Intracellular Na\(^+\) Regulates Epithelial Na\(^+\) Channel Maturation*

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Background: ENaC activity decreases when intracellular Na\(^+\) is elevated.

Results: High intracellular Na\(^+\) inhibits N-glycan remodeling and prevents proteolytic processing by endogenous and exogenous proteases.

Conclusion: Na\(^+\) inhibits ENaC maturation within the biosynthetic pathway.

Significance: This work provides novel insights into the mechanisms underlying Na\(^+\) feedback inhibition and demonstrates that intracellular Na\(^+\) regulates ENaC biogenesis.

The epithelial sodium channel (ENaC) is the rate-limiting step of electrogenic Na\(^+\) absorption in the distal nephron, intestine, and lung (1–3). In these tissues, the basolateral sodium/potassium ATPase generates an electrochemical gradient that favors Na\(^+\) entry into the cell when active ENaCs are present on the apical membrane. By manipulating ENaC activity, organisms are able to maintain optimal extracellular fluid volume despite wide fluctuations in dietary Na\(^+\) intake. Interestingly, Na\(^+\) itself is a strong inhibitor of the channel. Exocellular Na\(^+\) rapidly (in seconds) decreases the ENaC open probability through a process known as sodium self-inhibition. Additionally, high intracellular Na\(^+\) ([Na\(^+\)]) decreases the activity of ENaC through a process known as Na\(^+\) feedback inhibition (4–11). In contrast to Na\(^+\) self-inhibition, Na\(^+\) feedback inhibition occurs over minutes to hours.

In general, Na\(^+\) feedback inhibition has been characterized by a reduction in whole-cell Na\(^+\) current when [Na\(^+\)], is elevated. Previous studies have identified potential mechanisms by which [Na\(^+\)]\(_i\) inhibits ENaCs. Channel surface density has been shown to be reduced by Nedd4-2-dependent endocytosis when [Na\(^+\)]\(_i\), is elevated (5, 6). Likewise, [Na\(^+\)]\(_i\), seems to regulate the open probability of the channel (9). In this regard, recent evidence suggests that intracellular Na\(^+\) regulates the cleavage state of ENaC so that the channel undergoes increased proteolytic activation when [Na\(^+\)]\(_i\), is low (10, 11). Conversely, channel processing is decreased when [Na\(^+\)]\(_i\), is increased. Interestingly, Knight et al. (10) observed that increased [Na\(^+\)]\(_i\), reduces the ability of exogenous trypsin to cleave surface ENaC, suggesting that high Na\(^+\) induces a conformational change in the channel that prohibits proteolysis.

In this work, we examined the hypothesis that [Na\(^+\)]\(_i\), regulates ENaC activity by modulating posttranslational processing during the biogenesis of the channel. Previous work from Hughey et al. (12–14) has demonstrated that ENaC maturation involves modification of immature N-glycans to complex mature oligosaccharides and proteolytic processing of the α and γ ENaC subunits by furin (12–14) (Fig. 1). Because these two modifications occur together in an “all or none event,” we hypothesized that [Na\(^+\)]\(_i\), reduces the proportion of channels that undergo maturation in the Golgi and trans-Golgi network. To examine this hypothesis, the biochemical and electrophysiological effects of [Na\(^+\)]\(_i\), were examined in Fisher rat thyroid (FRT) epithelia transiently transfected with human ENaCs.
EXPERIMENTAL PROCEDURES

cDNA Constructs—Human α-, β-, and γ ENaC cDNAs were provided by Prof. Douglas Eaton (Emory University). Human hepatocyte growth factor activator inhibitor type 1 (HAI-1) cDNA was cloned from human bronchial epithelial cells and subcloned into pcDNA3.1. The following mutations were introduced using site-directed mutagenesis (QuikChange II site-directed mutagenesis kit, Agilent Technologies): αCl (R175A, R177A, R178A, R201A, and R204A), βCl (R135KRR-AKAA and R178KRK-QQQQ), and γCl (del Glu-139-Lys-181). All constructs were verified by sequencing.

Cell Culture—FRT cells were provided by Prof. Peter Snyder (University of Iowa). FRT cells were cultured in DMEM/F-12 media supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. Three days prior to experimentation, FRT cells were seeded onto Costar transwell filters (0.33 cm² for electrophysiology, 4.67 cm² for biochemistry, 0.4-μm pore size, Corning, Lowell, MA). Following seeding onto the transwell, the cells were transfected with cDNAs as indicated using Lipofectamine 2000 according to the instructions of the manufacturer. Unless specified otherwise, the FRT cells were transfected with 0.25 μg/subunit/filter for electrophysiology or 3.3 μg/subunit/filter for biochemistry. Following transfection, 50 nM dexamethasone and 50 μM amiloride were added to the media where indicated. The cells were preincubated in 0 or 130 mM Na⁺ solutions (130 mM N-methyl-d-glucamine-Cl or 130 mM NaCl, 10 mM HEPES, 5 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, and 10 mM glucose (pH 7.4)) for 2 h prior to use where indicated.

Electrophysiology—FRT cultures were mounted in Ussing chambers (catalog no. P2300, Physiological Instruments) with custom sliders modified to fit the transwell inserts. The cultures were continuously short-circuited with an automatic voltage clamp (catalog no. VCC MC8, Physiological Instruments). The bathing Ringer’s solution was composed of 120 mM NaCl, 25 mM NaHCO₃, 3.3 mM KH₂PO₄, 0.8 mM K₂HPO₄, 1.2 mM MgCl₂, 1.2 mM CaCl₂, and 10 mM glucose. The chambers were constantly gassed with a mixture of 95% O₂/5% CO₂, which maintained a pH of 7.4. The temperature was maintained at 37 °C with a circulating perfusion pump. Transepithelial resistance was estimated using Ohm’s law by applying a 10-mV pulse/sec via an automated pulse generator. Acquire and Analyze 2.3 (Physiological Instruments) was used to control the voltage clamp and analyze the short circuit current (Isc) data. When the cells were treated with amiloride prior to Isc recording, the apical medium was removed from the transwell immediately before the cultures were mounted in the Ussing chamber. Therefore, the amiloride concentration present in the apical chamber was diluted to below the effective inhibitory...
range (estimated amiloride concentration ~10–100 nM when 3–30 μl remained on the apical surface). A typical \( I_{SC} \) recording included an equilibration period followed by stimulation with 1 μM trypsin. The amiloride-sensitive \( I_{SC} \) (\( I_{Na} \)) was determined by the addition of 10 μM amiloride to the apical hemichamber at the end of each recording. Unless noted otherwise, all chemicals were obtained from Sigma-Aldrich.

**Biochemistry**—Transiently transfected FRT cells expressing ENaC subunits, with a C-terminal V5 epitope tag on one subunit, were used for surface biotinylation experiments. Three days after transfection, the cells were placed into ice-cold PBS + Ca\(^{2+}\) (135 mM NaCl, 17.4 mM Na\(_2\)HPO\(_4\), 3.5 mM NaH\(_2\)PO\(_4\), 3.5 mM KCl, 0.9 mM CaCl\(_2\), and 0.9 mM MgCl\(_2\)) + 10% FBS and the apical surface was rinsed three times to remove accumulated debris. The apical surface was then treated with sulfo-NHS-SS-Biotin in borate buffer (85 mM NaCl, 4 mM KCl, and 15 mM Na\(_2\)B\(_4\)O\(_7\) (pH 9)) for 30 min and then quenched with PBS + 10% FBS. After several rinses with PBS, the cells were lysed by manual scraping in lysis buffer (10 mM Tris-Cl, 50 mM EGTA, 1% Nonidet P-40 (pH 7.4)). In some experiments, the apical surface was exposed to 10 μM trypsin for 30 min and then neutralized with 10 μM aprotinin prior to lysis. Following lysis, the tagged ENaC subunits were recovered by immunoprecipitation with 3 μl of goat anti-V5 antibody (Novus) and 100 μl of protein G conjugated to Sepharose beads (Life Technologies). The proteins were eluted in lysis buffer containing 2% SDS. 4% of the eluate was saved as the total cellular pool. 7-fold excess lysis buffer was then added to the remaining eluate to decrease the effective SDS concentration. Subsequently, the biotinylated proteins were recovered with 50 μl Avidin-agarose beads (Pierce). For the glycochemistry experiments, the recovered proteins were exposed to PNGase or Endo H according to the specifications of the manufacturer (New England Biolabs). The resultant proteins were separated by SDS-PAGE on 8% gels (Tris/glycine, Invitrogen) and transferred to nitrocellulose membranes for immunoblotting. The membranes were blocked with 5% nonfat dry milk, blotted with 1:2500 mouse anti-V5 antibodies (Invitrogen), and visualized with 1:2500 HRP-conjugated secondary antibodies (Bio-Rad). After blocking, V5 epitope tags were introduced into the cell. Subsequently, the cells were exposed to apical trypsin and surface biotinylations were performed to identify cleaved and full-length α subunits. When Na\(^{+}\) entry into the cell was inhibited by amiloride, increased amounts of cleaved αENaC (65 kDa) were present on the cell surface. Interestingly, the majority of the full-length α subunit at the cell surface (90 kDa) was resistant to cleavage by exogenous trypsin. Next, furin-mediated proteolysis was prevented by mutating the consensus furin cleavage sites on the α subunit (α\(_{C1\_V5}\)). A slower-migrating, 95-kDa α\(_{C1\_V5}\) subunit appeared on the cell surface when Na\(^{+}\) was reduced by amiloride. The slower migration of the 95-kDa form is consistent with an increase in processing by endogenous channel-activating proteases (Fig. 2C). Therefore, pre-exposure to amiloride increases the basal \( I_{Na} \) and reduces the sensitivity of the channel to trypsin.

To confirm that ENaC subunit proteolytic processing increases in response to pre-exposure to amiloride, surface biotinylation was performed to identify the molecular species of αβγ ENaC present on the apical membrane in the presence and absence of amiloride. V5 epitope tags were introduced into the carboxyl termini of each subunit to facilitate efficient biochemistry. The cells were transfected with αβγ ENaC, and one subunit contained a V5 tag. Apical cell surface biotinylation was performed as described under “Experimental Procedures.” In the absence of amiloride, primarily full-length channels were observed on the cell surface, as shown in Fig. 2D (see Fig. 1 for a review of the predicted subunit cleavage sizes). Variable amounts of cleaved α and γ subunits were also observed. When amiloride was included in the media, increased amounts of cleaved α and γ subunits were present. Furthermore, the β subunit migrated more slowly, consistent with an increase in N-glycan maturation. These results suggest that pre-exposure to amiloride and the resultant decrease in [Na\(^{+}\)]\(_i\) increase ENaC activity by facilitating proteolytic processing of the channel, consistent with the findings of Knight et al. (10).

**Intracellular Sodium Alters Channel Processing by Proteases**—ENaC subunits are processed by proteases that reside in distinct cellular compartments. Furin and related proprotein convertases reside primarily in the trans-Golgi network, where they interact with substrates as they pass through the biosynthetic pathway. Other proteases are believed to process the channel in post-Golgi compartments. A series of experiments was performed to determine whether relatively high [Na\(^{+}\)]\(_i\) prevents ENaC processing by furin and/or other proteases. As shown in Fig. 3, α\(_{C1\_V5}\)βγ ENaC-expressing cells were cultured in the presence and absence of amiloride to manipulate Na\(^{+}\) entry into the cell. Subsequently, the cells were exposed to apical trypsin, and surface biotinylations were performed to identify cleaved and full-length α subunits. When Na\(^{+}\) entry into the cell was inhibited by amiloride, increased amounts of cleaved αENaC (65 kDa) were present on the cell surface. Interestingly, the majority of the full-length α subunit at the cell surface (90 kDa) was resistant to cleavage by exogenous trypsin. Next, furin-mediated proteolysis was prevented by mutating the consensus furin cleavage sites on the α subunit (α\(_{C1\_V5}\)). A slower-migrating, 95-kDa α\(_{C1\_V5}\) subunit appeared on the cell surface when Na\(^{+}\) was reduced by amiloride. The slower migration of the 95-kDa form is consistent with increased terminal processing of the N-glycans on the non-cleaved α subunit compared with the 90-kDa form (addressed further below). This 95-kDa form of the α subunit was sensitive to trypsin, whereas the 90-kDa α\(_{C1\_V5}\) ENaC subunit remained relatively resistant to cleavage. Therefore, there is enhanced α subunit cleavage when
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**FIGURE 2. Effects of amiloride on ENaC activity and expression.** FRT cells were transfected with human \(\alpha\beta\gamma\) ENaC and cultured with or without 50 \(\mu\)M amiloride for 3 days. A, representative \(I_{SC}\) tracing of ENaC-expressing FRT cells with or without amiloride. Following a 30-min equilibration period, monolayers were treated with 1 \(\mu\)M trypsin, and then ENaC was inhibited with 10 \(\mu\)M amiloride. B, the effect of amiloride on basal and trypsin-stimulated \(I_{SC}\). Data are the mean \(\pm\) S.D. of normalized basal and trypsin-stimulated \(I_{SC}\) with or without amiloride pretreatment. *, \(p < 0.001\) different from amiloride control, \(n = 12\). C, the effect of amiloride on ENaC activation by trypsin. Data are the mean \(\pm\) S.D. of basal \(I_{SC}\) with or without amiloride pretreatment. *, \(p < 0.001\) different from amiloride control, \(n = 12\). D, the effect of amiloride on ENaC expression. FRT cells were transfected with ENaCs containing C-terminal V5 tags on the indicated subunit and cultured with or without 50 \(\mu\)M amiloride for 3 days prior to surface biotinylation. Data are representative immunoblots of the individual ENaC subunits that were immunoprecipitated with anti-V5 (Total) and then pulled down with avidin-agarose beads (Biotinylate).

Intracellular Na\(^+\), is reduced by amiloride, and a more slowly migrating (95-kDa), trypsin-sensitive, full-length form of the \(\alpha\) subunit appears when furin processing is blocked with the \(\alpha_{C1}\) mutation.

Interestingly, mutating the \(\alpha\) subunit furin consensus sites did not block subunit cleavage when the cells were pretreated with amiloride. Therefore, we next examined the contribution of other serine proteases on \(\alpha\) subunit processing. To inhibit selected serine proteases, the transmembrane Kunitz-type serine protease inhibitor HAI-1 was coexpressed. HAI-1 did not significantly change wild-type \(\alpha\) subunit processing both in the presence and absence of amiloride. However, the addition of HAI-1 prevented cleavage of the furin-insensitive \(\alpha_{C1-V5}\) subunit when the cells were pretreated with amiloride. Therefore, ENaC is sensitive to cleavage by furin, other endogenous proteases, or exogenous trypsin when the [Na\(^+\)]\(_i\) is relatively high. Because channel proteolysis by these channel-activating proteases is reduced when [Na\(^+\)]\(_i\) is elevated, ENaC is likely in a state that limits protease accessibility to the consensus cleavage sites.

**FIGURE 3. Intracellular Na\(^+\) regulates ENaC sensitivity to proteolysis by furin, HAI-1-sensitive serine proteases, and exogenous trypsin.** FRT cells were transfected with \(\alpha_{WT-V5}\) or furin-resistant \(\alpha_{C1-V5}\), along with \(\beta\gamma\) ENaC \(\pm\) HAI-1. Where indicated, amiloride was included in the media. The apical surface was biotinylated and subsequently exposed to 10 \(\mu\)M trypsin for 30 min as indicated. Following anti-V5 IP, the apically biotinylated proteins were recovered by streptavidin pulldown. The anti-V5-precipitated proteins (Total) and streptavidin-precipitated proteins (Biotinylate) were immunoblotted with an anti-V5 anti-body (\(\alpha\)ENaC).

ENaC N-glycan Maturation Is Regulated by [Na\(^+\)]\(_i\)—Several lines of evidence suggest that [Na\(^+\)]\(_i\) alters channel maturation. First, pretreatment with amiloride caused an increase in the expression of the mature higher molecular weight species of \(\beta\) ENaC (Figs 1 and 2D). Previous work has suggested that this represents channels that have passed through the Golgi complex allowing for maturation of the N-glycans from high mannose to complex-type (12). Second, when furin processing was blocked by the \(\alpha_{C1}\) mutations, a slower migrating, 95-kDa \(\alpha\) subunit appeared, consistent with N-glycan maturation. Third, the number of cleaved channels increased when [Na\(^+\)]\(_i\), was
reduced by amiloride (Fig. 2D). Hughley et al. (12–14) have demonstrated previously that both maturation of N-glycans and proteolytic processing within an individual channel complex occur as an all or none event. Therefore, we hypothesized that [Na\(^+\)], regulates ENaC maturation within the Golgi complex.

To determine whether [Na\(^+\)], regulates ENaC N-glycan maturation, the sensitivity of channel N-glycans to digestion by endoglycosidases was examined under low and high [Na\(^+\)]. Peptide N-glycosidase F (PNGase F) is an enzyme that removes both high-mannose (immature) and complex-type (mature) N-glycans. Conversely, endoglycosidase H (Endo H) only removes high-mannose N-glycans. Therefore, ENaCs containing Endo H-resistant glycans represent mature channels that have undergone maturation in the Golgi complex. As shown in Fig. 4A, full-length αENaC (90 kDa, closed arrowhead) was the predominant form on the cell surface in the absence of amiloride. These full-length α subunits contained immature high-mannose N-glycans, as evidenced by sensitivity to digestion by both PNGase F and Endo H. When amiloride was included in the media to reduce [Na\(^+\)], both full-length (90 kDa) and cleaved (Fig. 4A, 65 kDa, open arrowhead) α subunits were readily observed. Under these conditions, non-cleaved α subunits remained sensitive to both endoglycosidases. However, the cleaved α subunit was largely resistant to digestion by Endo H, demonstrating that the glycans were modified to a mature complex type when [Na\(^+\)], was reduced.

We next examined whether [Na\(^+\)], regulates N-glycan maturation on the β and γ subunits. V5 epitope tags were added to the β and γ subunits, and surface biotinylations were performed in cells cultured in the presence and absence of amiloride. As shown in Fig. 4B, the full-length β subunit developed more mature N-glycans when the cells were cultured in the presence of amiloride. Prior to digestion with the endoglycosidas, the electromobility of the β subunit was reduced when the [Na\(^+\)], was reduced with amiloride. Moreover, the sensitivity of the N-glycans to digestion by Endo H was reduced. Similarly, the γ subunit developed increased amounts of mature N-glycans when the [Na\(^+\)], was reduced by amiloride. As shown in Fig. 4C, increased amounts of the cleaved γ subunit were present on the cell surface when the cells were cultured in the presence of amiloride. Two distinct Endo H-resistant γ subunit cleavage products were observed in the presence of amiloride. Presumably, these cleavage fragments represent the C-terminal products of subunits that have been cleaved by either furin alone or by other channel-activating proteases at the polybasic tract that lies distal to the furin site (Fig. 1). Therefore, the α, β, and γ ENaC subunits all undergo increased N-glycan maturation when the [Na\(^+\)], is reduced by amiloride.

To ensure that the increase in ENaC maturation following amiloride treatment was specifically due to changes in [Na\(^+\)], the intracellular Na\(^+\) concentration was also manipulated by two additional methods. First, channel P\(_O\) was altered using the γ\(_{C\text{I}}\) and γ\(_{A\text{ID}}\) subunits, as shown in Fig. 1. γ\(_{C\text{I}}\) lacks the proximal furin and distal polybasic protease cleavage sites and retains the γ subunit inhibitory track. Channels with γ\(_{C\text{I}}\) have reduced activity compared with the wild type (15), presumably reflecting a reduced P\(_O\). γ\(_{A\text{ID}}\), has a deleted γ subunit inhibitory track and has a very high intrinsic P\(_O\) (15). Second, [Na\(^+\)], was manipulated by preincubating cells in 0 or 130 mM Na\(^+\) solution for 2 h. As shown in Fig. 4, D and E, channel maturation (as assessed by α subunit cleavage and Endo H resistance) was augmented when Na\(^+\) entry into cells was decreased by the lower P\(_O\) γ\(_{C\text{I}}\) subunit or when [Na\(^+\)], was reduced by 0 mM Na\(^+\) HEPES. Conversely, channel maturation was inhibited when [Na\(^+\)], was increased by the high P\(_O\) channel containing γ\(_{A\text{ID}}\) and was reduced when cells were preincubated in the 130 mM
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Channel Maturation Involves a Change That Facilitates Proteolytic Processing—The above results demonstrate that [Na\(^+\)]\(_i\) regulates the magnitude of the pool of ENaCs containing mature N-glycans and cleaved channels. Therefore, [Na\(^+\)]\(_i\) appears to regulate ENaC trafficking and/or processing as the channel passes through the Golgi complex. We hypothesized that ENaC undergoes a structural change that facilitates efficient proteolytic processing during channel maturation in the Golgi complex and trans-Golgi network and subsequent cellular compartments when [Na\(^+\)] is low. If this is the case, only the channels with mature N-glycans would be available for cleavage by exogenous trypsin. To test this hypothesis, we examined whether uncleaved surface pools of α ENaC are cleaved by exogenous trypsin under low or high [Na\(^+\)]\(_i\) conditions. The α\(_{\text{CI}}\) subunit was expressed along with HAI-1 to prevent the endoglycosidases from processing the subunit. Therefore, ENaC cleavage and activity are not dependent on the presence of mature N-glycans. Kifunensine is an inhibitor of α-mannosidase I that is required to remove mannose from immature N-glycans so that they can be replaced by complex glycans. Therefore, N-glycan maturation is blocked by kifunensine. Surface biotinylations and \(I_{\text{SC}}\) measurement were performed using \(\alpha_{\text{CI}}\beta\gamma ENaC\)-expressing FRT cells after overnight exposure to kifunensine. As shown in Fig. 6, exposure to kifunensine did not prevent \(\alpha\) ENaC proteolysis but effectively prevented N-glycan maturation. Moreover, kifunensine did not substantially alter baseline or trypsin-stimulated \(I_{\text{SC}}\). Therefore, ENaC cleavage and activity are not dependent on the presence of mature N-glycans.

\[\text{[Na}^+]\text{, Affects ENaC Maturation Early in the Biosynthetic Pathway—}\]

We have postulated previously that channels that bypass maturation in the Golgi complex could serve as a reserve pool of channels for activation in post-Golgi compartments (14, 16, 17). However, our results in FRT cells suggest that the immature surface channels are protease-resistant. Biotin chase experiments were performed to determine whether the immature full-length surface channels are cleaved by post-Golgi proteases and to determine the fate of the surface pool of channels after a reduction in [Na\(^+\)]. Cells expressing wild-type \(\alpha\beta\gamma ENaC\) were apically biotinylated and then cultured for 120 min under low or high [Na\(^+\)] conditions. As shown in Fig. 7, A

![Figure 5. Immature subunits are not substrates for proteolytic processing.](image)

![Figure 6. Subunit cleavage and activation by proteases is not dependent on N-glycan maturation.](image)
and B, \( [\text{Na}^+] \), was manipulated by culturing the cells in the presence or absence of amiloride (A) or in 0 or 130 mM Na\(^+\) solution (B). 120 min after surface biotinylation, there was a reduction in the amount of biotinylated \( \alpha \) ENaC subunits, presumably as a result of ongoing channel degradation. Similar amounts of channel degradation were observed in cells cultured in low or high extracellular Na\(^+\). To assure that increased amounts of cleaved channels were present on the cell surface when [Na\(^+\)]\(_i\) was reduced, matched cultures of transfected FRT cells were biotinylated at 0 or 120 min after the change in [Na\(^+\)]\(_i\) (serial biotinylation). These results suggest that the increased surface pool of cleaved channels after a reduction in [Na\(^+\)]\(_i\) primarily reflect trafficking of new channels to the apical surface rather than from processing pre-existing, full-length surface channels.

Next, a series of serial biotinylation studies was performed to identify the source of cleaved surface channels that arise in response to a reduction in [Na\(^+\)]\(_i\). As shown in Fig. 7C, [Na\(^+\)]\(_i\) was reduced by 2-h incubation in amiloride in the presence and absence of brefeldin A (BFA). BFA blocks trafficking through the Golgi complex by inhibiting ER-to-Golgi transport. In the absence of BFA, increased amounts of cleaved \( \alpha \) ENaC subunits accumulated on the cell surface when [Na\(^+\)]\(_i\) was reduced by amiloride. In support of the hypothesis that the Golgi complex is required for the trafficking of cleaved channels to the cell surface, BFA prevented the increase in cleaved \( \alpha \) subunits when [Na\(^+\)]\(_i\) was reduced. To determine whether new protein synthesis is required for the accumulation of cleaved channels on the cell surface in response to a reduction in [Na\(^+\)]\(_i\), similar serial biotinylations were performed in the presence and absence of the protein synthesis inhibitor cycloheximide. As shown in Fig. 7D, ENaC surface levels and total expression were decreased when the cultures were incubated in cycloheximide. However, cycloheximide only partially reduced the accumulation of cleaved \( \alpha \) subunits on the cell surface when [Na\(^+\)]\(_i\) was reduced by amiloride. Therefore, the increase in cleaved ENaCs in response to decreased [Na\(^+\)]\(_i\) appears to be dependent, in part, on trafficking of a pool of channels from early in the biosynthetic pathway to the cell surface.

**DISCUSSION**

The data presented in this work demonstrate that [Na\(^+\)]\(_i\) regulates the number of active ENaCs present on the cell surface. In agreement with previous reports, relatively high [Na\(^+\)]\(_i\), reduced the baseline amiloride-sensitive current and decreased the number of cleaved channels on the cell surface (4–11). The channels present on the cell surface when [Na\(^+\)]\(_i\) was increased were primarily uncleaved, contained immature, high-mannose-type N-glycans, and were resistant to cleavage by furin and other serine proteases, including exogenous trypsin. Conversely, a pool of active and processed channels accumulated on the cell surface when [Na\(^+\)]\(_i\) was reduced. The active channels contained mature N-glycans and were efficiently cleaved by furin and other serine proteases and by exogenous trypsin. The surface-resident immature channels were not converted to cleaved channels when [Na\(^+\)]\(_i\) was reduced, as shown by biotin-
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chase experiments. Therefore, [Na\(^+\)], regulates ENaC maturation within the biosynthetic pathway.

Furthermore, we demonstrate that ENaC maturation involves N-glycan maturation and a transformation that allows channel processing by proteases. Previous work has indicated that channel maturation during biosynthesis involves both N-glycan remodeling and cleavage by furin (12–14). Our data demonstrate that channels that bypass N-glycan maturation are not cleaved by furin, HAI-1-sensitive serine proteases, or exogenous trypsin. Therefore, we propose that channel maturation also involves a conformational change that exposes consensus protease cleavage sites. This conformational change is necessary for channel activation by proteases, and immature channels are in a conformation that prevents proteolysis of the ENaC subunits. On the basis of these findings, we propose that channels that bypass Golgi processing do not serve as substrates for proteolytic activation in post-Golgi compartments. An important question is whether these inactive channels contribute to physiological Na\(^+\) absorption. Sheng et al. (18) have demonstrated that channels with non-cleaved α subunits expressed in Xenopus oocytes exhibited markedly enhanced Na\(^+\) self-inhibition. These channels were active in the presence of a low extracellular [Na\(^+\)], but P\(_{\text{Na}}\) markedly decreased with a high extracellular [Na\(^+\)]. They have suggested that these immature channels contribute to Na\(^+\) transport in the distal nephron in states associated with enhanced renal Na\(^+\) absorption where urinary [Na\(^+\)] may fall to low levels.

What Is the Physiological Relevance of These Findings?—Recently, Patel et al. (11) have demonstrated that ENaC maturation and activity is reduced in rats in vivo following an oral sodium load despite plasma aldosterone clamping. Na\(^+\) loading was associated with a reduction in whole-cell Na\(^+\) current, a decrease in ENaC N-glycan maturation, and a decrease in ENaC cleavage in the rat cortical collecting duct. These in vivo findings are consistent with what we observed in FRT epithelia. FRT cells appear to be highly sensitive to manipulations that change [Na\(^+\)], relative to other epithelia. For example, Na\(^+\) currents were reduced in FRT cells transfected with the channel-activating protease prostatin (data not shown). Conversely, prostatin increases I\(_{\text{Na}}\) in oocytes as well as in airway-derived and renally derived epithelia (19–21). FRT cell sensitivity to Na\(^+\) may reflect the poor ability of these cells to handle the increased Na\(^+\) load caused by the expression of functional ENaCs. We propose that the ability of a given cell type or model system to handle an intracellular Na\(^+\) load has significant effects on ENaC maturation and activity. Therefore, although feedback inhibition regulates ENaC activity in the cortical collecting duct, it is possible that other ENaC-expressing tissues differ with regard to their capacity to handle intracellular Na\(^+\) loads. Further studies are required to compare the ability of various epithelia to regulate [Na\(^+\)], and ENaC processing.

How Does [Na\(^+\)] \textit{i} Influence ENaC Biogenesis?—Our studies suggest that [Na\(^+\)], alters ENaC maturation within the biosynthetic pathway. However, it remains unclear whether [Na\(^+\)], influences ENaC maturation by altering the activity of the enzymes involved in N-glycan maturation, inducing a conformational change in channel subunits that affects subunit processing, impairing the overall efficiency of the Golgi complex, altering the activity of a chaperone, or altering trafficking through the biosynthetic pathway. It is unlikely that the reduction in ENaC activity associated with relatively high [Na\(^+\)], is solely caused by altering the N-glycan maturation of the channel. This notion is supported by several findings. First, as shown in Fig. 6, kifunensine, an inhibitor of ER-localized α-mannosidase-I, did not inhibit channel activity or subunit proteolysis. These findings are in agreement with our prior work that demonstrated that glycan maturation and channel proteolysis is an all or none event but that the two processes are not interdependent (12–14). Second, Canessa et al. (22) have demonstrated previously that ENaC activity was not affected when all the potential N-glycan consensus sequence Asn residues within the α subunit were mutated to generate an α subunit that lacked N-glycans. Therefore, we speculate that a conformational change in the extracellular domain of ENaC subunits has occurred in response to [Na\(^+\)], and that this conformational change is a major determinant of the ability of the channel to be processed by proteases. Because the immature channels that reach the cell surface lack N-glycan processing, we speculate that these channels traffic to the surface via a route that bypasses the Golgi complex and trans-Golgi network, as we have suggested previously (14).

Although membrane proteins conventionally traffic to the cell surface via the canonical ER-Golgi system, there are numerous examples of unconventional protein trafficking. For example, the cystic fibrosis conductance regulator has been shown to traffic to the cell surface via a Golgi-independent route. During classic cystic fibrosis conductance regulator maturation, the N-glycan structures of the channel undergo extensive modification during transit through the ER-Golgi. Recently, an unconventional pathway has been described that allows the cystic fibrosis conductance regulator to bypass the Golgi and traffic to the cell surface with immature N-glycans (23–26). Interestingly, the cystic fibrosis conductance regulator with immature N-glycans that traffics to the cells surface via the unconventional pathway has a similar activity as channels that undergo conventional ER-Golgi maturation (25). Numerous other membrane proteins capable of bypassing the Golgi have been described (reviewed in Ref. 27). The role of unconventional protein trafficking in ENaC biogenesis remains to be determined.

Other factors could be affecting the accessibility of proteases and glycan-modifying enzymes in addition to conformational changes and trafficking itineraries. Further studies are needed to identify at which step in the biosynthetic pathway Na\(^+\) influences ENaC maturation, which chaperones mediate ENaC traffic though the Golgi complex, and how [Na\(^+\)], allows channels to bypass processing in the Golgi complex.

Several aspects of Na\(^+\) feedback inhibition can be explained by the unifying hypothesis that [Na\(^+\)], regulates ENaC folding, maturation, and trafficking within the biosynthetic pathway. Consistent with previous studies, our data indicate that the number of active channels present on the apical membrane decreased with increasing [Na\(^+\)]. When [Na\(^+\)], was low, ENaC underwent increased proteolytic cleavage and N-glycan maturation and was resistant to further activation by trypsin. The primary novel finding in this work is that all of these compo-
ments of feedback inhibition are attributable to a reduction in ENaC folding and maturation as a result of relatively high [Na\(^+\)]. The channels that escaped maturation within the biosynthetic pathway lacked complex N-glycans and were resistant to proteolytic processing. Therefore, changes occur when [Na\(^+\)] is low that facilitate proteolytic processing and N-glycan maturation.

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