Luminal flow modulates $\text{H}^+$-ATPase activity in the cortical collecting duct (CCD)

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Luminal flow modulates $\text{H}^+$-ATPase activity in the cortical collecting duct (CCD). Am J Physiol Renal Physiol 302: F205–F215, 2012. First published September 28, 2011; doi:10.1152/ajprenal.00179.2011.—Epithelial Na$^+$ channel (ENaC)-mediated Na$^+$ absorption and BK channel-mediated K$^+$ secretion in the cortical collecting duct (CCD) are modulated by flow, the latter requiring an increase in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$), microtubule integrity, and exocytic insertion of preformed channels into the apical membrane. As axial flow modulates HCO$_3^-$ reabsorption in the proximal tubule due to changes in both luminal Na$^+$/$\text{H}^+$ exchange 3 and $\text{H}^+$-ATPase activity (Du Z, Yan Q, Duan Y, Weinbaum S, Weinstein AM, Wang T. Am J Physiol Renal Physiol 290: F289–F296, 2006), we sought to test the hypothesis that flow also regulates $\text{H}^+$-ATPase activity in the CCD. $\text{H}^+$-ATPase activity was assayed in individually identified cells in microperfused CCDs isolated from New Zealand White rabbits, loaded with the pH-sensitive dye BCECF, and then subjected to an acute intracellular acid load. Basolateral AE1 pumping was Ca$^{2+}$ dependent and required microtubule integrity, whereas intercalated cells are decorated with abundant apical microvilli and microplacma (17, 36, 48). Principal cells, the majority cell type, reabsorb Na$^+$ through apical epithelial Na$^+$ channels (ENaC) and secrete K$^+$ via ROMK and BK channels. Intercalated cells are involved in transepithelial acid/base transport via polarized $\text{H}^+$-ATPases and Cl$^-$/HCO$_3^-$ exchangers and may also contribute to K$^+$ reabsorption via apical H$^+$-K$^+$-ATPases, composed of HK-1 (gastric) and HK-2 (colonic) isoforms, under certain conditions (10, 38, 54, 60, 66). Intercalated cell subtypes include type A (or α), which mediate $\text{H}^+$ secretion and possess apical $\text{H}^+$-ATPase and basolateral AE1 (1, 58, 60); type B (or β), which secrete HCO$_3^-$ via apical pendrin, a Na$^+$ independent Cl$^-$/HCO$_3^-$ exchanger, and express basolateral H$^+$-ATPase (1, 50, 60, 67); and non-A non-B with both apical H$^+$-ATPase and pendrin (15, 27), cells whose function is as yet unknown. Intercalated cells also possess apical conducting BK channels (31, 45, 55), which have been proposed to function in flow-induced K$^+$ secretion (34) as well as to recycle K$^+$ taken up by the apical H$^+$-K$^+$-ATPase back into the luminal fluid (63). Increases in luminal flow rate in the CCD stimulate ENaC-mediated net Na$^+$ absorption and BK channel-mediated K$^+$ secretion, and lead to a transient increase in [Ca$^{2+}$]$_i$ in both principal and intercalated cells (33, 36, 57, 81).

Based on the studies summarized above, and the observations that 1) increases in [Ca$^{2+}$]$_i$ in acid-secreting α-intercalated cells in turtle bladder lead to exocytosis of H$^+$-ATPase-containing vesicles (8, 71) and 2) chronic administration of diuretics enhances distal acidification by upregulating H$^+$-ATPase activity in intercalated cells in rat kidney (43), we hypothesized that the H$^+$-ATPase present in the mammalian CCD is regulated by variations in luminal flow rate. We tested this in microperfused rabbit CCDs loaded with a pH-sensitive fluorescent dye by measuring the effect of luminal flow rate on Na$^+$ independent bafilomycin-sensitive intracellular pH (pHi) recovery from an intracellular acid load.

METHODS

Animals. Adult (>6 wk) female New Zealand White rabbits (Covance, Denver, PA) were housed in the animal care facility at the Mount Sinai School of Medicine (Center for Comparative Medicine)
or the University of Pittsburgh. All animals were allowed free access to tap water and chow. Animals were euthanized in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal protocols were approved by the Institutional Animal Care and Use Committee at the Mount Sinai School of Medicine or University of Pittsburgh School of Medicine, as appropriate.

Solutions and chemicals. The compositions of the solutions used have been previously described (10) and are given in Table 1. All experiments were performed in the nominal absence of CO$_2$/HCO$_3^{-}$ using solutions buffered with HEPES, adjusted to pH 7.4, and 290 ± 2 mosmol/kgH$_2$O using NaOH in Na$^{+}$-containing solutions or N-methyl-d-glucamine in Na$^{+}$-free solutions. The intracellular calibration solution was titrated to pH 6.8, 7.3, and 7.8 using HCl or KOH.

The fluorescent probes used included the pH indicator BCECF-AM (Molecular Probes, Eugene, OR), the Ca$^{2+}$ indicator fura 2 acetoxyethyl ester (fura 2-AM; Calbiochem), and principal cell marker rhodamine Dolichos biflorus agglutinin (DBA; Vector Labs, Burlingame) (16). The 20 mM stock solutions of BCECF-AM and fura 2-AM, prepared in DMSO, were diluted into Na$^{+}$-Ringer solution to a final concentration of 20 μM. DBA was added directly to the Na$^{+}$-Ringer solution in a concentration of 10 μg/ml.

Bafilomycin A$_1$ (LC Laboratories, Woburn, MA), a potent and specific inhibitor of vacuolar-type H$^+$-ATPases (4), was prepared as a 1 μM stock solution in DMSO and diluted on the day of experimentation to a final concentration of 10 nM. As indicated, some tubules were pretreated with either the acetoxyethyl ester of BAPTA (20 μM final concentration prepared from 20 mM stock solution in DMSO; Molecular Probes) to chelate [Ca$^{2+}$], or colchicine (10 μM final concentration prepared from 2.5 mM stock solution in water; Sigma, St. Louis, MO) to disrupt microtubules, added to the bathing solution (32, 73). Nigericin (Sigma-Aldrich) was prepared as a 2 mM stock solution and diluted to 10 μM in each standard calibration solution. All dilutions of dyes and inhibitors yielded a final concentration of DMSO of ≤0.1%.

Microperfusion of isolated rabbit CCDs. The kidneys were removed via a midline incision, sliced into 2-mm coronal sections, and single mid-CCDs were dissected freehand in cold (4°C) Na$^{+}$-Ringer solution. A single tubule was studied from each animal. Isolated CCDs were microperfused in vitro as previously described (10, 35). Briefly, each isolated tubule was immediately transferred to a temperature-controlled specimen chamber, assembled with a no. 1 coverslip (Corning) painted with a 3-μl drop of poly-D-lysine hydrobromide (0.01%; BS Biosciences), and set on the stage of a Nikon inverted epifluorescence microscope (Eclipse TE300 or Diaphot) linked to a Cascade 512F (Photometrics) or cooled Pentamax charge-coupled device (Princeton Instruments) camera interfaced with a digital imaging system (MetaFluor, Universal Imaging, West Chester, PA). The CCD was mounted on concentric glass pipettes, cannulated, and then positioned directly on the poly-D-lysine to immobilize the segment for the duration of the experiment, as previously described (36). Tubules were initially perfused and bathed at 37°C in symmetrical Ringer solution for the 30-min equilibration period before each experiment. The bathing solution was continuously exchanged throughout the experiment at a rate of 10 ml/h using a syringe pump (Razel, Stamford, CT) and maintained at 37°C.

For measurements of pH$_i$, each CCD was incubated for 20 min in 20 μM BCECF-AM added to the bathing medium, as originally described by Weiner and Hamm (77). The luminal perfusate was then replaced with the Na$^{+}$- and K$^{+}$-free solution (0Na, 0K; Table 1), to which 10 nM bafilomycin was added in some experiments, and the bath with a Na$^{+}$-Ringer solution. As indicated, BAPTA-AM (20 μM) was added to the bath at this point; in all experiments with colchicine (10 μM), the inhibitor was present in all bathing solutions, including the dissection solution. pH$_i$ measurements were begun after at least a 15-min washout of residual BCECF-AM from the bath.

Measurement of pH$_i$. BCECF-loaded cells were visualized using a Nikon 5 Fluor ×40 objective (numerical aperture 0.9, working distance 0.3). Autofluorescence was not detected at the camera gains utilized. Tubules were alternately excited at 490 and 440 nm using an excitation wavelength switcher (DG-4 or LAMBDA 10–2; Sutter); images of the fluorescence emission at 530 nm were acquired at intervals ranging from 2 to 15 s using MetaFluor image acquisition software (Universal Imaging, West Chester, PA) and were stored on a Digital Instruments computer. The 490 nm/440 nm fluorescence intensity ratios (FIRs) were subsequently calculated using our commercially available digital image-analysis system (MetaFluor).

We (10) and others (77) have previously reported that esterase-rich intercalated cells can be distinguished from principal cells by their preferential accumulation of BCECF and brighter appearance when viewed under epifluorescence illumination. Confirmation of the identity of principal cells was sought by their selective apical binding of DBA added to the luminal perfusate at the conclusion of each experiment before the intracellular calibration was performed (Fig. 1).

An intracellular calibration was performed in each CCD using the nigericin technique and high-K$^+$ intracellular calibration buffers (Table 1) (69). Linear regression analysis was used to generate a calibration curve that was then used for conversion of calculated FIRs to pH$_i$ using standard equations.

At least two intercalated and two principal cells residing in the lateral wall of the midregion of each perfused CCD (to capture the fluorescence signal from a single cell) were randomly selected for final analysis. The steady-state pH$_i$ of a single cell was calculated based on the average of at least six FIR readings; the steady-state pH$_i$ value for principal or intercalated cells in a single tubule was calculated as the mean pH$_i$ of at least two cells along the wall of each tubule. All pH$_i$ results are reported as the mean of $n$ tubules.

Kinetic assay for H$^+$-ATPase activity. The sensitivity of H$^+$ pumps to fluid flow rate was examined by measuring the effect of variations in luminal flow rate on Na$^{+}$-independent pH$_i$ recovery from an

Table 1. Composition of solutions (in mM) used for pH$_i$ assays

<table>
<thead>
<tr>
<th></th>
<th>Na$^{+}$-Ringer</th>
<th>NH$_4$Cl</th>
<th>0Na, 0K</th>
<th>0Na, 5K</th>
<th>Calibration pH</th>
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<tr>
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<tr>
<td>l-Alanine</td>
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<tr>
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<td>5.5</td>
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<tr>
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<tr>
<td>KCl</td>
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<td>125</td>
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pH$_i$, intracellular pH; NMDG, N-methyl-d-glucamine.
in intracellular acid load, accomplished by a ~5-min peritubular exposure of BCECF-loaded CCDs to a 20 mM NH₄Cl solution (Table 1). Measurement of luminal flow rates was performed before the NH₄Cl pulse, after each change in luminal perfusate, or at least three times during a single experiment by timed filling of a precalibrated volumetric pipette. Rapid washout of the basolateral NH₄Cl solution with a Na⁺-free solution (0Na, 5K; Table 1) led to a fall in pHi to ~6.2 (Figs. 2 and 3); these manual washes were accomplished by fully replacing the volume of the bath (~1.5 ml) at least three times within 10 s.

Recovery of pHi from its nadir was monitored initially in the absence of Na⁺ and K⁺ (0Na, 0K; Table 1) in the lumen and Na⁺ (0Na, 5K; Table 1) in the bath. We have previously reported that replacement of luminal Na⁺-Ringer solution with a 0Na, 0K solution has no significant effect on steady-state pHi in microperfused CCDs (10). Once pHi stabilized, 5 mM K⁺ was added to the luminal perfusate (0Na, 5K solution; Table 1), and pHi was again monitored. On reaching a new steady state, Na⁺-Ringer solution was restored to the bath and pHi recovery was again followed.

After each change in solution, pHi was followed for at least 10 min or, if cell alkalization was observed, until pHi stabilized (Fig. 2). In one set of experiments designed to identify the nature of the Na⁺- and luminal K⁺-independent pHi recovery routinely observed at pHi values less than ~6.6, the experimental protocol described above was modified to include the addition of bafilomycin A₁ to the 0Na, 0K luminal perfusate (Table 1). The initial rate of change in pHi (dpHi/dt) observed in response to a change in luminal or bathing solution was calculated by linear regression analysis of the rate of recovery over ~30–60 s, as previously described (10). Mean slopes of pHi recovery for principal and intercalated cells were calculated for each CCD.

Experiments were rejected if we observed a ≥60% loss in the 450-nm fluorescence intensity from the initial value. Also excluded from the present study were those experiments in which peritubular Na⁺ restoration failed to restore pHi to baseline, except when the steady-state pHi after luminal K⁺ restoration was already near baseline.

We did not attempt to differentiate intercalated cell subtypes in this study, as initial studies revealed little variability in pHi recovery rates among intercalated cells examined at slow and fast flow rates. Similarly, Silver et al. (62) also found a normal distribution of pHi recovery rates after an acid load among intercalated cells in the rat, thus leading these investigators not to distinguish α-type from β-type intercalated cells for the functional portion of their study. We thus grouped values from all intercalated cells analyzed together for statistical analysis. Note that we focused our studies on the mid-CCD, which we have previously characterized in terms of intercalated cell composition. Specifically, we have reported, based on functional assays of this segment in the rabbit, that ~78% of intercalated cells, defined as cells concentrating the pH-sensitive dye 6-carboxyfluorescein, bind peanut lectin agglutinin to their apical surfaces and are thus considered to be β cells (61).

Measurement of buffer capacity. To test whether flow rate alters buffer capacity in CCD cells, we calculated intrinsic buffer capacity, as we have previously described (54), in individually identified cells in BCECF-loaded tubules perfused and bathed in a Na⁺- and HCO₃⁻-free solution and subject to the rapid NH₃/NH₄⁺ withdrawal as described above. Buffer capacity (mM/pH U) was calculated as B = Δ[NH₄⁺]/ΔpHi, where [NH₄⁺] is the concentration of intracellular NH₄⁺. Using a pKₐ of 8.9 (5), we calculated the concentration of [NH₄⁺], as [NH₄⁺] = [NH₄⁺]₀ × 10⁶⁺⁻[pHi], where [NH₄⁺]₀ is the

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**Fig. 1.** Fluorescence micrographs of a single mid-cortical collecting duct (CCD), captured at slightly different focal planes, showing selective accumulation of BCECF (left) and apical binding of the principal cell marker rhodamine-conjugated Dolichos biflorus agglutinin (DBA; right) to discrete and nonoverlapping cells. The white asterisks identify cells (presumably intercalated) that preferentially accumulate BCECF loaded from the bath, and the white arrowheads identify principal cells with apical DBA caps.

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**Fig. 2.** Representative tracings of changes in intracellular pH (pHi) following an NH₄Cl-induced acute intracellular acid load in principal (PC; solid lines) and intercalated (IC; dotted lines) cells in CCDs perfused at a slow (~1 nl/min·mm⁻¹; left) or fast (~5 nl/min·mm⁻¹; right) luminal flow rate. L identifies the solutions in the lumen, and B the solutions in the bath. In both panels, cytoplasmatic acidification from an initial pHi of ~7.3 in Na⁺-Ringer (NR) solution to a nadir pHi of ~6.2 was accomplished by a brief exposure to a peritubular 20 mM NH₄Cl pulse. In the absence of luminal Na⁺ and K⁺ (0Na, 0K), washout of the peritubular NH₄Cl with a 0Na, 5K solution led to a minimal (at a slow flow rate) or partial (at a fast flow rate) pHi recovery in both PC and IC, pHi recovery in the absence of luminal K⁺ and bilateral Na⁺ is predominantly mediated by the vacuolar H⁺-ATPase. Readdition of 5 mM K⁺ (0Na, 5K) to the luminal perfusate led to a further increase in pHi; pHi recovery observed following restoration of K⁺ to the luminal perfusate is presumably mediated by the H⁺-K⁺-ATPase. Restoration of extracellular Na⁺ (NR) to provide substrate for the basolateral Na⁺/H⁺ exchanger led to full recovery of pHi, in both cell types to ~7.3 in all CCDs studied.
extracellular NH$_4^+$ concentration. $\Delta$P$_{\text{H}}$ was calculated as the difference in pHi between that measured just before and after the rapid NH$_4$Cl washout.

Measurement of $[\text{Ca}^{2+}]$. Following equilibration, microperfused tubules were loaded with 10 $\mu$M fura 2-AM added to the bath for 20 min. $[\text{Ca}^{2+}]$ was measured in individually identified fura 2-loaded cells subject to the identical in vitro acid loading and recovery protocol depicted in Fig. 2 and visualized using the imaging system described above. Autofluorescence was not detected at the camera gains utilized.

For acquiring data in fura 2-loaded CCDs, tubules were alternately excited at 340 and 380 nm and images acquired every 15 s were digitized for subsequent analysis. At the conclusion of each experiment, an intracellular calibration was performed using 10 $\mu$M EGTA-AM in a Ca$^{2+}$-free bath and then a 2 mM Ca$^{2+}$-bath containing ionomycin (10 $\mu$M) (36). Standard equations were used to calculate experimental values of $[\text{Ca}^{2+}]$. The mean $[\text{Ca}^{2+}]$ values for principal and intercalated cells, distinguished by their differing fluorescent intensities (36), were calculated.

Immunolocalization of H$^+$-ATPase in rabbit kidneys. Freshly harvested kidneys were cut into ~5-mm slices, and fixed using 4% paraformaldehyde-lysine-periodate buffer (40, 46) by immersion for 3 h at room temperature (RT). The fixed kidney tissues were washed in PBS and quenched in NH$_4$Cl, followed by washes in PBS. Tissues were cryoprotected by immersion in a 30% sucrose solution in PBS at $-27^\circ$C and 4-$\mu$m cryosections were obtained and placed onto polylysine-coated slides (Fisher). After 30-min rehydration in PBS, the slides were subject to a 1% SDS in PBS antigen retrieval step for 4 min in a wet chamber. After several washes in PBS, the sections were blocked in 1% BSA in PBS for 15 min (6). Then, the sections were incubated with DBA at a 1:100 dilution for 1 h at RT, followed by a 5-min wash in PBS and then by incubation in the presence or absence (no-primary control) of an antibody directed against the E subunit of the V-ATPase (raised in chickens, 1:500 dilution; GenWay) (21) for 75 min at RT, followed by two high-salt-PBS (2.7% NaCl) and one normal-strength PBS wash. Thereafter, both slides were incubated with a secondary goat-anti-chicken antibody coupled to FITC (1:100; Jackson ImmunoResearch) for 1 h at RT. After washes as above, the slides were mounted with Vectashield (Vector Laboratories). The tissues immunostained with the antibody against the V-ATPase E subunit and the no-primary control were imaged using a Leica TSC confocal microscope with a $\times 100$ objective, with identical laser acquisition settings, then imported into Adobe Photoshop for identical adjusting of levels of both the H$^+$-ATPase and the no-primary control images. Labels were added in Indesign (Adobe).

Control studies with the antibody preincubated with its respective immunogen were not performed, as the immunogen was not available from the original manufacturer. However, the antibody was tested by Western blotting by the manufacturer, and we (21, 24, 25) and others (82) have further shown cell-specific labeling of clear and intercalated cells in rat tissues using this antibody, as has been reported using other antibodies against the E subunit.

In addition, we also performed immunofluorescence labeling of rabbit kidneys using an antibody directed against the V-ATPase A subunit, raised in chickens, at a 1:100 concentration (Genway; immunogen not available from the manufacturer). The secondary antibody used was a goat anti-chicken antibody coupled to Alexa Fluor 488 at a 1:800 dilution. Images were obtained using a $\times 63$ objective.

Statistics. Results are expressed as means ± SE; n represents the number of tubules. At least four randomly chosen cells were functionally analyzed in each CCD. Significant differences between paired data were determined by a paired t-test. Comparisons of unpaired data were performed by t-test and analysis of variance, as appropriate. Commercially available statistical software (SigmaStat; SPSS Chicago, IL) was used for all statistical analyses. Significance was asserted if $P < 0.05$.

RESULTS

Effect of flow on steady state intercalated and principal cell pHi. In four CCDs perfused and bathed in Na$^+$-Ringer solution and studied at a slow luminal flow rate of 1.0 ± 0.1 nl·min$^{-1}$·mm$^{-1}$, steady-state pHi (baseline) in intercalated cells (7.27 ± 0.01) did not differ from that measured in principal cells (7.26 ± 0.03; $P = $ not significant (NS)) (Figs. 2 and 3). In five CCDs perfused at a fast flow rate of 5.5 ± 0.3 nl·min$^{-1}$·mm$^{-1}$, baseline steady-state pHi was similar in both intercalated (7.34 ± 0.04) and principal (7.27 ± 0.05; $P = $ NS) cells and was not different from that observed in the respective cell type at the slow flow rate ($P = $ NS) (Figs. 2 and 3). Buffer capacity in was similar in both intercalated (28.1 ± 5.7 mM/pH U) and principal (29.3 ± 6.0 mM/pH U; $P = $ NS) cells perfused at the slow flow rate and was unchanged by a fivefold increase in flow rate (26.8 ± 8.7 and 24.8 ± 8.3 mM/pH U, respectively; $P = $ NS vs. same cell at slow flow rate).

As changes in cell volume could alter the correlation between H$^+$ secretion and changes in pHi recovery, we measured the inner and outer diameters of CCDs perfused at slow and fast flow rates and calculated cell height. In tubules perfused and bathed in Na$^+$-Ringer solution at the slow flow rate of 1 nl·min$^{-1}$·mm$^{-1}$, these values were 15.9 ± 1.6, 37.5 ± 1.0, and 10.8 ± 0.3 $\mu$m, respectively. In tubules perfused at the fast flow rate of 5.5 ± 0.3 nl·min$^{-1}$·mm$^{-1}$, inner and outer diameters and cell height (19.6 ± 1.4, 36.9 ± 2.6, and 11.1 ± 0.8 mm, respectively) were not significantly different from those measured at the slow flow rate ($P = $ NS).
Effect of flow on apical H+-ATPase activity in DBA-negative cells. In response to the rapid basolateral washout of a 20 mM NH₄Cl pulse with a 0Na, 5K solution, pHᵢ fell to 6.17 ± 0.09 in DBA-negative and thus presumed intercalated cells in four CCDs perfused at a slow flow rate of 1.0 ± 0.1 nl·min⁻¹·mm⁻¹ with a 0Na, OK solution (Figs. 2 and 3). From this nadir pHᵢ, these cells exhibited an alkalization at an initial rate of 0.059 ± 0.009 pH U/min to reach a plateau pHᵢ of 6.46 ± 0.15 over a period of 6.0 ± 0.6 min (Figs. 2–4). We have previously reported that in the presence of 5 mM K⁺ in the bath but not luminal perfusate, Na⁺-independent pHᵢ recovery is mediated primarily by the vacuolar H⁺-ATPase, which appears to “turn off” at a pHᵢ of 6.6 (10). Restoration of this nadir pHᵢ, these cells exhibited an alkalization at an initial rate of 0.088 ± 0.016 pH U/min to stabilize at a pHᵢ of 6.75 ± 0.18 within 5.8 ± 1.0 min (Figs. 2–4). We have previously reported that this luminal K⁺-dependent, Na⁺-independent pHᵢ recovery is mediated by an apical H⁺-K⁺-ATPase as it is effectively inhibited by 10 μM SCH28080, an inhibitor of the H⁺-K⁺-ATPase (10). Thereafter, restoration of Na⁺ to the bathing solution resulted in a further cell alkalinization, presumably the result of basolateral Na⁺/H⁺ exchange, to reach a pHᵢ of 7.27 ± 0.08 (Figs. 2 and 3).

In a similar series of studies in which five CCDs were perfused at a fast luminal flow rate of 5.5 ± 0.3 nl·min⁻¹·mm⁻¹ with a 0Na, 0K solution and bathed in a 0Na, 5K solution, the initial alkalization from the nadir pHᵢ of 6.14 ± 0.09 averaged 0.146 ± 0.023 pH U/min (P < 0.03 vs. rate at 1 nl·min⁻¹·mm⁻¹) (Figs. 2–4). Once pHᵢ stabilized at 6.62 ± 0.06 within 4.3 ± 0.8 min, restoration of 5 mM K⁺ to the lumen led to a further increase in pHᵢ at a rate of 0.094 ± 0.020 pH U/min (P = NS vs. rate at 1 nl·min⁻¹·mm⁻¹) to reach a plateau pHᵢ of 6.95 ± 0.16 within 6.8 ± 1.4 min (Figs. 2–4). Addition of Na⁺ to the bathing solution at this point led to a further cell alkalinization to 7.38 ± 0.09, a value not significantly different from baseline (Figs. 2 and 3).

To confirm that the flow-stimulated, Na⁺-independent pHᵢ recovery from the nadir observed in the absence of luminal K⁺ was due predominantly to an H⁺-ATPase, as our previous studies suggested (10), pHᵢ recovery from a nadir of 6.14 ± 0.10 was monitored in a separate group of six CCDs pretreated with and perfused in the continuous presence of luminal bafilomycin. In these CCDs, perfused at a luminal flow rate of 5.6 ± 0.2 nl·min⁻¹·mm⁻¹, the initial rate of pHᵢ recovery was only 0.052 ± 0.014 pH U/min, a rate significantly less than that observed in CCDs perfused at the same flow rate in the absence of inhibitor (P < 0.01) and not significantly different from that observed in untreated CCDs perfused at the slow flow rate, to a plateau pHᵢ of 6.31 ± 0.11 (P < 0.03 vs. plateau pHᵢ of 6.62 ± 0.06 observed in untreated CCDs perfused at a fast flow rate) (Figs. 4 and 5). We propose that this residual bafilomycin-insensitive rate of pHᵢ recovery is due, at least in part, to a basolateral H⁺-K⁺-ATPase, as we have previously identified in the CCD (10).

Effect of flow on apical H⁺-ATPase activity in DBA-positive cells. In response to the rapid basolateral washout of a 20 mM NH₄Cl pulse with a 0Na, 5K solution, pHᵢ fell to 6.22 ± 0.06 in DBA-positive and thus presumed principal cells in four CCDs perfused at a slow flow rate of 1.0 ± 0.1 nl·min⁻¹·mm⁻¹...
with a 0Na, 0K solution (Figs. 2 and 3). From this nadir pH_i, these cells exhibited an alkalization at an initial rate of 0.050 ± 0.099 pH U/min to reach a pH_i of 6.45 ± 0.11 over a period of 5.6 ± 0.6 min (Figs. 2–4). Restoration of 5 mM K^- to the lumen led to a further increase in pH_i at a rate of 0.084 ± 0.016 pH U/min to stabilize at a pH_i of 6.72 ± 0.13 within 5.7 ± 1.3 min (Figs. 2–4). Thereafter, addition of Na^+ to the bathing solution resulted in a further cell alkalinization, presumably the result of basolateral Na^+/H^+ exchange, to reach a pH_i of 7.29 ± 0.11 (Figs. 2 and 3).

In a similar series of studies in which five CCDs were perfused at a fast luminal flow rate of 5.5 ± 0.3 nl·min^-1·mm^-1 with a 0Na, 0K solution and bathed in a 0Na, 5K SK solution, the initial alkalization from the nadir pH_i of 6.23 ± 0.06 averaged 0.126 ± 0.004 pH U/min (P < 0.001 vs. rate at 1 nl·min^-1·mm^-1) (Figs. 2–4). Once pH_i stabilized at 6.68 ± 0.06 within 5.0 ± 1.0 min, restoration of 5 mM K^- to the lumen led to a further increase in pH_i at a rate of 0.062 ± 0.016 pH U/min (P = NS vs. rate at 1 nl·min^-1·mm^-1) to reach a pH_i of 6.94 ± 0.15 within 7.5 ± 1.7 min (Figs. 2–4). Addition of Na^+ to the bathing solution at this point led to a further cell alkalinization to 7.35 ± 0.05, a value not significantly different from baseline (Figs. 2 and 3).

To discern whether the flow-stimulated Na^+-independent pH_i recovery from the nadir observed in the absence of luminal K^- was due to an H^+-ATPase, pH_i recovery from a nadir of 6.09 ± 0.09 was monitored in a separate group of six CCDs pretreated with and perfused in the continuous presence of luminal bafilomycin. In these CCDs, perfused at a luminal flow rate of 5.6 ± 0.2 nl·min^-1·mm^-1, the initial rate of pH_i recovery was 0.054 ± 0.013 pH U/min, a rate significantly less than that observed in CCDs perfused at the same flow rate in the absence of inhibitor (P < 0.001) and not significantly different from that observed in CCDs perfused at the slow flow rate, to a plateau pH_i of 6.33 ± 0.11 (P < 0.03 vs. plateau pH_i of 6.68 ± 0.06 observed in untreated CCDs perfused at a fast flow rate) (Figs. 4 and 5). As for the DBA-negative cells studied above, we propose that this residual rate of bafilomycin-insensitive pH_i recovery is due, at least in part, to a basolateral H^+-K^+-ATPase (10).

Effect of acute acid-loading protocol on steady-state cell [Ca^{2+}]_i. Na^+-free solutions have previously been shown to induce changes in [Ca^{2+}]_i, in microperfused OMCDs, which in turn modulated H^+-ATPase-mediated recovery from an acid load (26). To ensure that the effects of flow on H^+-ATPase activity were not due to wide variations in steady-state [Ca^{2+}]_i, induced by our acid-loading protocol, [Ca^{2+}]_i was examined in CCDs perfused at a fast flow rate of 5.2 ± 0.2 nl·min^-1·mm^-1 at baseline (initial value), 3–5 min after addition of NH_4Cl to the bath, immediately and then 10 min after washout of the NH_4Cl pulse with a 0Na, 5K bathing solution, 10 min after readadministration of K^+ to the lumen, and 10 min after restoration of Na^+ to the bathing medium. As shown in Fig. 6, the NH_4Cl prepulse was not associated with a significant change in [Ca^{2+}]_i, although [Ca^{2+}]_i, progressively increased in both principal and intercalated cells with assumed pH_i recovery.

Flow-stimulation of H^+-pumping requires an increase in [Ca^{2+}]_i. An acute increase in tubular fluid flow rate in the microperfused rabbit CCD leads to a rapid transient increase in [Ca^{2+}]_i from ~100 to 350 nM within ~10 s, followed by a gradual decay to a plateau [Ca^{2+}]_i, value that significantly exceeds baseline for at least 10 min during a period of sustained high flow (32, 36). To examine whether flow-stimulation of H^+-pumping in the CCD is dependent on an initial transient flow–induced increase in [Ca^{2+}]_i, CCDs were pretreated with 20 μM BAPTA-AM, a membrane-permeant Ca^{2+} chelator, and the rates of pH_i recovery from an acute acid load were measured at fast (5.1 ± 0.2 nl·min^-1·mm^-1) flow rates. Chelation of intracellular Ca^{2+} inhibited flow-stimulated Na^-independent pH_i recovery from the nadir in both principal and intercalated cells (Figs. 4 and 7; tracing not shown). These data suggest that flow stimulation of the H^+-ATPase requires an increase in [Ca^{2+}]_i.

Flow stimulation of H^+-pumping requires microtubule integrity. Intracellular Ca^{2+} regulates exocytosis and secretion in epithelial cells via, at least in part, microtubule-dependent movement of secretory vesicles (2, 7). To examine the role of the microtubules in flow stimulation of H^+-pumping, CCDs (n = 4) were pretreated with colchicine (10 μM), a microtubule inhibitor

![Fig. 6. Effect of NH_4Cl pulse on intracellular Ca^{2+} concentration ([Ca^{2+}]_i) in PC and IC cells in CCDs (n = 4) perfused at a fast flow rate and subject to the in vitro acid loading and recovery protocol described in Fig. 2. [Ca^{2+}]_i was examined in CCDs at baseline (initial value), 3–5 min after addition of NH_4Cl to the bath, immediately and then 10 min after washout of the NH_4Cl pulse with a 0Na, 5K bathing solution, 10 min after readadministration of K^+ to the lumen, and after restoration of Na^+ to the bathing medium. Values are means ± SE. *P < 0.05 vs. initial value.

![Fig. 7. Effect of the intracellular Ca^{2+} chelator BAPTA-AM (10 nM) on pH_i recovery of CCDs perfused at a fast flow rate following an NH_4Cl-induced acute intracellular acid load. Steady-state pH_i values of PC and IC in 4 CCDs pretreated with BAPTA-AM and microperfused at fast luminal flow rates before and during recovery from an in vitro acid load. The pH_i values indicated are described in detail in the legend to Fig. 3. Means ± SEM. * P < 0.05 vs. initial value; # P < 0.05 vs. nadir value; @ P < 0.05 vs. 5K (B) value.]
That also inhibits vesicle transport between the trans-Golgi network (TGN) and the plasma membrane of polarized epithelial cells (22, 72). CCDs were exposed to colchicine during microdissection as well as throughout the entire experiment, and the rates of pHi recovery from an acute acid load were measured at fast (5.5 H11006 0.2 nl·min−1·mm−1) flow rates. Colchicine inhibited flow-stimulated Na+/H+-independent pHi recovery from the nadir in both principal and intercalated cells (Figs. 4 and 8; tracing not shown). These data suggest that flow stimulation of the H+-ATPase requires microtubule integrity.

**Immunolocalization of H+-ATPase in rabbit CCD.** Immunodetectable V-ATPase E subunit was observed by indirect immunofluorescence confocal microscopy in rabbit CCD (Fig. 9). The α-intercalated cells revealed characteristic apical labeling for the V-ATPase E subunit (Fig. 9C, arrowheads). Principal cells were identified by their apical labeling with DBA coupled to CY3 (Fig. 9, B–E). Note that principal cells in the rabbit collecting duct also exhibited weak apical immunolabeling for the V-ATPase E subunit (Fig. 9C, arrows); these cells showed partial colocalization (yellow) of the V-ATPase E subunit (green) and DBA (red). In the absence of primary antibody against the V-ATPase E subunit, neither intercalated nor principal cells labeled specifically with the antibody (Figs. 9, D and F).

Moreover, we also detected the V-ATPase A subunit (ATP6V1A) by indirect immunofluorescence labeling and confocal microscopy in rabbit CCD (Fig. 10). We identified three distinct patterns of A subunit immunolabeling in the epithelial cells (Fig. 10): 1) intense immunolabeling at the luminal/apical membrane, in a pattern consistent with α-intercalated cells (arrowheads); 2) diffuse labeling, presumably of β-intercalated cells (arrowheads); and 3) weak immunolabeling at the apical pole of other cells, presumably principal cells.

**DISCUSSION**

Axial flow modulates HCO3− reabsorption in the proximal tubule due, at least in part, to stimulation of luminal H+-ATPase activity (11). Recent evidence indicates that fluid shear stress induces the translocation of cytoplasmic H+-ATPases to

![Figure 8](https://example.com/figure8.png)

**Fig. 8.** Effect of the microtubule disrupter colchicine (10 µM) on pHi recovery of CCDs perfused at a fast flow rate following an NH4Cl-induced acute intracellular acid load. Steady-state pHi values of PC and IC cells in 4 CCDs pretreated with colchicine and microperfused at fast luminal flow rates before and during recovery from an in vitro acid load. The pHi values indicated are described in detail in the legend to Fig. 3. Values are means ± SE. *P < 0.05 vs. initial value. #P < 0.05 vs. nadir value. @P < 0.05 vs. 5K (B) value.

![Figure 9](https://example.com/figure9.png)

**Fig. 9.** Immunolocalization of H+-ATPase E subunit in rabbit CCD. A–C: confocal images of a rabbit kidney collecting duct labeled with both an anti-H+-ATPase E subunit antibody (Ab; green channel; A) and PC marker lectin, DBA coupled to CY3 (red channel; B). C: merged image of A and B. Arrowheads identify IC and arrows PC. D–F: confocal images of a rabbit kidney collecting duct, no-primary antibody control for the H+-ATPase E subunit (green channel; D) and in the presence of the principal cell marker DBA (red channel; D). F: merged image of D and E. Arrows identify PC. Scale bars = 15 µm.
transmitted to the intracellular cytoskeleton and thereby regulate distant (e.g., basolateral) membrane transport proteins remains to be explored. The observation that luminal fluid shear stress (FSS) in the proximal tubule regulates peritubular Na-K-ATPase activity by stimulating the translocation of intracellular pumps into the basolateral membrane (14) sets a precedent for our finding of flow-stimulated H⁺ pumping in presumed β-intercalated cells.

The Ca²⁺ dependence of this process was intriguing. An acute elevation of PCO₂ rapidly stimulates the exocytotic fusion of vesicles containing H⁺-ATPases with the luminal membrane of intercalated cells (20, 59, 68), a response induced by a transient increase in [Ca²⁺]i (8, 71). Indeed, it is well established that [Ca²⁺]i regulates exocytosis and secretion in epithelial cells via, at least in part, microtubule-dependent movement of secretory vesicles (2, 3, 7). Our finding that intracellular Ca²⁺ chelation inhibited flow-stimulated, H⁺-ATPase-mediated H⁺ pumping suggests a role for exocytosis in this response. In support of this speculation is our finding that flow-stimulated H⁺ extrusion also appears to depend on microtubule integrity, as it was abolished in CCDs pretreated with colchicine.

Mathematical models for collecting duct acid excretion predict a strong flow dependence of urinary pH (80). In fact, functional studies in microperfused rabbit CCDs suggest that acid-base transport is regulated by tubular fluid flow. In microperfused CCDs poised for HCO₃⁻ secretion (e.g., isolated from alkali-loaded rabbits), an increase in tubular fluid flow rate from 1 to 5 nl·min⁻¹·mm⁻² leads to a reduction in the rate of net HCO₃⁻ secretion from −6 to 0 pmol·min⁻¹·mm⁻² (37). Other studies in which JHCO₃⁻ was measured in rabbit CCDs microperfused with a 25 mM HCO₃⁻, −120 mM Cl⁻ perfusate at variable flow rates also provides evidence for a flow dependence of acid-base transport (18, 19, 56, 70); specifically, these studies suggest that an increase in tubular fluid flow rate reduces net HCO₃⁻ secretion in rabbit CCDs. It should be noted that in contrast to rabbits, an increase in flow rate in rat CCD is associated with an increase, no change, or reduction in JHCO₃⁻ (summarized in Ref. 9).

In principle, a flow-induced change in net HCO₃⁻ transport in the CCD can reflect a change in either the rate of HCO₃⁻ secretion (by type B or β cells), H⁺ secretion (by type A or α cells), or both. Thus the apparent flow-induced reduction in JHCO₃⁻ in the rabbit CCD may be due to a reduction in HCO₃⁻ secretion by type B cells, an increase in H⁺ secretion by type A cells, or both. It is also possible that flow enhances both HCO₃⁻ secretion by type B cells as well H⁺ secretion by type A cells, but the flow-induced increase in H⁺ secretion exceeds that of HCO₃⁻ secretion. Furthermore, changes in transepithelial voltage (Vₑ) may affect the rate of H⁺ secretion; both luminal amiloride (39) and peritubular ouabain (29), inhibitors of apical ENaC and the basolateral Na⁺-K⁺-ATPase, respectively, decrease the rates of HCO₃⁻ absorption in rabbit CCDs that were initially absorbing HCO₃⁻. Increases in tubular fluid flow rate tends to reduce the lumen negative Vₑ (57).

Although principal cells in rabbit collecting tubule, albeit studied in a segment-specific manner, variably express all of the critical transport proteins necessary for H⁺ secretion and HCO₃⁻ reabsorption, including apical H⁺-ATPase and H⁺-K⁺-ATPase activities (75, 79) and basolateral Cl⁻/HCO₃⁻ exchange activity (76, 79), principal cells in the mid-CCD have
not traditionally been considered to have V-ATPase activity. We must thus acknowledge that our finding of a bafilomycin-sensitive H\(^+\) extrusion pathway in principal cells in this segment may be species and segment specific, unique to the rabbit mid-CCD. Additional support for species- and/or segment-specific expression of this pump is provided by the studies of Miller et al. (41). These investigators generated an intercalated cell-specific Cre-expressing transgenic mouse using an ATP6V1B1 promoter, which had previously been used to drive expression of enhanced green fluorescent protein (EGFP) (42), to drive expression of Cre recombinase. The ATP6V1B1-Cre transgenic mouse demonstrated active Cre in all intercalated cells (type A, B, and non-A/B cells) within the kidney, whereas it was not active in any other cell type except in \(\sim 50\%\) of principal cells within the connecting tubule only, a result similar to that observed in the B1-driven EGFP transgenic mice.

However, in support of functional evidence for H\(^+\)-ATPase activity in principal cells in rabbit CCD was the finding by Silver and Frindt (63), based on functional studies similar to those performed in the present study, that principal cells in this species have a mechanism for H\(^+\) extrusion that is not blocked by Sch-28080, an inhibitor of the H\(^+\)-K\(^+\)-ATPase. While the molecular identity of the H\(^+\) extrusion pathway in principal cells was not determined in the latter study, the authors proposed the presence of an electrodiffusive pathway for protons in these cells, a speculation supported by the observation that the imposition of a chemical driving force for protons by altering extracellular pH alters principal cell pH\(_i\) in a reversible manner (64) or that the Na\(^+\)-K\(^+\)-ATPase can substitute H\(^+\) for Na\(^+\) under Na\(^+\)-free conditions (47). It should also be noted that we detected bafilomycin-sensitive H\(^+\)-ATPase activity in principal cells acidified to a pH\(_i\) of \(\sim 6.2\) and that this Na\(^+\)- and K\(^+\)-independent recovery “turned off” at a pH\(_i\) of \(\sim 6.6\). Studies by Silver and Frindt (63) and others (76), in which principal cells were acid loaded to pH\(_i\) values \(>6.7\), would not have been expected to detect this transport pathway. A role for principal cells in maintaining acid-base balance has been further demonstrated by the finding that mice with targeted deletion of Rhcg in intercalated cells alone, subject to chronic acid loading to generate metabolic acidosis, demonstrate increased Rhcg immunolabel in principal cells in both the CCD and the OMCD (30), an adaptation proposed to contribute to acidosis-stimulated ammonia excretion.

Our detection of apical immunolabeling of principal cells, albeit less intense than that observed in intercalated cells, in rabbit CCDs using an antibody directed against the V-ATPase, provides additional support for the presence of an H\(^+\) pump in principal cells, as suggested by our finding of bafilomycin-sensitive pH\(_i\) recovery from an intracellular acid load in DBA-positive cells. While our detection of immunoreactive V-ATPase in principal cells in this study may reflect species-specific differences in V-ATPase subunit expression or differences between investigators in the methods used for immunolocalization, a careful review of the literature reveals evidence for immunoreactive H\(^+\)-ATPase in mammalian principal (or connecting tubule) cells. Alper et al. (1) reported a “delicate” labeling of the apical membranes of connecting tubule cells in rat cortex using an anti-V-ATPase E subunit antibody. Sabolic et al. (51) reported apical immunofluorescence labeling of principal cells in the rat cortical connecting segment using a polyclonal antibody directed against the vacuolar V-ATPase 31-kDa subunit. The 56-kDa B1 (ATP6V1B1) subunit was detected in principal cells in rat kidney (52), associated with endocytic vesicles that are involved in the vasopressin-stimulated recycling of water channels to and from the apical membrane. However, the endosomes in those principal cells did not acidify their lumen, leading the authors to suggest that the 56-kDa subunit might be involved in the recycling of aquaporin-2 (AQP2) without contributing to endosomal acidification. To add to this controversy, Gustafson et al. (23) reported that in LLC-PK\(_1\) cells, bafilomycin A1 blocks the recycling of AQP2, in the presence and absence of vasopressin, implicating a role for vesicle acidification via the V-ATPase in trafficking of AQP2, a process localized to principal cells. Based on the above, we propose that our current work brings important information to a controversial field, as we have confirmed the presence of a functional bafilomycin-sensitive H\(^+\) extrusion pathway in principal cells in the rabbit CCD and demonstrate immunoreactive V-ATPase subunits in these same cells.

In summary, increases in tubular (urinary) flow rate not only stimulate ENaC-mediated Na\(^+\) absorption and BK channel-mediated K\(^+\) secretion (53) but also, as demonstrated in the present study, H\(^+\)-ATPase activity in the rabbit mid-CCD. We speculate that flow stimulation of H\(^+\) secretion in the distal nephron may contribute to diuretic-induced metabolic alkalosis. The mechanosensors and mechanisms underlying flow stimulation of the vacuolar H\(^+\)-ATPase have yet to be explored.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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FLOW-DEPENDENT H⁺-ATPase ACTIVITY IN CCD


