A New Subunit of the Epithelial Na\(^+\) Channel Identifies Regions Involved in Na\(^+\) Self-inhibition*

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The epithelial Na\(^+\) channel (ENaC) is the apical entry pathway for Na\(^+\) in many Na\(^+\)-reabsorbing epithelia. ENaC is a heterotetrameric protein composed of homologous \(\alpha\), \(\beta\), and \(\gamma\) subunits. Mutations in ENaC cause severe hypertension or salt wasting in humans; and consequently, ENaC activity is tightly controlled. According to the concept of Na\(^+\) self-inhibition, the extracellular Na\(^+\) ion itself can reduce ENaC activity. The molecular basis for Na\(^+\) self-inhibition is unknown. Here, we describe cloning of a new ENaC subunit from *Xenopus laevis* (exENaC). exENaC can replace \(\alpha\)ENaC and formed functional, highly selective, amiloride-sensitive Na\(^+\)-channels when coexpressed with \(\beta\)xENaC and \(\gamma\)xENaC. Channels containing exENaC showed strong inhibition by extracellular Na\(^+\). This Na\(^+\) self-inhibition was significantly slower than for \(\alpha\)xENaC-containing channels. Using site-directed mutagenesis, we show that the proximal part of the large extracellular domain controls the speed of self-inhibition. This suggests that this region is involved in conformational changes during Na\(^+\) self-inhibition.

The epithelial Na\(^+\) channel (ENaC)\(^{3}\) is the apical entry pathway for Na\(^+\) in many Na\(^+\)-reabsorbing epithelia. ENaC is a heterotetrameric protein (1) composed of homologous \(\alpha\), \(\beta\), and \(\gamma\) subunits (2). Gain-of-function mutations in ENaC cause severe hypertension and hypokalaemia, whereas loss-of-function mutations cause a life-threatening salt-wasting syndrome (pseudohypoaldosteronism type I) in humans (3). This underlines the importance of ENaC for body Na\(^+\) balance. Consequently, ENaC activity is tightly controlled (4). Besides control by hormones such as aldosterone, vasopressin, and insulin, different mechanisms related to Na\(^+\) concentration work together to regulate ENaC activity.

According to the concept of Na\(^+\) feedback regulation, a rising intracellular Na\(^+\) concentration inhibits ENaC activity (5). Molecular mechanisms mediating this negative feedback regulation possibly include retrieval of surface-expressed channels by ubiquitin-mediated endocytosis. This feedback mechanism depends on interaction of a "PY" motif at the carboxyl terminus of ENaC subunits with the ubiquitin ligase Nedd4-2 (6, 7). However, the way the intracellular Na\(^+\) concentration is sensed is unknown.

In contrast, the model of Na\(^+\) self-inhibition proposes that the extracellular Na\(^+\) ion itself can reduce the ENaC activity (5, 8–10). Na\(^+\) self-inhibition has been best described in amphibian tissues such as *Rana* skin (8–11) and *Necturus* urinary bladder (12). In these tissues, an increase in the extracellular Na\(^+\) concentration leads to a decline of the Na\(^+\) current within <5 s. The speed of self-inhibition with no measurable increase in intracellular Na\(^+\) concentration has led to the proposal that the extracellular Na\(^+\) ion itself mediates self-inhibition. Self-inhibition leads to efficient reduction of Na\(^+\) transport whenever the extracellular Na\(^+\) concentration rises. This would protect the cell from Na\(^+\) overload and distribute Na\(^+\) transport more evenly along an epithelium such as the collecting duct. However, the molecular basis for Na\(^+\) self-inhibition is unknown.

ENaC has been best characterized in *Xenopus laevis* among amphibian species. In *Xenopus*, five ENaC subunits are known. Besides the \(\alpha\), \(\beta\), and \(\gamma\) subunits (13), which assemble into the classical heteromeric ENaC with an \(\alpha\)\(\beta\)\(\gamma\) stoichiometry (1), two isoforms of \(\beta\)xENaC and \(\gamma\)xENaC have been identified (\(\beta\)xENaC and \(\gamma\)xENaC, respectively) (14). These additional isoforms are probably due to polyplody of *Xenopus*. Channels containing the \(\gamma\) subunit are characterized by a relatively high affinity for Na\(^+\), leading to current saturation at low (20 mM) Na\(^+\) concentration (14). However, Na\(^+\) self-inhibition is not easily observed with any of the possible combinations of subunits. Very recently, using fast solution exchange, Chraibi and Horisberger (15) observed self-inhibition with recombinant \(\alpha\)\(\beta\)\(\gamma\)ENaC channels. However, self-inhibition was too fast to be reliably determined.

Here, we report the cloning of a new ENaC subunit from *X. laevis* (exENaC). exENaC-containing channels displayed strong Na\(^+\) self-inhibition with a reduction of the current amplitude of \(-70\%\). Na\(^+\) self-inhibition of exENaC-containing channels was significantly slower than that of \(\alpha\)xENaC-containing channels, facilitating the investigation of this process. In this study, we identify a region in the extracellular domain of the channel protein that controls the speed of inactivation.

**EXPERIMENTAL PROCEDURES**

Cloning of exENaC—We cloned a 220-bp PCR product showing homology to other members of the DEG/ENaC gene family from stage V–VI oocytes of *X. laevis* as described (16). This PCR product was used to design primers for rapid amplification of 5'- and 3'-cDNA ends (RACE) (exENaC-5' RACE, 5'-CCGAATTCGAGTGCATCATAGACGCTC-3' and exENaC-3' RACE, 5'-GGCACTGTCGCCCTCAACTGCTC-3') and for the nested PCRs (exENaC-5' RACE, 5'-GAGCACTGGACCGGA-
\( \varepsilon \text{ENaC} \) and \( \text{Na}^+ \) Self-inhibition

RESULTS

Cloning of \( \varepsilon \text{ENaC} \)—We isolated a cDNA for a new \( \text{ENaC} \) subunit by homology cloning from \( X. \text{laevis} \) (see “Experimental Procedures”). It encodes a 604-amino acid protein that displays 42% identity to \( \alpha \text{ENaC} \) and 29–31% identity to \( \beta \text{ENaC} \) and \( \gamma \text{ENaC} \), respectively (Fig. 1A). Identity to \( \delta \text{ENaC} \), which has been identified only in humans thus far and has properties similar to those of \( \varepsilon \text{ENaC} \) (17), is 36%. \( \beta \text{ENaC} \) and \( \gamma \text{ENaC} \) are \( >90\% \) identical to \( \beta \text{ENaC} \) and \( \gamma \text{ENaC} \), respectively (14); \( \text{ENaC} \) orthologs from rat and \( X. \text{laevis} \) are 57–61% identical. Thus, the new subunit does not represent the isoform or species ortholog of an already known \( \text{ENaC} \) subunit (Fig. 1B); and therefore, we named it \( \varepsilon \text{ENaC} \). \( \varepsilon \text{ENaC} \) has two predicted transmembrane domains similar to other \( \text{ENaC} \) subunits and a large loop between these domains, containing 16 conserved cysteines and seven potential glycosylation sites (Fig. 1A). A proline-rich motif (PPXY) that is conserved in the carboxyl termini of \( \alpha \text{ENaC} \), \( \beta \text{ENaC} \), and \( \gamma \text{ENaC} \) is missing in \( \varepsilon \text{ENaC} \) (Fig. 1A). The tissue expression pattern of \( \varepsilon \text{ENaC} \) was investigated by reverse transcription-PCR, revealing predominately expression in kidney, bladder, and faint expression in brain and skeletal muscle (Fig. 2).

Functional Characterization of \( \varepsilon \text{ENaC} \)—We investigated the electrophysiological characteristics of \( \varepsilon \text{ENaC} \) by functional expression in \( X. \text{laevis} \) oocytes. When injected alone in oocytes, \( \varepsilon \text{ENaC} \) elicited only small (<10 nA) amiloride-sensitive \( \text{Na}^+ \) currents. Coexpression with either \( \beta \text{ENaC} \) or \( \gamma \text{ENaC} \) increased the current amplitude, but only coexpression with both \( \beta \text{ENaC} \) and \( \gamma \text{ENaC} \) resulted in full expression with amplitudes on the order of several \( \mu \text{A} \) (Fig. 3A). This feature resembles \( \alpha \text{ENaC} \) and \( \delta \text{ENaC} \) (2, 17) and identified \( \varepsilon \text{ENaC} \) as an \( \alpha \)-like subunit, which efficiently co-assembles with \( \beta \text{ENaC} \) and \( \gamma \text{ENaC} \) to form surface-expressed heteromeric channels. Substitution of extracellular \( \text{Na}^+ \) with \( \text{Li}^+ \) or \( \text{K}^+ \) revealed that channels containing \( \varepsilon \text{ENaC} \) are highly selective for \( \text{Na}^+ \) over \( \text{K}^+ \), with a \( \text{Li}^+ \geq \text{Na}^+ \geq \text{K}^+ \) permeability sequence, similar to \( \alpha \text{ENaC} \)-containing channels (Fig. 3B). \( \varepsilon \text{ENaC} \)-containing channels were blocked by amiloride with an apparent \( \text{IC}_{50} \) of 2.50 \( \pm \) 0.82 \( \mu \text{A} \) (\( n = 12 \)), which is 10 times higher than the \( \text{IC}_{50} \) for \( \alpha \text{ENaC} \)-containing channels (0.22 \( \pm \) 0.01 \( \mu \text{A} \); \( p < 0.05 \)) (Fig. 3C). Together, \( \varepsilon \text{ENaC} \)-containing channels show the electrophysiological hallmarks of \( \text{ENaC} \)-s: high selectivity for \( \text{Na}^+ \) and block by amiloride at a low \( \mu \text{M} \) concentration. As illustrated in Fig. 4A, the time dependence of the current after washout of a saturating amiloride concentration (50 \( \mu \text{M} \)) was very different for \( \alpha \beta \gamma \text{ENaC} \) and \( \alpha \beta \varepsilon \text{ENaC} \)-expressing oocytes. For both channels, the current amplitude rose within seconds due to unblocking of the ion pore. However, whereas \( \alpha \beta \varepsilon \text{ENaC} \)-expressing oocytes showed a current inward current with no strong time dependence over a time period of minutes, the inward current of \( \alpha \beta \varepsilon \text{ENaC} \)-expressing oocytes strongly declined within a few seconds to a quasi-steady state (Fig. 4A). At this quasi-steady state (25 s after amiloride wash-out; see “Experimental Procedures”), the current amplitude was only 28 \( \pm \) 7\% (\( n = 10 \)) of the peak amplitude (Table I). In addition to this fast current decline of \( \alpha \beta \varepsilon \text{ENaC} \)-expressing oocytes, both \( \alpha \varepsilon \text{ENaC} \) and \( \alpha \beta \varepsilon \text{ENaC} \)-expressing oocytes displayed a linear “rundown” of channel activity on a time scale of minutes (Fig. 4A). The time course of this rundown was much slower than the initial fast decline of \( \alpha \beta \varepsilon \text{ENaC} \)-expressing oocytes, and both processes can be clearly distinguished. The initial fast decline was well fitted to an exponential function with a time constant of 8.2 \( \pm \) 1.3 s (\( n = 10 \)).

We investigated the dependence of both the initial and steady-state currents of \( \alpha \beta \varepsilon \text{ENaC} \)-expressing oocytes on-
tracellular Na⁺ by equimolar replacement of increasing amounts of Na⁺ with N-methyl-D-glucamine. The initial current after amiloride washout saturated at ~100 mM Na⁺ with an apparent EC₅₀ of 15.3 ± 5.3 mM (n = 14), similar to αENaC-containing channels (20.5 ± 7.2 mM (n = 8); p = 0.03) (Fig. 4B). However, after the time-dependent decline in the current...
The rapid inhibition with high concentrations of extracellular Na\(^+\) was also observed after switching from an extracellular solution containing a low (1 mM) Na\(^+\) concentration to one containing a high concentration (115 mM). Initially, the current amplitude rapidly rose due to the strongly increased driving force for the inward Na\(^+\) current. But then, the current of εβγxENaC-expressing oocytes relaxed by 70% with a time constant of 5.9 ± 1.5 s to a quasi-steady-state value (n = 10) (Fig. 4C), comparable to the behavior after amiloride washout. However, rapidly changing the extracellular Na\(^+\) concentration uncovered this fast inhibition also for αβγxENaC-expressing oocytes. Currents relaxed by ~40% with a time constant of 1.6 ± 0.2 s (n = 10) (Fig. 4C). Thus, inhibition of channels containing αENaC is significantly faster than that of channels containing εxENaC (p < 0.05). The speed of inhibition of channels formed by αβγxENaC probably leads to an underestimation of the peak current. Therefore, the apparent ratio of steady-state to peak current may be overestimated for these channels.

Such a fast inhibition with high concentrations of extracellular Na\(^+\) is known as Na\(^+\) self-inhibition (8–10). The characteristic feature of self-inhibition is that it is mediated by the extracellular Na\(^+\) ion itself and not by a rise in the intracellular Na\(^+\) concentration. However, due to the large current amplitude on the order of several μA in our experiments, a rapid increase in the intracellular Na\(^+\) concentration was difficult to prevent. To show that a rise in the intracellular Na\(^+\) concentration is not responsible for the rapid current decline, we therefore used an experimental setup that allowed us to use different concentrations of extracellular Na\(^+\) leading to an inward current of comparable size. This was achieved by using a low extracellular Na\(^+\) concentration together with a large negative holding potential and a high extracellular Na\(^+\) concentration together with a small positive holding potential. These experiments were performed successively on the same αβγxENaC-expressing oocyte. As shown in Fig. 4D, amiloride washout gave rise to an inward current in both cases. The initial amplitude of this current was 1.48 ± 0.20-fold larger in the 2 mM Na\(^+\) solution (n = 5; p = 0.05), indicating a larger Na\(^+\) influx under this condition. However, despite the smaller Na\(^+\) influx, a rapid current decline was observed only in the 115 mM Na\(^+\) solution (the current amplitude 30 s after the peak was 43 ± 3% of the peak amplitude (n = 5); p < 0.05). In the 2 mM Na\(^+\) solution, no significant current decline was registered (the current amplitude 30 s after the peak was 101 ± 2% of the peak amplitude (n = 5); p = 0.5) (Fig. 4D). These results strongly suggest that the rise in the extracellular (and not intracellular) Na\(^+\) concentration was the basis of the rapid current decline.

Moreover, we observed self-inhibition of a comparable degree for current amplitudes ranging from 1 to 50 μA. Finally, it is well documented that feedback inhibition, which is due to a rise in the intracellular Na\(^+\) concentration, has a slower time course on the order of minutes (6). Therefore, feedback inhibition probably underlies the slow linear rundown described above, which follows Na\(^+\) self-inhibition. At least one mechanism of feedback inhibition is internalization of membrane-expressed channels. However, the fast current decline was partially but rapidly reversible after application of an amiloride analog (see below), most likely too fast for a substantial extrusion of the Na\(^+\) load. Together, our results suggest that the extracellular Na\(^+\) ion itself was responsible for the fast current decline seen in εxENaC-expressing oocytes.
exENaC and Na\(^+\) Self-inhibition

Fig. 4. exENaC mediates Na\(^+\) self-inhibition. A, representative traces of inward currents after amiloride washout (50 \(\mu\)M) for \(\alpha\beta\gamma\)ENaC (black trace) and \(\alpha\beta\gamma\)xENaC (gray trace)-expressing oocytes. For better comparison, traces have been overlaid. B, left, representative traces of inward currents after amiloride washout with different Na\(^+\) concentrations (0, 3, 10, 35, 90, and 140 \(\mu\)M). Current traces have been normalized for channel rundown. Right, dependence on extracellular Na\(^+\) of the initial current amplitude for \(\alpha\beta\gamma\)ENaC (open circles) and \(\alpha\beta\gamma\)xENaC (closed circles) and of the current amplitude after 2 min (quasi-steady state) for \(\alpha\beta\gamma\)xENaC (closed squares). C, representative traces of inward currents after switching from a solution of low (1 \(\mu\)M) to high (115 \(\mu\)M) Na\(^+\) concentration. Channels were activated by washout with 50 \(\mu\)M amiloride. The extracellular Na\(^+\) concentration was either 2 \(\mu\)M with a holding potential of -120 mV (gray trace) or 115 \(\mu\)M with a holding potential of 10 mV (black trace). Experiments were performed successively on the same oocyte. For better comparison, traces have been overlaid. E, BIG relieves inhibition by extracellular Na\(^+\). Oocytes were superfused with a solution containing 1 mM NaCl, and channels were then “activated” by fast wash-in of a solution containing 115 mM NaCl. 1 mM BIG was applied after the current had relaxed to its steady state.

with an active state (A) and an inhibited state (I) linked by an inactivation rate constant (\(k_i\)) and an activation rate constant (\(k_a\)), the rate constant \(k_i\) would determine the speed of self-inhibition. The steady-state level that we measured as \(I_{\text{steady-state}}/I_{\text{peak}}\) depends on both \(k_i\) and \(k_a\). Because, in exENaC-containing channels, the kinetics of self-inhibition changed inversely to the steady-state level, this would imply that the exENaC subunit changes \(k_a\) as well as \(k_i\). Compared with the exENaC subunit, the \(k_i\) would be reduced, but the \(k_a\) would be even more reduced, leading to the increased but slower inhibition.

To identify regions in the channel protein responsible for this differential behavior of exENaC and exENaC, we constructed a set of chimeric channels with parts of exENaC exchanged with the corresponding parts of exENaC. Self-inhibition of these channels was then examined by rapidly switching from solutions of low (1 \(\mu\)M) to high (115 \(\mu\)M) Na\(^+\) concentrations (Fig. 5 and Table I). As is shown in Table I, the kinetics of self-inhibition was significantly different (\(p < 0.05\)) between either the wild-type exENaC or exENaC subunit and all of the chimeric channels. Channels in which only the N terminus was exchanged showed slow self-inhibition, as seen with wild-type exENaC subunits (C1, \(\tau = 4.07 \pm 1.42\) s). However, all of the chimeras in which also the first transmembrane domain was exchanged showed self-inhibition that was significantly faster than the self-inhibition of even wild-type exENaC-containing...
channels (C2–C5, $\tau = 0.22 \pm 0.14$ to $1.16 \pm 0.17 \text{ s}$) (Fig. 5 and Table I), suggesting unspecific effects in these chimeric constructs. Therefore, we constructed variants that retained both intracellular termini and both transmembrane domains from $\alpha$ENaC and in which only parts of the extracellular loop were exchanged with the corresponding parts of $\alpha$ENaC (C3ex–C5ex) (Fig. 5). A variant in which approximately the first third of the extracellular loop was exchanged showed self-inhibition with an intermediate time course (C3ex, $\tau = 2.69 \pm 1.05 \text{ s}$), whereas exchange of bigger parts led to self-inhibition that was not significantly different (p < 0.05) from $\alpha$ENaC.

Table I

| Construct | \(\tau_{\text{self-inhibition}}\) | \(I_{\text{steady-state}}/I_{\text{peak}}\) | \(\tau_{\text{rise time}}\) | \(n\) |
|-----------|-------------------------------|---------------------------------|----------------|-----|
| $\varepsilon$ | $5.93 \pm 1.46$ | $0.30 \pm 0.06$ | $0.24 \pm 0.07^{a}$ | 10 |
| C1 | $4.07 \pm 1.42$ | $0.16 \pm 0.08$ | $0.17 \pm 0.06$ | 10 |
| C2 | $1.16 \pm 0.17$ | $0.10 \pm 0.03$ | $0.10 \pm 0.03$ | 8 |
| C3 | $0.45 \pm 0.22$ | $0.69 \pm 0.06$ | $0.11 \pm 0.04$ | 10 |
| C4 | $0.22 \pm 0.14$ | $0.85 \pm 0.08$ | $0.11 \pm 0.04$ | 10 |
| C5 | $0.27 \pm 0.05$ | $0.65 \pm 0.05^{a}$ | $0.11 \pm 0.04$ | 10 |
| C3ex | $2.69 \pm 1.05$ | $0.78 \pm 0.12$ | $0.24 \pm 0.12^{a,b}$ | 8 |
| C4ex | $1.79 \pm 0.67$ | $0.85 \pm 0.07$ | $0.23 \pm 0.15^{a,b}$ | 7 |
| C5ex | $1.27 \pm 0.49$ | $0.67 \pm 0.09^{a}$ | $0.15 \pm 0.11$ | 10 |
| $\alpha$ | $1.61 \pm 0.16$ | $0.62 \pm 0.06$ | $0.29 \pm 0.11^{b}$ | 10 |

| Construct | \(\tau_{\text{self-inhibition}}\) | \(I_{\text{steady-state}}/I_{\text{peak}}\) | \(\tau_{\text{rise time}}\) | \(n\) |
|-----------|-------------------------------|---------------------------------|----------------|-----|
| $\varepsilon$ | $8.20 \pm 1.12$ | $0.28 \pm 0.07$ | $0.92 \pm 0.28$ | 10 |
| C1 | ND | ND | ND | |
| C2 | $2.22 \pm 0.59$ | $0.17 \pm 0.03$ | $1.5 \pm 0.42^{b}$ | 5 |
| C3 | $0.88 \pm 0.58$ | $0.48 \pm 0.16$ | $0.69 \pm 0.41^{b}$ | 9 |
| C4 | $1.72 \pm 0.43$ | ND | ND | |
| C5 | $0.90 \pm 0.72^{b}$ | ND | ND | |
| C3ex | ND | ND | ND | |
| C4ex | ND | ND | ND | |
| C5ex | ND | ND | ND | |

\(a\) Not significantly different from $\varepsilon$ENaC.

\(b\) Not significantly different (p < 0.05) from $\varepsilon$ENaC.
**exENaC and Na⁺ Self-inhibition**

**Fig. 6. Identification of the amino acid responsible for the low apparent amiloride affinity of exENaC.** Upper, sequence alignment of the second transmembrane domains of different ENaC subunits; lower, representative current traces of the exENaC mutant W513L after amiloride washout (left) or after switching from a solution of low (1 mM) to high (115 mM) Na⁺ concentration (right). The substitution-containing ε subunit was coexpressed with βxENaC and γxENaC. r, rat; h, human.

However, rapidly changing from a low to high Na⁺ concentration also uncovered self-inhibition for this mutant channel (right trace). Self-inhibition was as slow as in wild-type exENaC-containing channels ($\tau = 5.95 \pm 1.66$ s ($n = 10$); $p = 0.5$), confirming that Trp⁵¹³ is at the basis of differential amiloride affinity, but not of differential speed of self-inhibition.

**DISCUSSION**

**Variety of ENaCs in X. laevis—**The electrophysiological characteristics of ENaCs have been characterized in different tissues from various species. These characteristics vary with respect to selectivity, amiloride affinity, and single channel conductance (21). Here, we have reported the cloning of exENaC, a new ENaC subunit from *X. laevis*. We have demonstrated that exENaC-containing channels show a similarly high Na⁺/K⁺ selectivity as oxENaC-containing channels. Although we did not determine single channel conductance, most of the highly selective ENaCs have a single channel conductance of ~5 picoSiemens (21). However, in addition to differences in Na⁺ self-inhibition (see below), exENaC-containing channels are characterized by a 10-fold lower apparent affinity for amiloride. This value matches best with the apparent $K_i$ (4.3 μM) of an ENaC from *Rana* skin (22). Our results demonstrate that the lower apparent amiloride affinity is due to the presence of a unique tryptophan at the outer mouth of the ion pore. This finding confirms the amiloride-binding site identified in previous studies by site-directed mutagenesis (20, 23).

exENaC is already the sixth ENaC subunit identified in *Xenopus*. All subunits are expressed in kidney (13, 14) and could, assuming that channels contain an α-like, a β, and a γ subunit, theoretically assemble in eight different combinations. Channels formed by only two different subunits would increase the variety of ENaCs in *Xenopus* even more. Why this remarkable diversity evolved is unclear. Mammalian ENaCs seem to be less variable. βxENaC and γxENaC subunits do not appear to be present in mammalian genomes. Moreover, the closest exENaC homolog in the draft version of the human genome is αxENaC, suggesting that there is no exENaC subunit in humans.

**Na⁺ Self-inhibition—**Na⁺ self-inhibition had been best described in frog tissues (5). It has, however, been indirectly shown for native ENaC in rat cortical collecting tubules (24). Here, an apparent Na⁺ affinity of 9 mM was measured for the whole cell Na⁺ current ($I_{Na}$), whereas the current through single channels ($I_{Na}$) saturates with an apparent $K_i$ of 48 mM (24). Because $I_{Na} = (\gamma_{Na} N P_o)$, this implies that either the channel density ($N$) or the open probability ($P_o$) decreases as [Na⁺] increases (24). Thus, Na⁺ self-inhibition seems to be a general characteristic of ENaCs. Strikingly, however, most of the studies characterizing recombinant ENaC in heterologous expression systems did not reveal self-inhibition. It was only very recently that Chraibi and Horisberger (15) convincingly showed that human ENaC, which is formed by αβγ subunits, shows self-inhibition when expressed in *Xenopus* oocytes. The self-inhibition was observable only after fast amiloride washout or a change in the extracellular Na⁺ concentration, and the current relaxed to a quasi-steady state with a time constant of 3 s. Thus, self-inhibition is fast and can be observed only with a high time resolution. At room temperature, ~40% of the initial current was inhibited at steady-state with human ENaC and ~20% with rat ENaC. For αβγxENaC, self-inhibition was too fast to be reliably determined (15).

Due to the speed of self-inhibition, the time constants we determined should be taken only as estimates. Indeed, it seems that for all of the native channels, self-inhibition is faster than for exENaC-containing channels. The time course of self-inhibition has been reported in detail for ENaC from *Rana* skin and...
is on the order of 2–4 s (9, 11, 18). However, the solution flow rate in these experiments was as fast as 40 ml/s (9). Such fast flow rates and ensuing step changes in ionic concentrations can, unfortunately, not be applied to *Xenopus* oocytes. Considering that the constant we determined is probably limited by a comparably slow flow rate, the time course of self-inhibition of eENaC is in reasonable agreement with that of ENaC from *Rana* skin. However, self-inhibition of eENaC seems to be considerably faster. Moreover, self-inhibition of ENaC from *Rana* skin can be relieved by 1 mM BIG (11, 18), which has a dominant inhibitory effect on channels composed of αβγ subunits (Ref. 15 and this study). The time course of self-inhibition, the stimulatory effect of BIG, and the low amiloride affinity suggests that eENaC is the molecular correlate of the channel from *Rana* skin. Because eENaC is not expressed in *Xenopus* skin (Fig. 2), this would then imply species differences in eENaC tissue expression patterns.

**Molecular Mechanism of Na⁺ Self-inhibition**—It appears that upon exposure to a high extracellular Na⁺ concentration, ENaC passes into an inactive state. According to a model proposed by Palmer and Frindt (25), ENaC exists either in a gating mode characterized by a high \( P_o \) or in a gating mode characterized by a low \( P_o \). It may be that an increase in the extracellular Na⁺ concentration favors the low \( P_o \) mode. In this model, Na⁺ self-inhibition endows ENaC with a regulatory mechanism to respond to high extracellular Na⁺ concentrations. Because other regulatory mechanisms also act on \( P_o \), the extracellular Na⁺ concentration cannot, however, be the sole determinant of the open probability of ENaC.

Most likely, ENaC possesses a site in the extracellular loop that senses the extracellular Na⁺ concentration. Interaction of Na⁺ with this site would entail a conformational change, which would then reduce channel \( P_o \), leading to the apparent decrease of the whole cell current. The comparatively slow self-inhibition with eENaC-containing channels probably reflects a slower inactivation rate constant for the transition from the active (\( A \), high \( P_o \)) to the inactive (\( I \), low \( P_o \)) state. This may be due to a higher activation energy for this transition and indicates less favorable conformational changes during self-inhibition in eENaC-containing channels. Our approach using subunit chimeras has identified crucial determinants for the speed of self-inhibition in the extracellular loop, indicating conformational transitions during self-inhibition in this region.

Because self-inhibition could also be observed after amiloride washout, amiloride binding to the channel apparently interferes with self-inhibition. Either it masks the Na⁺ sensor, which should then be located distal to the amiloride-binding site, or it stabilizes the active (high \( P_o \)) state. This would also explain why amiloride analogs with a short dwell time such as BIG release the channel from self-inhibition. 1 mM BIG led to partial macroscopic blockade of the current mediated by eENaC-containing channels. But BIG activity would also interfere with self-inhibition, leading to the paradoxical increase in the macroscopic current amplitude. Using an exponential fit, we calculated a time constant for the rising phase of the current after amiloride washout that was significantly larger for αβγENaC-expressing oocytes than for εβγENaC-expressing oocytes (3.6 ± 2.5 s compared with 0.9 ± 0.3 s; \( p < 0.05 \)) (Table I). This suggests that the dissociation rate constant (\( k_{diss} \)) for amiloride for channels containing the ε subunit is significantly higher than for those containing the α subunit. A higher off-rate could account for the reduced apparent affinity of eENaC-containing channels for amiloride. Moreover, because amiloride washout was slower than self-inhibition for αENaC-containing channels (Table I), this could explain why self-inhibition could not be observed for these channels after amiloride washout. In agreement with this notion, amiloride affinity was increased and self-inhibition could no longer be observed for eENaC containing the single substitution W513L. Thus, Na⁺ self-inhibition can be more easily observed with eENaC-containing channels for two reasons: self-inhibition is slower, and amiloride washout is faster probably due to a higher off-rate.

Our analysis showed that the majority of eENaC-containing channels passed into the inactivated state at room temperature, suggesting a lower energy level for the inactive state compared with the active state. Due to the speed of self-inhibition, the proportion of αENaC-containing channels that passed into the inactive state could not be determined with precision. However, chimeric channels that contained the intracellular N terminus and the first transmembrane domain of αENaC showed fast self-inhibition and a high proportion of inactive channels at steady state (C2) (Fig. 5), suggesting that \( I_{steady-state}/I_{peak} \) is not overestimated for eENaC-containing channels. Moreover, channels containing human or rat αENaC also show a low proportion of inactive channels during steady state at room temperature (15). Thus, it is likely that, compared with αENaC-containing channels, a significantly larger proportion of eENaC-containing channels undergo self-inhibition. Our approach using subunit chimeras suggests that the proximal part of the extracellular loop is most crucial in determining the ratio of the active and inactive states and consequently in determining the energy levels of these states. Therefore, it seems that the same regions in the extracellular loop control the rate constant for inactivation (\( k_a \)) as well as for activation (\( k_o \)).

Only –10–40% of human or rat αβγENaC passes into the inactive state at room temperature (15), compared with 70% of εβγENaC. However, due to a strong temperature dependence of the inactivation rate, a fraction of the mammalian channels comparable to εβγENaC pass into the inactive state at 35 °C (15). Thus, because *Xenopus* is an ectothermic organism with a temperature preference of ~20 °C, both channel types have comparable equilibria of active and inactive states at the respective body temperatures, suggesting similar physiological implications for Na⁺ self-inhibition by εβγENaC and mammalian αβγENaC.

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A New Subunit of the Epithelial Na\(^+\) Channel Identifies Regions Involved in Na\(^+\) Self-inhibition

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