A central role for Pyk2-Src interaction in coupling diverse stimuli to increased epithelial NBC activity

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1Section of Nephrology, Department of Medicine, University of Illinois at Chicago, Chicago 60612–7315; 2Department of Physiology and Biophysics, College of Medicine, University of Illinois at Chicago, Chicago 60612–7342; and 3West Side Division, Veterans Administration Chicago Health Care System, Chicago, Illinois 60612

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Espiritu, Doris Joy D., Angelito A. Bernardo, R. Brooks Robey, and Jose A. L. Arruda. A central role for Pyk2-Src interaction in coupling diverse stimuli to increased epithelial NBC activity. Am J Physiol Renal Physiol 283: F663–F670, 2002. First published May 7, 2002; 10.1152/ajprenal.00338.2001.—Regulation of renal Na-HCO3 cotransporter (NBC) activity by cholinergic agonists, ANG II, and acute acidosis (CO2) requires both Src family kinase (SFK) and classic MAPK pathway activation. The nonreceptor tyrosine kinase proline-rich tyrosine kinase 2 (Pyk2) couples discrete G protein-coupled receptor and growth factor receptor signaling to SFK activation. We examined the role of Pyk2-SFK interaction in coupling these stimuli to increased NBC1 activity in opossum kidney cells. Carbachol increased tyrosine autophosphorylation of endogenous Pyk2 and ectopically expressed wild-type Pyk2 and were abrogated by kinase-dead mutant (Pyk2-KD) overexpression. Pyk2 phosphorylation was calcium/calmodulin dependent, and Pyk2 associated with Src by means of SH2 domain interaction. Pyk2 phosphorylation and Pyk2-Src interaction by carbachol were mimicked by both ANG II and CO2. To correlate Pyk2 autophosphorylation and Pyk2-Src interaction with NBC activity, cotransporter activity was measured in untransfected and in cells overexpressing Pyk2-KD in the presence or absence of carbachol, ANG II, or CO2. In Pyk2-KD-overexpressing cells, the effect of carbachol, ANG II, and CO2 was abolished. We conclude that Pyk2 plays a central role in coupling carbachol, ANG II, and CO2 to increased NBC activity. This coupling is mediated by Pyk2 autophosphorylation and Pyk2-Src interaction.

carbachol; angiotensin II; acute acidosis; autophosphorylation; sodium-bicarbonate cotransporter; proline-rich tyrosine kinase 2

WE HAVE PREVIOUSLY CHARACTERIZED the regulation of Na-HCO3 cotransporter (NBC) activity in renal epithelial cells by carbachol, ANG II, and CO2 (16–18, 20). Interestingly, these diverse stimuli have been shown to share a number of common signaling requirements, including both Src family kinase (SFK) and classic MAPK pathway activation (14, 22). In the case of carbachol and ANG II, we have demonstrated a specific role for calcium/calmodulin in this regulation (Ruiz OS and Arruda JAL, unpublished observations, and Ref. 14). However, a common unifying mechanism whereby these diverse stimuli activate these common signaling intermediates and increase proximal tubule cell NBC activity has not been elucidated.

The nonreceptor tyrosine kinase (NRTK) proline-rich tyrosine kinase 2 (Pyk2) (2, 10), also referred to as related adhesion focal tyrosine kinase (1), cellular adhesion kinase-β (26), and calcium-dependent tyrosine kinase (34), is known to interact with SFKs, an association that requires autophosphorylation and thereby plays an important role in coupling both G protein-coupled receptors (GPCR) and growth factor receptors to classic MAPK pathway activation in a number of cell types. Calcium-dependent Pyk2 activation and Src interaction have been demonstrated after GPCR stimulation by carbachol and ANG II (8). We therefore examined whether Pyk2 could play a similar role in the calcium-dependent carbachol and ANG II stimulation of NBC1 activity in renal epithelial cells.

The role of Pyk2 in epithelial cells has been incompletely explored (9, 11) and, to our knowledge, has not been examined in the kidney. Pyk2 exhibits renal expression (10, 12, 30) and has been immunohistochemically localized to the renal proximal tubule (12). We have recently reported, in preliminary form, the presence of endogenous Pyk2 in the opossum kidney (OK) proximal tubule cell line (6). In the present study, we examined both Pyk2 phosphorylation and Pyk2-SFK interaction for roles in the regulation of basolateral NBC activity in these cells. This cotransporter is a major determinant of HCO3− reabsorption by the proximal tubule and may serve as a prototype for the characterization of the role of Pyk2 in renal epithelial transport, especially the regulation of intracellular pH (pHi).

We have previously demonstrated a general role for G proteins in the regulation of proximal tubule cell...
NBC activity (19). More recently, we have shown that activation of specific GPCRs for both muscarinic cholinergic agonists (14) and ANG II (15) increase NBC activity by means of the sequential activation of Src family tyrosine kinases (SFKs) and the classic MAPK pathway. Although a general requirement for calcium/calcmodulin has been demonstrated for cholinergic stimulation of NBC activity (16) and calcium is a known effector of many of angiotensin’s cellular actions, the specific mechanisms coupling GPCR activation to SFK activation, and ultimately to increased NBC activity, have not been defined. We therefore sought to examine whether Pyk2 might play a role in these processes.

In addition to the GPCR-mediated processes above, acute acidosis has also been shown to activate both SFKs (22, 32, 33) and the classic MAPK pathway (22) in this cell type, thereby increasing NBC activity (22). We therefore also examined the role of Pyk2 in this unrelated, non-GPCR-mediated process.

MATERIALS AND METHODS

Reagents. The pH-sensitive fluorophore BCECF was obtained from Molecular Probes (Eugene, OR) as the membrane-permeable AM (BCECF-AM). Amiloride was purchased from Research Biochemicals (Natick, MA). Src-specific antibodies (GD11) were obtained from Upstate Biotechnology (Lake Placid, NY), as were the SKF and Src assay kits and ERK kinase kits employed herein. Phosphotyrosine-specific antibodies were obtained from Zymed (PY Plus; South San Francisco, CA) or BD Transduction Laboratories (RC20, Lexington, KY). Pyk2-specific monoclonal antibodies were also obtained from BD Transduction Laboratories. Rabbit polyclonal anti-Pyk2 phosphospecific antibodies (α-Pyk2-pTyr405) were obtained from Biosource (Camarillo, CA). Agarose-immobilized Src-SH2 domain peptides were purchased from Calbiochem (La Jolla, CA). Immunoblots were analyzed with a commercially available chemiluminescence detection system (Phototope) from New England BioLabs (Beverly, MA). All other immunoblotting reagents, including nitrocellulose membranes, were routinely obtained from Bio-Rad (Hercules, CA). Cell culture reagents, including serum and additives, were purchased from GIBCO BRL (Grand Island, NY). All other reagents, including carbachol, ANG II, the calmodulin antagonist N-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-13), and murine monoclonal antibodies directed against the c-myc epitope (MEQKLISEEDL, clone 9E10) (7) were obtained from Sigma (St. Louis, MO).

Cell culture. Mycoplasma-free American OK cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 37 and were maintained in Eagle’s minimum essential medium containing Earle’s balanced salts and supplemented with 10% fetal bovine serum as described previously (14). Cells were routinely serum deprived for 24 h before and during testing and were typically preincubated with inhibitors for at least 30 min where appropriate.

Fluorometric assays of pH1 and NBC activity. Confluent cell monolayers cultured on uncoated plastic coverslips were made quiescent, loaded with BCECF, and continuously monitored for pH-dependent changes in fluorescence at 37°C as described previously (14, 23). In brief, cells were perfused at 37°C with a Cl-free physiological solution (in mM) 25 NaHCO3, 110 sodium gluconate, 5 potassium gluconate, 2 CaSO4, 0.5 MgSO4, 1 KH2PO4, 10 glucose, and 9 HEPES, pH 7.40 containing 1 mM amiloride to minimize the contributions of cellular Cl/HCO3 and Na/H exchange activities at a rate of 20 ml/min. Extracellular pH was maintained constant at 7.40. After a stable basal fluorescence signal was established, Na removal by equimolar substitution with choline resulted in an immediate decrease in pH1 and pH-sensitive BCECF fluorescence that rapidly and fully recovered on the reintroduction of Na. NBC activity was taken as the initial rate of pH recovery after the addition of NaHCO3 and was calculated from the slope of the line drawn tangentially to the initial deflection over a period of 1 min. pH-sensitive BCECF fluorescence at 530 nm was routinely calibrated at the completion of each experiment in the presence of elevated extracellular potassium and the ionophore nigericin (to equilibrate pH1 and extracellular pH). All measurements were performed by dual-wavelength monitoring and ratiometric analysis at pH-sensitive (490 nm) and -insensitive (440 nm) excitation wavelengths (Ex/EmF490/F440).

Phosphorylation and kinase assay activities. Src phosphorylation was assessed by quantitative immunoblot analysis of whole cell lysates by using both Src-specific polyclonal antisera and recombinant monoclonal anti-phosphotyrosine antibodies (RC20) as described previously (14, 22). Src kinase activity was evaluated in parallel by using a commercially available immunoprecipitated kinase activity assay (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer’s recommendations. In brief, we tested the in vitro ability of Src immunoprecipitates to phosphorylate a synthetic oligopeptide substrate (KVEKGEGTYGGVYK) corresponding to residues 6–20 of p34cdc2 (3). Samples were incubated in (in mM) 25 Tris-HCl (pH 7.2), 31.3 MgCl2, 25 MnCl2, 0.5 EGTA, and 0.5 DTT, as well as 62.5 μM Na2VO4 and 115 μM ATP containing 10 μCi [γ-32P]ATP at 30°C for 10 min before the reaction was stopped by the addition of tri-chloroacetic acid. After application to P81 phosphocellulose paper, unincorporated radiocuclides were eluted with 7.5% (vol/vol) phosphoric acid. Phosphotransferase activity was then assayed as specific 32P incorporation into the substrate by liquid scintillation counting.

ERK1/2 activity assay. Total ERK1/2 kinase activity was measured by using a commercially available nonradioactive in vitro immunoprecipitated kinase activity assay (Upstate Biotechnology) according to the manufacturer’s recommendations. In brief, ERK1/2 immunoprecipitates prepared from whole cell lysates were tested for the ability to phosphorylate myelin basic protein in the presence of inhibitors of PKA, PKC (PKC inhibitor peptide), and calmodulin kinase II (R24571). The total concentration mixture consisted of (in mM) 15 MOPS, pH 7.2, 18.75 β-glycerophosphate, 18.75 MgCl2, 3.75 EGTA, 0.75 sodium orthovanadate, 0.75 DTT, and 115.2 ATP, and (in μM) 5 PKC inhibitor, 0.5 PKA inhibitor, and 0.5 compound R24571, as well as 20 mg of dephosphorylated myelin basic protein. Reactions were carried out at 30°C for 20 min. Five microliters of the reaction were resolved in 12% SDS-PAGE and probed with anti-PKB. Carbachol and ANG II findings were also confirmed with a radioactive immunoprecipitated ERK1/2 activity assay as described previously (14).

Ectopic Pyk2 expression. Mammalian expression vectors encoding wild-type Pyk2 (Pyk2-WT) mutant (pCMV-Pyk2-WT) and a K457A-kinase-dead Pyk2 (Pyk2-KD) mutant (pCMV-Pyk2-KD) were the generous gift of Dr. J. Thomas Parsons (University of Virginia) and have been described previously (31). These vectors express full-length Pyk2-WT or Pyk2-KD fused to an NH2-terminal c-myc epitope tag.
Transient gene transfer was achieved in subconfluent OK cells by using LipofectAMINE lipofection reagent (Life Technologies, Rockville, MD) according to the manufacturer’s recommendations and as described previously (14). Transiently transfected cells were routinely tested within 48 h of gene transfer. Ectopic Pyk2-WT or Pyk2-KD (K457A) expression was routinely confirmed by immunoblot analysis with the 9E10 murine monoclonal antibody directed against the c-myc epitope tag (7). For fluorometric assay, stable transfection of OK cells with Pyk2-KD was performed by using the same method and was selected with 400 μg/ml G418.

Phosphorylation and immunoprecipitation. Whole cell lysates (500 μg) were incubated with antibodies specific for Pyk2, phosphotyrosine, or Src for 6 h at 4°C. The resulting immunocomplexes were precipitated with immobilized protein G/protein A (Calbiochem). Constituent proteins were eluted from these immunocomplexes by boiling for 10 min in 2× SDS sample buffer and were resolved by denaturing PAGE before transfer to nitrocellulose for immunoblotting. To examine the effect of W-13 on Pyk2 phosphorylation, quiescent cells transiently transfected with Pyk2-WT were treated with 100 μM of W-13 for 30 min and stimulated with carbachol. The cells were lysed and 500 μg of lysates were immunoprecipitated with anti-Pyk2 or anti-phosphotyrosine and immunoblotted with anti-phosphotyrosine and anti-Pyk2, respectively.

Pyk2 autophosphorylation was determined by probing an equal amount of carbachol stimulated and paired control cell lysates with α-Pyk2-pTyr<sup>102</sup>, an antibody specific to the Pyk2 autophosphorylation site. Pyk2 autophosphorylation was also measured in Pyk2-WT and Pyk2-KD overexpressing cells with the same method.

Immunoblot analysis. Whole cell lysates were routinely prepared in radioimmunoprecipitation assay buffer (50 mM Tris·Cl, 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, and 1 mM EDTA) supplemented with both protease inhibitor and phosphatase inhibitor cocktails (Sigma). Lysate proteins were electrophoretically resolved and transferred to nitrocellulose as described previously (14). After incubation in Tris-buffered saline with 0.1% Tween 20, 20 mM Tris·Cl, and 500 mM NaCl containing 10% (wt/vol) immunoblot grade nonfat dry milk for 1 h at room temperature to block nonspecific binding, primary antibodies were routinely incubated.

Pyk2-Src coprecipitation assays. Pyk2-Src interaction was assayed by precipitating 500 μg of stimulated and unstimulated Pyk2-WT-overexpressing cell lysates with α-Src or α-Pyk2 antibodies. The eluted proteins were analyzed by immunoblotting with α-Pyk2 or anti-Src, respectively. Coprecipitation with SH2 domain linked to protein A-agarose was also performed.

Statistical analysis. Densitometric analysis of Western blots was carried out by using NIH Image software. All data are expressed as means ± SE for a series of n experiments. Student’s t-tests or ANOVA were used. P values <0.05 were considered to represent significant differences.

RESULTS

Cholinergic stimulation of Src kinase activity is calcium/calmodulin dependent. We have previously demonstrated a general requirement for increased intracellular calcium in the stimulation of NBC activity by both carbachol and ANG II. We have also shown that carbachol- and ANG II-stimulated NBC activity share a common requirement for sequential SFK and classic MAPK pathway activation (14, 21). The mechanism whereby intracellular calcium is coupled to these other signaling intermediates, however, has not been defined. The ubiquitous calcium-binding protein calmodulin is known to couple intracellular calcium to the activation of a number of signaling intermediates, including SFKs. Thus, to better define the relationship between intracellular calcium and other signaling intermediates implicated in NBC regulation, we examined the calcium chelator BAPTA and the calmodulin antagonist W-13 for the ability to prevent activation of both SFKs and the classic MAPK pathway. As shown in Table 1, both carbachol and ANG II increased the activities of SFK and Src, as well as ERK, the distal element of the classic MAPK pathway. We have examined the effect of ANG II, carbachol, or 10% CO<sub>2</sub> on MAPK activity by using ERK1/2 immunoprecipitates for their ability to phosphorylate myelin basic protein. As can be seen in Fig. 1, ANG II, carbachol, or 10% CO<sub>2</sub> all increased ERK1/2 activity. These effects were inhibited by intracellular calcium chelation (+ BAPTA), as well as by calmodulin antagonism (+ W-13). Neither BAPTA nor W-13 had a significant effect on the basal activities of these signaling intermediates, suggesting that neither agent directly inhibits the measured kinase activities.

Cholinergic stimulation increases endogenous Pyk2 tyrosine phosphorylation. Cholinergic stimulation of SFK activation is coupled to calcium/calmodulin and Pyk2 activation in PC12 cells. Similar coupling has been observed for ANG II stimulation in myocardial cells. We therefore studied a possible role for Pyk2 in coupling intracellular calcium to the regulation of NBC activity in renal epithelial cells. As depicted in Fig. 2A, Pyk2 is strongly expressed in OK cells. Cholinergic stimulation increased tyrosine phosphorylation of a protein that comigrates with Pyk2 (Fig. 2B) but not total Pyk2 abundance in quiescent OK cells stimulated with 100 μM carbachol for 5 min.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Src Kinase Activity</th>
<th>ERK1/2 Kinase Activity</th>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Carbachol</td>
<td>+BAPTA, 100 μM</td>
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</tr>
<tr>
<td>Carbachol</td>
<td>+W-13</td>
<td>82 ± 7</td>
</tr>
<tr>
<td>ANG II</td>
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Table 1. Calcium/calmodulin dependence of cholinergic- and ANG II-stimulated Src and ERK1/2 kinase activation

Values are means ± SE; n = 5. Intracellular calcium chelation (+ BAPTA, 5 μM) and calmodulin antagonism (+ W-13, 10<sup>-4</sup> M) were individually examined for the ability to attenuate both cholinergic and angiotensin stimulation of Na/HCO<sub>3</sub><sup>−</sup> (NBC)1 activity. Both Src and ERK1/2 activities are expressed as percent activity relative to paired unstimulated control cells. *P < 0.02.
Ectopic expression of both Pyk2-WT and Pyk2-KD in OK cells and Pyk2 phosphorylation. To determine whether a Pyk2 catalytic site plays a role in phosphorylation and association, we transiently transfected OK cells with Pyk2-WT and Pyk2-KD. After 48 h of transfection, cells were lysed and Pyk2 were analyzed by using Pyk2 and c-myc antibodies. An intense band of ~110 kDa for c-myc was noted in Pyk2-WT and Pyk2-KD transfectants, confirming successful overexpression because Pyk2-WT and Pyk2-KD were both targeted with c-myc epitope (Fig. 3A). Phosphorylation assays were also performed on cells overexpressing Pyk2-WT and Pyk2-KD with and without carbachol stimulation. Figure 3B shows that Pyk2 phosphorylation of cells transfected with Pyk2-WT was increased by carbachol compared with control, and transfection of Pyk2-KD prevented phosphorylation. Quantitative analysis of Pyk2 phosphorylation showed a significant increase in phosphorylation by carbachol (Fig. 3C).

Cholinergic stimulation increases Pyk2 autophosphorylation. Increased phosphorylation was shown in Fig. 3. To examine whether autophosphorylation is involved, we used Pyk2-pTyr402 antibodies to detect phosphorylation of Tyr402 residue. Figure 4A shows that endogenous Pyk2 is phosphorylated by cholinergic stimulation. The same result was obtained in cells overexpressing Pyk2-WT, and autophosphorylation was completely abolished in Pyk2-KD-overexpressing cells (Fig. 4B).

Pyk2 autophosphorylation requires calcium/calmodulin. To examine the calcium/calmodulin dependence of Pyk2 autophosphorylation, we preincubated cells overexpressing Pyk2-WT with W-13 (100 μM × 30 min) before carbachol stimulation. At 5 min, cells were harvested and lysed. Whole cell lysates were precipitated with anti-Pyk2 or anti-phosphotyrosine and probed with anti-phosphotyrosine and anti-Pyk2, respectively. There was an increase in Pyk2 phosphorylation with carbachol, but this effect was totally blocked with W-13 (Fig. 5A), consistent with a proximal effector function for calcium/calmodulin in Pyk2 phosphorylation. Identical results were observed for endogenous Pyk2 (Fig. 5B).

Autophosphorylated Pyk2 physically associates with Src. In previous experiments, Pyk2 autophosphorylation (Pyk2-Tyr402) in response to carbachol stimulation was shown, and the result was consistent with increased total tyrosine phosphorylation of Pyk2 in response to carbachol. Thus we sought to determine whether autophosphorylation plays a role in Pyk2 and Src association as previously shown in PC12 cells. Cells overexpressing Pyk2-WT were stimulated with carbachol, and Src-Pyk2 association was analyzed by coprecipitation assays. Figure 6A shows that in the presence of carbachol, there was an increased Src-Pyk2 association. This association was shown to be mediated by Src-SH2 domains. Pyk2 was found to physically interact with Src under conditions associated with Pyk2 autophosphorylation. The ability of immobilized Src-SH2 domains to mimic Src in coprecipitating Pyk2 suggests specific contributions by SH2-phosphotyrosine interactions. Figure 6B shows that carbachol

![IB: α- pMBP](image-url)
increases SH2-phospho-Pyk2 interaction compared with unstimulated cells. This association was enhanced in cells overexpressing Pyk2-WT and was prevented in cells expressing Pyk2-KD. We have previously shown that 10% CO₂ increased SFK activity (19), and this effect was mimicked by 100 μM carbachol and 0.1 nM ANG II. Also depicted in Fig. 1 is that these diverse stimuli increased ERK activity in parallel. To determine whether the association between Src and Pyk2 was restricted to carbachol stimulation, we studied the effects of 0.1 nM ANG II and 10% CO₂ on Pyk2-Src interaction. Figure 7 shows that similar to carbachol, both ANG II and 10% CO₂ also increased Pyk2 phosphorylation and Src-Pyk2 association.

**Disruption of Pyk2 function abrogates stimulated, but not basal, NBC activity.** We have previously shown that OK cells express the proximal tubule NBC1 isoform (15), and NBC activity is increased by ANG II, carbachol, and 10% CO₂ (14, 15, 20). We have shown here that Pyk2-KD overexpression decreased both Pyk2 autophosphorylation and Src-Pyk2 association in carbachol-stimulated cells. As an indirect test of the hypothesis that Pyk2 activation and Pyk2-Src association play a central role in the transduction of diverse extracellular signals that serve to increase NBC activity, we examined ectopic Pyk2-KD expression for the ability to attenuate the stimulatory effects of carbachol (10 μM), ANG II (0.1 nM), and acute acidosis (10% CO₂). Table 2 shows that Pyk2-KD overexpression prevented the effect of carbachol, ANG II, or 10% CO₂ to

Fig. 3. Cholinergic stimulation of Pyk2 phosphorylation. Overexpression of wild-type Pyk2 (Pyk2-WT) enhances Pyk2-phosphorylation and kinase-dead Pyk2 (Pyk2-KD) prevents phosphorylation. A: OK cells were grown to 80% confluence and transfected with Pyk2-WT (+Pyk2-WT) or Pyk2-KD (+Pyk2-KD) with the lipid-mediated transfection method. Anti-c-myc detected an ~110-kDa protein in transfected cells, which is not present in untransfected cells. B: quiescent untransfected cells and cells transiently transfected with Pyk2-WT or Pyk2-KD were treated with carbachol and 500 μg of cell lysates were immunoprecipitated (IP) with α-pyr and RC20 and immunoblotted with α-Pyk2 or vice versa. Carbachol increases phospho-Pyk2 and overexpression of Pyk2-KD diminishes phosphorylation. C: quantitative analysis of Pyk2 phosphorylation obtained by using densitometric analysis of 5 independent paired experiments showed a significant increase of Pyk2 phosphorylation by carbachol. Baseline phosphorylation of endogenous Pyk2 is so low that it did not permit quantitative analysis.

Fig. 4. Cholinergic stimulation increased Pyk2 autophosphorylation. A: 50 mg of carbachol-stimulated and -paired, unstimulated untransfected OK lysates were probed with antibody specific for the Pyk2 autophosphorylation site (αPyk2-pTyr402). Carbachol induced phosphorylation of endogenous Pyk2-Tyr402. B: similar results were obtained with cells overexpressing Pyk2-WT. Overexpression of Pyk2-KD prevented Pyk2-Tyr402 phosphorylation. Values are from a single, paired experiment that was repeated 3 times with identical results.
stimulate NBC activity without altering baseline activity.

**DISCUSSION**

Pyk2 is a recently described NRTK with structural similarities to focal adhesion kinase (FAK) that associates with SFK (2, 5, 8) through phosphorylation of Tyr^402 and creates a binding site for SH2 domains of SFKs (29). Despite close structural homology to FAK, which also interacts with Src, Pyk2 is activated by various stimuli and has been shown to couple both GPCRs and growth factor receptors to classic MAPK pathway activation (8). Pyk2 has been shown to mediate different functions from those associated with FAK in a number of cell types (31). Pyk2 is also distinguished from FAK by means of its association with stimuli capable of increasing free intracellular calcium, activation of PKC, and exposure to stress factors (28, 29).

Although Pyk2 is predominantly expressed in the central nervous system and best characterized in PC12 cells, it is expressed in the kidney (10, 12, 30) and has been immunohistochemically localized to the proximal tubule (12). In the present work, we have established the renal epithelial cell expression of Pyk2, as well as its function in coupling diverse stimuli to NBC activation and signal transduction mechanisms associated with NBC activation in an OK renal epithelial cell line. We demonstrated that calcium/calmodulin and the classic MAPK pathway serve as important signaling effectors proximal and distal to Pyk2-SFK interaction,
respectively. This also showed a common requirement of calcium/calmodulin and Pyk2-SFK interaction in the cholinergic and ANG II stimulation of epithelial cell NBC activity.

The presence of endogenous Pyk2 and its activation by acute acidosis in the OK renal epithelial cell line have also previously been reported in preliminary form (27), but Pyk2 activation in this cell type has not been characterized and its functional consequences are not known. In the present work, we confirmed the endogenous expression of Pyk2 in OK cells and have shown that this NRTK plays an important role in coupling the effect of carbachol, ANG II, and acute acidosis to SFK activation and ultimately to increased NBC activity. To our knowledge, this represents the first description of a functional interaction between Pyk2 and Src in renal epithelial cells.

Pyk2 autophosphorylation plays an important role in the transduction of diverse GPCR signals (5, 8) and was recently implicated in the translocation of GLUT4 (25). Both carbachol (4–8–10) and ANG II (13, 24) have been shown to stimulate Pyk2 activation in a variety of cell types. In cardiac fibroblasts, this activation has been shown to be calcium/calmodulin sensitive (13) and the first description of Pyk2 activation by acute acidosis. Our findings suggest a specific mechanism whereby SFK activation may be coupled to diverse GPCR and non-GPCR stimuli through Pyk2 and show a central role of Pyk2 in transducing a number of physiologically important signals that regulate pH, by means of coincident effects on NBC.

Ectopic expression of the kinase-inactive K457A-Pyk2 mutant (Pyk2-KD) completely abrogated Pyk2 tyrosine phosphorylation in both stimulated and unstimulated cells. This mutant contains a single Lys for Ala mutation in the ATP binding site of the kinase domain of Pyk2 (31). The absence of detectable tyrosine phosphorylation for both unstimulated and stimulated cells ectopically expressing Pyk2-KD is consistent with this result and emphasizes the importance of autophosphorylation. Overexpression of Pyk2-KD totally prevented Pyk2-Src association by means of the SH2 domain. Taken together, these findings suggest that Pyk2 autophosphorylation is required for both activity and interaction with Src.

Table 2. Central role for Pyk2 in the stimulation of NBC1 activity by ANG II, carbachol, and CO2

<table>
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<tr>
<th></th>
<th>ANG II</th>
<th>Carbachol</th>
<th>CO2</th>
</tr>
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<tbody>
<tr>
<td>OK cells</td>
<td>100±15</td>
<td>115±12</td>
<td>107±11</td>
</tr>
<tr>
<td>Pyk2-KD</td>
<td>104±15</td>
<td>115±12</td>
<td>107±11</td>
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Values are means ± SE; n = 6. ANG II (0.1 nM), carbachol (100 μM), and CO2 (10%) were each examined for the ability to increase NBC activity in opossum kidney (OK) cells and kinase-dead Pyk2-overexpressing (+Pyk2-KD) OK cells. NBC is expressed as percent activity relative to paired unstimulated control cells *P < 0.04.

These findings are compatible with those reported previously in other cell types (31).

We have previously shown that the glucocorticoid stimulation of NBC activity was not inhibited by overexpression of the negative regulator of Src, Csk (23), indicating that the effect of ANG II, carbachol, and CO2 on the Src/MAPK pathway is specific. The exact mechanism whereby acute changes in pH activate NRTKs, such as Pyk2 and Src, is not known, but acute acidosis has been variably associated with both increased and decreased free intracellular calcium content in a variety of cell types. A previous study has suggested that proximal tubule cell Src activation by acute intracellular acidosis is calcium independent (33). In our laboratory, CO2 stimulation of NBC1 activity appears to be calcium/calmodulin dependent (Ruiz OS and Arruda JAL, unpublished observations), suggesting that this may serve as a common mechanism of initiating the signaling cascade examined herein and culminating in increased NBC activity. The present studies show that the ability of both ANG II and CO2 to stimulate ERK1/2 was also inhibited by BAPTA and W-13 (Fig. 1). The ability of CO2 to mimic the effects of ANG II and carbachol in activating the calcium-dependent NRTK Pyk2 is compatible with this hypothesis.

In the present work, we have attempted to define the molecular basis for SFK activation and signaling in the stimulation of NBC activity by acute acidosis, cholinergic agonists, and ANG II. These stimuli have been individually shown to signal by means of both SFK-dependent and SFK-independent mechanisms in a variety of cell types. It is therefore of considerable interest that these diverse stimuli share a common mechanism of NBC activation involving both SFK activation and SFK-MAPK coupling in renal epithelial cells (6, 21). We have clearly demonstrated that Pyk2-SFK interaction serves as a unifying coupling mechanism for these diverse stimuli and thereby plays an important role in the integration of a wide range of signals known to increase epithelial cell NBC activity. In addition to providing important mechanistic insights into the regulation of epithelial cell NBC activity, our findings provide new regulatory and functional insights into the role of Pyk2 in these cells. We have demonstrated the novel activation of Pyk2 by acute acidosis, and our findings also suggest a novel functional role for this NRTK in the regulation of pH. A common requirement for Pyk2 in both the activation of SFKs and the subsequent stimulation of NBC activity is compatible with a pivotal role for Pyk2-SFK coupling in the regulation of vectorial ion transport in this cell type.

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