Feedback inhibition of ENaC: Acute and chronic mechanisms

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Abbreviations: ENaC, epithelial Na channel; CCD, cortical collecting duct; [Na]i, intracellular Na concentration; Po, open probability; INa, amiloride-sensitive current.

Intracellular [Na+] ([Na+]i) modulates the activity of the epithelial Na channel (ENaC) to help prevent cell swelling and regulate epithelial Na+ transport, but the underlying mechanisms remain unclear. We show here that short-term (60–80 min) incubation of ENaC-expressing oocytes in high Na+ results in a 75% decrease in channel activity. When the β subunit was truncated, corresponding to a gain-of-function mutation found in Liddle’s syndrome, the same maneuver reduced activity by 45% despite a larger increase in [Na+]i. In both cases the inhibition occurred with little to no change in cell-surface expression of γENaC. Long-term incubation (18 hours) in high Na+ reduced activity by 92% and 75% in wild-type channels and Liddle’s mutant, respectively, with concomitant 70% and 52% decreases in cell-surface γENaC. In the presence of Brefeldin A to inhibit forward protein trafficking, high-Na+ incubation decreased wt ENaC activity by 52% and 88% after 4 and 8 hour incubations, respectively. Cleaved γENaC at the cell surface had lifetimes at the surface of 6 hrs in low Na+ and 4 hrs in high Na+, suggesting that [Na+]i increased the rate of retrieval of cleaved γENaC by 50%. This implies that enhanced retrieval of ENaC channels at the cell surface accounts for part, but not all, of the downregulation of ENaC activity shown with chronic increases in [Na+]i.

Introduction

The epithelial Na+ channel (ENaC) serves as the apical entry pathway for Na+ in many epithelia. When Na+ influx through the channels is acutely raised, it can exceed the ion’s efflux across the basolateral membrane through the Na+, K+-ATPase resulting in increased intracellular [Na+]i ([Na+]i). In response, epithelial cells decrease ENaC activity to limit Na+ entry. This process was initially studied in frog skin where serosal application of ouabain to inhibit the Na+ pump was found to decrease mucosal Na+ permeability.1 Later experiments in frog skin,2 toad bladder,3 and rat cortical collecting duct (CCD)4 showed that this decrease in apical Na permeability was at least indirectly related to increased [Na+]i. This phenomenon, termed “feedback inhibition,” has also been observed in colon,5,6 mammalian salivary ducts7,8 and the A6 cell line.9

After ENaC was cloned,10 the Xenopus oocyte has become a convenient expression system to study feedback inhibition of ENaC.11–14 Abriel and Horisberger utilized the cut-open oocyte technique to find that [Na+]i does not directly inhibit ENaC but requires other unidentified intracellular factors.11 This inhibition by [Na+]i was found to be biphasic with the initial phase occurring in 3 minutes and a second phase requiring 30 minutes. Anantharam et al. investigated longer-term effects of elevated [Na+]i, and directly measured decreases in Po.12 However, decreased currents were still observed when channel Po was increased to 1 with cysteine modification of a degenerin mutant, implicating other mechanisms of inhibition under these conditions.

Kellenberger et al.15 showed that channels with mutations found in Liddle’s syndrome – a monogenic form of hypertension involving gain of function of ENaC16—were resistant to feedback inhibition in oocytes. Liddle’s mutations alter or truncate the C-termini of β or γENaC, preventing ubiquitination via neural precursor cell expressed, developmentally down-regulated 4–2 (Nedd 4–2), an E3 ubiquitin ligase.17–19 Binding of Nedd 4–2 at PY motifs on C-termini of ENaC subunits leads to ubiquitination of lysine residues on the N-termini of α and γENaC.20,21 In salivary gland ductal cells, both the WW domains of Nedd 4–2 and the C termini of α, β, and γENaC are necessary for feedback inhibition of ENaC by [Na+]i, suggesting that the Nedd 4–2 ubiquitination pathway is critical in this process.22–24

Ubiquitination is thought to enhance the rate of retrieval of ENaC from the cell surface.20,25–27 This implicates decreased surface expression in the feedback-inhibition process.
Kellenberger et al measured changes in surface expression after chronic elevation of $[Na^+]$, that could partially explain the down-regulation of ENaC. This would be consistent with the findings of Anatharam et al of an effect of $[Na^+]$-independent of $P_o$. However this mechanism has not been directly tested with acute changes in $[Na^+]$. In this manuscript we evaluate the role of surface expression in the feedback inhibition of ENaC observed under both acute and chronic conditions.

**Results**

To determine the initial time course of feedback inhibition of ENaC in Xenopus oocytes, rENaC-expressing oocytes were incubated overnight in low-$Na^+$ solution. They were placed in the recording chamber and superfused with high-$Na^+$ solution starting at $t = 0$. Control oocytes were maintained in the low-$Na^+$ solution except for brief exposures to high-$Na^+$ to measure their electrical properties. The time courses of amiloride-sensitive current ($I_{Na}$) and intracellular $Na^+$ concentration ($[Na^+]_i$) are shown in Figure 1. Typical $I_{Na}$-$V$ curves at various time points are shown in Fig. 1A. $I_{Na}$ decreased by 80% over the course of 80 minutes (Fig. 1B) while $[Na^+]_i$ increased from 18 to 55 mM in the same period (Fig. 1C). Both $I_{Na}$ and $[Na^+]_i$ were constant in control oocytes.

We also studied channels with a truncation at the C-terminus of $\beta$ENaC (BR564X), mimicking a Liddle’s syndrome mutation. In these oocytes $I_{Na}$ decreased by 59%, less than for wt ENaC-expressing oocytes. In particular, the initial rate of decline in $I_{Na}$ was about 1/3 that of the wt channels despite a significantly larger rise in $[Na^+]_i$. Again, $I_{Na}$ and $[Na^+]_i$ were stable in control oocytes. Fig. 1D shows the fractional decrease in $I_{Na}$ as a function of $[Na^+]_i$ for the 2 channel types. While the wild-type channel activity decreased sharply as $[Na^+]_i$ exceeded $\sim 25$ mM, currents

![Figure 1](https://www.landesbioscience.com/Channels/445/channels_445_02042007_fig1.jpg)

**Figure 1.** High Na$^+$ reduces ENaC activity over 80 minutes. (A) Representative current-voltage plots of wt ENaC-expressing oocytes incubated in high Na$^+$ solution for 0, 15, 30, 45, and 60 minutes. (B) $I_{Na}$ of wt ENaC (squares) and Liddle’s mutant (triangle) expressing oocytes exposed to high-Na$^+$ solution for 80 minutes. Control oocytes were maintained in low-Na$^+$ solution except for brief (30 second) exposures to high-Na$^+$ to measure $I_{Na}$ and $[Na^+]_i$. Currents were normalized to values at $t = 0$. Data represent means ± SEM for 6 oocytes. (C) $[Na^+]_i$ measured in the same oocytes as in (b). (D) Normalized current vs. $[Na^+]_i$ for wt and Liddle’s mutant ENaC.
though the mutants were not strongly affected until [Na\(^+\)], reached \(\sim 75 \text{ mM}\).

To assess the role of surface expression in mediating these effects, we assessed cell-surface γENaC by labeling with a cell-impermeant biotin reagent and isolation of biotinylated proteins with neutravidin beads. Figure 2 shows a control experiment. Neutravidin eluates contained significant amounts of γENaC only when the oocytes were surface-labeled with biotin. In contrast, non-specific binding of α and βENaC to neutravidin beads prevented reliable measurements of cell-surface α and βENaC.

In whole-oocyte extracts, γENaC appeared in 2 forms. The 75–80 kDa band presumably represents intact subunits, while the relatively broad band at \(\sim 65 \text{ kDa}\) reflects a product of cleavage by cellular proteases. Cell-surface γENaC appeared predominantly in the cleaved form. In this case 2 bands could be resolved: an intermediately cleaved form (\(\sim 64 \text{ kDa}\)) and a maximally cleaved form (\(\sim 60 \text{ kDa}\)). These cleaved products may correspond respectively to γENaC cleavage by intracellular proteases only, and by intracellular plus extracellular proteases.\(^{28,29}\) Very little full-length γENaC was detected at the cell surface.

To assess the role of ENaC trafficking in the acute response to a Na\(^+\) load, ENaC-expressing oocytes were incubated in high-Na\(^+\) or low-Na\(^+\) solution for 1–1.5 hrs. Whole-oocyte current recordings were made on a subset of oocytes. The remaining oocytes were subjected to the biotinylation protocol to assess cell-surface expression of γ ENaC. In this set of experiments wild-type ENaC currents decreased by 75% while a smaller decrease (45%) was observed in the Liddle’s mutant, similar to those in Fig. 1 (Fig. 3A). The cell-surface expression of γENaC changed very little for either wild-type or Liddle’s mutant ENaC with short-term incubation in high Na\(^+\) (23% and 13% decrease, respectively, not significant) (Figs. 3B, C). Furthermore there was no detectable change in the ratio of intermediate to maximal-cleaved γENaC during this time (Fig. 3D). The lack of changes in cell-surface γENaC suggests that a decrease in ENaC open probability and/or the fraction of channels at the surface in the active state largely accounts for the decrease in wild-type, and the smaller reduction in the Liddle’s mutant.

We next investigated more chronic effects of increased [Na\(^+\)]. Eighteen-hour incubation in high Na\(^+\) decreased ENaC currents by 92% for wild-type and by 75% for the Liddle’s mutant (Fig. 4A). In contrast to the short-term experiments, the cell-surface expression of cleaved γENaC decreased significantly, 70% for wild-type and 52% for the Liddle’s mutant (Figs. 4B, C). In each case, both the intermediate and maximal cleavage bands declined with high Na\(^+\). However, the maximal-cleaved species decreased more, leading to a two-fold increase in the intermediate/maximal cleaved ratio (Fig. 4D). The changes in this ratio did not reach statistical significance for either WT or mutant channels individually, but were highly significant when the results from the 2 similar data sets were combined.

We next investigated the roles of trafficking through the ER and Golgi versus changes in retrieval of channels from the cell surface in regulating the surface expression of ENaC. Brefeldin A (BFA) was used to inhibit protein trafficking from the ER to the Golgi.\(^ {30–32}\) Under these conditions, any changes in ENaC activity and/or cell-surface expression between high- and low-Na\(^+\) solutions would result from alterations in the rates of channel internalization from and/or recycling to the cell surface. Oocytes were incubated for 4 or 8 hours with 5 μM BFA in presence of low- or high-Na\(^+\) solution. Values of I_{Na} measured at these time points are shown in Figs. 5A and C. In low-Na solution with BFA, there was a significant decrease in I_{Na} after 8 hours but not 4 hours. In the presence of BFA, high Na\(^+\) caused a 50% and 96% reduction in I_{Na} relative to those in low-Na after 4 hours and 8 hours incubations, respectively.

The decline in cell-surface expression of γENaC of oocytes in the presence of BFA was used to determine rates of channel internalization in the presence of high- or low-Na\(^+\) solution. As expected, BFA decreased the amount of γENaC at the surface, reflecting channel retrieval in the nominal absence of channel insertion. In low-Na, the decrease in the cleaved form relative to initial values was 48% at 4 hrs and 74% at 8 hrs. This is consistent with an exponential decay process with a mean lifetime (\(\tau\)) of 6 hrs at the surface (Fig. 5D). In high Na\(^+\) the fall was faster, with a \(\tau\) of 4 hrs. This indicates a 50% increase in the rate of channel retrieval in the presence of high Na. The difference in \(\tau\) was statistically significant, but the fractional change was smaller than that of either the overall surface expression or of I_{Na}.

**Discussion**

We investigated the ability of [Na\(^+\)], to regulate ENaC in oocytes by assaying activity through voltage clamp and cell-surface expression with biotinylation over different time domains. Novel findings were: (1) Short-term (60–80 min) feedback inhibition, which is reduced in Liddle’s mutants, does not require a change in surface expression. (2) More chronic feedback inhibition (18 hr) correlates with surface
expression in both wild-type and Liddle’s mutant channels (3). Cell-surface γENaC is observed in 2 different states of cleavage, with a relative abundance that depends on [Na⁺]i. (4) The lifetime of γENaC at the plasma membrane is reduced by [Na⁺]i.

These studies, along with previous results, suggest that 3 different mechanisms contribute to the phenomenon of feedback inhibition of ENaC.

1. Changes in Pₒ. ENaC activity measurements showed large decreases in I Na with short-term incubation in high Na⁺ that did not correlate with changes in ENaC cell-surface expression. Kellenberger et al.15 found a similar decrease in wt but not in Liddle’s mutant ENaC currents with a shorter 25-minute incubation in high Na⁺. Cell-surface expression was not assessed under these conditions. They did show, however, that for overnight Na⁺ loading, currents decreased more than surface expression, consistent with a fall in Pₒ. We found very similar results using a different method of estimating surface expression (Fig. 4). Anantharam et al. found a 50% decrease in Pₒ measured directly by patch clamp after overnight incubation in high Na⁺.12 A similar decrease in Pₒ could explain at least in part the acute decreases in I Na, shown in Figures 1A and 3A. The mechanisms by which increases in [Na⁺]i decrease Pₒ have yet to be determined. However the reduced sensitivity of the Liddle’s mutant to this process indicates that the C-terminus of the β ENaC subunit is involved.

2. Changes in subunit cleavage. Previous studies have found that increases in [Na⁺]i can decrease the sensitivity of ENaC subunits to proteolytic cleavage.14 In our experiments, cell-surface γENaC appeared as both intermediately cleaved and maximally cleaved forms (Figs. 2-4). After long-term high Na⁺ incubation, this subunit was mostly in the intermediately cleaved state, while with low Na⁺ there was a larger proportion in the maximally cleaved state. Since this is presumed to be the state of highest activity,28,29 this effect of [Na⁺]i on cleavage

Figure 3. Incubation in high Na⁺ for 60–90 min reduces ENaC currents but has little effect on cell-surface γENaC expression. (A) I Na in oocytes incubated in low or high-Na⁺ for 60–90 min. I Na was reduced by 75% in wt ENaC (P < 0.0001, n = 40 for high Na⁺ and n = 43 for low Na⁺) and by 45% in the Liddle’s mutant (P = 0.0016, n = 44 for high Na⁺ and n = 50 for low Na⁺). (B) γENaC in whole-cell extracts and neutravidin eluates. (C) Cell-surface expression of cleaved γENaC in high vs. low Na⁺ was not statistically different in wt (P = 0.4, n = 7) or Liddle’s mutant (P = 0.8, n = 7). (D) Ratio of intermediately cleaved to maximally cleaved γENaC. Ratios were similar in high- and low-Na⁺ incubated oocytes. * indicates statistically significant difference.
could contribute to feedback inhibition not accounted for by cell-surface expression with long-term incubation in high Na⁺. 15

Ubiquitination has also been suggested to decrease the sensitivity of ENaC to proteolytic cleavage. 33,34 However we observed similar effects on cleavage products with a Liddle’s mutant β-subunit in which ubiquitination is at least partially disrupted. Other possible mechanisms include Na⁺-dependent changes in the activity of proteases, 14 or preferential retrieval of specific cleaved products of ENaC from the cell surface.

3. Effects on channel trafficking. The cell-surface expression of γENaC decreased substantially (by 70%) with long-term incubation in high Na⁺ (Figs. 4B, D). The change was smaller than the decrease in I_Na (92%), consistent with additional effects on P_o as described above. Similar results were reported by Kellenberger et al., 15, who used radiola- beled-antibody binding to measure surface expression.

Using BFA to block channel insertion into the membrane, we found that high [Na⁺]i increased the apparent rate of channel retrieval from the membrane. However the magnitude of the increase of 50% predicts, with the simplest model of insertion and retrieval, a change in surface expression of 33%, substantially less than the measured 75% decrease. It is possible that [Na⁺]i also affects the rate of channel insertion into the membrane.

We recently reported a similar decrease in ENaC surface expression in response to increased [Na⁺]i in vivo. During Na⁺ repletion of salt-deprived rats, the surface expression of γENaC decreased by 80% after 4 hours, and this effect was prevented by treating animals with amiloride to block Na⁺ entry into the ENaC-expressing cells. Thus the effects observed in oocytes appear to be relevant to renal function.

Rates of ENaC retrieval may depend on the process of Nedd4–2 dependent ubiquitylation of the channels. 20, 21

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**Figure 4.** Incubation for 18 hrs in high Na⁺ incubation reduces ENaC activity and cell-surface γ ENaC expression. (A) 18-hr incubation in high Na⁺ reduced I_Na by 92% in wt (p = 0.0001, n = 10) and by 75% in Liddle’s mutant ENaC (p = 0.03, n = 11). (B) γENaC in whole-cell extracts and neutravidin eluates. (C) High Na⁺ decreased cell-surface expression of cleaved γENaC. Ratios of expression in high Na⁺ to that in low Na⁺ for total cleaved, intermediate-cleaved and maximal-cleaved γENaC were all significantly less than 1. Data represent means ± SEM for 3 batches of oocytes. (D) Ratio of intermediate-cleaved to maximally cleaved γENaC. The ratios were larger in high-Na⁺ incubated oocytes. The differences were not quite statistically significant (WT p = 0.08; mutant p = 0.08). However the 2 data sets were similar and when combined the effect of Na⁺ was highly significant (p = 0.005, n = 6). *Indicates statistically significant difference.
Consistent with this idea, Kellenberger et al.\textsuperscript{15} found that Liddle’s mutants showed little to no change in cell-surface expression after overnight incubation in high Na. In contrast, in our experiments with a Liddle’s syndrome mutant the reductions in both current (75%) and cleaved \(\gamma\)ENaC surface expression (52%) were substantial, albeit less than those of the wild-type channels. As the Liddle’s mutant has defective binding of Nedd 4–2, this suggests a Nedd 4–2-independent method of ENaC retrieval from the cell surface that is sensitive to \([\text{Na}^+]_c\).

In summary, we provide evidence for 2 \([\text{Na}^+]_c\)-dependent processes that regulate ENaC activity. One, involving acute changes, is dependent on modulation of channel PO4 or activation of channels at the plasma membrane. The other, observed after longer-term elevations of \([\text{Na}^+]_c\), entails decreased surface expression including, in part, acceleration of channel retrieval from the membrane.

\textbf{Materials and Methods}

Oocyte Isolation - All animal procedures were carried out according to the guidelines of and with the approval of the Institutional Animal Use and Care Committee of Weill Cornell Medical College. Xenopus laevis were anaesthetized in water containing tricaine methanesulphonate. Oocytes were removed through a 1 cm incision in the abdomen and incubated in OR2 solution (mM): 82.5 NaCl, 2.5 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 1 Na\(_2\)HPO\(_4\), 5 HEPES at pH 7.4 with 2 mg/ml collagenase type II and 2 mg/ml hyaluronidase type II for 60 minutes with gentle shaking. Oocytes were then washed with OR2 solution and incubated for 1 hr at 18°C prior to being injected.

cRNA Injection – \(\alpha, \beta, \gamma\) rat ENaC (rENaC) subunits cloned into the pSPORT1 vector were linearized with NotI restriction enzyme. Linearized plasmids were isolated using QIAquick PCR purification kit (Qiagen). cRNAs were transcribed using the T7 RNA polymerase and the mMESSAGE mAMACHINE kit (Ambion). cRNA was dissolved in nuclease-free water and stored at \(-80^\circ\text{C}\). Oocytes were injected with 4 ng of cRNA from each ENaC subunit being expressed. The oocytes were then incubated overnight at 18°C in either high-\(\text{Na}^+\) solution: (mM): 110 NaCl, 2 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 1 Na\(_2\)HPO\(_4\), 5 HEPES at pH 7.4 with 2 mg/ml collagenase type II and 2 mg/ml hyaluronidase type II for 60 minutes with gentle shaking. Oocytes were then washed with OR2 solution and incubated for 1 hr at 18°C prior to being injected.

Electrophysiology — Oocytes were perfused with high-\(\text{Na}^+\) solution (mM): 110 NaCl, 2 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 5 HEPES at pH 7.4. Whole-cell currents were measured using the 2-electrode voltage clamp (OC-275, Warner Instrument Corp.).
with an ITC-16 interface (Instrutech) running Pulse software (Heka Elektronik). Pipettes were made from hemocytic capillary tubes using a 3-step vertical pipette puller (David Kopf Instruments) and backfilled with 3M KCl. Pipettes had resistances of 0.5–3 MΩ.

For electrophysiological measurements oocytes were placed in a fast-exchange chamber (OPC-1, AutoMate Scientific, Berkeley, CA). Current-voltage curves were generated using a protocol of 15 pulses of 50 msec each separated by 10 mV starting at −80 mV and ending at +60 mV while holding at −20 mV between pulses. Leak currents measured during brief administration of 10 μM amiloride were subtracted. Channel-mediated current (I\(_{\text{Na}}\)) was calculated as the difference in currents in the absence and presence of amiloride at −60 mV. Changes in I\(_{\text{Na}}\) will differ from changes in Na\(^+\) permeability determined from the constant-field equation by less than 10%. [Na\(^+\)], was estimated from the reversal potential of I\(_{\text{Na}}\) as described previously. 12

Oocyte Biotinylation – 25 oocytes expressing α, β, and γ ENaC subunits were incubated in high-Na\(^+\) PBS (mM): 95 NaCl, 5 NaH\(_2\)PO\(_4\) at pH 8.0 or low-Na\(^+\) PBS (mM): 95 KCl, 5 NaH\(_2\)PO\(_4\) at pH 8.0 with 0.5 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce) at 4°C for 30 minutes. The biotin reaction was then quenched with high-Na\(^+\) TBS (mM): 80 NaCl, 20 TRIS at pH 8.0 or low-Na\(^+\) TBS (mM): 80 KCl, 20 TRIS at pH 8.0 at 4°C for 30 minutes. The oocytes were then washed twice more with TBS and lysed by trituration with Pasteur pipettes in 200 μL of lysis buffer (mM): 100 NaCl, 50 TRIS, 5 EDTA, plus 1% Triton X-100, 1 μg/mL leupeptin (Sigma-Aldrich), 0.1 mg/mL PMSF (Sigma-Aldrich) at pH 7.4. The lysate was centrifuged for 10 minutes at 10,000 g.

For whole-lysate samples, 20 μL of the supernatant was added to 25 μL of LDS sample buffer (Invitrogen) and 45 μL of H\(_2\)O. Ten μL of Sample Reducing Agent (Sigma-Aldrich) was added and the samples were heated for 5 minutes at 95°C.

For the avidin pulldown, 80 μL of supernatant was incubated with 75 μL of NeutrAvidin Agarose Resin (Pierce) and 200 μL of lysis buffer in 1.5 mL Eppendorf centrifuge tubes overnight at 4°C with gentle shaking. The agarose resin was then washed once with lysis buffer, twice with high-salt lysis buffer: 500 mM NaCl, 50 mM TRIS, 5 mM EDTA, 0.1% Triton X-100, 1 μg/mL leupeptin, 0.1 mg/mL PMSF at pH 7.4, and twice with no-salt lysis buffer: 10 mM TRIS, 1 μg/mL leupeptin, 0.1 mg/mL PMSF at pH 7.4. 45 μL of LDS sample buffer and 10 μL of Sample Reducing Agent were added and the tubes were heated for 10 minutes at 80°C.

Samples were separated by electrophoresis on 4–12% Bis-TRIS gels (Invitrogen). For immunoblotting, the proteins were transferred electrophoretically to PVDF membranes. After blocking with BSA, membranes were incubated overnight at 4°C with polyclonal antibodies raised against the C-terminus of the γ subunit of rENaC at 1:1,000 dilution as described. 35 Anti-rabbit IgG conjugated with alkaline phosphatase was used as a secondary antibody. Bound antibody was visualized on autoradiography film (Biomax ML, Kodak) using a chemiluminescence substrate (Western Breeze, Invitrogen).

Statistics – Statistical significance was assessed by unpaired Student t-tests. Data are reported as means ± SEM.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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