Sodium Transport Is Modulated by p38 Kinase–Dependent Cross-Talk between ENaC and Na,K-ATPase in Collecting Duct Principal Cells

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ABSTRACT
In relation to dietary Na+ intake and aldosterone levels, collecting duct principal cells are exposed to large variations in Na+ transport. In these cells, Na+ crosses the apical membrane via epithelial Na+ channels (ENaC) and is extruded into the interstitium by Na,K-ATPase. The activity of ENaC and Na,K-ATPase must be highly coordinated to accommodate variations in Na+ transport and minimize fluctuations in intracellular Na+ concentration. We hypothesized that, independent of hormonal stimulus, cross-talk between ENaC and Na,K-ATPase coordinates Na+ transport across apical and basolateral membranes. By varying Na+ intake in aldosterone-clamped rats and overexpressing γ-ENaC or modulating apical Na+ availability in cultured mouse collecting duct cells, enhanced apical Na+ entry invariably led to increased basolateral Na,K-ATPase expression and activity. In cultured collecting duct cells, enhanced apical Na+ entry increased the basolateral cell surface expression of Na,K-ATPase by inhibiting p38 kinase-mediated endocytosis of Na,K-ATPase. Our results reveal a new role for p38 kinase in mediating cross-talk between apical Na+ entry via ENaC and its basolateral exit via Na,K-ATPase, which may allow principal cells to maintain intracellular Na+ concentrations within narrow limits.

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The fine-tuning of Na+ balance is critical for the homeostasis of body fluid compartments. A variety of disorders and diseases, such as hypertension and edema, result at least partly from disturbances of Na+ homeostasis.1 The final regulation of renal Na+ reabsorption takes place in aldosterone-responsive distal tubules and collecting ducts.2 The bulk of Na+ transport in the collecting duct (CD) occurs in principal cells, where Na+ enters the cell via the epithelial sodium channel (ENaC) and is extruded into the interstitial compartment via Na,K-ATPase.3 Thus, tight control of vectorial Na+ transport must be exerted on CD principal cells to achieve whole-body Na+ homeostasis.

According to dietary Na+ intake and aldosterone levels, CD principal cells are exposed to large physiologic variations of Na+ transport.2,3 Meanwhile, intracellular Na+ concentration must be maintained within narrow ranges, which is essential for vital cellular functions, such as control of osmolality, ionic strength, and membrane potential. Therefore, apical Na+ entry and basolateral Na+ extrusion must be rapidly and tightly coordinated in order to match variations of Na+ transport while minimizing fluctuations of intracellular Na+ concentration. The mechanisms mediating this coordination remain largely unknown.
Control of exocytosis/endocytosis is a common mechanism for modulating the abundance and function of membrane proteins. For example, increasing the activity of the AMP-activated protein kinase (AMPK), as a result of increased ATP consumption, modulated Na,K-ATPase endocytosis in cultured renal epithelial MDCK cells. Among several actions, activation of p38 kinase, a member of the MAP kinase family, regulates the endocytosis of a variety of cell surface proteins. We reported previously that aldosterone treatment which stimulates active transcellular Na⁺ reabsorption reduced p38 kinase activation, but not that of ERK1/2, in renal CD principal cells. Activation of p38 kinase is essential for EGFR endocytosis and lysosomal degradation. Interestingly, p38 kinase controls the phosphorylation and ubiquitinylation of aquaporin-2 (AQP2), triggering its endocytosis and degradation in renal CD principal cells.

We hypothesized that CD principal cells exhibit tight coordination of apical and basolateral Na⁺ transport, putatively through modulation of Na,K-ATPase cell surface expression by Na⁺ apical entry. AMPK and/or p38 kinase signaling pathways may control Na,K-ATPase endocytosis involved in cross-talk between ENaC and Na,K-ATPase. In this study, we describe a cross-talk between apical ENaC and basolateral Na,K-ATPase in a physiologic context. We identified p38 kinase-regulated endocytosis and degradation of cell surface Na,K-ATPase as a key player in this cross-talk.

**RESULTS**

**Enhanced Apical Na⁺ Delivery Increases Na,K-ATPase Activity and Expression in Isolated Rat Cortical Collecting Ducts**

To investigate whether ENaC-mediated Na⁺ entry is coordinated with Na,K-ATPase-dependent Na⁺ exit in *vivo*, we assessed Na,K-ATPase activity and protein expression in isolated cortical CDs (CCDs) from aldosterone-clamped rats fed with a low- or a normal-Na⁺ diet. All rats were infused with a high dose of aldosterone and fed with a low-Na⁺ diet for the first 3 days. They were then switched to a low- or a normal-Na⁺ diet for an additional 4 days in the continuous presence of high aldosterone levels (Figure 1A). The supraphysiologic plasma level of aldosterone forces the recruitment of active ENaC to the apical membrane of CD principal cells. Under this condition, apical ENaC expression is maintained at high levels, and hence variations in Na⁺ entry into CCD principal cells mostly result from changes in Na⁺ delivery. This experimental setting allows an *in vivo* investigation of coordination between apical ENaC and basolateral Na,K-ATPase that occurs independently of variations of aldosterone levels. Higher apical Na⁺ entry via ENaC in rats fed with the normal Na⁺ diet compared with rats fed the low-Na⁺ diet was associated with an increase in Na,K-ATPase activity (Figure 1B). The observed stimulation of Na,K-ATPase activity was associated with a proportional increase of the Na,K-ATPase α-subunit expression assessed by Western blotting in total lysates of isolated CCDs (Figure 1, C and D). Therefore, the stimulation of Na,K-ATPase activity most likely relies on an increased number of active Na,K-ATPase units at the plasma membrane. In rat CCDs, Na,K-ATPase activity measured as ouabain-sensitive currents was upregulated by exogenous aldosterone under a Na⁺-rich diet. This effect was eliminated by inhibition of ENaC-mediated Na⁺ entry with coinfused amiloride, suggesting cross-talk between ENaC and Na,K-ATPase. Here our results show that, *in vivo*, modulation of ENaC-mediated Na⁺ entry in a physiologic context induces parallel changes of Na⁺, K⁺-ATPase activity and expression independently of aldosterone level, confirming the occurrence of intrinsic cross-talk between ENaC and Na,K-ATPase.

**Enhanced Apical Na⁺ Entry Increases Total and Cell Surface Na,K-ATPase Expression in Cultured CD Cells**

For further investigation of coordination between ENaC and Na,K-ATPase, we manipulated apical Na⁺ entry in the absence of pharmacologic and hormonal treatment in cultured mCD, mpkCD, and mpkCD, cells, two well-differentiated mouse CCD principal cell models. These cells, grown on a polycarbonate filter, establish a robust transepithelial resistance together with an amiloride-sensitive vectorial transport of Na⁺. In addition, such a setting allows independent manipulation of the apical ion concentration of the medium. Recent experimental evidence indicated that γ-ENaC expression is a rate-limiting factor for amiloride-sensitive Na⁺ transport in CD cells. Therefore, we established an inducible γ-ENaC TetOn-mCCD cell line that conditionally over-expresses wild-type mouse γ-ENaC in response to doxycycline
Total and cell surface expression of both noncleaved and cleaved forms of γ-ENaC revealed by Western blotting (Figure 2A), as well as the benzamil-sensitive current (Figure 2C), increased in γ-ENaC-TetOn-mCCD cells cultured in the presence of Dox. As revealed by Western blotting, Dox-induced overexpression of γ-ENaC led to a parallel increase in total and cell surface expression of the Na,K-ATPase α-subunit, but not E-cadherin, (Figure 2, A and B), another abundant basolateral membrane protein. Accordingly, the ouabain-sensitive (Na,K-ATPase-mediated) transepithelial current was proportionally increased in response to Dox (Figure 2, B and C). Of note, Dox treatment had no effect on transepithelial Na⁺ current or Na,K-ATPase expression in the parental cells overexpressing the reverse tetracycline-controlled transactivator protein alone (Supplemental Figure S1). These results show that changes in vectorial Na⁺ transport that rely only on variations of apical ENaC expression and activity induce coordinated changes in basolateral Na,K-ATPase expression and activity. Importantly, this coordination does not require any external input, such as corticosteroid hormones.

Such a coordination between ENaC and Na,K-ATPase activity was further confirmed by altering apical Na⁺ availability in mpkCCD₃4 cells. Enhanced Na⁺ entry was confirmed by measuring the benzamil-sensitive Na⁺ current in mpkCCD₃4 cells incubated with 150 mM compared with 30 mM apical Na⁺ (supplemented with 120 mM choline-Cl to maintain isosmolality) (Figure 2D). Total and cell surface expression of Na,K-ATPase was higher in the presence of 150 mM apical Na⁺ than in the presence of 30 mM apical Na⁺ (Figure 2, E and F). This set of experiments further demonstrates a hormone-independent coordination between apical ENaC-mediated Na⁺ entry and basolateral Na,K-ATPase-mediated Na⁺ extrusion in CD principal cells.

Modulation of Constitutive Lysosomal Degradation of Na,K-ATPase Is Involved in the Response to Variations of Apical Na⁺ Entry

By real-time PCR, we found that enhanced Na⁺ entry in response to Dox did not alter Na,K-ATPase α1 mRNA level in γ-ENaC-TetOn-mCCD cells (Supplemental Figure S2), suggesting post-transcriptional control of Na,K-ATPase abundance. The amount of a given protein is determined by the balance between the rate of its synthesis and the rate of degradation. Inhibition of lysosomal degradation by chloroquine increased Na,K-ATPase abundance in total cell lysate, an effect that was not additive to that of Dox-induced increase of apical Na⁺ entry (Figure 3, A and B). These results strongly suggest that modulation of lysosomal degradation is the major factor underlying the observed increase of Na,K-ATPase abundance in response to variations of ENaC-mediated Na⁺ entry.

This conclusion was further tested by morphologic analyses. By immuno-electron microscopy, we found that Na,K-ATPase labeling along the plasma membrane was, as expected, strictly restricted to the basolateral area of rat CCD principal cells. Na,K-ATPase was also detected in a restricted intracellular compartment that may represent multivesicular bodies (Figure 3C). In this intracellular compartment, Na,K-ATPase labeling was predominantly associated with internal vesicles rather than with the surrounding membrane (Figure 3, C and insert), suggesting that this compartment belongs to the degradation pathway of Na,K-ATPase internalized from the plasma membrane. In cultured mCCD cells, fluorescence imaging revealed colocalization of Na,K-ATPase
Figure 3. Lysosomal degradation of Na,K-ATPase is involved in cross-talk between ENaC and Na,K-ATPase. (A) Representative immunoblot showing the effect of chloroquine (Chloro, 10^{-5} M, 24 hours) on the Na,K-ATPase α-subunit expression in γ-ENaC-TetOn-mCCD cells pretreated or not pretreated with Dox for 24 hours. GAPDH was used as a loading control. (B) Densitometric quantification of immunoblots shown in A. (C) Rat kidney cortices were processed and the Na,K-ATPase labeling was enriched in a distinct population of intracellular organelles in addition to its distribution along the basolateral membrane in rat CCD principal cells. The CCD lumen is denoted as “L.” Two different magnifications are shown. The inset shows a high magnification of a labeled intracellular organelle from a neighboring principal cell. Black arrows show plasma membrane and white arrows show intracellular membranes. (D) Colocalization of Na,K-ATPase and LysoTracker Red in γ-ENaC-TetOn-mCCD cells grown on transparent filters in the presence or absence of chloroquine. Results are means ± SEM of five independent experiments; **P<0.01.

AMPK Does Not Mediate Na,K-ATPase Endocytosis in CD Cells

Increasing active transcellular Na⁺ reabsorption by renal epithelial cells consumes more ATP via its hydrolysis by Na,K-ATPase and thus results in an elevated cytosolic AMP:ATP ratio that may activate AMPK and modulates Na,K-ATPase recycling, as previously suggested in MDCK cells.⁴ We therefore assessed the possibility that increased activity of Na,K-ATPase activates a feedback loop that inhibits its internalization from the plasma membrane and subsequent lysosomal degradation. Enhanced Na⁺ entry in γ-ENaC-TetOn-mCCD cells increased phosphorylation levels of AMPK α-subunit and of acetyl-CoA carboxylase (ACC), its downstream target,¹⁸ indicating AMPK activation (Figure 5, A and B). However, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an activator of AMPK that increased phosphorylation of AMPK α-subunit and ACC, did not alter Na,K-ATPase expression level (Figure 5C). Furthermore, AICAR did not alter the rate of disappearance of biotinylated cell-surface Na,K-ATPase assessed under basal conditions or in response to increased Na⁺ entry via γ-ENaC overexpression (Figure 5D). Therefore, AMPK does not play a significant role in mediating regulated endocytosis and degradation of Na,K-ATPase that underlies coordinated ENaC-mediated Na⁺ entry and Na,K-ATPase-mediated Na⁺ extrusion.

p38 Kinase Inhibition Mediates Decreased Na,K-ATPase Endocytosis in Response to Enhanced Apical Na⁺ Entry

Activation of p38 kinase is required for endocytosis and lysosomal degradation of a variety of cell surface proteins, such as E-cadherin, in MDCK cells. However, p38 kinase inhibition did not alter the rate of disappearance of biotinylated cell-surface Na,K-ATPase assessed under basal conditions or in response to increased Na⁺ entry via γ-ENaC overexpression (Figure 5E). Therefore, p38 kinase does not play a significant role in mediating regulated endocytosis and degradation of Na,K-ATPase that underlies coordinated ENaC-mediated Na⁺ entry and Na,K-ATPase-mediated Na⁺ extrusion.
as EGFR5,7–9 and aquaporin-2.10 We assessed whether p38 kinase modulates endocytosis and degradation of Na,K-ATPase in response to enhanced apical Na+ entry in γ-ENaC-TetOn-mCCD cells. We found that enhanced Na+ entry decreased phosphorylation levels of p38 kinase and of ATF2, its downstream target (Figure 6, A and B). In contrast, the phosphorylation level of ERK1/2, which also belong to the MAP kinase family, was unchanged (Supplemental Figure S3A), indicating the specificity of the inhibition of p38 kinase in response to increased transcellular Na+ transport. By performing the pulse-chase biotinylation assay as described above (Figure 4A), we found that anisomycin, an activator of p38 kinase (Supplemental Figure S3B),20 prevented the inhibition of endocytosis and degradation of cell surface Na+,K-ATPase by enhanced Na+ entry (Figure 6, C and D). Conversely, PD169316 and SB203580, two specific inhibitors of p38 kinase,21 inhibited endocytosis and degradation of cell surface Na+,K-ATPase in a nonadditive manner to γ-ENaC phosphorylation (Figure 6, E and F). This recruitment of Na+ reabsorption by stimulating amiloride-sensitive Na+ entry via apical ENaC as well as basolateral Na+ extrusion via Na,K-ATPase in a coordinated manner.23 Our results show that increased apical Na+ entry triggers signaling events that lead to increased expression and activity of Na,K-ATPase, implying that aldosterone may, at least in part, control Na,K-ATPase expression via stimulation of ENaC. This mechanism may also at least partially explain coordinated regulation of ENaC and Na,K-ATPase in response to other hormones, such as vasopressin,24 or in response to increased Na+ delivery, such as observed under diuretic treatment.25 However, we cannot rule out the possibility that aldosterone may also separately regulate ENaC and Na,K-ATPase, and this dual regulation by aldosterone may coexist with sequential cross-talk between ENaC and Na,K-ATPase, each contributing to the coordination of Na+ transport in a context-dependent manner.

Increased activity and cell surface expression of Na,K-ATPase in response to increased intracellular Na+ concentration following treatment with ionophores or extracellular K+ removal have been shown both in isolated rat CD26,27 and in cultured mouse CD principal cells.28 This recruitment of Na+ reabsorption by stimulating amiloride-sensitive Na+ entry via apical ENaC as well as basolateral Na+ extrusion via Na,K-ATPase in a coordinated manner.23 Our results show that increased apical Na+ entry triggers signaling events that lead to increased expression and activity of Na,K-ATPase, implying that aldosterone may, at least in part, control Na,K-ATPase expression via stimulation of ENaC. This mechanism may also at least partially explain coordinated regulation of ENaC and Na,K-ATPase in response to other hormones, such as vasopressin,24 or in response to increased Na+ delivery, such as observed under diuretic treatment.25 However, we cannot rule out the possibility that aldosterone may also separately regulate ENaC and Na,K-ATPase, and this dual regulation by aldosterone may coexist with sequential cross-talk between ENaC and Na,K-ATPase, each contributing to the coordination of Na+ transport in a context-dependent manner.

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Na,K-ATPase units has been linked to an increase of cell volume associated with increased intracellular Na⁺ concentration.29,30 The role of variations of intracellular Na⁺ concentration and cell volume in cross-talk between ENaC and Na,K-ATPase remains to be investigated.

We clearly showed that Na,K-ATPase undergoes constitutive endocytosis and lysosomal degradation in CD cells. Enhanced apical Na⁺ entry increases Na,K-ATPase activity and cell surface expression by inhibiting this process, demonstrating that the degradation rate of Na,K-ATPase is subjected to physiologic modulation. E-cadherin endocytosis and degradation were not altered in response to enhanced Na⁺ entry, showing that this modulation is specific for Na,K-ATPase and that basolateral membrane area most likely remains unchanged. Our results indicate that regulation of Na,K-ATPase cell surface expression occurs via the control of a constitutively high rate of internalization and degradation. This represents a very efficient mode of regulation allowing rapid adaptations of large amplitude of the basolateral Na⁺ extrusion capacity compared with modulation of de novo protein synthesis alone. Such a mechanism allows efficient adjustment of Na⁺ transport according to body demands without causing significant fluctuations of intracellular Na⁺ concentration.

We found that inhibition of p38 kinase activation mediates regulated endocytosis of Na,K-ATPase that underlies cross-talk between ENaC and Na,K-ATPase. Phosphorylation at Ser,19 putatively by protein kinase C, induces Na,K-ATPase endocytosis and degradation.31–33 Phosphorylation and ubiquitination, among other post-translational modifications, may also play a role in p38 kinase-mediated endocytosis and degradation of Na,K-ATPase that underlies cross-talk between ENaC and Na,K-ATPase in renal CD cells. Nevertheless, deciphering specific mechanisms involved in p38 kinase regulation of Na,K-ATPase endocytosis requires further investigation.

One important aspect of Na⁺ homeostasis is to maintain the blood volume and BP within a narrow range, which entails coordination between Na⁺ reabsorption and water reabsorption. Vasopressin, upon binding to vasopressin V2 receptors, inhibits p38 kinase activity by stimulating the cAMP/PKA pathway.10 This inhibition of p38 kinase by vasopressin was shown to increase cell surface expression of AQP2 by reducing its endocytosis and degradation. Therefore, p38 kinase may act as a molecular hub in controlling extracellular volume and thereby blood pressure by coordinating Na⁺ and water reabsorption by the CD in response to both hormonal and nonhormonal stimuli.

In conclusion, this study identified cross-talk between ENaC and Na,K-ATPase that may play a role in the correlation between distal Na⁺ delivery and reabsorption independently of hormonal influence. This nonhormonal control mechanism of Na⁺ reabsorption is potentially important for the control of extracellular volume and therefore BP.

**CONCISE METHODS**

**Chemicals**

Benzamil, ouabain, chloroquine, and anisomycin were from Sigma-Aldrich (St. Louis, MO). AICAR was from Cell Signaling Technology (Danvers, MA). PD169316 and SB203580 were from Enzo Life Sciences (Farmingdale, NY).

**Animal Experimental Protocol and Isolation of Kidney Tubules**

After 1 week of acclimation, 24 male Sprague-Dawley rats (150–200 g body weight; Charles-River, Saint Germain de l’Arbresle, France) were implanted with osmotic minipumps (model 2001; Alzet) containing aldosterone (200 μg/d) in 0.9% saline and 30% DMSO (vol/vol). The rats were kept in metabolic cages for the next 7 days. All rats were fed with low-Na⁺ diet for 3 days and then pair-fed with 7.5 g synthetic rat chow/100 g body weight per day supplemented with low- Na⁺ (0.005 mmol

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**Figure 5.** AMPK is not involved in Na,K-ATPase endocytosis that mediates cross-talk between ENaC and Na,K-ATPase. (A) Representative immunoblots showing the effect of enhanced apical Na⁺ entry on total and phosphorylated AMPK and its downstream target, ACC. GAPDH was used as a loading control. (B) Densitometric quantification of phosphorylated AMPK from immunoblots shown in A. Results are means ± SEM of six independent experiments; *P<0.05. (C and D) Confluent γ-ENaC-TetOn-mCCD cells grown on filters were pretreated or not pretreated with Dox for 48 hours before biotinylation of cell surface proteins. Biotinylated cell surface proteins were then chased for 12 hours at 37°C in the presence or absence of AICAR (1 mM), an activator of AMPK. E-cadherin was used as a loading control.
Na⁺/100 g body weight per day) or normal- Na⁺ (1.0 mmol Na⁺/100 g body weight per day) diet for the following 4 days. CCDs were isolated by microdissection from collagenase-treated kidneys as described previously. The protocol was approved by the local ethics committee. The equivalent short-circuit current across polarized cell monolayers was measured at the end of 48-hour Dox treatment and calculated according to Ohm’s law from the values of transepithelial potential and resistance measured with a Millicell device (Millipore), as previously described. Amiloride-sensitive and ouabain-sensitive short-circuit current were determined as the difference between values measured before and after incubation for 30 minutes with 10⁻⁵ M benzamil added to the apical medium or 5×10⁻⁵ M ouabain added to the basal medium, respectively.

**Immunoblotting**

Equal amounts of protein from lysed cultured cells or pools of 50 microdissected rat CCDs were separated by 4%-12% SDS-PAGE (Invitrogen, Basel, Switzerland), and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The equivalent short-circuit current across polarized cell monolayers was measured at the end of 48-hour Dox treatment and calculated according to Ohm’s law from the values of transepithelial potential and resistance measured with a Millicell device (Millipore), as previously described. Amiloride-sensitive and ouabain-sensitive short-circuit current were determined as the difference between values measured before and after incubation for 30 minutes with 10⁻⁵ M benzamil added to the apical medium or 5×10⁻⁵ M ouabain added to the basal medium, respectively.

**Cell Culture**

mCCD₁Δt and mpkCCD₁Δt cells are two immortalized mouse CCD cell lines expressing endogenous ENaC and exhibiting amiloride-sensitive Na⁺ currents. We used mCCD₁Δt cells to generate γ-ENaC-TetOn-mCCD cells that conditionally overexpress wild-type mouse γ-ENaC in response to Dox. mCCD₁Δt cells were first stably transfected with a plasmid encoding the tetracycline transactivator under the control of the human cytomegalovirus promoter (a gift of H Bujard, University of Zürich, Switzerland); anti–glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody, diluted 1:10⁵ (Millipore); anti–E-cadherin antibody diluted 1:10³ (BD Biosciences); and anti–phospho p38 kinase (Thr180/Tyr182) and anti–AMPK antibodies diluted 1:10³ (BD Transduction Laboratories); phospho-ATF-2 (Thr71) antibody diluted 1:10⁵ (Cell Signaling Technology); phospho-AMPK (Thr172) and anti-AMPK antibodies diluted 1:10⁵ (Themo Scientific). The signal was quantified by densitometry and normalized with respect to GAPDH taken as a loading control.

**Biotinylation and Pulse-Chase of Cell Surface Proteins**

After labeling using 1.0 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Themo Scientific), cell surface proteins were precipitated by
Data were analyzed according to the 2-ΔΔCt method.

Statistical Analyses
Results are given as the mean ± SEM from at least three independent experiments. Comparisons between two groups were performed by Mann-Whitney U test and comparisons between more than two groups were performed by Kruskal-Wallis test. A P value < 0.05 was considered to represent a statistically significant difference. GraphPad Prism v5 was used.

Immunoelectron Microscopy
The subcellular distribution of Na,K-ATPase in CCD cells was analyzed by immunoelectron microscopy in rats. Kidneys were perfusion-fixed in situ with 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH, 7.2). The tissue was postfixed for 2 hours, infiltrated with 2.5% sucrose, and frozen in liquid nitrogen. Ultrathin cryosection sections were cut on a Reichert Ultracut S cryoultramicrotome (Leica), blocked in PBS containing 0.05 M glycine and 0.1% skim milk, and incubated overnight at 4°C with polyclonal rabbit anti-Na,K-ATPase antibodies diluted 1:3,000. Immunolabeling controls were conducted with nonimmune rabbit IgG or without primary antibody. The Na,K-ATPase labeling was visualized using a polyclonal goat antirabbit antibody conjugated to 2 nm colloidal gold particles (GAR EM2; BioCell Research Laboratories, Cardiff, United Kingdom). The gold particles were silver enhanced (Aurion R-Gent SE-EM, Wageningen, The Netherlands), and the cryosections were stained with 0.3% uranyl acetate in 1.8% methylcellulose and examined with a FEI Morgagni electron microscope.

RNA extraction, reverse transcription, and Real-Time PCR analysis were performed as previously described. Primers used for RNA detection of the Na,K-ATPase α1-subunit were 5′-TCCGTTCAACTCCACCAACAA-3′ and 5′-TTTGCTGATGCGATTGG-3′, and those for GAPDH, used as an internal standard, were 5′-GTGTGGGTAGTGGCAGTG-3′ and 5′-GTTCCTGGTTCACCACCT-3′. Data were analyzed according to the 2-ΔΔCt method.

Immunocytochemistry
Colocalization of Na,K-ATPase and LysoTracker Red (Invitrogen) was performed by immunocytochemistry as previously described. γ-ENaC-TetOn-mCCD cells grown on Transwell-Clear (Corning Costar) were incubated with LysoTracker diluted 1:1000 for 30 minutes at 37°C. Then the cells were rinsed three times with PBS and fixed in 2% formaldehyde/150 mM NaCl/20 mM Hepes (pH, 7.8) for 30 minutes at room temperature. After quenching with 50 mM Tris in staining buffer (0.1% Triton X-100/100 mM NaCl/20 mM Hepes [pH, 7.8]) for 1 hour and rinsing with staining buffer, the cells were then incubated overnight at 4°C with a polyclonal rabbit anti-Na,K-ATPase antibody diluted 1:10 in staining buffer. After rinsing in staining buffer, the cells were incubated for 1 hour at room temperature with Alexa Fluor 488-conjugated goat antirabbit secondary antibody (Invitrogen) diluted 1:10 in staining buffer. Filters were excised from the supports, mounted on microscope slides using ProLong Gold antifade reagent (Invitrogen). Fluorescence was detected using an LSM 510 Meta confocal laser-scanning microscope (Carl Zeiss, Feldbach, Switzerland).
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DISCLOSURES

None.

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