents, which have a more transient form compared with those generated by αβγ-ENaC. This is the only ENaC ortholog characterized to date that displays such characteristics. The transient nature is due to a slow but profound inhibition of ENaC activity by extracellular sodium ions, a mechanism called sodium self-inhibition (SSI) (17, 18). Various cues such as acidic pH (14) or proteases (19) have been shown to increase ENaC activity by uncoupling channels from SSI. Because Xenopus ENaCs containing the δ-ENaC subunit are insensitive to proteolytic activation (18), we hypothesized that the enhanced SSI of Xenopus δβγ-ENaC enables channel activation through extracellular acidification. This ENaC isoform might therefore exhibit functional characteristics of a proton-stimulated ENaC ancestor.

We demonstrate that, in contrast to canonical Xenopus αβγ-ENaC, δ-ENaC-containing channels are profoundly activated by extracellular acidification. Modulation of δβγ-ENaC activity within physiological pH ranges (pH 8.0–6.0) involves changes in single-channel open probability and SSI. Substitutions of single aspartates in an acidic cleft located at the interface between the δ-ENaC knuckle and finger domains significantly alter δβγ-ENaC sensitivity to pH and SSI, suggesting convergence of channel regulation by extracellular protons and sodium within this region. These findings suggest that Xenopus δβγ-ENaC might be a functional model that represents the junction between constitutively active ENaCs and cognate proton-gated ion channels within the degenerin/ENaC family. Consequently, this amphibian ENaC isoform may provide insight into the evolutionary transition between gating modes that may have occurred during water–to–land transition of tetrapod vertebrates.

**Results**

**Activity of Xenopus δβγ-ENaC is sensitive to changes in the extracellular pH**

The effect of extracellular acidification on Xenopus δβγ-ENaC activity was functionally characterized by microelectrode recordings, employing the Xenopus oocyte expression
system at a holding potential (\(V_{\text{hold}}\)) of \(-60\) mV. We and others have previously demonstrated a transient nature of transmembrane currents (\(I_{\text{M}}\)) mediated by Xenopus ENaC containing the \(\delta\)-subunit after washout of the ENaC blocker amiloride or a rapid increase in the extracellular sodium concentration (17, 18). As illustrated in Fig. 1, this characteristic decay of \(I_{\text{M}}\) in oocytes expressing Xenopus \(\delta\beta\gamma\)-ENaC was altered depending on the extracellular pH. Changing the extracellular sodium concentration ([Na\(^+\)] from 1 to 90 mM at pH 7.4, elicited a rapid increase in \(I_{\text{M}}\) that was followed by an initial, fast decline and a subsequent slow but continuous rundown (Fig. 1a). Current levels normalized to the initial peak in \(I_{\text{M}}\) followed a two-phase decay function (Fig. 1b), where 39.6 ± 3.1% \((n = 14)\) of the total current decline was attributable to the initial fraction of \(I_{\text{M}}\) decay (Fig. 1c). Although this two-phase current decline was also observed at an alkaline pH of 8.0, the fraction of the initial \(I_{\text{M}}\) decay under these conditions was significantly enhanced \((58.5 ± 3.1\%; p = 0.0058; n = 17)\). In contrast, acidification of the extracellular solution to pH 6.0 markedly reduced the fraction of this initial \(I_{\text{M}}\) decay \((15.9 ± 6.9\%; p = 0.0048; n = 8)\) culminating in increased amiloride-sensitive current fractions (\(\Delta I_{\text{amp}}\)) mediated by Xenopus \(\delta\beta\gamma\)-ENaC under acidic conditions (Fig. 1d). Changes in the extracellular pH did not affect the magnitude of the initial peak in \(I_{\text{M}}\) \((I_{\text{M, peak}}): \text{pH} 8.0, -11.0 ± 1.6 \mu A; \text{pH} 7.4, -10.3 ± 1.1 \mu A; \text{pH} 6.0, -12.5 ± 1.1 \mu A\); one-way ANOVA, \(F = 0.7822, p = 0.4632\)). These results indicate that the extracellular pH influences the activity of Xenopus \(\delta\beta\gamma\)-ENaC and alters current kinetics at the whole-cell level.

We next examined the effect of extracellular acidification on \(\delta\beta\gamma\)-ENaC at the single channel level. Cell-attached patch-clamp recordings lasting for 120–180 s at \(V_{\text{hold}} = -100\) mV were performed using pipette solutions with pH values (pH\(_{\text{pip}}\)) of 8.0, 7.4, and 6.0 (Fig. 2). Individual channels displayed a low open probability \((P_o)\) at pH\(_{\text{pip}}\) 8.0 \((0.12 ± 0.02; n = 15)\), whereas \(P_o\) increased to a moderate level at pH\(_{\text{pip}}\) 7.4 \((0.24 ± 0.04; p = 0.011; n = 18)\) and was further enhanced at pH\(_{\text{pip}}\) 6.0 \((0.52 ± 0.04; p = 0.0003; n = 15)\) (Fig. 2b). The number of visible channels was increased in recordings performed at pH\(_{\text{pip}}\) 6.0 when compared with alkaline conditions at pH\(_{\text{pip}}\) 8.0 (Fig. 2c); however, a low \(P_o\) at pH\(_{\text{pip}}\) 8.0 might lead to underestimation of channel abundance. The slope conductance (\(G_{\text{slope}}\)) of \(\delta\beta\gamma\)-ENaC.

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**Figure 2. Extracellular pH affects gating of Xenopus-\(\delta\beta\gamma\)-ENaC.** a, representative current traces from cell-attached patch-clamp recordings of oocytes expressing Xenopus \(\delta\beta\gamma\)-ENaC at a holding potential of \(-100\) mV. Recordings were performed using pipette solutions at pH 8.0, 7.4, or 6.0 (pH\(_{\text{pip}}\)). Dashed lines indicate the number of individual open channel levels or the current baseline (c). b, open probability of individual channels increases with decreasing pH\(_{\text{pip}}\) (one-way ANOVA, \(F = 5.83, p = 0.0056\); Tukey’s multiple comparisons test). c, number of visible channels in cell-attached recordings as depicted in a (one-way ANOVA, \(F = 1.854, p = 0.1725\)), which was calculated from the linear regression of unitary channel conductance (mean of at least three single channel amplitudes per \(n\) at \(-40\) to \(-100\) mV). e, estimation of the number of channels in the patch. \(P_{\text{n}}\) is the probability of \(n\) channels out of the total number of channels within the patch being opened. The observed probability for each \(n\) (i.e. observed current levels) was compared with a theoretical estimation of the number of channels present in the patch. There is no significant difference between observed and predicted \(P_{\text{n}}\) values under the employed pH\(_{\text{pip}}\) conditions (Kruskal-Wallis test with Dunn’s multiple comparisons test in each panel, \(p > 0.9999\) between each pair of \(P_{\text{n}}\) observed and \(P_{\text{n}}\) predicted). Statistical evaluation is based on individual recordings lasting for 120–180 s with a maximum of eight channels per patch. Please note that single-channel characteristics at pH\(_{\text{pip}}\) 7.4 include data, which have been reported earlier (18). Patch-clamp data for both studies were collected simultaneously using the same oocyte batches for all pH conditions and a\(\beta\)\(\gamma\)-as well as \(\delta\beta\gamma\)-ENaC.

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**J. Biol. Chem. (2019) 294(33) 12507–12520**

12509
ENaC was derived from linear regression of unitary channel conductance at $V_{hold}$ from $-40$ to $-100$ mV. The $P_{o,0}$ did not significantly affect the $G_{slope}$ of the channel (Fig. 2d; one-way ANOVA, $F = 1.854$, $p = 0.1725$). Taken together, these results indicate that pH-mediated modulation of Xenopus $\delta\beta\gamma$-ENaC activity is based on changes in channel $P_{o}$.

The $\delta$-subunit confers a pronounced pH-sensitivity to Xenopus ENaC

The magnitude of stimulation of Xenopus $\delta\beta\gamma$-ENaC by extracellular acidification was compared with channels containing the $\alpha$-ENaC subunit ($\alpha\beta\gamma$) from X. laevis as well as both human channel orthologs ($\alpha\beta\gamma$-ENaC). As depicted in Fig. 3a, stepwise acidification of the extracellular solution from pH 8.0 to 6.0 in increments of pH 0.2 resulted in a successive increase of $I_{M}$ in oocytes expressing Xenopus $\delta\beta\gamma$-ENaC that reached a maximum between pH 6.4 and 6.0 and was sensitive to amiloride. The dose-response relationship of individual current levels normalized to $I_{M,0}$ at pH 7.4 indicated a maximal activation of the channel by a factor of 3.9 ± 0.2 under acidic conditions, whereas alkaline pH reduced ENaC activity to a factor of 0.5 ± 0.2 ($n = 15$). The sigmoidal dose-response curve exhibited a Hill-slope of 1.6 ± 0.4 and a half-maximal channel activation ($EC_{50}$) at pH 6.9 (Fig. 3b). Alkaline inhibition, maximal acidic activation, as well as the $EC_{50}$ and Hill-slope of Xenopus $\delta\beta\gamma$-ENaC were not significantly different when H$_2$SO$_4$ was employed for pH titration instead of HCl (data not shown). In Xenopus ENaC containing the $\alpha$-subunit, maximal acid-induced channel activation was significantly reduced to a factor of $1.2 \pm 0.06$ ($n = 12$), while there was no channel inhibition at alkaline pH. Moreover, pH-mediated channel regulation between pH 8.0 and 6.0 was also reduced in human ENaC isoforms (Fig. 3, c and d). Human $\delta\beta\gamma$-ENaC displayed a maximal acid-induced fold-activation of $1.05 \pm 0.01$ and an alkaline inhibition to a factor of $0.93 \pm 0.04$ ($n = 12$), whereas in $\alpha\beta\gamma$-ENaC these values were $1.03 \pm 0.01$ and $0.9 \pm 0.1$ ($n = 11$), respectively. These results demonstrate that the presence of the $\delta$-subunit facilitates a markedly pronounced pH-sensitivity in Xenopus ENaC that is not found in either isoform of orthologous human channels. As $I_{M}$ recorded in water-injected oocytes was not reduced at alkaline pH or enhanced under acidic conditions, the involvement of endogenous channels or transporters in the observed pH-mediated changes of $I_{M}$ can be excluded.

Changes in the extracellular pH modulate ENaC sodium self-inhibition

Transmembrane currents mediated by ENaC are generally subject to a time-dependent decay that is attributable to two distinct mechanisms. SSI describes an immediate, allosteric reduction of ENaC $P_{o}$, in the presence of high extracellular sodium concentrations (20). The more slowly-progressing feedback inhibition involves a reduction of channel $P_{o}$ and internalization of membrane resident ENaCs due to accumulation of intracellular Na$^{+}$ (21). Because previous reports have suggested that ENaC activation by acidic pH is predominantly associated with a reduced SSI (14), we hypothesized a similar correlation in Xenopus $\delta\beta\gamma$-ENaC, which is characterized by a markedly pronounced SSI (17, 18). Fig. 4 illustrates a similar reversibility and magnitude of acid-induced activation and SSI of $\delta\beta\gamma$-ENaC as well as an apparent independence of both mechanisms from the slowly progressing decline of $I_{M}$ over time. Increasing extracellular [Na$^{+}$] from 1 to 90 mm at pH 7.4 induced a peaked increase in $I_{M}$ that followed by a rapid, initial current decay and a subsequent slow current rundown (Fig. 4a, also see Fig. 1). During this current rundown, extracellular acidification (pH 6.0 for 30 s) reversibly activated a consistent fraction of $I_{M}$ that did not decrease but slightly increased over time (Fig. 4b). The observed increase of pH 6.0 sensitive $I_{M}$ fractions can be mainly attributed to a reduced magnitude of the first acid-induced current increase, which may originate from an overlap with the strong, initial current decay. Notably, the magnitudes of SSI (measured as current fractions lost after 3 min of SSI) were constant over time when SSI was repetitively induced (Fig. 4, c and d) supporting a functional correlation between ENaC activation by acidic pH and inhibition through SSI. To examine a functional interdependence between Na$^{+}$-mediated inhibition and acid-induced activation of ENaC, we assessed the Na$^{+}$ dependency of channel activation due to extracellular acidification. As presented in Fig. 4, e and f, the half-maximal proton-induced activation (pH $EC_{50}$) of Xenopus $\delta\beta\gamma$-ENaC depended on the extracellular Na$^{+}$ concentration. Reduction of the extracellular [Na$^{+}$] dose-dependently shifted the channel’s pH $EC_{50}$ to more alkaline values, suggesting that
lower concentrations of protons are necessary to maximally activate ENaC at reduced extracellular \([\text{Na}^+\text{]}. These results indicate that pH-mediated activation of Xenopus \(\delta\beta\gamma\)-ENaC is affected by the extracellular \(\text{Na}^+\) concentration, thus suggesting that protons alter channel activity by modulating SSI. Because SSI of ENaC is only visible during experimental conditions as employed in this study, we additionally examined whether proton-sensitivity is maintained in prolonged recordings, when currents have reached a quasi-steady-state level. Indeed, extracellular acidification (pH 6.0) led to a strong increase of \(I_M\) (Fig. 4g), indicating that pH-sensitivity might also represent a physiologically relevant stimulus activating Xenopus \(\delta\beta\gamma\)-ENaC.

To further examine how protons and SSI interact, we determined the effect of extracellular pH on the magnitude and apparent \(\text{Na}^+\) affinity of SSI in Xenopus \(\delta\beta\gamma\)-ENaC (Fig. 5). SSI was assessed by successively increasing extracellular \([\text{Na}^+\text{]}) from 1 mM to either 3, 10, 30, 60, 90, or 120 mM for 3 min. Fractional inhibition of \(I_M\) from the initial peak current \((I_{M,\text{peak}})\) to current levels after 3 min \((I_{M,3\text{ min}})\) was defined as SSI \((I_{M,\text{peak}}/I_{M,3\text{ min}})/I_{M,\text{peak}}\). Plotting the magnitude of SSI against the respective \([\text{Na}^+\text{]}) allowed for estimation of maximal inhibition \((V_{\text{max}})\) and apparent \(\text{Na}^+\) affinity \((K_m)\) of SSI as values followed a Michaelis-Menten relation. Under alkaline conditions (pH 8.0), Xenopus \(\delta\beta\gamma\)-ENaC exhibited a maximal SSI of \(0.57 \pm 0.03\), which was half-maximally reached at \(2.1 \pm 0.4\) mM \(\text{Na}^+\) \((n = 10; \text{Fig. 5a})\). Although the magnitude of maximal SSI reached a similar value in the presence of pH 7.4 \((0.55 \pm 0.03\), \(p = 0.9502\)), the \(\text{Na}^+\) affinity of SSI was significantly reduced under these conditions \((K_m = 8.1 \pm 2.2\) mM \(\text{Na}^+\), \(p = 0.0347\), \(n = 16\); Fig. 5b). Compared with pH 7.4, further extracellular acidification (pH 7.0, Fig. 5c) led to a reduction of maximal SSI \((0.4 \pm 0.05\), \(p = 0.017\)), whereas the apparent \(\text{Na}^+\) affinity \((K_m = 16.8 \pm 6.8\) mM \(\text{Na}^+\), \(p = 0.9999\), \(n = 13\)) was not significantly changed. In line with results presented in Fig. 1, SSI of Xenopus \(\delta\beta\gamma\)-ENaC was altered at pH 6.0 (Fig. 5d). Although the magnitude of channel inhibition was moderate in the presence of 3 mM \(\text{Na}^+\) \((0.29 \pm 0.04\), it did not increase with rising sodium concentrations (SSI at 120 mM \(\text{Na}^+\) = 0.14 \pm 0.02, \(n = 10\)). Because these data did not converge with a Michaelis-Menten fit, estimation of maximal SSI and apparent \(\text{Na}^+\) affinity was not feasible. Nevertheless, these data clearly demonstrate that changes in the extracellular pH affect the magnitude and \(\text{Na}^+\) affinity of SSI in Xenopus \(\delta\beta\gamma\)-ENaC, thus indicating that extracellular protons activate ENaC by antagonizing \(\text{Na}^+\)-mediated channel inhibition. This is additionally supported by the synergy/antagonism estimation using an interaction matrix composed of \(\text{Na}^+\) - and proton-dependent SSI values of Xenopus \(\delta\beta\gamma\)-ENaC (Fig. 5e). The landscape visualizes deviations of the observed values for SSI from a reference model (Bliss model (22)) that assumes a functional independence between the two factors. Negative Bliss values with an average of -23.4 indicate an antagonistic combinational effect, which protons exert on the \(\text{Na}^+\)-dependent magnitude of SSI in Xenopus \(\delta\beta\gamma\)-ENaC.
Mutations in the “acidic cleft” of the δ-ENaC subunit affect pH-sensitivity and sodium self-inhibition

To identify molecular determinants that facilitate ENaC inhibition by extracellular Na⁺ ions, Kashlan et al. (23) previously reported the presence of a putative binding site for Na⁺ in the extracellular loop of the α-subunit in mouse ENaC, which was termed the acidic cleft. Site-directed mutagenesis of several acidic residues within this region altered SSI of mouse αβγ-ENaC and in one instance additionally affected sensitivity to extracellular acidification. Hypothesizing the presence of an analogous site of interaction in Xenopus δ-ENaC, we aimed to determine the effect of mutating key acidic residues within the δ-subunit of Xenopus ENaC on channel modulation by pH and Na⁺. Fig. 6a shows a 3D model of a Xenopus δβγ-ENaC trimer based on the cryo-EM–derived structure of human αβγ-ENaC (PDB code 6BQN (7)). The region corresponding to the acidic cleft in mouse α-ENaC is located at the interface between the β2-α1 and β6-β7 loops at the top of the δ-ENaC extracellular domain (Fig. 6a, red area). This region contains three negatively charged aspartates (δAsp-105, δAsp-293, and δAsp-296) that provide potential sites for protonation and potentially contribute to the coordination of Na⁺ ions (Fig. 6b and c) (24, 25).

Within the corresponding structure of Xenopus α-ENaC, two of these aspartates are replaced by a lysine (αLys-105) and a glutamate (αGlu-296), respectively (Fig. 6c). In line with previous observations (23), these structural discrepancies may sufficiently alter the Na⁺- and proton-sensitivity of the α-ENaC acidic cleft. To examine a putative role of the acidic cleft in proton-mediated activation and SSI of Xenopus δβγ-ENaC, we generated mutant channels containing either single (δD105K, δD293N, and δD296N), double (δD105K,D296N and δD293N,D296N), or triple (δD3: δD105K,D293N,D296N) substitutions of aspartates within this region. The charge-reversing δD105K mutation was chosen, as the presence of a negatively charged δAsp-105 in place of a lysine in α-ENaC (αLys-105) represents the primary difference between both subunits’ acidic clefts. Because negatively charged side chains in positions δAsp-293 and δAsp-296 are conserved in the acidic cleft of α-ENaC, we decided to investigate their impact on pH- and Na⁺-mediated channel regulation through introduction of charge-neutralizing mutations.

The magnitude of acid-induced activation of WT or mutant channels was assayed by successively decreasing extracellular pH from 8.0 to 6.0 in pH 0.2 increments (see Fig. 3). Introduction of single aspartate substitutions within the δ-ENaC acidic cleft significantly reduced maximal acid-induced channel activation when compared with WT δβγ-ENaC (Fig. 7, a and b, and Table 1). This effect was not cumulative in double-mutant channels, as maximal acid-induced activation of δD105K,D296Nβγ and δD293N,D296Nβγ was not significantly different from that of ENaC containing only single aspartate substitutions. However, mutation of all aspartates in the δ-ENaC acidic cleft (δΔA3) further decreased the maximal acid-induced channel activation when compared with double-mutant channels (Table 1). Reduction of ENaC activity at alkaline pH was curtailed in all mutant channels except those containing the δD293N mutation (Table 1). The SSI of WT and

Figure 5. Extracellular acidification antagonizes sodium self-inhibition of Xenopus δβγ-ENaC. a, representative recording of Iₘ in oocytes expressing Xenopus δβγ-ENaC. SSI was determined by rapidly changing extracellular [Na⁺] from 1 to 3–120 mM at pH 8.0. The magnitude of Na⁺-self inhibition (Vₘₐₓ – Vₘₜₐₚₕ)/Vₘₜₐₚₕ) was plotted against the respective [Na⁺] and fitted to the Michaelis-Menten equation, allowing estimation of maximal inhibition (Vₘₐₓ) and apparent affinity for Na⁺ (Kₐ). b–d, current recordings and Michaelis-Menten plots of SSI in Xenopus δβγ-ENaC at pH 7.4, 7.0, and 6.0. At pH 8.0, Vₘₐₓ of SSI is similar to values at pH 7.4 (b), but SSI displays an enhanced Na⁺ affinity (Kruskal-Wallis test, p = 0.035; Dunn’s multiple comparisons test). Compared with values at pH 7.4, further acidification (pH 7.0, c) decreases Vₘₐₓ (one-way ANOVA, F = 5.508, Tukey’s multiple comparisons test, p = 0.017), whereas the apparent Na⁺ affinity of SSI is not significantly changed (Kruskal-Wallis test, Dunn’s multiple comparisons test, p > 0.999). Irrespective of the extracellular [Na⁺], SSI of Xenopus δβγ-ENaC is markedly reduced at pH 6.0 (d) and does not converge to a Michaelis-Menten fit. e, interaction landscape for Na⁺-dependent SSI at different extracellular H⁺. The Bliss score indicates deviations of the measured combinatorial responses from a reference model that assumes independence between Na⁺- and H⁺-mediated responses. Negative Bliss values across the interaction landscape indicate antagonism of Na⁺-dependent SSI by increasing H⁺.
mutant Xenopus δβγ-ENaC was assessed at extracellular [Na\textsuperscript{+}] from 3 to 120 mM as described above. Interestingly, ENaCs containing the δD296N mutation (δβ296ND296γ, δD293N,D296γ, and δD3βγ) exhibited a strongly reduced SSI with moderate inhibition of channel activity in the presence of 3 mM Na\textsuperscript{+} (δD296ND296γ, 0.23 ± 0.04) that did not increase with [Na\textsuperscript{+}] (δD296ND296γ, 0.23 ± 0.04) that did not increase with [Na\textsuperscript{+}] (δD296ND296γ, 0.23 ± 0.04) that did not increase with [Na\textsuperscript{+}]. Consistent with the results shown in Fig. 7, c and d). Although introduction of δD105K or δD292N also decreased maximal SSI compared with WT channels, the considerably reduced effect of these mutations indicates an essential role for the δAsp-296 residue in SSI of Xenopus δβγ-ENaC.

Functional convergence of ENaC regulation by protons and Na\textsuperscript{+} within the acidic cleft was further investigated through assessment of the half-maximal proton-induced activation (pH EC\textsubscript{50}) in the presence of high (90 mM) and low (3 mM) [Na\textsuperscript{+}] (Fig. 7, e and f). Consistent with the results shown in Fig. 4, e and f, the pH EC\textsubscript{50} of WT δβγ-ENaC significantly shifted from 6.93 ± 0.02 (n = 11) in the presence of 90 mM [Na\textsuperscript{+}] to 7.49 ± 0.05 (n = 12; p < 0.001) under 3 mM [Na\textsuperscript{+}]. This shift was lost in δβγ-ENaC containing the single δD296N or triple δD3 mutations. Instead, the pH EC\textsubscript{50} of channels containing acidic cleft mutations was shifted toward more alkaline values in the presence of 90 mM [Na\textsuperscript{+}] when compared with WT δβγ-ENaC.

Taken together, these results suggest a contribution of negatively charged residues within the acidic cleft of Xenopus δ-ENaC to the regulation of channel activity by pH and SSI. Notably, the charge-neutralizing δD296N mutation seems sufficient for almost complete ablation of SSI, while also profoundly decreasing acid-induced ENaC activation. Because substitutions at the δAsp-105 and δAsp-293 positions mimicked the δD296N effect on pH-mediated regulation of Xenopus δβγ-ENaC but induced a less profound reduction of SSI, distinct contributions of these positions to channel regulation by protons and Na\textsuperscript{+} may be hypothesized.

The acidic cleft is necessary but not sufficient for enhanced pH-sensitivity of Xenopus δβγ-ENaC

To test whether the acidic cleft of δ-ENaC is sufficient for enhanced acid-induced ENaC activation, we mutated corresponding residues within Xenopus αβγ-ENaC. Neither partial (α\textsubscript{K105D}) nor full (α\textsubscript{K105D,E296D,Q297L}) reconstitution of the δ-ENaC acidic cleft in the α-ENaC subunit conveyed an enhanced pH-sensitivity to the channel (Fig. 7g), thus deeming the acidic cleft necessary but not sufficient for proton-mediated activation of Xenopus ENaC. Previous work on vertebrate ASICs revealed that a segment connecting the first two β-sheets within the proximal part of the channel’s ectodomain modifies ASIC-gating kinetics and pH-sensitivity independently of the proton sensor within the acidic pocket (26, 27). Comparison of the corresponding peptid sequence in vertebrate ENaCs revealed that a leucine residue is conserved in the β1–β2 linker of α-ENaC orthologs, whereas δ-ENaC contains a lysine at this position (Fig. 9). Our homology model of Xenopus δ-ENaC indicates δLys-89 to be located in the subunit’s extracellular loop, where it is oriented toward the β9-sheet of the δ-ENaC palm domain (Fig. 8a). Substitution of this lysine by a leucine (δK89L) significantly shifted the pH dependence of the channel to more acidic values (Fig. 8b). Interestingly, baseline currents generated by δK89Lβγ-ENaC were strongly reduced under alkaline to neutral pH conditions, but still susceptible to strong acid-induced activation, leading to an enhanced stimulus-activated character of this mutant channel.

In summary, we have demonstrated that incorporation of the δ-subunit confers a pronounced pH-sensitivity to Xenopus ENaC, generating acid-activated ion channels. Protons antagonize SSI and induce ENaC activation by increasing channel open probability. Mutational analysis of single aspartates within the δ-ENaC acidic cleft suggests a functional convergence of channel regulation by pH and SSI within this region. Although necessary, the presence of an acidic cleft is not sufficient to enhance proton-sensitivity of ENaC. Analogous to vertebrate ASIC1, the β1–β2 linker in ENaC further modifies the apparent pH-sensitivity of Xenopus δβγ-ENaC.

Discussion

This study aimed to determine whether functional traits of ancestral proton-activated ion channels are retained in the δβγ-ENaC isofrom of the anuran X. laevis. Indeed, our results demonstrate that Xenopus δβγ-ENaC, unlike αβγ-ENaC, is profoundly sensitive to changes in the extracellular pH. Extracellular acidification from pH 8.0 to 6.0 resulted in a 7–8-fold increase of amiloride-sensitive transmembrane currents in...
oocytes expressing *Xenopus* δβγ-ENaC (Fig. 3). This stimulatory effect of extracellular protons is much stronger compared with *Xenopus* αβγ-ENaC (Fig. 3) (14), or rodent ENaCs (23, 28). The reversibility and relatively fast time course of acid-induced ENaC activation suggest that protons interfere with ENaC gating, rather than altering membrane abundance of the channel. This is in line with previous studies that either demonstrated a decreased pH-sensitivity of ENaCs containing gating-mutations (14, 29) or reported a direct influence of protons on ENaC *P* _o_ (30, 31). Consistently, cell-attached patch-clamp recordings presented in this study indicate an inverse correlation between pH and ENaC *P* _o_ (Fig. 2b). Previous studies reported a significant inhibition of rat αβγ-ENaC by cytoplasmic acidification (31, 32), but changes in the extracellular pH within a range that was employed in our study have a slow and marginal effect on the cytoplasmic pH of *Xenopus* oocytes (33, 34). Although we cannot completely exclude a contribution of changes in the intracellular pH to modulation of *Xenopus* δβγ-ENaC activity, our results suggest that channel gating is predominantly affected by extracellular protons.

Previous studies have suggested that acid-induced ENaC activation involves a relief from SSI, which describes an allosteric reduction of ENaC activity, our results suggest that channel gating is predominantly affected by extracellular protons.

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tional changes that affect ENaC gating downstream of Na\(^+\) binding. The results we presented in this study indicate a similar functional interdependence between pH and SSI in Xenopus δβγ-ENaC, because extracellular acidification (pH 8.0–6.0) also reduced the V\(_{\text{max}}\) of SSI in Xenopus δβγ-ENaC in a dose-dependent manner (Fig. 5). However, we observed an increased potency of acidic pH in curtailing SSI of this amphibian ENaC isoform, which is potentially caused by a proton-mediated antagonism of Na\(^+\) binding at a conserved Na\(^+\) coordination site within an acidic cleft of the δ-ENaC ectodomain.

Additional evidence for functional convergence of ENaC regulation by pH and SSI comes from mutations within the δ-ENaC acidic cleft, which decreased both acid-induced channel activation as well as ENaC inhibition through SSI (Fig. 7). This is consistent with previous findings that demonstrated a contribution of conserved negatively charged residues in the acidic cleft of mouse α-ENaC to both SSI and pH (23). The authors suggested the acidic cleft as a potential Na\(^+\)-binding site that initiates SSI and proposed that protonation of a central aspartate (Asp-296 in Xenopus δ-ENaC at acidic pH would obstruct Na\(^+\) coordination. Consistent with this hypothesis, the δD296N mutation led to a full disruption of SSI in Xenopus δβγ-ENaC, while also partially impeding acid-induced channel activation (Fig. 7). A model involving a proton-mediated antagonism of Na\(^+\) binding, which would contribute to subsequent channel activation by disruption of SSI, is consistent with the observed shift in half-maximal proton-induced activation of δβγ-ENaC due to a reduction of the extracellular [Na\(^+\)] (Fig. 4, e and f) as well as δ-ENaC acidic cleft mutations that mimic this effect (Fig. 7, e and f). Furthermore, we observed a decrease in the apparent Na\(^+\) affinity of SSI as a result from extracellular acidification (Fig. 5). It thus seems reasonable to propose that protonation of acidic residues within the δ-ENaC acidic cleft shifts the Na\(^+\)-binding equilibrium of this coordination site and thereby impedes initiation of SSI. Substitutions of nearby located aspartates in the δ-ENaC acidic cleft (δD105K and δD293N), however, did not lead to a full ablation of SSSI, whereas acid-induced channel activation was reduced to a similar extent as observed with the δD296N mutation (Fig. 7). This accentuates a central role for the negative charge of the δAsp-296 moiety in ENaC SSI, potentially involving coordination of Na\(^+\) at this position. Interestingly, as indicated in Fig. 9, the negative charge present in Xenopus δAsp-296 is strongly conserved throughout the α-ENaC lineage as well as in other members of the degenerin/ENaC family (7, 35), whereas it is lost in most mammalian δ-ENaC orthologs. Although conservation of this residue would imply a central role in channel functionality, its presence does not seem to correlate with an increased pH-sensitivity or SSI of ENaC. However, we found that introduction of the neighboring δD105K or δD293N mutations in Xenopus δ-ENaC led to a small but significant reduction in the magnitude of ENaC SSI while also significantly reducing proton-induced channel activation. Thus, it may be speculated that the proximate molecular environment of δAsp-296 additionally modifies Na\(^+\) coordination within the acidic cleft, allowing for modification of Na\(^+\)- and pH-sensitivity without altering basic channel functionality. Consistently, the structural similarities between the acidic cleft of Xenopus δ-ENaC and α-ENaC subunits (Fig. 6; also see Fig. 9) suggest that subtle amino acid alterations within this extracellular region may have profound effects on the channel’s pH-sensitivity. This is interesting from an evolutionary perspective, because mutations within the acidic cleft might have the potential to transform a proton-stimulated ENaC ancestor into a less proton-sensitive and rather constitutively active channel. Indeed, it was recently highlighted that functional diversification within the degenerin/ENaC protein family is associated with a high variability in the extracellular domains contributing to the formation of the acidic cleft, including the knuckle and finger domains as well as the β6–β7 loop (2). Three acidic residues within that region are conserved in α-like ENaC in lamprey as well as δ-ENaC in amphibians and reptiles, whereas the number of acidic residues decreases within the mammalian δ-ENaC lineage (Fig. 9). Human δβγ-ENaCs have a largely reduced SSI (36) and lack strong pH-sensitivity (Fig. 3), suggesting a potential correlation between altered acidic cleft structure and constitutive-ENaC activity within the δ-ENaC lineage. It is, however, unclear whether the altered structure of the acidic cleft in mammals caused a reduction in SSI or the cleft mutated as a result of functional uncoupling from gating mechanisms. We also note that proton-sensitivity of Xenopus δβγ-ENaC does not necessarily represent a functional trait that was retained during channel evolution but may have developed secondarily in the secondarily aquatic, pipid frog (37). Future studies might focus on

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**Table 1**

| pH-mediated channel activation | Na\(^+\) self-inhibition | | | | |
|--------------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| **Maximal activation** | **Minimal activity** | *n* | **V\(_{\text{max}}\)** | **Km (mm Na\(^+\))** | *n* |
| δβγ-ENaC | 3.48 ± 0.31* | 0.5 ± 0.03* | 22 | 0.72 ± 0.01 | 3.8 ± 0.7 | 16 |
| δD105Kδβγ | 2.04 ± 0.11* | 0.62 ± 0.02* | 14 | 1.94 ± 0.07* | 0.49 ± 0.03* | 14 |
| δD105Kδγ | 1.94 ± 0.07* | 0.49 ± 0.03* | 14 | 1.81 ± 0.10* | 0.60 ± 0.02* | 14 |
| δD293Nδβγ | 1.92 ± 0.09* | 0.54 ± 0.02* | 19 | 1.81 ± 0.10* | 0.60 ± 0.02* | 14 |
| δD293Nδγ | 1.7 ± 0.04* | 0.65 ± 0.02* | 17 | 1.92 ± 0.09* | 0.54 ± 0.02* | 19 |
| δD293Nδβγδ296N | 1.38 ± 0.04* | 0.74 ± 0.03* | 12 | 1.92 ± 0.09* | 0.54 ± 0.02* | 19 |

* | p < 0.05 when compared with δD105KδD293Nδ296Nδ-ENaC in the respective column.  
* | p < 0.05 when compared with wildtype δβγ-ENaC in the respective column.  

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comparing pH-mediated regulation of ENaC orthologs from species representing early tetrapod ancestors to attain a better understanding of this conundrum. In contrast to δ-ENaC, proteolytic processing has exclusively been established in the α-ENaC lineage, as represented by the presence of two furin cleavage sites in subunit orthologs from terrestrial tetrapods (Fig. 9) (38). Furin cleavage activates ENaC by uncoupling the channel from SSI (19). This might provide a potential explanation for the strong conservation of the acidic cleft structure and residual pH-sensitivity in the α-ENaC lineage (Fig. 9).

The recently resolved structure of human αβγ-ENaC identified extensive contacts between the knuckle and finger domains of neighboring subunits, forming a collar of inter-subunit interactions at the top of the extracellular loop (7). The α-helices from the knuckle (α6) and the finger domains (α1 and α2) of human ENaC were shown to enclose the β6–β7 loop that has been proposed to form part of the acidic cleft in mouse α-ENaC (23) as well as Xenopus δ-ENaC (Fig. 6). The homology model of Xenopus δβγ-ENaC presented in this study indicates the β6–β7 loops of individual subunits to be located between the β2–α1 loop preceding the finger domain and the α6–β11 loop of the knuckle domain within the same subunit, generating individual intra-subunit conjunctions. Conformational changes due to the coordination of Na⁺ in the δ-ENaC acidic cleft may therefore be transmitted by means of the knuckle–finger collar to drive structural reorganizations that eventually lead to the adoption of a low Pₒ, ENaC-gating mode. Conversely, protonation of the acidic cleft could oppose Na⁺ coordination at this site and thereby shift the channel’s gating equilibrium toward a high Pₒ state. Although a precise mechanistic interpretation of how the acidic cleft contributes to regulation of Xenopus δβγ-ENaC gating by pH and SSI may not be feasible based on the present results, they clearly demonstrate that both modulatory factors converge within the acidic cleft where they likely interact in a competitive manner.

Additionally, protons likely modulate conformational changes, which would induce a transition into a low Pₒ-gating mode, downstream from the binding of extracellular Na⁺. The presence of a component in acid-induced activation of δβγ-ENaC that is independent of Na⁺ coordination at the acidic cleft is indicated by the residual pH-sensitivity of δ296Nδβγ mutant channels, which do not exhibit SSI (Fig. 7). Previous studies by Snyder and co-workers (14, 28) have identified a crucial role of titratable residues within the βγ-ENaC subunits in facilitating acid-induced activation of human αβγ-ENaC. Further investigations by the same group (39) convincingly demonstrated that key pH-sensitive residues located at the interfaces between adjacent channel subunits affect ENaC gating by altering intersubunit distances through electrostatic interactions. The subunit composition of Xenopus ENaC thus may alter pH-sensitivity, not only by introduction of the δ-ENaC acidic cleft but also through the formation of intersubunit interactions in lower parts of the channel’s extracellular loop. pH-sensitive residues in human αβγ ENaC have been proposed to facilitate acid-induced channel activation through interactions with the β1–β2 linker in the lower palm domain of adjacent subunits (39). This is in good agreement with our results suggesting that modulatory sites distal to the acidic cleft, such as the δ-ENaC β1–β2 linker, affect proton-induced activation of Xenopus δβγ-ENaC (Fig. 8). These observations also suggest interesting similarities to the molecular mechanisms determining pH-sensitivity of ASICs. For example, mutations in the β1–β2 linker conveyed pH-sensitivity to lamprey ASIC1 (26), largely increased macroscopic and acid-induced current amplitudes of elephant shark ASIC1 (27), and decreased the velocity of Xenopus ASIC1.1 activation. Analogous to the proposed evolutionary adaptation of pH-sensitivity in ASICs from early diverging vertebrates (27), structural modifications in the β1–β2 linker could have enabled diversification of proton-sensitivity in ENaC isoforms due to changes within the palm domain and independently of the structure of the acidic cleft. Furthermore, pH-sensitivity of mouse ASIC1α was reported to be affected by mutations of Lys-211, which links the palm and thumb domains of neighboring subunits (16). Conservation of the positive charge at the corresponding position in Xenopus δ-ENaC (δArg-279) could additionally account for pH-sensitivity of this channel isoform, whereas its absence in the α-ENaC subunit (αMet-282) might prevent acid-induced channel activation. Taken together, we propose that extracellular acidification activates Xenopus δβγ-ENaC through modulation of SSI by means of at least two distinct mechanisms. Protonation of acidic residues within the δ-ENaC acidic cleft likely shifts the Na⁺-binding equilibrium of a Na⁺ coordination site and thus impedes initiation of SSI. Additionally, protons potentially interfere with conformational changes, which would induce the transi-
**pH-sensitivity of Xenopus δ-ENaC**

by Balinsky and Baldwin (44) reported that the urinary pH in *X. laevis* is alkaline (pH 8.0), which might result from ammonotelic nitrogen excretion in this species. Based on our study, this would suggest δβγ-ENaC activity to be generally low. We have previously shown that currents generated by δβγ-ENaC are consistently larger than those generated by αβγ-ENaC (18), suggesting that ENaCs containing the δ-subunit might play a role under conditions where maximum Na⁺ absorption is necessary. *X. laevis* can survive droughts by aestivation, a physiological state where the animals absorb water via the urinary bladder. Nitrogen excretion switches from ammonotelia to ureotelia under aestivation, which might trigger changes in urinary pH. Indeed, McBean and Goldstein (45) reported a reduction in urine flow as well as an increase of plasma Na⁺ levels preventing dehydration of *X. laevis* in an hyposmosic environment. Similar physiological responses due to salt stress have been observed the aestivating toad *Scaphiopus couchii* (46) and the semiaquatic *B. marinus* (47). However, because the cellular localization of δβγ-ENaC in the urogenital tract is still unknown, further exploration is warranted to dissect the precise physiological role of this ENaC isoform under such conditions.

**Experimental procedures**

**Synthesis of cRNA**

Con structs encoding full-length human or *X. laevis* δ-α-β- and γ-ENaC were present in the pTNT expression vector (Promega, Mannheim, Germany). Site-directed mutagenesis (primers listed in Table 2) was performed using the QuikChange Lightning kit (Agilent Technologies, Waldbronn, Germany) according to the manufacturer’s instructions. Successful mutagenesis was verified by sequencing (SeqLab, Goettingen, Germany). Constructs were linearized with BamHI (δ- and α-ENaC) or Nael (γ-ENaC, both enzymes purchased from Promega). Plasmids containing β-ENaC were not linearized due to the presence of BamHI and Nael restriction sites in the coding sequence. Synthesis of m7G-capped cRNA was performed using the T7 RNA polymerase system (RiboMAX large scale RNA production system, Promega) according to the manufacturer’s protocol. cRNAs were diluted in diethylpyrocarbonate-treated H₂O to a final concentration of 10 ng/μl per subunit for two-electrode voltage-clamp (TEVC) recordings and 20 ng/μl for patch-clamp recordings.

**Expression in Xenopus oocytes**

Stage V/VI oocytes were isolated from adult frogs as described previously (18). Animals were anesthetized in 0.2% MS-222/H₂O (Pharmaq, New Hampshire, UK) at pH 6.0 for 15 min prior to euthanasia by decapitation and sounding of the spinal cord. The methods used to humanely euthanize the ani-
**pH-sensitivity of Xenopus δ-ENaC**

**Table 2**

| Primer Sequence | Primer sequence (5 ’–3’) |
|-----------------|--------------------------|
| δ293N           | Forward  |
| δ306N           | Forward  |
| δ105K           | Forward  |
| δ293N,296N      | Forward  |
| δK105L          | Forward  |
| αE105D          | Forward  |
| αL293D,Q297L    | Forward  |

Site-directed mutagenesis primer sequences

Single nucleotide exchanges for site-directed mutagenesis are highlighted in underlined, bold letters. Generation of constructs containing single amino acid substitutions (δ293N, δ306N, δ105K, and δK105L), as well as the δ293N,296N double mutant was achieved using wildtype δ- or α-ENaC constructs. The δ105K primers were employed in combination with constructs encoding δ293N- or δ293N,296N-ENaC for generation of δ293N,296N- and δ105K,293N,296N-ENaC respectively. Primers introducing the αE105D, mutation were used in combination with constructs containing the δ293N,297L double mutation to create αE105D,293N,297L triple mutants.

Electrophysiological recordings

TEVC and cell-attached patch-clamp recordings were essential performed as described previously (18). In brief, TEVC recordings of oocyte whole-cell transmembrane currents (I

For patch-clamp experiments, mechanically devitellinized oocytes were placed in a recording chamber containing bath solution (in mM: 145 KCl, 1.8 CaCl2, 10 HEPES, 2 MgCl2, and 5.5 glucose at pH 7.4). Patch pipettes (6–9 meqhn resistant) were pulled from borosilicate glass capillaries, heat-polished, and filled with pipette solution (in mM: 145 NaCl, 1.8 CaCl2, 10 HEPES, 2 MgCl2, and 5.5 glucose). The pH of the pipette solution was adjusted using HCl and NaOH. Current signals were amplified using an LM-PC patch-clamp amplifier (List-Medical, Darmstadt, Germany), low-pass filtered at 100 Hz (Frequency Devices, Haverhill, IL), and recorded at 2 kHz with Axon Clampex software (Axon Instruments, Foster City, CA) using an Axon 1200 interface. Single-channel analysis was performed with Clampfit version 10.7 (Axon Instruments). The amount of channels in each recording was stochastically estimated by comparing the number of visible channels with a theoretical amount derived from binomial distribution as described previously (21). All electrophysiological recordings were performed at room temperature.

Homology modeling

Homology modeling of Xenopus ENaC isoforms was performed using the I-Tasser web server (48) and PyMol (The PyMOL Molecular Graphics System, Version 2.0, Schrödinger, LLC) to generate the heterotrimERIC complexes based on the cryo-EM derived structure of human αβγ-ENaC (PDB code 6BQN (7)). Sequence alignments were generated employing the Clustal Omega online tool (49).

Vertebrate ENaC sequence analysis

Protein sequences of different vertebrate α- and δ-ENaC orthologs were collected from the UniProt and NCBI Protein databases. Because many protein sequences are in silico translation products from genomic DNA of sequencing efforts from different animals that have yet not been validated by cDNA cloning, verification of available sequence information was required. To verify, or complete, a coding DNA sequence, the genomic sequence information was compared with known cod-
ing sequences from other species such as human or Xenopus. If available, genomic sequence information was retrieved from the linked sources of the respective protein sequences (Table S1). In other cases, sequence information from closely related species was submitted to BLASTN (NCBI) with default parameters for megablast/blast algorithms choosing the database for whole-genome shotgun contigs and specifying the respective organism or family. Depending on the size of the output sequence, all sequences were immediately or iteratively compared with confirmed exon sequences using MultAlin (http://multalin.toulouse.inra.fr/multalin/) (51) with parameters set for DNA alignment. The comparison included determination of the exon boundaries and the quality of the splice donor and acceptor sites, exon length (as a consistent feature across various species and isoforms), as well as potential Kozak consensus sequences for the translational start. The verified or completed cDNA sequence information was finally translated into the respective amino acid sequence (https://web.expasy.org/translate/; Table S1). Generation of sequence alignments using the ClustalW algorithm was achieved employing the Mega X suite.

Data analysis

Data are presented in column scatter plots or x–y graphs with lines/symbols and error bars representing mean ± S.E. Oocytes for each experiment were derived from at least three different donors, and the number of individual experiments (n) is noted in parentheses. Statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software Inc., La Jolla, CA). Gaussian distribution of individual data were assessed with the D’Agostino and Pearson omnibus normality test. Multiple groups were compared by one-way ANOVA followed by Dunn’s multiple-comparison test (two-tailed) for normally distributed data or a Kruskal-Wallis test followed by Dunn’s multiple-comparison test (two-tailed) for data not following Gaussian distribution. A p value ≤ 0.05 was considered significant. Whole-cell current decays as depicted in Fig. 1a were fit to the built-in equation for a two-phase exponential decay provided by GraphPad Prism, using the constraints \( Y_0 \left( I_{Mf}/I_{M,initial} \right) after 0 min) = 1 \) and \( Y_{plateau} \left( I_{Mf}/I_{M,initial} \right) after infinite minutes) > 0 \). Evaluation of synergy or antagonism between extracellular H\(^+\) and Na\(^+\) on ENaC sodium self-inhibition (SSI) was performed with the R-based synergyfinder package (https://synergyfinder.fimm.fi) (50). Oocytes were exposed to six concentrations of Na\(^+\) (3, 10, 30, 60, 90, and 120 mM) at four different extracellular pH values (pH 8.0, 7.4, 7.0, and 6.0), and the degree of SSI was determined as relative fractions of ENaC inhibition. pH values were expressed as proton concentration in millimolar. To quantify the degree of H\(^+\)/Na\(^+\) interaction, SSI “inhibitory values” ranging from 0 (no inhibition) to 100 (full inhibition) were used as input for the synergy finder tool employing the Bliss synergy reference model (22). Interaction scores were visualized as three-dimensional interaction surfaces according to Ref. 50 with negative Bliss values indicating antagonism and positive values indicating synergy. All figures were assembled and finalized using Inkscape (version 0.92.3; https://inkscape.org).3

Author contributions—L. W. and M. A. conceptualization; L. W. and S. M. data curation; L. W., J. M.-W., S. M., P. P. S., and M. A. formal analysis; L. W. and S. M. validation; L. W., J. S. D., J. M.-W., and S. M. investigation; L. W. and J. M.-W. visualization; L. W., P. P. S., and M. A. methodology; L. W. and M. A. writing-original draft; L. W., J. S. D., J. M.-W., S. M., P. P. S., I. M., and M. A. writing-review and editing; I. M. and M. A. resources; M. A. supervision; M. A. funding acquisition; M. A. project administration.

Acknowledgments—We thank Sean Gettings for generating ENaC cRNA and Dr. Tim Boswell as well as Dr. Peter Simmons for critical comments on the manuscript.

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