Pyk2 regulates H\(^+\)-ATPase-mediated proton secretion in the outer medullary collecting duct via an ERK1/2 signaling pathway

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Fisher KD, Codina J, Petrovic S, DuBose TD Jr. Pyk2 regulates H\(^+\)-ATPase-mediated proton secretion in the outer medullary collecting duct via an ERK1/2 signaling pathway. Am J Physiol Renal Physiol 303; F1353–F1362, 2012. First published July 18, 2012; doi:10.1152/ajprenal.00008.2012.—Acid-secreting intercalated cells (A-ICs) respond to changes in systemic pH through regulation of apical H\(^+\) transporters. Little is known about the mechanism by which these cells sense changes in extracellular pH (pHe). Pyk2 is a nonreceptor tyrosine kinase activated by autophosphorylation at Tyr402 by cell-specific stimuli, including decreased pH, and is involved in the regulation of MAPK signaling pathways and transporter activity. We examined whether the Pyk2 and MAPK signaling pathway mediates the response of transport proteins to decreased pH in outer medullary collecting duct cells. Immunoblot analysis of phosphorylated Pyk2 (Tyr402), ERK1/2 (Thr202/Tyr204), and p38 (Thr180/Tyr182) was used to assay protein activation. To examine specificity of kinase activation and its effects, we used Pyk2 siRNA and PP 1 prevented Pyk2 phosphorylation as well as increased the phosphorylation of Pyk2, ERK1/2, and p38. Consistent with our previous studies, we found that mOMCD1 cells exhibit H\(^+\)-ATPase and H\(^+\)-ATPase activity. Pyk2 inhibition by Pyk2 siRNA and PP 1 prevented Pyk2 phosphorylation as well as H\(^+\)-ATPase-mediated recovery in mOMCD1 cells. In addition, ERK1/2 inhibition by U0126 prevented acid-induced ERK1/2 phosphorylation and H\(^+\)-ATPase-mediated pH\(_i\) recovery but not phosphorylation of p38. We conclude that Pyk2 and ERK1/2 are required for increasing H\(^+\)-ATPase, but not H\(^+\)-ATPase, activity at decreased pH in mOMCD1 cells.

intracellular pH; kinase; regulation of H\(^+\) transport; acidosis; outer medullary collecting duct; Pyk2

THE KIDNEY PLAYS A MAJOR ROLE in maintaining acid-base homeostasis. Metabolism of dietary protein from a typical Western diet in a 70-kg subject produces ~70 mmol of nonvolatile acid per day, which is released into the extracellular space (15). Epithelial cells of the kidney collecting duct “sense” small decreases in systemic pH and respond by increasing H\(^+\) secretion and HCO\(_3\)\(^-\) absorption to defend against metabolic acidosis (MA; Refs. 6, 15, 19). The underlying condition or disease causing MA can vary, but typically MA accompanies and accelerates the progression of chronic kidney disease (55). The kidney collecting duct consists of three segments: cortical, outer medullary (OMCD), and inner medullary collecting duct, and is responsible for final regulation of net acid excretion (9, 15, 58) and maintenance of acid-base homeostasis. The OMCD exhibits the highest rate of acid secretion, which is mediated specifically by type A acid-secreting intercalated cells (A-IC; Refs. 15, 46). A-ICs respond to metabolic acidosis by increasing apical proton secretion via H\(^+\)-ATPase (14, 56, 57), via the gastric and colonic H\(^+\)-ATPases (9, 11, 31, 61), and by increasing basolateral bicarbonate absorption via the bicarbonate/chloride exchanger anion exchanger 1 (AE1; Refs. 1, 7). Distal renal tubular acidosis (RTA type 1) is a specific example of abnormal net H\(^+\) transport and is characterized clinically, in the complete form, by an inability of the kidney to excrete net acid appropriately, resulting in positive net acid balance and decreased systemic pH. One example of the inherited forms of distal RTA has been linked to mutations in the apical H\(^+\)-ATPase in the collecting duct (18, 48).

In view of the role of the collecting duct in final adjustments of the overall acid–base status and fine tuning of kidney net acid secretion, it seems reasonable to assume that A-IC cells “sense” and respond to increases in acid load by increasing acid secretion. However, the precise mechanism involving pH sensor and afferent signaling pathways that mediate upregulation of acid secretion via the H\(^+\)-ATPase and/or H\(^+\)-ATPase have not been completely elucidated.

One of the putative pH sensors in the kidney, Pyk2, is a 116-kDa nonreceptor tyrosine kinase that is expressed in the central nervous system (35), cartilage (30), vascular smooth muscle (41), and kidney (28, 49). Pyk2 activation by autophosphorylation at Tyr402 occurs in response to a variety of extracellular cell-specific stimuli, such as neuronal membrane depolarization (47), the inflammatory cytokine TNFα in hematopoietic cells (12), cartilage-destroying fibronectin fragment in chondrocytes (30), and decreased pH in proximal renal tubule cells (28, 40). Pyk2-mediated signaling pathways have a wide range of physiological effects, depending on cell type, including regulation of ion transport (27, 28, 40), cell movement via focal adhesion spreading (44), cell proliferation (50), and vasoconstriction (32). Activated Pyk2 leads to phosphorylation of mitogen-activated protein kinases (MAPK) including ERK1/2 (21, 35, 41), p38 (33, 38, 49), and c-Jun NH\(_2\)-terminal kinase (JNK; Refs. 20, 63), which mediate the physiological effects of Pyk2.

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Pyk2 has been shown to regulate the coupling of receptor proteins to MAPK signaling pathways, such as integrins (5, 30), G-protein-coupled receptors (13, 35), vascular endothelial growth factor receptor (33), and epidermal growth factor receptor (45, 51). It has been reported previously that Pyk2 is involved in signaling pathways initiated by endothelin (20) and angiotensin (52). Both hormones are involved in the regulation of H+/ATPase-mediated acid secretion in the collecting duct, for example (39, 43, 59).

In the proximal tubule, ERK1/2 signaling pathways regulate acid stimulation of the sodium-proton exchanger NHE3 and sodium-coupled bicarbonate/citrate cotransporter NaDC-1 (28, 40, 53). In the collecting duct, ERK has been shown to activate H⁺,K⁺-ATPase activity in a cAMP-dependent manner (25) and also activates the Na⁺,K⁺-ATPase (34) but inhibits the ROMK channel (60). In bicarbonate-secreting (type B) intercalated cells (B-ICs) of the cortical collecting duct, ERK1/2 is involved in calcitonin-induced activation of H⁺,K⁺-ATPase through a cAMP-dependent, PKA-independent pathway involving Epac1, Rap1, and B-Raf (26).

The purpose of this study was to determine whether the acid-activated Pyk2 signaling pathway underlies the upregulation of H⁺ transporter activity in mouse-derived OMCD (mOMCD1) cells. Our results indicate that Pyk2 is expressed in mOMCD1 cells. We demonstrate further that the adaptation of mOMCD1 cells to a reduction in pH may be facilitated by a Pyk2-mediated ERK1/2 signaling pathway that regulates the apical H⁺-ATPase but not the H⁺,K⁺-ATPase.

### MATERIALS AND METHODS

#### Cell Culture

The mOMCD1 cell line is an immortalized cell line cultured from a microdissected tubule isolated from the inner stripe of the OMCD of a mouse transgenic for the early region of SV40. This cell line was previously characterized by our laboratory (17) and others (31). In the present study, mOMCD1 cells were grown at 37°C in a 5% CO₂ humidified incubator in DMEM/F-12 (1:1) media supplemented with 100 U/l penicillin, 100 µg/ml streptomycin, and 10% FBS. Cells were confluent and incubated in serum-free media for 24 h before starting an experiment.

**Incubation of cells in acid media (extracellular pH 6.7).** Confluent, quiescent mOMCD1 cells were incubated for 2 h in antibacterial-free and serum-free DMEM/F-12 media, buffered using 35 mM HEPES and calibrated to pH 7.4 using NaOH. The ionic strength was corrected by addition of NaCl. With the exception of the cells used for the zero time-point, each dish was incubated in acid media (pH 6.7) for one of the following time periods: 1, 3, 5, 10, or 15 min, and then lysed. Cells, growing in 3.5-cm Petri dishes, were lysed using 200-µl 2× Laemmli sample buffer (23) containing 10% β-mercaptoethanol, scraped, and sonicated.

### Intracellular acidification using an NH₄Cl prepulse.

We employed the NH₄Cl prepulse, a widely accepted technique to study the effect of decreased intracellular pH (pHi) on H⁺ transport (17, 29, 42). The solutions used to manipulate pH, (for the NH₄Cl prepulse) are shown in Table 1. Renal epithelial cells recover from an acid load by increasing the rate of proton transport and acid extrusion. The rate of pHi recovery (dpHi/dt) varies depending on the presence and activity of specific transporters (6, 17). To measure changes in pHi, confluent monolayers of mOMCD1 cells grown on coverslips were incubated for 10 min in physiological saline solution (PSS) containing 10 µM BCECF-AM, followed by incubation in PSS (see Table 1) for 10 min at 37°C. Coverslips were then transferred into a quartz cuvette and placed into the chamber of a fluorospectrophotometer [Deltascan Photon Technology International, South Brunswick, NJ] at a 45° angle to the light beam of the high-speed multiwavelength illuminator. The coverslip was then perfused with PSS, and an intracellular acid load was induced using an NH₄Cl prepulse in Na⁺, K⁺, HCO₃⁻-free solutions, as previously described by our laboratory and others (17, 22).

The solutions used for NH₄Cl prepulse are shown in Table 1. They were sequentially and continuously perfused through the cuvette and changed at the following time-points: PSS for 5 min, NH₄Cl solution for 5 min, 0K⁺/0Na⁺ or 5K⁺/0Na⁺ solution for 10 min, followed by PSS for 10 min. BCECF was alternately excited at 488 nm (pH-sensitive wavelength) and 440 nm (pH-insensitive wavelength, the isosbestic point for BCECF). Fluorescence intensity was measured at 535 nm. Felix software (Photon Technology International, NJ) was used for data acquisition and analysis. At the end of each experiment, the ratio of 488/440 was converted to pHi, using a fluorescence-pHi ratio calibration curve obtained with pH 7.5, 7.0, and 6.5 solutions containing 10 µM nigericin and high K⁺ solutions, as previously described (17). pHi recovery was measured as the rate of pHi change, expressed as ΔpHi/min, and interpreted as reflecting apical proton secretion.

For parallel immunoblots of the phosphorylation of Pyk2 and MAPKs (ERK1/2 and p38) during an NH₄Cl prepulse, 3.5-cm dishes of confluent mOMCD1 cells were lysed at the following time points during an NH₄Cl prepulse: 2-min incubation in PSS, after 5 min in the NH₄Cl solution, 1-min incubation in the 0K⁺/0Na⁺ solution, 5-min incubation in the 5K⁺/0Na⁺ solution, and after 5 min of the final incubation in PSS solution. For immunoblot analysis of cells at “normal pHi,” cells were incubated in PSS for 2 min and lysed. For immunoblot analysis of cells at “acid pHi,” cells were lysed after 5 min in PSS, followed by 5 min in NH₄Cl solution and then 1 min in 0K⁺/0Na⁺ solution (Table 1). This point in the NH₄Cl prepulse corresponds to the minimum pHi achieved during the prepulse (see representative tracing in Fig. 2A at 10 min).

### Small Interfering Pyk2 Small Interfering RNA

Following the approach described by Nicodemo et al. (35) and Preisig (40), 80% confluent mOMCD1 cells, in 3.5-cm Petri dishes, were transiently transfected with 25 nM (final concentration) ON-TARGETplus nontargeting pool (control) small interfering (si)RNA (Dharmacon, Lafayette, CO. no. D-001810–10) or ON-TARGETplus SMARTpool mouse PTK2B siRNA (Dharmacon no.

### Table 1. Composition of solutions used in the NH₄Cl prepulse

<table>
<thead>
<tr>
<th>Solution</th>
<th>CaCl₂</th>
<th>MgSO₄</th>
<th>Glucose</th>
<th>HEPES</th>
<th>NaCl</th>
<th>KCl</th>
<th>NH₄Cl</th>
<th>NMDG</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSS</td>
<td>1.8</td>
<td>0.8</td>
<td>5.5</td>
<td>10</td>
<td>135</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>1.8</td>
<td>0.8</td>
<td>5.5</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0K⁺/0Na⁺</td>
<td>1.8</td>
<td>0.8</td>
<td>5.5</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>120</td>
</tr>
<tr>
<td>5K⁺/0Na⁺</td>
<td>1.8</td>
<td>0.8</td>
<td>5.5</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>140</td>
</tr>
</tbody>
</table>

Final composition of each solution is shown in mM. All solutions were adjusted to pH 7.4 and maintained at 37°C. PSS, physiological salt solution; NMDG, N-methyl-d-glucamine chloride.

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inhibitors on the phosphorylation of Pyk2 and ERK1/2 on pHi recovery. PP1 is known to inhibit Src family kinases, including Pyk2 (21, 29).

**Antibodies**

- Monoclonal anti-p-Pyk2 (the antibody specifically detects phosphorylated Pyk2 at Tyr 402; Santa Cruz Biotechnology no. sc-101790); monoclonal anti-ERK (Santa Cruz Biotechnology no. sc-135900); monoclonal anti-β-actin (Sigma-Aldrich no. A5316); Alexa Fluor 680 goat anti-mouse (Invitrogen no. A-21058); and Alexa Fluor 680 goat anti-rabbit (Invitrogen no. A-21109).

**Results**

Acute Decrease in pHr, Induces a Decrease in pH1, and an Increase in Pyk2 and MAPK Phosphorylation

Incubation of mOMCD1 cells at decreased pHr, 6.7 decreased pH1 from 7.4 to 7.1 ± 0.1, as illustrated in a representative tracing in Fig. 1A (n = 4). We next tested whether acid pH elicits phosphorylation of Pyk2 and MAPKs. Each 3.5-cm Petri dish of confluent and quiescent mOMCD1 cells was lysed after incubation at media pH1, 6.7 for the time indicated above each lane of the representative immunoblot, from 0 to 15 min (Fig. 1B). The data were normalized using β-actin. We observed a rapid increase in Pyk2 phosphorylation (210% of control) that peaked after a 1-min incubation in acid media and lasted through a 3-min incubation (130% of control; P < 0.05; n = 7) as illustrated in the line graph in Fig. 1C. Total Pyk2 did not change in response to decreased pH (Fig. 1B). ERK1/2 phosphorylation increased after a 3-min incubation in acid media (420%), peaked at 5 min (480%), and lasted through 10 min (400%; P < 0.05, n = 9), as shown in a representative immunoblot in Fig. 1B and summarized in a line graph in Fig. 1D. ERK1/2 expression did not change in response to decreased pH (Fig. 1B). Finally, phosphorylation of p38 also increased after a 5-min incubation in acid media (650%; P < 0.05; n = 4; Fig. 1, B and E). The antibody used to detect phosphorylated Pyk2 is specific for phosphorylated Tyr402, which is the widely accepted autophosphorylation site on Pyk2. Increased autophosphorylation at Tyr402 is generally interpreted as Pyk2 activation (24, 28, 40).

**Acute Decrease in pHr, Using an NH4Cl Prepulse Increases Pyk2 and MAPK Phosphorylation**

The second, more direct method to manipulate pHr is the NH4Cl prepulse. Figure 2A illustrates a representative tracing of changes in pHr during a NH4Cl prepulse obtained using BCECF-AM loaded mOMCD1 cells. Since the NH4Cl prepulse produced a sustained decrease in pHr and allowed measurement of dpH/dr, providing a functional “readout” of the various conditions examined, we used the NH4Cl prepulse in the remainder of the experiments.

The tracing in Fig. 2A aligns with representative immunoblots of p-Pyk2, p-ERK1/2, and p-p38 shown in Fig. 2B. Data were normalized using β-actin. Bar graphs in Fig. 2, C–E, summarize mean band intensities and compare the first lane (normal pH1) to the third lane (acid pH1) of the immunoblot (Fig. 2B). These graphs demonstrate that acid pH elicits phosphorylation of Pyk2 (Fig. 2C), ERK1/2 (Fig. 2D), and p38 (Fig. 2E). Pyk2 phosphorylation increased 333% of control at acid pH1 (P < 0.01; n = 10), ERK1/2 increased 220% of control (P < 0.01; n = 16), and p38 increased 230% of control (P < 0.05; n = 5). There was no significant change in JNK phosphorylation.
Fig. 1. An acute decrease in extracellular pH (pHₐ) reduces intracellular pH (pHᵢ) and increases Pyk2 and MAPK phosphorylation. Representative tracing in A shows that decreasing extracellular pH to 6.7 elicits a parallel decrease in intracellular pH in 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein-acetoxyethyl ester (BCECF-AM)-loaded mouse-derived outer medullary collecting duct (mOMCD1) cells. B: representative immunoblots of confluent mOMCD1 cells, which were lysed after incubation in acid media (pHₐ 6.7) for the time indicated above each lane. Data were normalized using β-actin. Line graphs show mean band intensities illustrating phosphorylation of Pyk2 (p-Pyk2; C, n = 7), ERK1/2 (p-ERK1 + p-ERK2; D, n = 9), and p38 (p-p38; E, n = 4) as a function of time (*P < 0.05, **P < 0.01 vs. untreated cells). In this and Figs. 2–6, molecular mass of Pyk2 and p-Pyk2 was ~116 kDa, ERK1 and p-ERK1 was ~44 kDa, ERK2 and p-ERK2 was ~42 kDa, p-p38 was ~38 kDa, and β-actin was ~45 kDa.

Pyk2 siRNA Blocks acid pHᵢ-Induced ERK1/2 Phosphorylation

mOMCD1 cells were transfected with control siRNA or Pyk2 siRNA. After 48 h, the cells were lysed at normal or acid pHᵢ, pHₐ was decreased using an NH₄Cl prepulse and protein phosphorylation and expression were analyzed by immunoblot. Knockdown of Pyk2 was confirmed by assessing the level of total Pyk2 expression in cells transfected with Pyk2 siRNA. Pyk2 siRNA decreased expression of total Pyk2 84% (P < 0.05; n = 4), as illustrated in the representative immunoblot in Fig. 3A. Compared with the cells transfected with the control siRNA, a decrease in the level of total Pyk2 resulted in attenuation of Pyk2 phosphorylation at normal pHᵢ (P < 0.05; n = 4; Fig. 3B). Compared with the cells transfected with the control siRNA and incubated at acid pHᵢ, Pyk2 phosphorylation was significantly reduced in cells transfected with Pyk2 siRNA and incubated at acid pHᵢ (88% inhibition; P < 0.01; n = 4), as shown in Fig. 3A and in the bar graph summarizing mean band intensities in Fig. 3B. We next examined the effect of Pyk2 knockdown on MAPK phosphorylation at acid pHᵢ. The representative blots are shown in Fig. 3A. A decrease in Pyk2 levels caused by Pyk2 siRNA significantly attenuated ERK1/2 phosphorylation at normal pHᵢ (60% inhibition) as well as at acid pHᵢ (75% inhibition; P < 0.05; n = 4; Fig. 3A, summarized in Fig. 3C) without affecting total ERK1/2 expression (Fig. 3A). Pyk2 knockdown had a marginal effect on the acid-induced phosphorylation of p38 (n = 4; Fig. 3A, summarized Fig. 3D). Taken together, these data indicate that Pyk2 is required for ERK1/2 phosphorylation by acid pHᵢ in mOMCD1 cells. Table 2 displays the same data for which p-Pyk2 and p-ERK1/2 expression was corrected by total Pyk2 and total ERK1/2 kinase expression, respectively.
PP 1 Inhibits Acid pH-induced Pyk2 and ERK1/2 Phosphorylation

In the next set of experiments, we used the Src kinase inhibitor PP 1 (50 μM) to inhibit Pyk2 and determine its effect on MAPK phosphorylation by acid pH in mOMCD1 cells. A representative immunoblot of the effects of PP 1 pretreatment on Pyk2, ERK1/2, and p38 phosphorylation at acid pH is shown in Fig. 4A. Compared with the control cells, PP 1 pretreatment significantly reduced phosphorylation of Pyk2 at acid pH (91% inhibition; \( P < 0.05; n = 3 \); Fig. 4B). PP 1 pretreatment, attenuated ERK1/2 phosphorylation at both normal pH (99% inhibition; \( P < 0.05; n = 3 \)) as well as at acid pH (99% inhibition; \( P < 0.01; n = 3 \); Fig. 4C). PP 1 pretreatment attenuated p38 phosphorylation in acid pH by 56% (\( P < 0.05; n = 3 \); Fig. 4D). In summary, inhibition of Pyk2 phosphorylation with PP 1 abolished Pyk2 and ERK1/2 phosphorylation at acid pH, as well as p38 phosphorylation, although to a lesser degree. Table 2 displays the

Table 2. Summary of data displayed in Figs. 2–5 showing normalization of phosphorylation band intensity (Pyk2 and ERK1/2) by total kinase expression

<table>
<thead>
<tr>
<th>Condition</th>
<th>Normal pH</th>
<th>Acid pH</th>
<th>Normal pH</th>
<th>Acid pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cells</td>
<td>0.433 ± 0.076</td>
<td>1.810 ± 0.086*</td>
<td>0.397 ± 0.021</td>
<td>0.920 ± 0.093*</td>
</tr>
<tr>
<td>Pyk2 siRNA</td>
<td>0.487 ± 0.037</td>
<td>0.532 ± 0.018</td>
<td>0.337 ± 0.058</td>
<td>0.542 ± 0.0759</td>
</tr>
<tr>
<td>PP 1</td>
<td>0.444 ± 0.012</td>
<td>0.482 ± 0.081</td>
<td>0.413 ± 0.043</td>
<td>0.473 ± 0.114</td>
</tr>
<tr>
<td>U0126</td>
<td>0.482 ± 0.082</td>
<td>1.709 ± 0.282*</td>
<td>0.304 ± 0.045</td>
<td>0.258 ± 0.041</td>
</tr>
</tbody>
</table>

Values are means ± SE. Data are essentially identical to corresponding data normalized by β-actin expression as displayed in Figs. 2-5. siRNA, small interfering RNA; PP 1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine. *\( P < 0.01 \) vs. normal pH.
summary, inhibition of ERK1/2 with U0126 abolished most of ERK1/2 phosphorylation, without affecting Pyk2 or p38 phosphorylation. Table 2 displays the same data for which p-Pyk2 and p-ERK1/2 expression were normalized by total Pyk2 and total ERK1/2 kinase expression, respectively.

**Inhibition of Pyk2 and ERK1/2 Blocks H⁺-ATPase-Mediated pHᵢ Recovery**

To correlate the phosphorylation profile of Pyk2 and ERK1/2 with the rate of ATPase-mediated pHᵢ recovery, we used the pH-sensitive probe BCECF-AM in mOMCD1 cells. The average rate of K⁺⁻ and Na⁺⁻independent recovery after an NH₄Cl prepulse, indicating H⁺⁻ATPase-mediated proton secretion, was 0.022 ± 0.002 pH U/min, (n = 4). A representative tracing is shown in Fig. 6A, and the average rate is summarized in Fig. 6C. To confirm that the observed proton secretion was mediated by H⁺⁻ATPase, we used bafilomycin A1 (10 nM) as a specific H⁺⁻ATPase inhibitor (17, 36, 37, 54). Incubation of mOMCD1 cells with bafilomycin A1 resulted in a 90% inhibition of the rate of Na⁺⁻ and K⁺⁻independent pHᵢ recovery (0.002 ± 0.001 pH U/min; n = 3; P < 0.05 vs. 0.02 ± 0.001 pH U/min).

**U0126 Blocks Acid pHᵢ-Induced ERK1/2 Phosphorylation**

In the next set of experiments, we used U0126, an inhibitor of MEK1/2 that blocks ERK1/2 phosphorylation. A representative immunoblot of the effects of U0126 on Pyk2, ERK1/2, and p38 phosphorylation at acid pHᵢ in mOMCD1 cells is shown in Fig. 5A. Compared with control cells, U0126 pretreatment did not affect phosphorylation of Pyk2, as expected, and marginally decreased acid induced phosphorylation of p38 at acid pHᵢ (Fig. 5A, B, and D). As expected, U0126 significantly inhibited ERK1/2 phosphorylation at both normal pHᵢ (98% inhibition; P < 0.05; n = 3) and acid pHᵢ (99% inhibition; P < 0.01; n = 3), as shown in Fig. 5, A and C.
Acid activation of Pyk2 stimulates the H⁺-ATPase in the OMCD.

To ascertain the specificity of Pyk2 effects on the rate of ATPase-mediated pH recovery, we used BACEF-AM to measure proton secretion in mOMCD1 cells transfected with control or Pyk2 siRNA. H⁺-ATPase-mediated proton secretion measured as the average rate of K⁺ and Na⁺-independent recovery after an NH₄Cl prepulse in cells transfected with Pyk2 siRNA was 0.0017 ± 0.0005 pH U/min, which represents 92% inhibition compared with 0.02 ± 0.002 pH U/min in the cells transfected with the control siRNA (n = 4; P > 0.05; Fig. 6, B and C). The rate of pHᵢ recovery in cells transfected with Pyk2 siRNA increased in the presence of extracellular K⁺ (5 mM) to an average rate of 0.05 ± 0.01 pH U/min, representative of H⁺,K⁺-ATPase activity. Compared with the cells transfected with control siRNA (0.072 ± 0.001 pH U/min), this represents 31% inhibition but was not statistically significant (P > 0.05; n = 4), (Fig. 6, B and C). Cells transfected with control siRNA exhibited rates of pHᵢ recovery similar to the nontransfected, control mOMCD1 cells (data not shown). These data demonstrate involvement of Pyk2 in the stimulation of H⁺-ATPase-mediated pHᵢ recovery.

To further correlate the phosphorylation of Pyk2 and ERK1/2 with the rate of ATPase-mediated pHᵢ recovery, we inhibited Pyk2 and ERK1/2 substantially decreased the activity of H⁺-ATPase, measured as K⁺ and Na⁺-independent pHᵢ recovery. The activity of H⁺-ATPase was nearly abolished in cells treated with PP 1 (from 0.020 ± 0.002 pH U/min in control cells pretreated with DMSO to 0.002 ± 0.0002 pH U/min in cells treated with PP 1; n = 3; P < 0.01, 99% inhibition; Fig. 6, B and C). The ERK1/2 inhibitor U0126 inhibited 61% of H⁺-ATPase activity (from 0.025 ± 0.005 pH U/min in the control cells pretreated with DMSO to 0.0078 ± 0.003 pH U/min in cells treated with U0126; n = 3; P < 0.05; Fig. 6, B and C). Inhibition of Pyk2 by 50 µM PP 1, had a significant, but much less dramatic effect on H⁺-K⁺-ATPase. The activity of H⁺,K⁺-ATPase, measured as Na⁺-independent, K⁺-dependent pHᵢ recovery, decreased from 0.065 ± 0.004 pH U/min in the control cells to 0.04 ± 0.008 pH U/min in cells treated with PP 1 (n = 3, P < 0.05), a decrease of 33%, (Fig. 6C). Inhibition of ERK1/2 with U0126, however, did not affect H⁺,K⁺-ATPase activity, (pHᵢ recovery declined from 0.060 ± 0.003 pH U/min, in control cells, to 0.047 ± 0.01 pH U/min in cells treated with U0126; n = 3; P > 0.05 or 22% inhibition; Fig. 6C). Taken together, these data demonstrate that the Pyk2/Src complex and phosphorylated ERK1/2 mediate acid activation of the H⁺-ATPase.

**DISCUSSION**

Acid-secreting type A-IC cells of the OMCD are known to respond to changes in systemic pH through parallel regulation of luminal proton secretion and basolateral bicarbonate absorption (15, 46, 54), but the afferent pathways that sense acid pH and augment H⁺ transport are not completely understood. In our study, we report that Pyk2, a putative pH sensor in the kidney (28, 40), is expressed in mOMCD1 cells and responds rapidly to a reduction in pHᵢ by enhanced phosphorylation of Pyk2 at Tyr402 (Figs. 1, B and C; and 2, B and 2C), ERK1/2 at Thr180/Tyr182 (Figs. 1, B and D, and 2, B and D) and p38 at Thr202/Tyr204 (Figs. 1, B and E, and 2, B and E). This increase in phosphorylation is observed independently of the method used to normalize the phosphorylation data (correcting for expression of either β-actin or total kinase). This finding supports the possibility that phosphorylation of these kinases mediates an increase in proton secretion via the bafilomycin A1-sensitive H⁺-ATPase (Fig. 6).

To confirm the specificity of Pyk2 and ERK1/2 phosphorylation for the activation of the H⁺-ATPase...
ACID-ACTIVATION OF Pyk2 STIMULATES THE H⁺-ATPase IN THE OMCD

and H⁺,K⁺-ATPase by a reduction in pH, we examined the effects of molecular inhibition of Pyk2 mRNA stability (siRNA), chemical inhibition of Pyk2 phosphorylation (PP 1), and chemical inhibition of ERK1/2 phosphorylation (U0126). By knockdown of Pyk2 expression using Pyk2 siRNA or by inhibition of Pyk2 phosphorylation with PP 1 we blocked acid induced Pyk2 phosphorylation (Figs. 3, A and B, and 4, A and B) and acid induced ERK1/2 phosphorylation (Figs. 3, A and C, and 4, A and C). Each of these maneuvers inhibited stimulation of acid secretion by the H⁺-ATPase similarly (Fig. 6). The inhibition of p38 phosphorylation elicited by a reduction in pH was less evident (Figs. 3, A and D, and 4, A and D), suggesting that p38 is not downstream of Pyk2/ERK1/2. Taken together, our results indicate that the signaling pathway is initiated by the activation of Pyk2, followed by the activation of ERK1/2, and stimulation of the H⁺-ATPase. This interpretation is in agreement with the observations of Li et al. (28) and Preisig (40) who demonstrated Pyk2-mediated acid-activation of NHE3 in opossum kidney proximal tubule cells. In our studies using mOMCD1 cells, and in the studies by Li et al. (28) using opossum kidney proximal cells, Pyk2 phosphorylation increased within 2 min after reduction in pH, and was required for MAPK-mediated acid secretion (Fig. 6, B and C); however, the time frame to detect an increase in proton excretion was very different. In studies of Li’s et al., the experiments were performed after 6 h of acidosis induced by reduction of pH, and were consistent with an increase in NHE3 mRNA, as demonstrated by Amemiya et al. (3). Our experiments were performed a few minutes after the NH₄Cl prepulse, a time frame that suggests that mRNA synthesis was not involved.

Our studies demonstrate that Pyk2/ERK1/2 signaling plays an important role in acute stimulation of H⁺-ATPase-mediated acid secretion, providing further information on the mode of activation of the H⁺-ATPase in the OMCD. Alzamora et al. (2) and Gong et al. (16) have shown that pH-sensitive activation of H⁺-ATPase in the collecting duct involves phosphorylation of the pump subunits and is mediated by cAMP and PKA. Winter et al. (62) demonstrated that aldosterone regulates H⁺-ATPase by the PKA/cAMP pathway and by the PKC/Ca²⁺-mediated ERK signaling pathway that facilitates the nongenomic, more rapid effects of aldosterone on H⁺-ATPase activity. Our results appear to be consistent with the later possibility; however, the assumption that acid and aldosterone regulate apical H⁺-ATPase activity via a common pathway cannot be supported at this time. Further studies will be needed to determine the phosphorylation sites and ATPase subunits involved in the Pyk2-mediated upregulation of proton secretion by the H⁺-ATPase in OMCD in response to a decrease in pH.

In summary, we have shown for the first time that Pyk2 and ERK1/2 are activated by an acute reduction in pH in the OMCD in vitro (mOMCD1 cells). We corroborated that pH recovery after an NH₄Cl prepulse involves the regulation of both the H⁺-ATPase and the H⁺,K⁺-ATPase in these cells.

![Graph A](image1.png)

**A** Overlapping representative tracings of pH recovery. A: Overlapping representative tracings of pH recovery. B: Tracings in B show control cells in green, cells pretreated with U0126 in red and PP 1 in purple, and cells expressing Pyk2 siRNA in orange. Bar graph in C summarizes the average rates of pH recovery under a variety of inhibitory conditions as depicted along the x-axis. In each group, bar 1 from left should be compared with bar 3 (indicates H⁺-ATPase response to the inhibitor) and bar 2 to bar 4 (indicates H⁺,K⁺-ATPase response to the inhibitor). Otherwise, the effect of 5K⁺/0Na⁺ is evident when comparing bar 1 to 2 and 3 to 4. *P < 0.05 compared with control (5K⁺/0Na⁺) in the experiment designed to test the effect of PP 1) and **P < 0.01, ***P < 0.001, compares the H⁺-ATPase activity in each specific group.

![Graph B](image2.png)

**B** Representative tracings of pH recovery under a variety of conditions. A: Overlapping representative tracings of pH recovery under a variety of conditions. B: Representative tracings of pH recovery under a variety of conditions. C: Bar graph in C summarizes the average rates of pH recovery under a variety of inhibitory conditions as depicted along the x-axis. In each group, bar 1 from left should be compared with bar 3 (indicates H⁺-ATPase response to the inhibitor) and bar 2 to bar 4 (indicates H⁺,K⁺-ATPase response to the inhibitor). Otherwise, the effect of 5K⁺/0Na⁺ is evident when comparing bar 1 to 2 and 3 to 4. *P < 0.05 compared with control (5K⁺/0Na⁺) in the experiment designed to test the effect of PP 1) and **P < 0.01, ***P < 0.001, compares the H⁺-ATPase activity in each specific group.

![Graph C](image3.png)

**C** Overlapping representative tracings of pH recovery. A: Overlapping representative tracings of pH recovery. B: Tracings in B show control cells in green, cells pretreated with U0126 in red and PP 1 in purple, and cells expressing Pyk2 siRNA in orange. Bar graph in C summarizes the average rates of pH recovery under a variety of inhibitory conditions as depicted along the x-axis. In each group, bar 1 from left should be compared with bar 3 (indicates H⁺-ATPase response to the inhibitor) and bar 2 to bar 4 (indicates H⁺,K⁺-ATPase response to the inhibitor). Otherwise, the effect of 5K⁺/0Na⁺ is evident when comparing bar 1 to 2 and 3 to 4. *P < 0.05 compared with control (5K⁺/0Na⁺) in the experiment designed to test the effect of PP 1) and **P < 0.01, ***P < 0.001, compares the H⁺-ATPase activity in each specific group.
Moreover, our studies demonstrate that Pyk2-mediated MAPK signaling pathways stimulate H⁺-ATPase activity in mOMCD1 cells in response to a reduction in pH, (Fig. 6, B and C). That there was an inhibitory effect of Pyk2 siRNA, PP 1, and U0126 on ERK1/2 phosphorylation and K⁺-independent pH recovery demonstrates that the H⁺-ATPase is regulated by Pyk2 and ERK1/2. These results also suggest that acid activation of the H⁺,K⁺-ATPase may be regulated by a different signaling cascade than the H⁺-ATPase and will require further investigation. In conclusion, our studies demonstrate that Pyk2 may function as a pH sensor in mOMCD1 cells to increase the activity of the H⁺-ATPase, most likely via an ERK1/2-mediated signaling pathway.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

30. Loeser RF, Forsyth CB, Samarel AM, Im HJ. Fibroenectin fragment activation of proline-rich tyrosine kinase PYK2 mediates integrin signals.


