ENaC inhibition stimulates HCl secretion in the mouse cortical collecting duct. II. Bafilomycin-sensitive H⁺ secretion

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Submitted 18 March 2015; accepted in final form 26 May 2015

In cortical collecting ducts (CCDs) from aldosterone-treated mice and rats, robust NaCl absorption is observed. Following epithelial Na⁺ channel (ENaC) blockade with amiloride analogs, NaCl absorption falls >50% (20, 27), due to inhibition of ENaC-mediated Na⁺ absorption. However, since ENaC does not transport Cl⁻, how amiloride reduces Cl⁻ absorption is unclear. In the companion paper (17a), we observed that Cl⁻ secretion increases either with ENaC gene ablation or with chemical inhibitors of ENaC. This ENaC-sensitive Cl⁻ flux is eliminated with stilbene analogs, such as DIDS, which are nonselective Cl⁻ transporter blockers. Benzamil-stimulated Cl⁻ secretion is also reduced with inhibitors of the Na⁺-K⁺-2Cl⁻ cotransporter, NKCC1. Since NKCC1 localizes to the basolateral membrane of type A intercalated cells, ENaC blockade may stimulate Cl⁻ secretion by type A intercalated cells.

In addition to reducing renal Cl⁻ absorption, some studies indicate that ENaC blockade in the CCD reduces net H⁺ secretion (16). Since type A intercalated cells are the primary mediator of electrogenic H⁺ secretion in the CCD, amiloride likely inhibits the apical H⁺-ATPase of the type A intercalated cell. In the turtle urinary bladder and in the rabbit CCD, amiloride application lowers H⁺ secretion (10, 15, 16) by reducing lumen-negative transepithelial voltage, which attenuates the electromotive force for H⁺ secretion (24, 37). In rats, dogs, and in humans, amiloride administration in vivo increases urinary pH and reduces serum HCO₃⁻ concentration, (1, 3, 23), consistent with apical H⁺-ATPase inhibition. As such, administration of amiloride in vivo generates a distal renal tubular acidosis that is thought to be voltage dependent (1). This amiloride-induced fall in net H⁺ secretion may modulate Cl⁻ flux.

To further characterize amiloride-sensitive Cl⁻ secretion, the purpose of this study was twofold: 1) to determine the co- or countertransported ion that accompanies benzamil- and DIDS-sensitive Cl⁻ transport, and 2) to determine whether inhibiting this co- or countertransport mechanism attenuates DIDS-sensitive changes in Cl⁻ transport.

METHODS

Animals. Unless otherwise indicated, experiments were performed in male and female wild-type mice on a 129S6/SvEvTac background (Taconic Farms). In some experiments, male or female Slc26a4 (−/−) mice on a 129S6/SvEvTac background, developed by Everett et al. (4), were compared with wild-type mice from the same strain (129S6/SvEvTac), bred in parallel. Unless otherwise stated, mice were fed a balanced diet (53881300; Zeigler Brothers) prepared as a gel (0.6% agar, 74.6% water, and 24.8% mouse chow) supplemented with NaCl (∼0.8 meq NaCl/day, treatment 1) and were given aldosterone by minipump (250 μg·kg body wt⁻¹·day⁻¹, treatment 2) for 5–7 days before euthanasia (11). The Institutional Animal Care and Use Committee at Emory University approved all treatment protocols.

In vitro perfusion of isolated CCD. For transepithelial flux studies, CCDs were dissected from medullary rays and perfused at flow rates of 2–3 nl/min in the presence of a symmetric, HCO₃⁻-buffered physiological solution (solution 1, Table 1) or in HEPES-buffered solutions (solution 2, Table 1). Tubules were equilibrated at 37°C for 30 min before the collections began. A stock solution of benzamil...
amounts of the solution components, such as NaCl, were calculated according to the equation:

\[
J_{\text{Cl}} = \frac{(C_0 - C_L)Q}{L}
\]

where \(C_0\) and \(C_L\) are perfusate and collected fluid Cl concentrations, respectively, \(Q\) is the flow rate (in nl/min), and \(L\) is tubule length. Net fluid transport was taken to be zero since net fluid flux has not been observed in CCDs when perfused in vitro in the presence of symmetric solutions and in the absence of vasoressin (12, 13). \(J_{\text{Cl}}\) was expressed as picomoles per millimeter per minute.

Measurement of transepithelial \(\text{HCO}_3^-\) flux. Transepithelial \(\text{HCO}_3^-\) flux, \(J_{\text{HCO}_3^-}\), was calculated according to the equation:

\[
J_{\text{HCO}_3^-} = \frac{(C_0 - C_L)Q}{L}
\]

where \(C_0\) and \(C_L\) are perfusate and collected fluid \(\text{HCO}_3^-\) concentrations, respectively, \(Q\) is the flow rate (in nl/min), and \(L\) is the tubule length.

Measurement of intracellular \(\text{pH}\). Intracellular \(\text{pH}\) was measured in intercalated cells from CCDs perfused in vitro using the esterified \(\text{HCO}_3^-\)-sensitive fluorophore 6 methoxy-(3-sulfopropyl) quinolinium (SPQ; Molecular Probes, Eugene, OR), as described previously (29, 32, 33). Transepithelial \(\text{HCO}_3^-\) flux, \(J_{\text{HCO}_3^-}\), was measured in the perfusate and collected samples using a continuous-flow fluorimeter and the \(\text{Cl}^-\)-sensitive fluorophore 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ; Molecular Probes, Eugene, OR) with a xenon light source connected to an Olympus inverted microscope and a Hamamatsu ORCA ER digital camera. Imaging Workbench 6 (INDEC BioSystems, Santa Clara, CA) software was used for the unit control and image analysis. In each CCD, four to nine intercalated cells were identified by their characteristic shape and preferential loading with BCECF, and circumscribed as regions of interest (36).

Every 15 s, emission intensity was measured continuously at an excitation wavelength of 495 for 20 ms and then at 440 nm for 50 ms under basal conditions and then following the addition and then the withdrawal of benzamil to the perfusate. The perfusate contained \(\text{Cl}^-\), with or without Na\(^+\) (Table 1, solutions 3 and 4). Tubules were bathed in a NaCl-free solution (Table 1, solution 5). A three-point standard curve was constructed for each tubule at the end of each experiment by perfusing the lumen and peritubular bath with a high-K\(^+\)-containing solution, pH 6.7–7.6 (Table 1, solution 6). The bath calibration solution also contained the K\(^+\)/H\(^+\) ionophore nigericin (14.7 \(\mu\)mol/l).

Statistics. All data are presented as means ± SE. Each “n” used in the statistical analysis represents data from separate mice. To test for statistical significance between two groups, a paired or an unpaired Student’s \(t\)-test was used. When results from more than two groups were compared, an ANOVA was used followed by Tukey’s protected \(t\)-test. The criterion for statistical significance was \(P < 0.05\).

RESULTS

DIDS inhibits \(H^+\) secretion mediated by the \(H^+/\text{ATPase}\), which increases the lumen-negative transepithelial voltage. In the companion paper (17a), we observed that ENaC inhibition stimulates conductive \(\text{Cl}^-\) secretion, which is reversed with the application of stilbene inhibitors (DIDS). Further experiments examined the effect of DIDS on transepithelial voltage during ENaC inhibition. We reasoned that stimulating conductive anion secretion should increase the lumen-negative transepithelial voltage, whereas inhibiting conductive anion secretion should reduce the lumen-negative transepithelial voltage. As such, we predicted that when conductive \(\text{Cl}^-\) secretion is inhibited with the nonselective \(\text{Cl}^-\) transport blocker DIDS, the lumen-negative transepithelial voltage will fall, i.e., becomes less lumen negative. To test this prediction, we examined the effect of the DIDS on transepithelial voltage in CCDs from aldosterone-treated mice. Figure 1 shows that in the absence of ENaC blockade, the lumen-negative transepithelial voltage is either unchanged or slightly reduced with DIDS application (Fig. 1, A and B), which is consistent with inhibition of a conductive \(\text{Cl}^-\) secretory pathway. However, when ENaC activity is eliminated either with a chemical inhibitor (benzamil, Fig. 1C) or with ENaC gene ablation (Fig. 1D), DIDS application increased the lumen-negative transepithelial voltage. This DIDS-induced increase in the lumen-negative voltage observed during ENaC blockade occurred in mice given diet alone or diet and aldosterone (Fig. 1).

Further experiments explored the mechanism behind the unexpected rise in lumen-negative transepithelial voltage observed with stilbene inhibitor application. We hypothesized that during ENaC blockade, DIDS increases the lumen-negative transepithelial voltage by inhibiting secretion of a cation, such as \(H^+\), rather than by stimulating secretion of an anion. To test this hypothesis, we examined the effect of DIDS on total CO\(_2\) (\(\text{HCO}_3^- + \text{CO}_2 + \text{H}_2\text{CO}_3\)) flux. Total CO\(_2\) flux generally reflects \(\text{HCO}_3^-\) flux, \(J_{\text{HCO}_3^-}\), since total CO\(_2\) is primarily \(\text{HCO}_3^-\) under most physiological conditions (34). Figure 2 shows that during ENaC blockade, DIDS application changed total CO\(_2\) flux from net absorption to net total CO\(_2\) secretion. The fall in total CO\(_2\) absorption observed with DIDS application could be from reduced \(H^+\) secretion or increased secretion of OH\(^-\) equivalents, such as \(\text{HCO}_3^-\). To discriminate between these possibilities, we tested whether the DIDS-induced

### Table 1. Solution composition (in mM)

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<tr>
<th>Solution</th>
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<th>3</th>
<th>4</th>
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NMDG, N-methyl-D-glucamine.
change in total CO₂ flux is eliminated with H⁺-ATPase inhibitor (bafilomycin) application. We observed that during ENaC blockade, application of DIDS to the perfusate changed total CO₂ flux from net absorption to net secretion. However, during blockade of both ENaC and the apical H⁺-ATPase, DIDS application did not change total CO₂ flux [vehicle, 2.2 ± 4.3 vs. 3.6 ± 3.3 pmol-mm⁻¹-min⁻¹ with DIDS, n = 4, P = not significant (NS)]. We conclude that during ENaC blockade, DIDS application inhibits H⁺ secretion mediated by the apical H⁺-ATPase.

DIDS reduced total CO₂ absorption during ENaC blockade, most likely by inhibiting H⁺ secretion. Since a fall in electrogenic H⁺ secretion should increase the lumen-negative transepithelial voltage (26), we asked whether DIDS increases the lumen-negative transepithelial voltage by inhibiting apical H⁺-ATPase-mediated H⁺ secretion. If so, H⁺-ATPase inhibitors should blunt the increment in the lumen-negative transepithelial voltage observed with DIDS application. To test this hypothesis, we examined the effect of DIDS on transepithelial voltage in the presence or absence of an H⁺-ATPase inhibitor (bafilomycin 5 nM, Fig. 3A). As shown, during ENaC blockade, inhibiting the H⁺-ATPase blunted the DIDS-induced change in lumen-negative transepithelial voltage (DIDS-sensitive transepithelial voltage).

During ENaC blockade, DIDS inhibits HCl secretion. Since DIDS reduces secretion of both H⁺ and Cl⁻ during ENaC blockade, we asked whether DIDS-sensitive changes in H⁺ and Cl⁻ flux are coupled. Therefore, we examined the effect of DIDS on Cl⁻ flux during apical H⁺-ATPase blockade. Whereas DIDS reduced net HCl secretion during ENaC blockade alone, Fig. 3B shows that with blockade of both ENaC and the apical H⁺-ATPase, the increment in Cl⁻ absorption observed with DIDS application (DIDS-sensitive Cl⁻ absorption) is blunted. We conclude that during ENaC blockade, DIDS-sensitive changes in H⁺ and Cl⁻ flux depend on the apical H⁺-ATPase.

**Fig. 1.** During epithelial sodium channel (ENaC) blockade, DIDS increases the lumen-negative transepithelial voltage, Vₜ. The effect of DIDS on Vₜ was measured in cortical collecting ducts (CCDs) from aldosterone-treated mice in the presence or absence of functional ENaC. The effect of DIDS on Vₜ was examined either with functional ENaC (A and B) or when ENaC function was eliminated with chemical inhibitors of ENaC (benzamil, C) or with ENaC gene ablation (D). In other experiments, we observed that during ENaC blockade, DIDS increased the lumen-negative Vₜ by 2.5 ± 0.5 mV (n = 4) in CCDs from mice receiving diet alone and by 7.6 ± 1.3 mV (n = 7) in mice receiving diet and aldosterone (P < 0.05).
ENaC blockade stimulates net H⁺ secretion. Since DIDS application reduced net H⁺ secretion during ENaC blockade, we explored the effect of ENaC blockade alone on net H⁺ secretion. Figure 4 shows that in the absence of inhibitors, CCDs from aldosterone-treated mice secrete total CO₂, as reported previously (22). However, total CO₂ flux changed from net secretion to net absorption following benzamil application. Therefore, benzamil either stimulates H⁺ secretion or inhibits OH⁻ secretion, such as through blockade of apical anion exchange.

To test whether ENaC blockade increases H⁺ secretion, we examined the effect of benzamil on total CO₂ flux, during apical H⁺-ATPase blockade (bafilomycin, Fig. 4B). As shown, blockade of the apical H⁺-ATPase eliminated the benzamil-sensitive change in total CO₂ flux. To test further whether benzamil stimulates H⁺ secretion or instead inhibits apical anion exchange, intracellular pH was measured in intercalated cells before and after the application of this diuretic to the perfusate (Fig. 5 and Table 2). With Na⁺ in the luminal fluid, intercalated cell intracellular pH rose with benzamil application, consistent with stimulation of net H⁺ efflux or inhibition of OH⁻ efflux (Fig. 5A). In contrast, benzamil had no effect on intracellular pH in the absence of luminal Na⁺ (Fig. 5B), suggesting that benzamil modulates H⁺ secretion through ENaC-mediated Na⁺ absorption.

Since benzamil may raise intracellular pH by inhibiting apical Cl⁻/HCO₃⁻ exchange (17, 35) and since most apical anion exchange is pendrin mediated (2, 33), we examined the effect of benzamil on intercalated cell intracellular pH in the absence of pendrin-mediated apical Cl⁻/HCO₃⁻ exchange (pendrin null mice). As shown, benzamil produced a similar increment in intracellular pH in wild-type and pendrin null mice (Fig. 5C and Table 2). We conclude that benzamil increases intracellular pH either by stimulating the apical H⁺-ATPase or by stimulating a H⁺ or HCO₃⁻ exchanger other than pendrin. However, Figs. 4 and 5 together indicate that benzamil most probably increases intercalated cell intracellular pH and net H⁺ secretion by stimulating the apical H⁺-ATPase.

The apical H⁺-ATPase modulates ENaC-stimulated Cl⁻ secretion. Since ENaC blockade stimulates secretion of H⁺ and Cl⁻ through associated pathways, we reasoned that inhibiting the H⁺-ATPase should reduce Cl⁻ secretion or increase Cl⁻ absorption. To test this hypothesis, we examined the effect of an H⁺-ATPase inhibitor (bafilomycin, 5 nM) on Cl⁻ absorption and transepithelial voltage. While application of H⁺-ATPase inhibitors to the perfusate did not change Cl⁻ flux in the absence of ENaC blockade (19), Fig. 6A shows that during ENaC blockade, Cl⁻ absorption increased with the application of bafilomycin to the perfusate, as expected when a pathway mediating Cl⁻ secretion is inhibited.

Since DIDS application increased the lumen-negative transepithelial voltage during ENaC blockade, we reasoned that DIDS increases the lumen-negative voltage, not by changing anion flux, but by reducing the secretion of a cation, i.e., H⁺. Further experiments therefore tested the effect of inhibiting H⁺ secretion on transepithelial voltage. Figure 6B shows that during ENaC blockade, apical H⁺-ATPase inhibition (bafilomycin) increased the lumen-negative voltage, as expected when conductive cation secretion falls. We conclude that during ENaC blockade, inhibiting electrogenic H⁺ secretion increases the lumen-negative transepithelial voltage and reduces conductive Cl⁻ secretion.

H⁺-ATPase inhibition reduces benzamil-sensitive transepithelial voltage. Since ENaC blockade stimulates the apical H⁺-ATPase, ENaC inhibitors may reduce the lumen-negative transepithelial voltage not only by reducing Na⁺ absorption but also by stimulating H⁺ secretion. As such, we examined the contribution of apical H⁺ secretion to the benzamil-sensitive change in transepithelial voltage. Figure 7 shows that benzamil reduces transepithelial voltage either in the presence (Fig. 7B) or the absence (Fig. 7A) of apical H⁺-ATPase blockade. However, the magnitude of benzamil-sensitive transepithelial voltage fell by ~60% with apical H⁺-ATPase blockade. We conclude that benzamil reduces the lumen-negative transepithelial voltage not only by reducing the absorption of Na⁺ but also by stimulating the secretion of H⁺.

Benzamil-sensitive transepithelial voltage falls with removal of CO₂/HCO₃⁻. Since H⁺-ATPase blockade reduced the benzamil-sensitive component of transepithelial voltage, further experiments asked whether H⁺-ATPase blockade reduces the benzamil-sensitive component of Cl⁻ absorption. To answer this question, H⁺ secretion was reduced not only by applying bafilomycin to the perfusate but also by eliminating CO₂ as a source of H⁺ for luminal acidification. To do so, HCO₃⁻/CO₂ was eliminated from the perfusate and bath (26, 31). We detected a benzamil-sensitive component of Cl⁻ absorption in both the presence and in the absence of H⁺-ATPase blockade (Fig. 8A). However, the magnitude of benzamil-sensitive Cl⁻ absorption was reduced when CO₂/HCO₃⁻ was removed. This suggests that a portion of the H⁺-ATPase-sensitive Cl⁻ absorption may be accounted for by the CO₂/HCO₃⁻ exchanger.
absorption (Fig. 8A) fell whether H⁺ secretion was reduced with chemical inhibitors (bafilomycin) or with elimination of CO₂ as a H⁺ source. We conclude that benzamil-sensitive Cl⁻ flux is dependent on the apical H⁺-ATPase.

Figure 8B shows, however, that under the same treatment conditions benzamil-induced Cl⁻ flux did not change in tandem with benzamil-sensitive transepithelial voltage. These data argue against the possibility that apical H⁺-ATPase inhibition reduces benzamil-sensitive Cl⁻ absorption exclusively by changing Cl⁻ transport across tight junctions.

**H⁺-ATPase inhibition blocks NKCC1-dependent Cl⁻ secretion.** We have shown previously that the basolateral membrane Na-K-2Cl transporter NKCC1 mediates Cl⁻ uptake across the basolateral plasma membrane of type A intercalated cells in the rat outer medullary collecting duct (OMCD) (32) and mouse CCD (20), which is then secreted across the apical membrane into the luminal fluid (Fig. 9). When NKCC1 is inhibited with bumetanide, Cl⁻ secretion in the CCD is reduced (20, 32). Because type A intercalated cells mediate the secretion of H⁺ in tandem with Cl⁻ secretion, we asked...
whether apical \( \text{H}^+ \)-ATPase blockade abolishes the effect of NKCC1 inhibitors (bumetanide) on transepithelial \( \text{Cl}^- \) transport (Fig. 10). To answer this question, we examined the effect of bumetanide application on \( \text{Cl}^- \) absorption in the presence and absence of \( \text{H}^+ \)-ATPase blockade. As shown, during ENaC blockade alone, bumetanide application to the bath increased \( \text{Cl}^- \) absorption from 2.7 ± 4.1 to 9.6 ± 3.2 pmol-mm\(^{-1}\)-min\(^{-1}\) (\( P < 0.05 \)), consistent with our previous observations in mouse CCD (20). However, during blockade of both ENaC and the apical \( \text{H}^+ \)-ATPase, \( \text{Cl}^- \) absorption was unchanged with application of this NKCC1 inhibitor (Fig. 10). We conclude that across type A intercalated cells, NKCC1 mediates \( \text{Cl}^- \) uptake across the basolateral membrane. \( \text{Cl}^- \) is then secreted across the type A intercalated cell apical plasma membrane through a yet to be identified apical conductive \( \text{Cl}^- \) transport pathway in tandem with \( \text{H}^+ \) secretion mediated by the apical plasma membrane \( \text{H}^+ \)-ATPase.

Model predictions of the bafilomycin-sensitive HCl secretion. In the companion study (Tables 2 and 3 in Ref. 17a), we used a CCD model in which CIC-5 (1\( \text{H}^+/2\text{Cl}^- \)) is present in the type A intercalated cell luminal membrane, to simulate the effect of bafilomycin on \( \text{Cl}^- \) and \( \text{HCO}_3^- \) transport. In the absence of CIC-5, the model predicts that \( \text{H}^+ \)-ATPase inhibition will increase the lumen-negative transepithelial voltage, which should slightly increase \( \text{Cl}^- \) absorption. With CIC-5 present, however, the model predicts a greater increase in \( \text{Cl}^- \) absorption with bafilomycin application and predicts that bafilomycin will reduce intracellular pH within type A intercalated cells, thereby activating CIC-5-mediated \( \text{Cl}^- \) absorption and \( \text{H}^+ \) secretion. The model also predicts that in the CCD, \( \text{H}^+ \)-ATPase inhibition will promote \( \text{Cl}^- \) absorption independently of ENaC inhibition. In the absence of bafilomycin, the model predicts that bafilomycin will increase \( \text{Cl}^- \) absorption by 9.0 pmol-mm\(^{-1}\)-min\(^{-1}\), due to a 10.7 pmol-mm\(^{-1}\)-min\(^{-1}\) increase in CIC-5-mediated \( \text{Cl}^- \) flux. However, during ENaC blockade, where \( \text{Cl}^- \) flux is secretory, bafilomycin should reduce this \( \text{Cl}^- \) secretion by 11.5 pmol-mm\(^{-1}\)-min\(^{-1}\). In both cases, bafilomycin depolarizes the luminal membrane of the intercalated cell, thus providing an electrical force favoring movement of \( \text{Cl}^- \) from lumen to cell.

However, the model predicts a different effect of ENaC inhibition on \( \text{H}^+ \) secretion from that observed experimentally. The model predicts that bafilomycin application will reduce the driving force for electrogenic \( \text{H}^+ \) secretion by the apical membrane \( \text{H}^+ \)-ATPase, due to the fall in lumen-negative voltage expected with the application of this diuretic. The predicted result is reduced \( \text{H}^+ \) secretion and reduced intracellular pH within type A intercalated cells (Table 3 in Ref. 17a). Experimental data displayed in Figs. 4 and 5 (the present study) show that bafilomycin instead alkalinizes intercalated cells and increases \( \text{H}^+ \) secretion. Our experimental data show that the primary mechanism by which bafilomycin increases \( \text{H}^+ \) secretion and increases intercalated cell intracellular pH is by stimulating the apical membrane \( \text{H}^+ \)-ATPase in the type A intercalated cell. Bafilomycin stimulates \( \text{H}^+ \) secretion through the apical \( \text{H}^+ \)-ATPase, despite the fall in lumen-negative transepithelial voltage, which reduces the driving force for electrogenic \( \text{H}^+ \) secretion. As such, this diuretic does not alter \( \text{H}^+ \) secretion through changes in transepithelial voltage. Therefore, our observation in the companion paper (17a) that DIDS-sensitive \( \text{Cl}^- \) transport occurs only in the presence of bafilomycin is consistent with model predictions that compared total \( \text{Cl}^- \) flux in the presence or absence of CIC-5. However, our observations regarding the impact of bafilomycin on \( \text{H}^+ \) flux show a lack of concordance between experiment and model.

**DISCUSSION**

We and others have shown that inhibiting ENaC reduces \( \text{Cl}^- \) absorption in CCDs from mice given aldosterone and a NaCl-replete diet (19, 20, 28). The mechanism of bafilomycin-sensitive \( \text{Cl}^- \) absorption is thought to occur through paracel-

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### Table 2. Effect of bafilomycin on intercalated cell pHi

<table>
<thead>
<tr>
<th>Condition</th>
<th>Basal pH</th>
<th>dpHi/dt, pH units/s</th>
<th>ΔpHi</th>
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<td>Wild-type mice, Na(^+) present</td>
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<tr>
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<td>0.015 ± 0.004(^*)</td>
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Values are means ± SE. pHi, intracellular pH. \( ^*P < 0.05 \), ANOVA.
lular Cl\textsuperscript{−} transport across tight junctions driven by the lumen-negative transepithelial voltage generated by ENaC (8). However, the present and previous (20) studies have shown that benzamil-sensitive changes in transepithelial voltage and Cl\textsuperscript{−} flux do not always change in tandem.

The apical H\textsuperscript{+}-ATPase generates a lumen-positive transepithelial voltage in the late distal convoluted tubule (DCT) (5) and in the OMCD (32). In the inner stripe of the OMCD, apical H\textsuperscript{+} and Cl\textsuperscript{−} secretion are dependent on HCO\textsubscript{3}/CO\textsubscript{2} since the lumen-positive voltage and H\textsuperscript{+} secretion fall markedly when HCO\textsubscript{3}/CO\textsubscript{2} is eliminated (31, 32). Whereas HCl secretion was observed in the absence of ENaC inhibitors in the inner stripe of the OMCD from rats given an aldosterone analog (31, 32), the present study observed HCl secretion in CCDs from aldosterone-treated mice only during ENaC blockade. The HCl secretion observed in the in the inner stripe of the rat OMCD in the absence of ENaC blockade (31) may reflect the lack of net Na\textsuperscript{+} absorption, and presumably the lack of significant ENaC-mediated Na\textsuperscript{+} absorption, in this segment when perfused in vitro (25, 31).

The present study demonstrates that in the CCD, inhibiting ENaC stimulates HCl secretion that is sensitive to H\textsuperscript{+}-ATPase inhibitors. Our observations are consistent with those of Stoner, Burg, and Orloff (26), who showed that adding the

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**Fig. 7.** Apical H\textsuperscript{+}-ATPase inhibition reduces benzamil-sensitive V\textsubscript{T}. V\textsubscript{T} was measured before and after ENaC blockade in CCDs from aldosterone-treated mice in the presence (B) and in the absence (A) of bafilomycin (5 nM) in the luminal fluid. Solid lines display results of experiments in which vehicle was applied in the first period and benzamil was applied in the second. Dashed lines indicate experiments where the order was reversed (i.e., bafilomycin was applied in the initial period and then removed in the second). The boxes display the means ± SE of all experiments performed. C: benzamil-sensitive component of V\textsubscript{T} measured in each group. *P < 0.05.

**Fig. 8.** H\textsuperscript{+}-ATPase inhibition attenuates benzamil-induced Cl\textsuperscript{−} secretion. The effect of benzamil on J\textsubscript{Cl} and V\textsubscript{T} was examined in the presence and absence of apical H\textsuperscript{+}-blockade achieved with bafilomycin (5 nM) added to the luminal fluid or with HCO\textsubscript{3}/CO\textsubscript{2} removed from the bath and perfusate. Benzamil-induced Cl\textsuperscript{−} secretion (i.e., the benzamil-sensitive component of J\textsubscript{Cl}) and benzamil-sensitive V\textsubscript{T} were calculated from data obtained under each of these treatment conditions. *P < 0.05, ANOVA.
ENaC inhibitor amiloride to the luminal fluid generates a lumen-positive transepithelial voltage in rabbit CCDs perfused in vitro. Since this amiloride-induced lumen-positive transepithelial voltage is eliminated with either carbonic anhydrase inhibition or with removal of CO₂, these authors concluded that amiloride stimulates H⁺ secretion. Later studies by McKinney and Burg (15, 16) showed, however, that in rabbit CCDs perfused in vitro, amiloride application to the perfusate increases net HCO₃⁻ secretion, or reduces net H⁺ secretion. In the turtle urinary bladder, Husted and Steinmetz (10) also observed that amiloride reduces H⁺ secretion. Because H⁺ secretion falls when the transepithelial voltage is less lumen negative (24), they concluded that amiloride reduces H⁺ secretion, in part, by hyperpolarizing the luminal membrane. However, a large increase in the lumen-negative transepithelial voltage produces only a small increment in net H⁺ secretion in the turtle bladder. As such, while the reason for the different results of the present study and those of Steinmetz is unclear, what is clear is that electrochemical driving force is not the major regulator of net H⁺ secretion along the distal nephron. The present study shows that ENaC inhibition does not stimulate H⁺ secretion through changes in driving force since ENaC inhibition increases H⁺ secretion while reducing the lumen-negative transepithelial voltage, which decreases the driving force for H⁺ secretion. ENaC inhibition may instead alter H⁺-ATPase activity through changes in downstream luminal fluid composition or through a paracrine mechanism.

It is well established that amiloride application in vivo reduces net H⁺ excretion in the mouse, rat, and human, thereby generating a metabolic acidosis (1, 9, 23, 37). What is not clear is why amiloride analogs increase H⁺ secretion in CCDs from aldosterone-treated mice perfused in vitro, but reduce H⁺ secretion in CCDs from rabbits, at least under basal conditions (15) or following NaHCO₃ ingestion (16). Amiloride may affect transport differently in the mouse and rabbit. Alternatively, the increase in H⁺ secretion observed during ENaC blockade may be more physiologically significant during high-aldosterone states. Chronic dietary NaCl restriction increases aldosterone release, which stimulates renal Cl⁻ transporters, such as pendrin, thereby increasing Cl⁻ absorption. If a NaCl-rich meal is then consumed, serum aldosterone concentration falls, which reduces apical plasma membrane pendrin abundance probably over a period of hours to days. Without a means to rapidly reduce Na⁺ and Cl⁻ absorption, a sudden increase in NaCl intake would markedly increase total body NaCl balance. The HCl secretion observed following ENaC blockade might represent a mechanism for rapidly reducing Cl⁻ absorption. High NaCl intake increases distal Na⁺ delivery, which rapidly downregulates ENaC through Na⁺ self-inhibition (7). This ENaC inhibition should stimulate HCl secretion, thereby minimizing net NaCl absorption.

In the present study, benzamil did not change Cl⁻ and H⁺ flux in a 1:1 relationship since the change in Cl⁻ flux observed

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**Fig. 9. Proposed mechanism of benzamil-induced Cl⁻ secretion.** The Na-K-2Cl cotransporter, NKCC1, mediates Cl⁻ uptake across the basolateral membrane in type A intercalated cells. Cl⁻ is then secreted across the apical plasma membrane through a yet to be identified pathway. H⁺ is secreted by type A cells through the apical H⁺-ATPase, which is associated with Cl⁻ secretion mediated by this pathway. However, there is evidence that NKCC1 localizes to the basolateral regions of not only type A intercalated cells (14, 20), but also to the basolateral regions of principal cells (14).

**Fig. 10.** H⁺-ATPase inhibition eliminates bumetanide-induced changes in Cl⁻ flux. Mice were fed the NaCl-replete gelled diet described in Methods and were given aldosterone by minipump (200 Î¼g·kg body wt·day⁻¹) for 5–7 days before euthanasia. The effect of bumetanide (100 Î¼M) on JCl was examined during ENaC blockade (3 Î¼M benzamil in the luminal fluid) in the presence (right) and absence (left) of apical H⁺ blockade (bafilomycin, 5 nM, the luminal fluid). As shown, H⁺-ATPase blockade prevented the increase in Cl⁻ absorption observed with bumetanide application. Solid lines display results of experiments in which bumetanide vehicle was applied in the first period and bumetanide (plus vehicle) was applied in the second. Dashed lines indicate experiments where the order was reversed (i.e., bumetanide was applied in the initial period and then removed in the second). The boxes display means ± SE of all experiments performed. *P < 0.05.
with benzamil application is about twofold higher than the change in CO₂ flux observed with the application of this diuretic. Changes in total CO₂ flux may, however, underestimate H⁺ secretion since a disequilibrium pH is observed in the CCD (12). Alternatively, this conductive pathway or conductive complex might secrete 2 Cl⁻ for each secreted H⁺ (Fig. 9). For example, if 2 H⁺ are secreted through the apical H⁺-ATPase in tandem with the secretion of 2 Cl⁻ and the absorption of 1 H⁺ through an electrogenic Cl⁻/H⁺ exchanger, net secretion of 1 H⁺ and 2 Cl⁻ would be observed, although other mechanisms are also possible. For example, we cannot exclude the possibility that cations other than H⁺ are cosecreted with benzamil-stimulated Cl⁻ secretion. However, K⁺ is not likely a cosecreted cation since amiloride reduces K⁺ secretion.

The benzamil-sensitive change in transepithelial voltage is commonly used as a measure of ENaC-mediated Na⁺ absorption. However, the present study shows that benzamil reduces transepithelial voltage not only by reducing ENaC-mediated Na⁺ absorption but also by stimulating H⁺ secretion. As such, benzamil-sensitive transepithelial voltage reflects, in part, apical H⁺-ATPase-mediated H⁺ secretion.

Eliminating the lumen-negative voltage of the CCD with benzamil increases the driving force for conductive Cl⁻ secretion. Conversely, inhibiting conductive Cl⁻ secretion with stilbene inhibitors should reduce the lumen-negative transepithelial voltage. However, we observed instead that application of stilbene inhibitors to the luminal perfusate increased the lumen-negative transepithelial voltage most likely by inhibiting apical H⁺-ATPase-mediated H⁺ secretion (18). DIDS likely inhibits conductive Cl⁻ secretion as an indirect consequence of H⁺-ATPase blockade.

In conclusion, in CCDs from aldosterone-treated mice the majority of Cl⁻ absorption occurs through a pathway that is modulated by ENaC. Inhibiting ENaC increases conductive, DIDS-sensitive Cl⁻ secretion, which is associated with apical H⁺-ATPase-mediated H⁺ secretion. While benzamil may modulate transepithelial Cl⁻ absorption by changing Cl⁻ flux across tight junctions through changes in transepithelial voltage, this study demonstrates that other transport mechanisms are possible.

ACKNOWLEDGMENTS

We thank Dr. Steve Sansom for helpful suggestions.

GRANTS

This study was supported by DK 46493 (to S. M. Wall), DK 29857 (to A. M. Weinstein), T32 DK07656 (to Y. Lazo-Fernandez), and by ASN Career Development Grant 145596 (to V. Pech).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: M.N., V.P., Y.-L.F., and A.M.W. performed experiments; M.N., V.P., Y.-L.F., A.M.W., and S.M.W. analyzed data; M.N., V.P., Y.-L.F., A.M.W., and S.M.W. interpreted results of experiments; M.N., V.P., A.M.W., and S.M.W. provided conception and design of research; V.P., A.M.W., and S.M.W. prepared figures; S.M.W. drafted manuscript.

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