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Angiotensin II Stimulation of Renal Epithelial Cell Na/HCO_3 Cotransport Activity: A Central Role for Src Family Kinase/Classic MAPK Pathway Coupling

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Abstract. Angiotensin II (AII) plays an important role in renal proximal tubular acidification via the costimulation of basolateral Na/HCO_3 cotransporter (NBC) and apical Na/H exchanger (NHE) activities. These effects are mediated by specific G protein-coupled AII receptors, but their corresponding downstream effectors are incompletely defined. Src family tyrosine kinases (SFKs) contribute to the regulation of both transport activities by a variety of stimuli and are coupled to classic mitogen-activated protein kinase (MAPK) pathway activation in this cell type. We therefore examined these signaling intermediates for involvement in AII-stimulated NBC activity in cultured proximal tubule cells. Subpressor concentrations of AII (0.1 nM) increased NBC activity within minutes, and this effect was abrogated by selective antagonism of AT_1 angiotensin receptors, SFKs, or the classic MAPK pathway. AII directly activated Src, as well as the proximal (Raf) and distal (ERK) elements of the classic MAPK module, and the activation of Src was prevented by AT_1 receptor antagonism. An associated increase in basolateral membrane NBC1 content is compatible with the involvement of this proximal tubule isoform in these changes. We conclude that AII stimulation of the AT_1 receptor increases NBC activity via sequential activation of SFKs and the classic MAPK pathway. Similar requirements for SFK/MAPK coupling in both cholinergic and acidotic costimulation of NBC and NHE activities suggest a central role for these effectors in the coordinated regulation of epithelial transport by diverse stimuli.

Key words: Angiotensin II — Proximal tubular epithelial cell — Src family kinases — Extracellular signal-regulated kinases — Signal transduction — Na/HCO_3 cotransport — Intracellular pH regulation — AT_1 receptor

Introduction

In addition to its classic role as a renal vasoconstrictor, angiotensin II (AII) is a potent stimulator of proximal tubule acidification and Na reabsorption (Harris & Young, 1977; Schuster, Kokko & Jacobson, 1984; Liu & Cogan, 1987). These tubular effects are observed at suppressor AII concentrations (<1 nM) and reflect the direct stimulation of transepithelial $NaHCO_3$ flux in this nephron segment. Both basolateral Na/HCO_3 cotransport (NBC) and apical Na/H exchange (NHE) play major roles in the regulation of this flux, and the corresponding transporters represent important target effectors of AII action in the proximal tubule. The ability of AII to concomitantly stimulate both NBC and NHE activities in isolated perfused proximal tubules (Geibel, Giebisch & Boron, 1990) is compatible with such an interpretation. AII increases NBC activity in both proximal tubule (Geibel et al., 1990; Coppola & Frömter, 1994*a*, 1994*b*) and cortical basolateral membrane vesicle preparations (Eiam-Ong et al., 1993; Ruiz et al., 1995*a*), effects that uniformly mimic those of AII on NHE3 activity in similar models

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(Arruda & Ruiz, 1991). AII has also been shown to stimulate NHE3 activity in both primary (Saccomani, Mitchell & Navar, 1990; Houillier et al., 1996) and continuous cultures of proximal tubule cells (Cano et al., 1994), but, to our knowledge, such a demonstration has not yet been reported for NBC. Thus, to better understand the molecular mechanisms underlying AII-stimulated NBC activity and to identify signaling intermediates contributing to this effect, we examined the ability of AII to stimulate NBC activity in the OK (American opossum kidney) proximal tubule cell line. We also confirmed the expression and basolateral localization of the proximal tubule NBC1 isoform in this cell culture model.

The parallel regulation of proximal tubule cell NBC and NHE activities by diverse stimuli suggests common regulatory mechanisms. Acute acidosis, for example, has been shown to increase both transport activities via activation of nonreceptor Src family tyrosine kinases (SFKs). In the case of NBC activity, the classic mitogen-activated protein kinase (MAPK) pathway has been shown to couple SFK activation by acidosis to increased transporter activity (Ruiz et al., 1999). We have also recently demonstrated that cholinergic stimulation is similarly coupled to NBC activation in these cells (Robey et al., 2001). These findings suggest a central role for SFKs and the classic MAPK pathway in the regulation of NBC activity by diverse stimuli, so we also examined the role of these signaling intermediates in AII-stimulated NBC activity in OK cells.

Materials and Methods

Reagents

The pH-sensitive fluorophore 2′7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) was obtained as the cell-permeable acetoxymethyl ester (BCECF-AM) from Molecular Probes (Eugene, OR), and amiloride was purchased from Research Biochemicals (Natick, MA). Irbesartan, an AT_1-selective non-peptide AII receptor antagonist (SR 47436; BMS 186295; 2-n-butyl-3-([2′-(1H-tetrazol-5-yl)-1,1′-biphenyl-4-yl]methyl)-1,3-diazaspiro [4,4] non-1-en-4-one), was obtained from Bristol-Myers Squibb (Princeton, NJ). Herbimycin A, PD98059 (2′-amino-3′-methoxyflavone), PP1 (4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]-pyrimidine), PP3 (4-amino-7-phenylpyrazolo[3,4-d]-pyrimidine), and hygromycin B were obtained from Calbiochem (San Diego, CA), and recombinant phosphotyrosine-specific RC20 antibodies were purchased from BD Transduction Laboratories (Lexington, KY). ERK1/2- and Src-specific antibodies were obtained from Upstate Biotechnology (Lake Placid, NY), as were the SFK, ERK1/2, and Raf-1 kinase assay kits employed herein. All immunoblots were analyzed using commercially available chemiluminescence detection systems from either New England Biolabs (Phototope®; Beverly, MA) or Amersham Pharmacia Biotech (ECL™; Arlington Heights, IL). Immunoblotting reagents, including nitrocellulose membranes, were routinely obtained from BioRad (Hercules, CA), and cell culture reagents, including serum and media additives, were obtained from Invitrogen (Grand Island, NY). All other reagents, including synthetic human AII, were obtained from Sigma (St. Louis, MO) unless noted otherwise.

Cell culture

Mycoplasma-free American opossum kidney (OK) cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD) at passage 37. Cell monolayers were maintained in a humidified 37°C/5% CO_2 incubator in Eagle's minimum essential medium containing Earle's salts and supplemented with 10% fetal bovine serum (FBS). All experiments were performed on newly confluent cell monolayers between passages 38 and 48 to minimize the effects of phenotypic variation in continuous culture. Cells were also routinely serum-deprived for 24 hr prior to and during testing.

Fluorometric Assays of pH_i and NBC Activity

Cell monolayers cultured on uncoated plastic coverslips were loaded with BCECF-AM and continuously monitored for pH-dependent changes in BCECF fluorescence as described previously (Robey et al., 1998). In brief, cells were perfused with a Cl-free physiologic solution (in mM): 25 $NaHCO_3$, 110 sodium gluconate, 5 potassium gluconate, 2 $CaSO_4$, 0.5 $MgSO_4$, 1 M KH_2PO_4, 10 glucose, and 9 HEPES, pH 7.40) supplemented with 1 mM amiloride to minimize the contributions of cellular Cl/HCO_3 and Na/H exchange activities. All experiments were performed at 37°C, and extracellular pH was maintained constant at 7.40 throughout. The equimolar substitution of choline for Na uniformly resulted in immediate decreases in both pH_i and pH-sensitive BCECF fluorescence. Upon the reintroduction of Na, fluorescence rapidly and fully recovered, and NBC activity was taken as the initial rate of this recovery. In the absence of chloride, recovery is primarily attributable to NBC activity (Alpern, 1985; Ruiz et al., 1995*b*). pH-sensitive BCECF fluorescence at 510 nm was routinely calibrated at the completion of each experiment in the presence of elevated extracellular potassium and the ionophore nigericin (to equilibrate intracellular and extracellular pH). All measurements were performed by dual-wavelength monitoring and ratiometric analysis at pH-sensitive (500 nm) and pH-insensitive (450 nm) excitation wavelengths (F_{500}/F_{450}). By convention, results are presented as both absolute ($\Delta pH/\Delta t$) and relative (% change in $\Delta pH/\Delta t$) rates of change in pH_i. However, it is pertinent to note that changes in pH are exponentially related to changes in $[H^+]$ ($pH = -\log[H^+]$), so the latter expressions of *per cent* change have limited quantitative utility.

Ectopic Carboxyterminal Src Kinase (CSK) Expression

OK cells stably overexpressing carboxyterminal Src kinase (Csk) have been characterized previously (Ruiz et al., 1999) and were uniformly grown to confluence in normal growth medium supplemented with selection antibiotic (100 μg/ml hygromycin B) before testing. Where appropriate, Csk overexpression was confirmed by immunoblot analysis as described previously (Ruiz et al., 1999).

Src Phosphorylation and Kinase Activity Assays

Src phosphorylation was assessed by quantitative immunoblot analysis of whole-cell lysates as described previously (Ruiz et al., 1999). In these studies, both total Src abundance and Src tyrosine

phosphorylation were evaluated in parallel using Src-specific polyclonal antisera and recombinant monoclonal anti-phosphotyrosine antibodies (RC20), respectively. Src kinase activity was evaluated using a commercially available immunoprecipitated kinase activity assay (Upstate Biotechnology) according to the manufacturer's recommendations. In brief, we tested the in vitro ability of Src immunoprecipitates to phosphorylate a synthetic oligopeptide substrate (KVEKIGEGTYGVVYK) corresponding to residues 6–20 of $p34^{cdc\ 2}$ (Cheng et al., 1992). Samples were incubated in (in mM) 25 Tris-HCl (pH 7.2), 31.3 $MgCl_2$, 25 $MnCl_2$, 0.5 EGTA, 0.5 dithiothreitol, 62.5 μM Na_3VO_4, and 112.5 μM ATP containing 10 μCi [γ-^{32}P]ATP at 30°C for 10 min before stopping the reaction by the addition of trichloroacetic acid. Aliquots were applied to P81 phosphocellulose paper and phosphotranferase activity was determined as specific ^{32}P incorporation by liquid scintillation counting following the elution of unincorporated radionuclide with 7.5% (v/v) phosphoric acid.

Extracellular Signal-regulated Kinases 1 and 2 (ERK1/2) Activity Assays

Total ERK1/2 kinase activity was measured using a commercially available 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 (PKI), PKC (PKC inhibitor peptide), and calmodulin kinase II (compound R24571). The final reaction mixture consisted of (in mM) 20 MOPS (pH 7.2), 25 β-glycerol phosphate, 16.9 $MgCl_2$, 5 EGTA, 1 Na_3VO_4, 1 dithiothreitol, 5 μM PKC inhibitor peptide, 0.5 μM PKI, 5 μM compound R24571, and 112.5 μM ATP containing 10 μCi [γ-^{32}P]ATP. Following incubation for 10 min at 30°C and application to P81 phosphocellulose paper, unincorporated ^{32}P was eluted with 7.5% (v/v) phosphoric acid and the remaining incorporated radioactivity was assayed by liquid scintillation counting.

Raf-1 Kinase Cascade Activity Assays

Raf-1 kinase activity was measured using a commercially available kit (Upstate Biotechnology) according to the manufacturer's recommendations. In brief, Raf-1 immunoprecipitates were prepared from whole-cell lysates and were examined for the ability to activate an in vitro phosphorylation cascade involving recombinant MEK1- and ERK2-GST fusion proteins. Activation of this cascade was monitored as specific ^{32}P incorporation into the substrate myelin basic protein in (mM) 20 MOPS (pH 7.2), 25 β-glycerol phosphate, 25 $MgCl_2$, 5 EGTA, 1 Na_3VO_4, 1 dithiothreitol, and 167 μM ATP supplemented with tracer quantities of [γ-^{32}P]ATP for 10 min at 30°C. Aliquots were spotted onto P81 phosphocellulose paper, and incorporated radioactivity was assayed by liquid scintillation counting following the elution of unincorporated ^{32}P with 7.5% (v/v) phosphoric acid. Positive control samples containing activated recombinant ERK2 were routinely assayed in parallel.

NBC1 Immunoblot Analysis

Immunoblot analysis of whole-cell lysates was performed using rabbit polyclonal antipeptide antibodies directed against the 20 carboxyterminal residues of human NBC1 (DSKPSDRERSPTFLERHTSC; 85% identity with the corresponding rat sequence). These antibodies (Alpha Diagnostic; San Antonio, TX) recognize NBC1 of both simian and human origin (Noboa et al., 2000, 2001; Weinman et al., 2001), so lysates of NBC1-expressing HK-2 human proximal tubule cells (Ryan et al., 1994; Racusen et al., 1997) were routinely examined in parallel as a positive control. HEK 293 human embryonic kidney cells (ATCC), which do not express immunodetectable NBC1 (*data not shown*), were employed as a corresponding negative control in these experiments.

Biotinylation and Affinity Purification of Cell Surface Proteins

Basolateral membrane localization of NBC1 was evaluated by cell surface biotinylation as described previously (Weinman et al., 2001) with minor modifications. For these experiments, OK cell monolayers were grown to confluence under normal growth conditions on semipermeable polycarbonate supports (Corning Transwell®, 0.4 μm pore size; Corning, NY). OK cells grown in this manner exhibit a polarized phenotype that permits directed examination of cell surface protein expression at both basolateral and apical cell interfaces (Noel, Roux & Pouysségur, 1996) (*data not shown*). After thorough washing with a cold physiological saline solution (PBS), basolateral cell surface proteins were labeled by direct application of the *N*-hydroxysulfosuccinimide active ester sulfo-NHS-LC-biotin (1.5 mg/ml; Pierce; Rockford, IL) for 25 min at 4°C per the manufacturer's recommendations. Glycine was routinely included as a quenching agent on the apical side, and cells were thoroughly washed to remove unreacted biotinylating agent before lysis. Biotinylated proteins were then precipitated from whole-cell lysates using immobilized streptavidin (Pierce), and the resulting eluates were probed for NBC1 content by immunoblot analysis as detailed above.

Statistical Analysis

Results were expressed as means ± SEM, and statistical comparisons were performed by *t*-testing for paired or unpaired data where appropriate, using a significance level of 95%. Where unequal variances of the data were observed, nonparametric comparisons were made using Kruskal-Wallis multiple-comparison tests with post-hoc analysis by the method of Dunn (1964).

Results

AII Stimulation of NBC Activity is Prevented by Both Nonselective and SFK-Selective Tyrosine Kinase Inhibition

As previously demonstrated in cortical basolateral membrane preparations (Ruiz et al., 1995*a*), 0.1 nM AII rapidly increased NBC activity in OK cells (Figs. 1*A* and *B*). In these experiments, the initial rate of pH_i recovery increased by over 30% in AII-treated cells. The nonselective tyrosine kinase inhibitor herbimycin A completely prevented this increase (Fig. 1*A*) at a concentration (1 μM) known to inhibit SFK activity (Uehara & Fukazawa, 1991), but had no corresponding effect on basal NBC activity at the same concentration (Fig. 1*A*). To specifically address the role of SFKs in AII-stimulated cotransport we also tested the tyrphostin PP1, an SFK-selective tyrosine kinase inhibitor (Hanke et al., 1996; Klint et al., 1999), for the ability to mimic the effects of

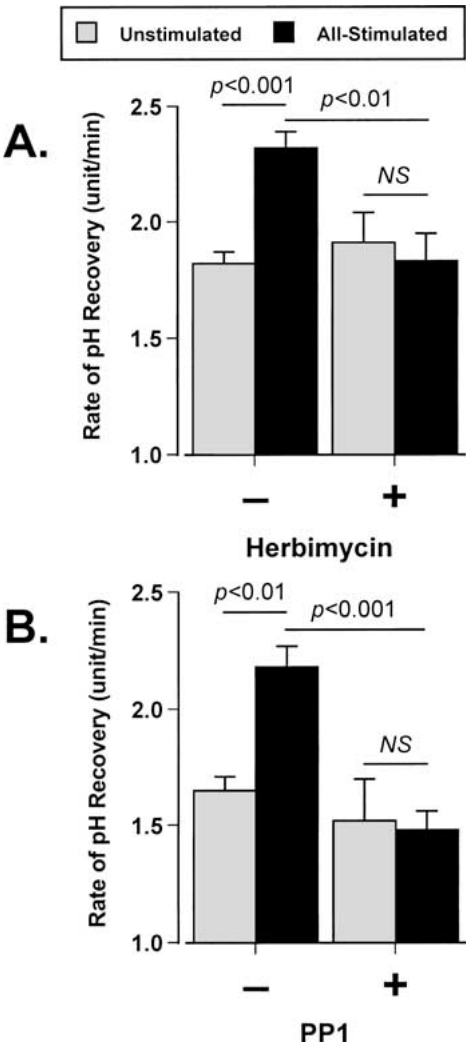

Fig. 1. AII stimulation of NBC activity is prevented by both nonselective (*A*) and SFK-selective (*B*) tyrosine kinase inhibitors. The influence of general tyrosine kinase inhibition (1 μM herbimycin A; panel *A*) and selective SFK inhibition (100 nM PP1; panel *B*) on basal and AII-stimulated (0.1 nM × 5 min) NBC activity is depicted. Both herbimycin (panel *A*) and PP1 (panel *B*) completely prevented the increase in NBC activity by AII (*black bars*), whereas neither inhibitor altered basal activity (*gray bars*; *NS vs.* unstimulated controls). All data are presented as the means ± SEM for at least six independent paired experiments.

nonselective tyrosine kinase inhibition. As shown in Fig. 1*B*, 100 nM PP1 similarly blocked AII-stimulated NBC activity without altering basal activity, which is further consistent with a role for SFKs in this effect.

AII-Stimulated NBC Activity is Inhibited by Carboxyterminal Src Kinase (CSK) Overexpression

To better define the involvement of SFKs in AII-stimulated NBC activity, we also examined Csk, a negative regulator of SFKs, for the ability to attenuate this response. In OK cells stably expressing

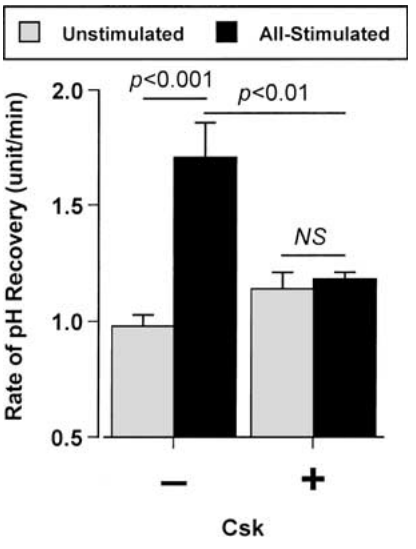

Fig. 2. Ectopic carboxyterminal Src kinase (Csk) expression prevents the stimulation of NBC activity by AII. NBC activity was measured in both unstimulated and AII-stimulated (0.1 nM × 5 min) Csk-overexpressing or wild-type control cells. AII (*black bars*) increased NBC activity normally in control cells (–), but this effect was completely prevented in Csk-overexpressing cells (+). In contrast, Csk overexpression had no independent effect on basal cotransporter activity (*gray bars*, *NS vs.* unstimulated controls), consistent with our previous report (Ruiz et al., 1999). Each data set is presented as the mean ± SEM for six independent paired experiments.

ectopic Csk (Ruiz et al., 1999), 0.1 nM AII did not increase NBC activity, whereas paired control cells responded normally to this stimulus and increased their initial rate of pH_i recovery by more than 50% (Fig. 2). Basal cotransporter activity was unaffected by Csk overexpression, consistent with our previous reports (Ruiz et al., 1999; Robey et al., 2001).

AII Activates Src and Increases NBC Activity via an AT_1 Receptor-dependent Mechanism

AII (0.1 nM) doubled the level of immunodetectable Src tyrosine phosphorylation within 5 min (Figs. 3*A* and *B*) without altering total Src abundance (Fig. 3*B*). Src kinase activity was similarly affected, and the observed increases in both Src phosphorylation and kinase activity were completely prevented by tyrosine kinase inhibition using either 1 μM herbimycin *A* (Fig. 3*A*) or 100 nM PP1 (*data not shown*). AT_1 receptor antagonism by 10 μM irbesartan (Fig. 3*B*) also prevented Src phosphorylation by AII, suggesting specificity for the AT_1 receptor. In separate experiments to examine the functional consequences of this effect, 0.1 nM AII increased the maximal apparent rate of OK cell NBC activity by 1 ± 1% and 23 ± 6% ($p < 0.04$) in the presence and absence of 10 nM irbesartan, respectively. Since NBC activity was always expressed by convention as a

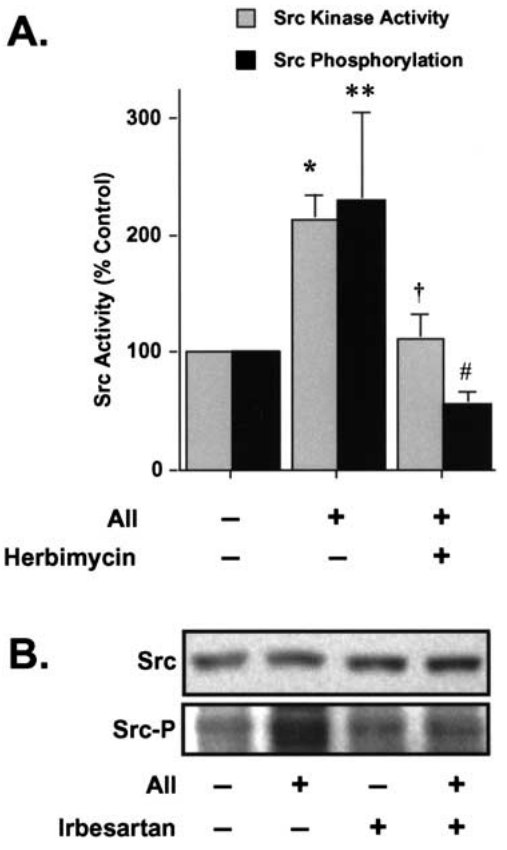

Fig. 3. AII increases Src tyrosine phosphorylation and kinase activity via an AT_1 receptor-dependent mechanism. As depicted in panel *A*, AII (0.1 nM × 5 min) increased Src kinase activity (*gray bars*; $^{*}p < 0.01$ *vs.* unstimulated controls) within 5 min as evaluated by an in vitro immunoprecipitated kinase activity assay. This effect was completely prevented by pretreatment with 1 μM herbimycin A ($^{\dagger}p < 0.02$ *vs.* AII alone). Src tyrosine phosphorylation (*black bars*) increased in parallel ($^{**}p < 0.05$ *vs.* unstimulated controls), and was similarly prevented by herbimycin A ($^{\#}p < 0.05$ *vs.* AII alone). The SFK-selective inhibitor PP1, at a concentration of 100 nM, was found to mimic the latter effect of herbimycin A (*data not shown*). The depicted data for Src kinase activity and tyrosine phosphorylation are presented as the means ± SEM for five and three independent paired experiments, respectively. As shown in panel *B*, the ability of AII to increase Src phosphorylation (Src-P) was completely prevented by pretreatment with the AT_1-selective AII receptor antagonist irbesartan (10 μM). Data from a single experiment, repeated with identical results, are depicted.

logarithmic function of Na/HCO_3 cotransport (*Δ*pH unit/min), the actual differences will be even greater. Irbesartan alone had no independent effect on NBC activity in these experiments (97 ± 2% of unstimulated control values).

MEK Inhibition Prevents AII Stimulation of NBC Activity

AII activates the classic MAPK pathway in a variety of cell types (Duff, Berk & Corson, 1992; Ishida et al., 1992; Aoki et al., 2000), including renal proximal

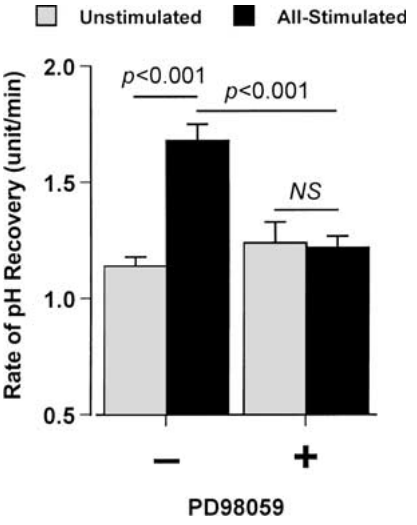

Fig. 4. MEK inhibition prevents AII stimulation of NBC activity. Classic MAPK pathway involvement in AII-stimulated (0.1 nM × 5 min) NBC activity was evaluated pharmacologically using the MEK-selective inhibitor PD98059. At 50 μM, PD98059 completely prevented the increase in NEC activity by AII (*black bars*) without altering basal activity (*gray bars*; *NS vs.* unstimulated controls). The data are depicted as the means ± SEM for nine independent paired experiments.

tubule cells (Terada et al., 1995*a*, 1995*b*). We have previously shown that this pathway couples SFK activation to increased NBC activity in both CO_2- and carbachol-stimulated OK cells (Ruiz et al., 1999; Robey et al., 2001). We therefore examined the involvement of this important signal transduction pathway in the stimulation of NBC activity by AII. To this end, we tested the MEK-selective inhibitor PD98059 for the ability to attenuate AII-stimulated cotransport activity. As depicted in Fig. 4, PD98059, at 10 μM, completely blocked AII-stimulated NBC activity. Consistent with our previous findings (Ruiz et al., 1999; Robey et al., 2001), PD98059 alone had no effect on basal cotransport activity, suggesting that this inhibitor does not exert its effect via direct transporter interaction.

AII Activation of the Classic MAPK Pathways is SFK-Dependent

To further examine the involvement of the classic MAPK pathway in AII-stimulated NBC activity, we directly tested the ability of AII to activate both the proximal (Raf) and distal (ERK) elements of the classic MAPK module. OK cell treatment with 0.1 nM AII nearly doubled the kinase activities of both Raf-1 (Fig. 5*A*) and ERK1/2 (Fig. 5*B*) within 5 min. PD98059 also completely prevented ERK1/2 activation by AII (Fig. 5*B*), consistent with the sequential activation of Raf-1, MEK1/2, and ERK1/2. Pretreatment with 100 nM PP1 completely prevented

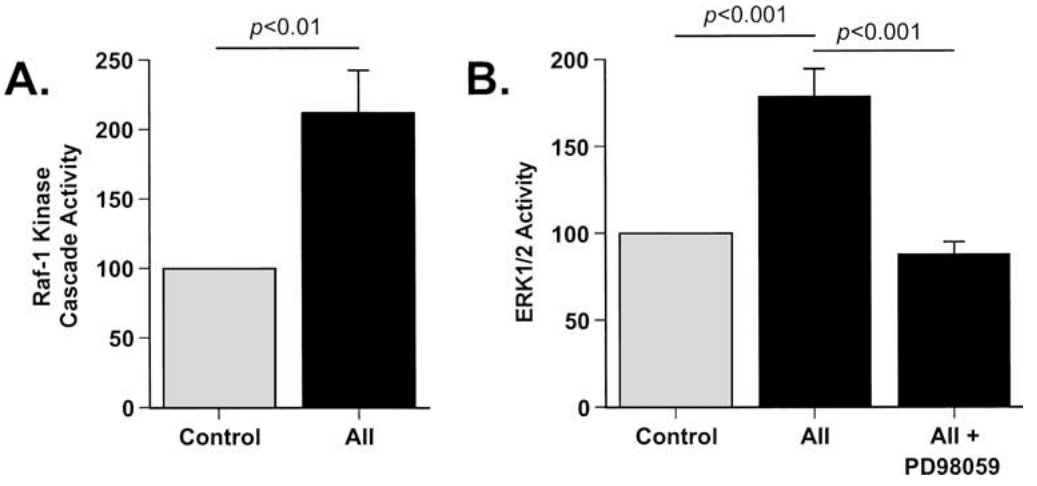

Fig. 5. AII increases classic MAPK pathway activation. AII (0.1 nM) increased Raf-1 kinase activity (panel *A*) within 5 min, and ERK1/2 kinase activity increased in parallel (panel *B*). The latter increase was completely prevented by pretreatment with the MEK-selective inhibitor PD98059 (50 μM). The ability of AII to activate both the proximal (Raf-1) and distal (ERK1/2) elements of the classic MAPK module—coupled with the ability of PD98059 to prevent ERK1/2 activation—is consistent with the sequential activation of all elements of this module (Raf→ MEK → ERK) by AII. All data represent the means ± SEM for at least six independent paired experiments.

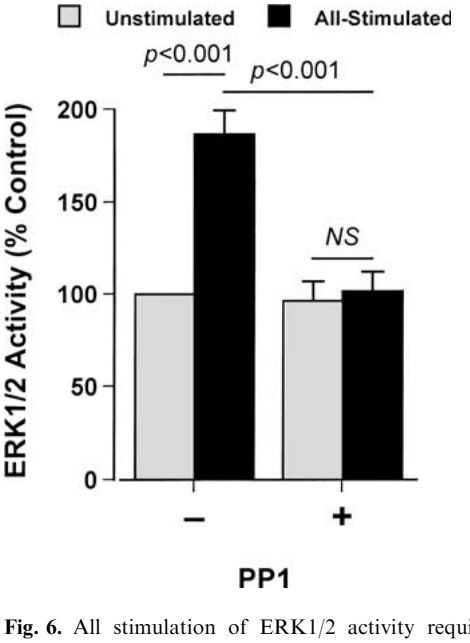

Fig. 6. All stimulation of ERK1/2 activity requires Src family kinase activity. AII stimulation (0.1 nM × 5 min) of classic MAPK pathway activation was completely prevented by the SFK-selective inhibitor PP1 (100 nM) and is consistent with SFK/MAPK coupling in these cells. The data are depicted as the means ± SEM for at least five independent paired experiments.

ERK1/2 activitation by AII, suggesting a requirement for SFK activation (Fig. 6).

OK Cells Express Immunodetectable NBC1

Although the proximal tubular origin of OK cells is widely accepted, we directly examined these cells for the expression of the NBC1 proximal tubule isoform.

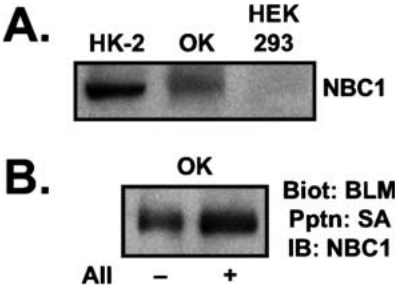

Fig. 7. AII increases the amount of immunodetectable NBC1 in the basolateral membrane. As shown in panel *A*, OK and HK-2 proximal tubule cells, but not HEK 293 cells, express immunodetectable NBC1. A representative immunoblot of whole-cell lysates from each cell line (50 μg total protein) is depicted, and this experiment was repeated twice with similar results. Although these results may be taken to suggest lower relative abundance of NBC1 in OK cells, the sequence of the opossum NBC1 homolog is not known, so contributions by differential immunoreactivity for human and opossum NBC1 cannot presently be excluded. As depicted in panel *B*, basolateral membrane NBC1 localization was also examined in polarized OK cell monolayers cultured on semipermeable supports. Basolateral cell surface proteins were labeled by direct application of the biotinylating agent sulfo-NHS-LC-biotin (Biot:BLM), and biotinylated proteins were affinity-purified from whole-cell lysates using immobilized streptavidin (Pptn:SA; 2.5 mg total protein) before probing eluants for immunoreactive NBC1 (IB:NBC1). AII (0.1 nM) increased basolateral NBC1 content within 15 min. A representative immunoblot, repeated twice with similar results, is depicted. Corresponding changes in the total amount of immunodetectable NBC1 content were not observed over the same time period (*data not shown*).

As shown in Fig. 7*A*, polyclonal antibodies directed against the carboxyterminus of human NBC1 recognized a band of appropriate size in lysates of NBC1-expressing human HK-2 proximal tubule cells (No-

boa et al., 2000, 2001), but not HEK 293 human embryonic kidney cells (ATCC). An immunodetectable band of lesser intensity with similar migration characteristics was also observed in OK cell lysates. These antibodies are specifically targeted to human NBC1, and the corresponding sequence of the opossum homolog is not known, so these differences may be attributable to either differential immunoreactivity to human and opossum homologs or lower levels of NBC1 abundance in OK cells. Functional studies would suggest the former possibility, although we cannot presently exclude contributions by other NBC isoforms in OK cells. The close agreement of our findings in this model with those in other proximal tubule cell models does not immediately suggest this possibility.

AII Increases Basolateral Membrane NBC1 Content

To evaluate basolateral membrane NBC1 localization, basolateral cell surface proteins were selectively labeled by the basolateral application of the biotinylating agent sulfo-NHS-LC-biotin to polarized OK cells grown on semipermeable supports. As depicted in Fig. 7*B*, immunodetectable basolateral cell surface NBC1 increased within 15 min of exposure to 0.1 nM AII without a corresponding change in the total cellular NBC1 content (*data not shown*), suggesting a selective increase in basolateral NBC1 content. We have reported similar results, in preliminary form (Noboa et al., 2000, 2001), in human proximal tubule cells, compatible with the contention that NBC1 translocation to the basolateral membrane contributes to changes in NBC activity

Discussion

Suppressor concentrations of AII (<1 nM) increase proximal tubular HCO_3 reabsorption by directly stimulating both basolateral Na/HCO_3 cotransport (NBC) and apical Na/H exchange (NHE). These parallel effects are mediated by the activation of specific G protein-coupled AII receptors on proximal tubule cells (Xie et al., 1990; Cogan et al., 1991). We have previously demonstrated a specific requirement for basolateral AT_1 receptor activation in the regulation of NBC activity by subnanomolar AII concentrations (Ruiz et al., 1995*a*), but the corresponding downstream signaling effectors are incompletely defined. To better understand the molecular mechanisms underlying the stimulation of NBC activity by AII, we examined this process in proximal tubule cell-like OK cells. We report here that AII rapidly increased NBC activity in these cells via the sequential activation of AT_1 receptors, SFKs, and the classic MAPK pathway. An associated increase in basolateral membrane NBC1 content is compatible with specific contributions by this known proximal tubular cell NBC isoform. The involvement of SFKs in this process is of particular interest, since this important family of nonreceptor tyrosine kinases is known to mediate both basolateral NBC (Ruiz et al., 1999) and apical NHE3 (Yamaji et al., 1995, 1997) activation by acidosis in this cell type. Similar requirements have been reported for cholinergic NBC activation in these cells (Robey et al., 2001). As demonstrated previously for both acidotic and cholinergic stimulation, SFK activation by AII was coupled to increased NBC activity via the classic MAPK pathway. These findings confirm and extend previous observations in both isolated perfused tubules (Geibel et al., 1990; Coppola & Frömter, 1994*a*; 1994*b*) and basolateral membrane preparations (Eiam-Ong et al., 1993; Ruiz et al., 1995*a*). They also represent the first clear demonstration of AII-stimulated NBC activity in a proximal tubule cell-culture model and establish the utility of using cultured cells to characterize AII signal transduction related to NBC regulation.

The classic biphasic functional response to AII in the proximal tubule was first described nearly 35 years ago (Barraclough, Jones & Marsden, 1967) and has subsequently been confirmed by a number of investigators (Steven, 1974; Harris & Young, 1977; Schuster et al., 1984). Subnanomolar concentrations of AII have been associated with the stimulation of proximal tubule transport functions, whereas higher concentrations (i.e., >1 nM) have shown corresponding inhibitory effects (Harris & Young, 1977; Schuster et al., 1984). Although not directly examined in the present study, these differences probably reflect the existence of multiple AII receptor subtypes with differing membrane distribution patterns and associated signal transduction mechanisms (Douglas & Hopfer, 1994; Dulin et al., 1994). It is also relevant to note that the 0.1 nM AII concentration employed in our studies has not only been associated with stimulation of proximal tubule function, but is also comparable in magnitude to normal circulating plasma levels (Oster, Hackenthal & Hepp, 1973; Schuster et al., 1984; Seikaly, Arant & Seney, 1990; Braam et al., 1993;). Many previous studies have examined the cellular effects of AII at higher concentrations associated with both inhibition of proximal tubular function and altered renal hemodynamics in vivo. The proximal tubule possesses a complete renin-angiotensin system (Marchetti, Roseau & Alhenc-Gelas, 1987; Inglefinger et al., 1990; Sechi et al., 1992), so local AII concentrations in vivo may actually exceed those observed in the systemic circulation. Reports of intraluminal proximal tubule AII concentrations that exceed circulating levels (Seikaly et al., 1990; Braam et al., 1993) are compatible

with this possibility, but the physiologic relevance of these observations remains to be established. Intraluminal AII concentrations in the nM range have been attributed to the tubular secretion of AII or its precursors (Seikaly et al., 1990; Braam et al., 1993) and have been shown to influence proximal tubular reabsorption (Harris & Young, 1977; Liu & Cogan, 1988; Seikaly et al., 1990). It is widely accepted that proximal tubule cells express both apical and basolateral AII receptors, but the μM range affinity of brush border membranes for AII binding is markedly lower than that observed for basolateral membranes. Fundamental differences in the identity and coupling of specific AII receptors have been suggested to account for differences in AII binding and action at the apical and basolateral interfaces (Douglas & Hopfer, 1994), and the general unresponsiveness of luminal AII concentrations to intravascular volume expansion sufficient to decrease circulating AII levels (Braam et al., 1993) argues against a major physiologic role for intraluminal AII. Our findings, including the demonstrated involvement of the AT_1 receptor at subnanomolar AII concentrations, are specifically applicable to signals emanating from the basolateral surface and do not directly address those originating at the apical interface. Thus, without additional direct measures of local AII concentrations in vivo, we can only speculate regarding the physiologic appropriateness — or, in many cases, the pathophysiologic significance — of the use of AII concentrations $\geq$1 nM to examine proximal tubule cell function in vitro. We have therefore restricted our studies to circulating concentrations that can clearly be regarded as physiologic.

The ability of SFK-selective tyrosine kinase inhibitors (e.g., PP1 and Csk) to prevent AII-stimulated NBC activity suggests an important role for SFKs in mediating this effect. The corresponding ability of AII to directly activate Src is compatible with this interpretation. We have previously shown that other stimuli as diverse as CO_2 and cholinergic agonists share a common requirement for SFK activation in the regulation of NBC activity (Ruiz et al., 1999; Robey et al., 2001). Similar effects of both AII and carbachol have been observed in human proximal tubule cells (*data not shown*), and these effects are non-additive, which is compatible with a common mechanism of action. Others have demonstrated similar requirements for SFKs in the regulation of NHE3 activity in OK cells (Yamaji et al., 1995; 1997), providing indirect support for the contention that NBC activity shares these common regulatory mechanisms with NHE3. The uniform association between Src activation and increased NBC activity also suggests a specific role for Src in these processes, although contributions by other SFKs expressed in this cell type, such as Fyn and Yes (Sugawara et al., 1991; Arreaza et al., 1994; Grandaliano et al., 2000), cannot presently be excluded.

AII activates the classic MAPK pathway (Raf $\rightarrow$ MEK $\rightarrow$ ERK) in a variety of cell types (Duff et al., 1992; Ishida et al., 1992; Aoki et al., 2000), including renal proximal tubule cells (Terada et al., 1995*a*; 1995*b*). This pathway can also serve as a downstream effector of SFK activation (Tsuda et al., 1992; Igishi & Gutkind, 1998; Daulhac et al., 1999) and has been shown to specifically couple SFK activation by CO_2 and carbachol to increased NBC activity in this cell type (Ruiz et al., 1999; Robey et al., 2001). We have demonstrated that subnanomolar AII is similarly capable of activating Src, Raf-1, and ERK1/2 in OK cells. We have also shown that associated increases in NBC activity can be prevented by selective antagonism of signaling via AT_1 receptors (irbesartan), SFKs (PP1 and Csk), or MEK1/2 (PD98059). These findings clearly suggest that these signaling intermediates couple subnanomolar AII stimulation to increased NBC activity in proximal tubule cells. It should be noted, however, that some investigators have reported maximal ERK activation by AII at concentrations as high as 100 nM in both vascular smooth muscle cells (Duff et al., 1992) and OK cells (Terada et al., 1995*a*). The corresponding inability of tyrosine kinase inhibition to prevent this effect in OK cells at higher, ostensibly pharmacologic, concentrations of AII (Terada et al., 1995*a*) suggests the involvement of signal transduction mechanisms additional or alternative to those described herein.

The specific mechanisms whereby MAPK pathway activation is coupled to increased NBC activity have not been defined. A large number of potential phosphorylation motifs—including consensus sites for protein kinase A (PKA), protein kinase C (PKC), and casein kinase II—have been reported in the deduced amino-acid sequence of the proximal tubule NBC1 isoform (Burnham et al., 1997; Romero et al., 1997). Additional candidate phosphorylation motifs are readily identifiable using internet-based prediction algorithms [NetPhos 2.0; http://www.cbs.dtu.dk/services/NetPhos/ (Blom, Gammeltoft & Brunak, 1999)]. It would therefore be attractive to speculate that direct phosphorylation of NBC1 plays a role in its acute regulation. Consistent with such a possibility, it has recently been reported that serine phosphorylation of a consensus site for PKA is associated with altered NBC1 transport stoichiometry that could contribute to the changes associated with PKA stimulation (Gross et al., 2001). This observation, made in NBC1-deficient cells reconstituted to express ectopic NBC1, contrasts markedly with the reported inability of PKA to phosphorylate endogenous NBC1 in BS-C-1 epithelial cells (Weinman et al., 2001). Although this disparity may simply reflect differences in the models examined, it is relevant to note that we have been unable to reproducibly demonstrate endogenous NBC1 phosphorylation in other NBC1-expressing epithelial cell models, including OK cells (*data not*

shown). Both the nature and significance of these discrepant observations remain to be defined. However, given the well-described similarities between apical NHE3 and basolateral NBC1 regulation in the proximal tubule, it is pertinent to add that direct phosphorylation is reportedly necessary, but not sufficient, for NHE3 regulation by PKC (Wiederkehr, Zhao & Moe, 1999). Taken together, we cannot presently exclude a regulatory role for direct NBC1 phosphorylation, but it appears that other regulatory mechanisms are involved. It has previously been shown that basolateral NBC1 activity, like apical NHE3 activity, is regulated by PKA through the associated regulatory factor NHE-RF (Bernardo et al., 1999; Weinman et al., 2001). The demonstrated involvement of NHE-RF suggests potential scaffolding or targeting functions, as well as the general possibility that other associated regulatory proteins may contribute to NBC regulation. The demonstrated ability of AII to rapidly increase basolateral membrane NBC1 content in OK cells also suggests specific contributions by intracellular translocation of this isoform. Consistent with this possibility, we have reported, in preliminary form (Noboa et al., 2000; 2001), that NBC1 trafficking between intracellular pools and the plasma membrane plays a role in the acute regulation of NBC activity by AII in human proximal tubule cells. The relative contributions of NBC1 phosphorylation, associated regulatory proteins, and intracellular trafficking remain to be established. However, when considered in toto, our findings and the studies cited above clearly suggest a central role for both SFKs and the classic MAPK pathway in the coordinated regulation of both NBC and NHE activities by stimuli as diverse as AII, cholinergic agonists, and acidosis. It is therefore tempting to speculate that these signaling intermediates may play similar roles in other important epithelial transport functions and in other epithelial cell types.

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