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

Kimberly D. Fisher,1 Juan Codina,1 Snezana Petrovic,1,2 and Thomas D. DuBose, Jr.1

1Sections on Nephrology and Molecular Medicine, Department of Internal Medicine, Wake Forest University School of Medicine, Winston-Salem, North Carolina; and 2Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, North Carolina

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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 exhibit 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 small interfering RNA to knockdown Pyk2 expression levels, the Src kinase inhibitor 4-amino-5-(4-methylphenyl)-7-((butyl)pyrazolo[3,4-d]-pyrimidine (PP 1) to inhibit Pyk2 phosphorylation, and the MEK inhibitor U0126 to inhibit ERK1/2 phosphorylation. The pH-sensitive fluorescent probe 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein-acetoxyethyl ester (BCECF-AM) was used to assay H⁺ transporter activity. The activity of H⁺ transporters was measured as the rate of intracellular pH (pHᵢ) recovery after an NH₄Cl prepulse. We show that Pyk2 is endogenously expressed and activated by acid pH in mouse-derived outer medullary collecting duct cells (mOMCD1). Incubation of mOMCD1 cells in acid media extracellular pH (pHₑ) 6.7 increased the phosphorylation of Pyk2, ERK1/2, and p38. Reduction in pHₑ induced by an NH₄Cl prepulse also increased the phosphorylation of Pyk2, ERK1/2, and p38. Consistent with our previous studies, we found that mOMCD1 cells exhibit H⁺-ATPase and H⁺,K⁺-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 pHᵢ recovery but not phosphorylation of p38. We conclude that Pyk2 and ERK1/2 are required for increasing H⁺-ATPase, but not H⁺,K⁺-ATPase, activity at decreased pHₑ 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 HCO₃⁻ 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⁺,K⁺-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⁺,K⁺-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 NH₂-terminal kinase (JNK; Refs. 20, 63), which mediate the physiological effects of Pyk2.

Address for reprint requests and other correspondence: T. D. DuBose, Jr., Dept. of Internal Medicine, Medical Center Boulevard, Winston-Salem, North Carolina 27157 (e-mail: tdubose@wakehealth.edu).

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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 the ROMK channel (60). In bicarbonate-secreting (type B) intercalated cells (B-ICs), ERK1/2 is involved in calcitonin-induced activation of H^+ ,K^+-ATPase activity has been shown to occur via a PKA-mediated pathway involving Ras and Raf-1 (25). In A-ICs, ERK1/2 is involved in calcitonin-induced activation of the 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 OMCD1 (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) (Sigma, St Louis, MO) supplemented with 100 U/l penicillin, 100 

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: 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.

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

<table>
<thead>
<tr>
<th></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>0</td>
<td>0</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>5</td>
<td>135</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.
L-040719-00). Transfection was performed in antibiotic-free and serum-free media using 8 μM (final concentration) Dharmafect transfection reagent (Dharmacon no. T-2001), as described by the manufacturer. After a 3-h incubation at 37°C, 1 ml media containing serum was added to each dish. Twenty-four hours after transfection, fresh serum-free media was added to cells. Forty-eight hours after transfection, cells were stimulated with or without an NH4Cl prepulse through incubation in 0K+/0Na+ solution for 1 min (normal or acid pH). Immunoblot was used to confirm knockdown of Pyk2 and analyze the effects of Pyk2 siRNA on the phosphorylation of Pyk2 and MAPKs. Equal loading of the gel was verified by β-actin quantification as described previously by our laboratory (10, 11); additionally, the quantification of phospho-kinases was normalized for total kinase expression.

Pyk2 and ERK1/2 Inhibition

4-Amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine (PP 1) is known to inhibit Src family kinases, including Pyk2 (21, 49, 62). U0126 is a highly selective inhibitor of the MEK1/2 kinases (4, 20). We tested the effect of these inhibitors on the phosphorylation of Pyk2 and ERK1/2 (4, 20). We followed the approach described previously by our laboratory (21, 49, 62). U0126 is a highly selective inhibitor of the MEK1/2 kinases (4, 20). We tested the effect of these inhibitors on the phosphorylation of Pyk2 and ERK1/2 on pH recovery in mOMCD1 cells. Cells were preincubated in serum-free media containing PP 1 (50 μM) for 30 min at 37°C before stimulation or in serum-free media containing U0126 (10 μM) for 30 min 2 h before stimulation. The potential effect of DMSO was verified by addition of DMSO to control groups. Inhibition of Pyk2 and ERK1/2 phosphorylation by PP 1 and U0126, respectively, was confirmed by an immunoblot. Equal loading was verified as described above.

Inhibition of H+•-ATPase

Confluent mOMCD1 cells were pretreated with bafilomycin A1 (10 nM), a specific inhibitor of the H+•-ATPase (17, 36, 37, 54), for 30 min at 37°C before stimulation using an NH4Cl prepulse. The same concentration of bafilomycin A1 was maintained during the 0K+/0Na+ or 5K+/0Na+ treatment during pHi recovery.

Immunoblots

We followed the approach described previously by our laboratory (8, 10). Briefly, 50 μl of each sample were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Equal loading of the gel was verified by staining the membranes in Ponceau S solution and later by immunoblot of phosphoblots shown in Fig. 1, B, C, and E. The antibody used to detect phosphorylated Pyk2 is specific for phosphorylated Tyr402, which is the widely accepted autophosphorylation site on Pyk2 (24, 28, 40).

Antibodies

Monoclonal anti-p-Pyk2 (the antibody specifically detects phosphorylation of Pyk2 at Tyr 402; Santa Cruz Biotechnology no. sc-101790); polyclonal anti-Pyk2 (Santa Cruz Biotechnology no. sc-1515); monoclonal anti-ERK (Santa Cruz Biotechnology no. sc-135900); monoclonal anti-p-ERK1/2 (Thr202/Tyr204) (Santa Cruz Biotechnology no. sc-7378); polyclonal anti-p-p38 (Thr180/Tyr182)-R (Santa Cruz Biotechnology no. sc-17852-R); 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).

Statistical Analysis

Significance of data was determined using t-tests of the mean value of multiple independent experiments indicated, n-values shown in RESULTS. Two-tailed two-sample t-tests assuming equal variance were used to analyze the significance of our data (in our data, *P < 0.05, **P < 0.01). Error bars represent means values ± SE. Statistical analyses were done using PSI-Plot Version 7.5 (Poly Software International, Pearl River, NY).

RESULTS

Acute Decrease in pHi, Induces a Decrease in pH, and an Increase in Pyk2 and MAPK Phosphorylation

Incubation of mOMCD1 cells at decreased pHi, 6.7 decreased pHi 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 pHi 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 = 5), 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 pH, Using an NH4Cl Prepulse Increases Pyk2 and MAPK Phosphorylation

The second, more direct method to manipulate pHi is the NH4Cl prepulse. Figure 2A illustrates a representative tracing of changes in pHi during a NH4Cl prepulse obtained using BCECF-AM loaded mOMCD1 cells. Since the NH4Cl prepulse produced a sustained decrease in pH (Fig. 2B), which is the widely accepted autophosphorylation site on Pyk2, increased autophosphorylation at Tyr402 is generally interpreted as Pyk2 activation (24, 28, 40).

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 pH) to the third lane (acid pH) 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 pH (P < 0.01; n = 10), ERK1/2 increased 220% of control (P < 0.01; n = 16), p38 increased 230% of control (P < 0.05; n = 5). There was no significant change in JNK
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, pHᵢ 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 phosphorylation 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, and in the bar graph summarizing mean band intensities at acid pHᵢ 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, 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, and in the bar graph summarizing mean band intensities in Fig. 3B.
PP 1 Inhibits Acid pH1-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 pH1 in mOMCD1 cells. A representative immunoblot of the effects of PP 1 pretreatment on Pyk2, ERK1/2, and p38 phosphorylation at acid pH1 is shown in Fig. 4A. Compared with the control cells, PP 1 pretreatment significantly reduced phosphorylation of Pyk2 at acid pH1 (91% inhibition; \(P < 0.05; n = 3\); Fig. 4B). PP 1 pretreatment, attenuated ERK1/2 phosphorylation at both normal pH1 (99% inhibition; \(P < 0.05; n = 3\)) as well as at acid pH1 (99% inhibition; \(P < 0.01; n = 3\); Fig. 4C). PP 1 pretreatment attenuated p38 phosphorylation in acid pH1 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 pH1, 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>p-Pyk2/Pyk2</th>
<th>p-ERK1,2/ERK1,2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal pH</td>
<td>Acid pH</td>
</tr>
<tr>
<td>Control cells</td>
<td>0.433 ± 0.076</td>
<td>1.810 ± 0.086*</td>
</tr>
<tr>
<td>Pyk2 siRNA</td>
<td>0.487 ± 0.037</td>
<td>0.532 ± 0.018</td>
</tr>
<tr>
<td>PP 1</td>
<td>0.444 ± 0.012</td>
<td>0.482 ± 0.081</td>
</tr>
<tr>
<td>U0126</td>
<td>0.482 ± 0.082</td>
<td>1.709 ± 0.282*</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-buty1)pyrazolo[3,4-d]-pyrimidine. *\(P < 0.01\) vs. normal pH.
Acid pH reduces acid-induced phosphorylation of p38, ERK1/2 (p-ERK1/2), and Pyk2. Representative immunoblot in A and the corresponding bar graphs show the effects of control siRNA and Pyk2 siRNA on acid-induced phosphorylation of Pyk2 (B), ERK1/2 (p-ERK1/2), and p38 (D). Cells were lysed at normal pHi (N) or acid pHi (A). The acid pHi was induced by an NH4Cl prepulse. Data were normalized using β-actin and the graphs illustrate the mean band intensity. In the statistical analysis of the data displayed in panels B-D, bar 1 from the left was compared with bar 3, and bar 2 with bar 4. These comparisons emphasize the effect of acute intracellular acidosis induced by an NH4Cl prepulse. Similar comparisons were done in Figs. 4 and 5. In Figs. 3–5, as in the experiments described above, *P < 0.05 and **P < 0.01. These same data were normalized as well for total kinase expression (Table 2), and the results are not statistically different.

**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 pre-treatment 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. 5A and C. In 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 ± 0.002 pH U/min for normal conditions). A comparison of the rate of ATPase-mediated pHi recovery with and without U0126 pretreatment is illustrated in Fig. 6B. These data were normalized and compared as described in Fig. 3. As expected, U0126 inhibited pHi recovery by 98% (P < 0.05, **P < 0.01, vs. control cells; n = 3). B-D were compared as described in Fig. 3. As these same data were normalized as well for total kinase expression (Table 2), and the results are not statistically different.
Acid activation of Pyk2 stimulates the H^+-ATPase in the OMCD

**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 pH_i by enhanced phosphorylation of Pyk2 at Tyr402 (Figs. 1, B and C) and 2) as the average rate of pH_i recovery in the presence of 5 mM K^+ and bafilomycin A1, which inhibited H^+-ATPase activity (0.045 ± 0.01 pH U/min; n = 3, Figs. 6, A and C). Taken together, these data demonstrate H^+-ATPase and H^+,-K^+-ATPase mediate pH_i recovery in mOMCD1 cells in response to an acute decrease in pH_i.

To ascertain the specificity of Pyk2 effects on the rate of ATPase-mediated pH_i recovery, we used BCECF-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 NH_4Cl 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 pH_i 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 pH_i recovery similar to the nontransfected, control mOMCD1 cells (data not shown). These data demonstrate involvement of Pyk2 in the stimulation of H^+,-ATPase-mediated pH_i recovery.

To further correlate the phosphorylation of Pyk2 and ERK1/2 with the rate of ATPase-mediated pH_i recovery, we inhibited Pyk2 and ERK1/2 substantially decreased the activity of H^+,-ATPase, measured as K^- and Na^-independent pH_i 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 0.0020 ± 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^+,-ATPase activity (from 0.025 ± 0.005 pH U/min in cells treated with PP 1; n = 3, P > 0.05 (99% inhibition; Fig. 6C). Inhibition of ERK1/2 with U0126, however, did not affect H^+,-ATPase activity, (pH_i 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.

**Fig. 5.** U0126 blocks acid pH_i induction of ERK1/2 phosphorylation. Representative immunoblot in A and the corresponding bar graphs show the effects of U0126 on acid-induced phosphorylation of Pyk2 (B), ERK1/2 (p-ERK1+ p-ERK2; C), and p38 (D). Cells were pretreated with 0.05% DMSO (control) or U0126 (10 μM) for 30 min before stimulation. Cells were lysed at normal pH_i (N) or acid pH_i (A). Acid pH_i was induced by an NH_4Cl prepulse. Data were normalized using β-actin and the graphs illustrate the mean band intensity. *P < 0.05, **P < 0.01, vs. control cells; n = 3. B-D were compared as described in Fig. 3. These same data were normalized as well for total kinase expression (Table 2), and the results are not statistically different.

0.002 in Fig. 6). Compared with Na^+ and K^-independent pH_i recovery, representing H^+-ATPase-mediated pH_i recovery, the rate of pH_i recovery increased in the presence of extracellular K^+ (5 mM) to an average rate of 0.072 ± 0.01 (n = 4) pH U/min (representative of H^+,-K^+-ATPase plus H^+-ATPase activity) vs. 0.022 ± 0.002 pH U/min (representing the H^+,-ATPase; n = 4, P < 0.001; Fig. 6, A and C). The activity of H^+,-K^+-ATPase was calculated as 1) the difference in the average rates of pH_i recovery before and after K^- addition (0.052 ± 0.0003 pH U/min; n = 4; Fig. 6, A and C); and 2) as the average rate of pH_i recovery in the presence of 5 mM K^- and bafilomycin A1, which inhibited H^+-ATPase activity (0.045 ± 0.01 pH U/min; n = 3, Figs. 6, A and C). Taken together, these data demonstrate H^+-ATPase and H^+,-K^+-ATPase mediate pH_i recovery in mOMCD1 cells in response to an acute decrease in pH_i.
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 pHi.

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.

Fig. 6. Inhibition of Pyk2 and ERK1/2 blocks H⁺-ATPase-mediated pH recovery. pH was measured in BCECF-AM loaded mOMCD1 cells during an NH₄Cl prepulse. A: overlapping representative tracings of pH in the presence or absence of extracellular K⁺ (5 mM) and in the presence or absence of bafilomycin A1 (10 nM). Na⁺-independent, K⁺-independent pH recovery (0K⁺/0Na⁺) is shown in green and the effect of 10 nM bafilomycin A1 on Na⁺-independent, K⁺-independent pH recovery (0K⁺ + Baf) in gray. Na⁺-independent, K⁺-dependent pH recovery (5K⁺/0Na⁺) is shown in blue and the effect of 10 nM bafilomycin A1 on K⁺-dependent pH recovery (5K⁺ + Baf) is shown in black. B: representative tracings of the effect of Pyk2 and ERK1/2 inhibitors on Na⁺-independent, K⁺-independent pH recovery (0K⁺/0Na⁺). 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⁺-ATPase response to the inhibitor). Otherwise, the effect of 5K⁺ 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 pHi (Fig. 6, A and C). That there was an inhibitory effect of Pyk2 siRNA, PP1, and U0126 on ERK1/2 phosphorylation and K\(^+\)-independent pHi 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


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