A role for ERK1/2 in EGF- and ATP-dependent regulation of amiloride-sensitive sodium absorption

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Submitted 29 April 2004; accepted in final form 29 December 2004

Falin, Rebecca, I. Elias Veizis, and Calvin U. Cotton. A role for ERK1/2 in EGF- and ATP-dependent regulation of amiloride-sensitive sodium absorption. Am J Physiol Cell Physiol 288: C1003–C1011, 2005. First published January 5, 2005; doi:10.1152/ajpcell.00213.2004.—Receptor-mediated inhibition of amiloride-sensitive sodium absorption was observed in primary and immortalized murine renal collecting duct cell (mCT12) monolayers. The addition of epidermal growth factor (EGF) to the basolateral bathing solution of polarized monolayers reduced amiloride-sensitive short-circuit current (Isc) by 15–25%, whereas the addition of ATP to the apical bathing solution decreased Isc by 40–60%. Direct activation of PKC with phorbol 12-myristate 13-acetate (PMA) and mobilization of intracellular calcium with 2,5-di-tert-butyl-hydroquinone (DBHQ) reduced amiloride-sensitive Isc in mCT12 monolayers by 46 ± 4% (n = 8) and 22 ± 2% (n = 8), respectively. Exposure of mCT12 cells to EGF, ATP, PMA, and DBHQ caused an increase in phosphorylation of p42/p44 (extracellular signal-regulated kinase; ERK1/2). Pretreatment of mCT12 monolayers with an ERK kinase inhibitor (PD-98059; 30 μM) prevented phosphorylation of p42/p44 and significantly reduced EGF, ATP, and PMA-induced inhibition of amiloride-sensitive Isc. In contrast, pretreatment of monolayers with a PKC inhibitor (bisindolylmaleimide I; GF109203x; 1 μM) almost completely blocked the PMA-induced decrease in Isc, but did not alter the EGF- or ATP-induced inhibition of Isc. The DBHQ-mediated decrease in Isc was due to inhibition of basolateral Na+/K+-ATPase, but EGF-, ATP-, and PMA-induced inhibition was most likely due to reduced apical sodium entry (epithelial sodium channel activity). The results of these studies demonstrate that acute inhibition of amiloride-sensitive sodium transport by extracellular ATP and PMA involves ERK1/2 activation and suggests a role for MAP kinase signaling as a negative regulator of electrogenic sodium absorption in epithelia.

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these studies demonstrate that phosphorylation and activation of ERK1/2 is an important step in ATP- and EGF-induced inhibition of amiloride-sensitive sodium transport.

METHODS

Cell isolation and culture. Experiments were carried out with primary cultures of mouse renal epithelial cells and an immortalized mouse collecting duct principal cell line (mCT12). The collecting duct cell line used in these studies was generated as described previously (32, 38, 39). To prepare primary cultures of mouse renal epithelial cells, the kidneys were removed from transgenic HoxB7/EGFP/+ mice (36) (ureteric bud and collecting duct specific expression of enhanced green fluorescent protein) under sterile conditions from CO2-euthanized animals. The renal capsule was removed and the kidneys were minced with a razor blade, rinsed with sterile HEPES-buffered Ringer solution, and suspended in 10 ml of collecting tubule (CT) media that contained collagenase (0.625 mg/ml, Type IV; Worthington Biochemical). The tissue was digested for 10–30 min at 37°C in a shaking water bath. The cellular material was collected by centrifugation, resuspended in CT media, plated onto plastic tissue culture dishes, and placed in a humidified tissue culture incubator (37°C and 5% CO2). The media were changed 24 h later and every 48 h thereafter. Primary cultures were expanded for 5–7 days, seeded onto collagen-coated permeable supports, and studied 5–10 days later. Some experiments with primary mouse renal epithelial cells were done on an enriched preparation of collecting duct principal cells (>95% purity) obtained by fluorescence-activated cell sorting for EGFP (collecting duct) cells (44). The cells isolated by fluorescence-activated cell sorting were seeded onto collagen-coated permeable supports and studied 5–10 days later. The studies described herein were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Case Western Reserve University School of Medicine.

CT media were composed of the following: a 1:1 mix of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (Life Technologies) supplemented with 1.3 μg/ml sodium selenite, 1.3 μg/l 3,3’-5-iodo-l-thyronine, 5 mg/l insulin, 5 μg/l transferrin, 25 μg/l prostaglandin E1, 2.5 mM glutamine, 50 nM dexamethasone, 50 μg/ml streptomycin, and 30 mg/ml penicillin G. Interferon-γ (10,000 U/l) was added to the CT media for mCT12 cells.

Transpapillary electrical measurements. Cells were seeded (2–5 × 10⁵ cells/filter) onto collagen-coated, permeable supports (modified 12 mm Millicell-CM filters; Millipore) as described previously (39). Confluent cell monolayers were mounted vertically in a thermostatically controlled Ussing chamber equipped with gas inlets and separate reservoirs for the perfusion of apical and basolateral compartments. Both sides of the cell monolayer were bathed with equal volumes of Krebs-Ringer bicarbonate solution containing (in mM): 115 NaCl, 25 NaHCO₃, 5 KCl, 2.5 NaHPO₄, 1.8 CaCl₂, 2 MgSO₄, and 10 glucose. Some experiments were done with cell monolayers bathed by CT media. The solutions were circulated through the water-jacketed glass reservoir by gas lifts (95% O₂/5% CO₂) to maintain solution temperature at 37°C and pH at 7.4. Transepithelial voltage difference (Vt) was measured between two Ringer-agar bridges, and calomel half-cells connected the bridges to a high impedance voltmeter. Current from an external direct current source was passed by silver-silver chloride electrodes and Ringer-agar bridges to clamp the spontaneous Vt to 0 mV. The current required [short-circuit current (Isc)] was corrected for solution and filter series resistance and recorded. Monolayers were maintained under short-circuit conditions except for brief 3- to 5-s intervals when the current necessary to clamp the voltage to a nonzero value (2–10 mV) was measured to calculate transepithelial resistance.

Western blots. Cell lysates were prepared from confluent culture filters (24 mm Transwell, Clear tissue culture treated; Corning Costar) in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 3 mM EDTA, and 1 mM EGTA. Protein concentrations were measured by bicinchoninic acid protein assay (Pierce). Western blot analysis for total and phosphorylated ERK1/2 (p42/p44) was performed as described previously (32).

Statistical analysis. All results are expressed as means ± SE. Statistical significance was evaluated by either paired or unpaired Student’s t-test. P < 0.05 was considered significant.

RESULTS

EGF and extracellular ATP inhibit amiloride-sensitive sodium absorption. Primary cultures of renal collecting duct principal cells form polarized epithelial monolayers and exhibit Isc. As illustrated in Fig. 1, the Isc is sensitive to amiloride (>95% inhibition), an ENaC blocker, and to EGF and extracellular ATP. Addition of EGF (20 ng/ml) to the basolateral bathing solution elicits, after a ~5-min delay, a monotonic decrease in Isc (~25%), and the remaining current is inhibited by subsequent addition of amiloride (Fig. 1A). Pretreatment of the monolayer with amiloride leads to almost complete inhibition of Isc and subsequent addition of EGF does not elicit a change in Isc (Fig. 1B). The addition of ATP (100 μM) to the apical bathing solution causes a transient increase in Isc (anion secretion), followed by a decline in Isc (Fig. 1C) to a value below the initial current. Pretreatment of the monolayer with amiloride does not block the transient stimulation of Isc by ATP, but does prevent a drop in current below the value recorded immediately before the addition of ATP (Fig. 1D). The inhibitory effects of EGF and ATP on amiloride-sensitive Isc in monolayers of primary mouse collecting duct principal cells and immortalized mCT12 are summarized in Fig. 2. Additional experiments were done with mCT12 to identify the signaling pathways responsible for inhibition of amiloride-sensitive sodium absorption by EGF and ATP.

ERK1/2 activation contributes to acute EGF- and ATP-induced inhibition of amiloride-sensitive sodium absorption. Previous studies have shown that acute exposure of isolated, perfused collecting ducts to either EGF or extracellular ATP causes inhibition of sodium absorption; however, the mechanism of inhibition is unknown. Furthermore, chronic exposure (12–48 h) of immortalized collecting duct principal cells to EGF reduces amiloride-sensitive Isc via an ERK1/2-dependent pathway. To determine whether activation of MAP kinase signaling is responsible for the acute effects of EGF on sodium absorption, the epithelial monolayers were treated with an inhibitor of ERK kinase (PD-98059) before exposure to EGF. Typical Isc traces (Fig. 3, A–D) and summary data (Fig. 3E) reveal that addition of PD-98059 (30 μM; apical and basolateral) to mCT12 epithelial monolayers had no significant effect on amiloride-sensitive Isc, yet completely prevented the EGF-induced inhibition of Isc. Similar results were obtained with another MEK inhibitor (U0126; 10 μM; data not shown).

Activation of the ERK signaling cascade by EGF elicits rapid phosphorylation of p42/p44 MAPK (ERK1/2). As illustrated in Fig. 4, exposure of mCT12 cells to EGF or extracellular ATP resulted in an increase in phospho-p42/p44 MAPK compared with unstimulated cells. Because extracellular nucleotide triphosphates, acting at purinergic receptors, are thought to signal through PKC and/or elevated calcium, the effects of PKC activation and calcium mobilization on ERK signaling were investigated. Exposure of mCT12 cells to phor-
bol 12-myristate 13-acetate (PMA; 0.5 μM; apical) to activate PKC or elevation of intracellular calcium by exposure to an endoplasmic reticulum Ca-ATPase inhibitor [2,5-di-tert-butylhydroquinone (DBHQ), 25 μM; apical] led to enhanced phosphorylation of p42/p44 (Fig. 4). In all cases, the increased phosphorylation of p42/p44 was almost completely prevented by pretreatment with PD-98059.

The inhibitory effects of EGF, ATP, PMA, and DBHQ on amiloride-sensitive $I_{sc}$ and the ability of an ERK kinase inhibitor (PD-98058) and a PKC inhibitor (BIM) to modulate the responses were evaluated in a series of experiments similar to those depicted in Fig. 3, A–D, and the results are summarized in Figs. 5 and 6. The inhibitory effects of EGF, ATP, and PMA on amiloride-sensitive $I_{sc}$ were completely (EGF) or partially (ATP and PMA) blocked by pretreatment of the monolayers with the ERK kinase inhibitor (PD-98059). In contrast, the DBHQ-induced inhibition of amiloride-sensitive $I_{sc}$ was completely insensitive to the ERK kinase inhibitor (Fig. 5). Similar experiments were conducted using a PKC inhibitor [bisindolylmaleimide I (BIM) GF109203x; 1 μM] to evaluate the role of PKC as a mediator of EGF- and ATP-induced downregulation of sodium absorption. As shown in Fig. 6, treatment of the monolayers with BIM almost completely prevented the PMA-induced inhibition of $I_{sc}$ but had no effect on EGF, ATP, or DBHQ-induced inhibition. Neither PD-98059 nor BIM caused significant changes in $I_{sc}$ compared with vehicle controls (Figs. 5 and 6). The addition of ATP to the apical bathing solution elicited a 40–50% decrease in amiloride-sensitive $I_{sc}$ that was prevented in part by PD-98059 but not by BIM (Fig. 6).

Fig. 1. Effect of EGF and extracellular ATP on amiloride (Amil)-sensitive short-circuit current ($I_{sc}$). A: EGF before Amil; B: Amil before EGF; C: ATP prior to Amil; D: Amil before ATP. Primary monolayer cultures of murine renal epithelial cells were mounted in Ussing chambers and bathed on both sides with collecting tubule (CT) media. Monolayers were maintained under short-circuit conditions, except during 3-s pulses, when transepithelial voltage was clamped to +2 mV (vertical deflection at 1-min intervals) to measure transepithelial resistance. Amiloride (100 μM; apical), EGF (20 ng/ml; basolateral), and ATP (100 μM; apical) were present at the times indicated by the bars. The time scale shown in A applies to all 4 traces.

Fig. 2. Summary of the effects of EGF and ATP on amiloride-sensitive $I_{sc}$. A: primary renal epithelial cell monolayers. B: immortalized murine collecting duct principal cell (mCT12) monolayers. Epithelial monolayers were exposed to vehicle, EGF, or ATP, as described in Fig. 1, and the amiloride-sensitive $I_{sc}$ was measured 20 min later. At this time, the ATP-induced secretory chloride current had largely dissipated and thus the amiloride-induced decrease in $I_{sc}$ is a good estimate of net electrogenic sodium transport. Values are means ± SE; n = 4–8 monolayers for each condition. *P < 0.05, treated monolayers exhibited significant reduction in amiloride-sensitive $I_{sc}$ (paired t-test).
channels and ouabain-sensitive Na\(^+\)-K\(^+\)-ATPase in the basolateral plasma membrane. The acute inhibitory effects of EGF, ATP, PMA, and DBHQ on \(I_{sc}\) could be due to an effect on apical and/or basolateral transport proteins. To electrically isolate the basolateral membrane and to evaluate the effects of EGF, ATP, PMA, and DBHQ on ouabain-sensitive pump activity, the apical plasma membrane of mCT12 monolayers was rendered permeable to monovalent ions by exposure to amphotericin B. As illustrated in Fig. 7A, the addition of amiloride to the apical bathing solution reduced \(I_{sc}\) to near zero, and subsequent addition of amphotericin to the apical bathing solution caused a rapid increase in \(I_{sc}\). Approximately 30 min later, ouabain was added to the basolateral bathing solution and the \(I_{sc}\) was rapidly reduced toward zero. If the epithelial monolayer was treated with amphotericin before exposure to amiloride, the inhibitory effect of amiloride on sodium absorption was prevented, but subsequent treatment with ouabain again significantly reduced \(I_{sc}\). The effects of EGF, ATP, PMA, and DBHQ on pump current were measured...
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Fig. 5. Effects of ERK kinase inhibitor on EGF-, ATP-, PMA-, and DBHQ-induced inhibition of amiloride-sensitive $I_{\text{sc}}$. The experiments were conducted as illustrated in Fig. 3. Sets of 4 mCT12 monolayers were exposed to either vehicle, kinase inhibitor (PD-98059, 30 μM apical and basolateral), agonist (EGF, ATP, PMA, DBHQ), or kinase inhibitor, followed by agonist. The amiloride-sensitive currents for each condition are normalized to the time-control monolayer (i.e., treated with vehicle) and expressed as amiloride-sensitive current of treated monolayer divided by amiloride-sensitive current in control monolayer (i.e., treated with vehicle) and expressed as amiloride-sensitive currents for each condition are normalized to the time-control monolayer (i.e., treated with vehicle) and expressed as amiloride-sensitive currents in the intact monolayer, suggesting that the primary effect of DBHQ-induced calcium mobilization on sodium absorption is via inhibition of Na⁺/K⁺-ATPase, whereas the inhibitory effects of EGF, ATP, and PMA are most likely due to inhibition of apical ENaC activity.

EGF-induced inhibition is reversible. In a final set of experiments, the reversibility of the EGF-induced ERK1/2 phosphorylation and inhibition of amiloride-sensitive sodium absorption were tested. As illustrated in Fig. 8E, EGF-induced phosphorylation of ERK1/2 was rapidly reversed by addition of an ERK kinase inhibitor (U0126) to monolayers. ERK1/2 phosphorylation level was essentially constant between 15 and 30 min after the addition of EGF to the basolateral bathing solution. The addition of U0126 to the monolayers during the plateau phase of EGF-induced ERK1/2 phosphorylation led to a dose-dependent reversal of ERK1/2 phosphorylation (Fig. 8E). Similarly, the decrease in $I_{\text{sc}}$ elicited by EGF was partially reversed by the addition of U0126, whereas addition of the ERK kinase inhibitor produced only a small decrease in $I_{\text{sc}}$ (3–4%) independent of the presence or absence of EGF treatment. Individual traces are shown in Fig. 8, A–C, and summary data are depicted in Fig. 8D.

DISCUSSION

Previous studies demonstrated acute inhibition of amiloride-sensitive sodium absorption and/or ENaC activity by a variety of receptor agonists, including EGF (10, 43), endothelin (15, 17), and extracellular nucleotide triphosphates (21, 24, 26). PKC (24), calcium (22, 43), arachidonic acid (47), and Src kinase (17) were implicated as intracellular signaling intermediates; however, there are conflicting data, and the precise mechanisms of inhibition have not been elucidated. Activation of MAP kinase signaling (ERK1/2) is a common event in renal cells exposed to EGF, acetylcholine, endothelin, and extracellular ATP as well as other receptor agonists (41). Garty and coworkers (8, 33) showed recently that ERK2 can phosphorylate well-conserved threonines in COOH terminal peptides from the β- and γ-subunits of ENaC and suggested that MAP kinases may play a role in regulating ENaC. We showed previously (32) that prolonged exposure of renal collecting duct cell lines to EGF or PMA leads to activation of MAP kinase signaling and subsequent downregulation of ENaC-mediated sodium absorption (50–60% decrease). Phosphorylation of ERK1/2 (p42/44) and inhibition of sodium absorption were prevented by pretreatment with an inhibitor of ERK kinase, and a transcriptional mechanism was proposed based on the finding that EGF caused a 70–85% decrease in the steady-state levels of the α-, β-, and γ-subunit mRNAs. The results of the experiments presented herein reveal an additional, nontranscriptional, ERK1/2-dependent mechanism for rapid downregulation of sodium absorption.

EGF-mediated inhibition of $I_{\text{sc}}$. Studies of in vitro perfused cortical collecting duct (43, 46) and primary cultures of endometrial epithelial cells (10) found that EGF caused rapid inhibition of sodium transport. On the basis of changes in membrane potential and direct measurements of apical membrane sodium conductance in permeabilized monolayers, the primary site of action of EGF-induced inhibition of sodium absorption was localized to the apical sodium channel (10, 46). We were able to reproduce the effects of EGF on sodium absorption in mouse collecting duct cells and in immortalized mouse collecting duct principal cells.
The studies cited above concluded that elevation of intracellular calcium directly inhibited sodium channels and/or activated PKC to cause EGF-induced inhibition of sodium absorption. The pore-forming antibiotic amphotericin B was added to the apical bathing solution at a final concentration of 30 μM to increase the permeability of the apical plasma membrane to monovalent ions. After \( I_{sc} \) stabilized (10–15 min), EGF (20 ng/ml; basolateral), ATP (100 μM, apical), PMA (500 nM, apical), or DBHQ (25 μM, apical) was added and the effect on \( I_{sc} \) (pump current) was monitored for 30 min. At the end of each experiment, ouabain (Ob; 10 mM) was added to the basolateral bathing solution to confirm that the \( I_{sc} \) was mediated by the sodium pump. A–H: representative traces showing the effects of various compounds added at the indicated times. I: summary of the effects of EGF, ATP, PMA, and DBHQ on pump currents. Vehicle-treated time controls were run in parallel and were used to express the data as a percentage of control current (i.e., monolayers treated with amphotericin B but not with EGF, ATP, PMA, or DBHQ). Values are means ± SE; \( n = 6–8 \) monolayers for each condition. *\( P < 0.05 \), significant agonist-induced decrease in pump current compared with untreated controls (unpaired t-test).
ATP-mediated inhibition of $I_{sc}$ Extracellular nucleotide triphosphates are known to inhibit amiloride-sensitive sodium absorption in airway epithelium (13, 21), endometrial cells (30), and distal renal tubules (24, 26, 40). A variety of studies have been conducted in freshly isolated tissues, primary cultures, and cell lines from multiple species, but there is not a consensus as to the mechanism(s) responsible for inhibition of sodium transport. Most of the results support a role for calcium mobilization (13, 21), but not PKC activation, despite evidence that direct activation of PKC with phorbol esters can lead to inhibition of amiloride-sensitive sodium absorption in airways (18, 25). O’Grady and coworkers (30) recently reported that extracellular UTP elicited a transient stimulation of Cl secretion, followed by a sustained inhibition of amiloride-sensitive sodium absorption in cultured porcine endometrial epithelial cells. They found that the inhibition of sodium transport was due to a PKC-dependent decrease in apical sodium conductance, mediated by both calcium-dependent and calcium-independent PKC isoforms. Studies of ATP- and UTP-mediated inhibition of sodium transport in renal connecting tubules and collecting duct cells have also yielded mixed results. Koster et al. (24) concluded that extracellular ATP-mediated inhibition of sodium absorption in primary cultures of rabbit cortical collecting duct (CCD) and connecting tubule was due to PKC activation; however, Leipziger and coworkers (26, 40) found no evidence of a role for calcium or PKC activation in ATP-induced inhibition of sodium transport in immortalized murine collecting duct principal cells (M1 cells) or in perfused mouse CCD. In contrast, the present studies of immortalized collecting duct principal cells (mCT12) clearly showed that elevation of intracellular calcium or activation of PKC (Figs. 5 and 6) can elicit inhibition of amiloride-sensitive sodium absorption. Furthermore, PKC-mediated inhibition is almost completely prevented by pretreatment with a PKC inhibitor (Fig. 6). The importance of these two pathways in ATP-induced inhibition of apical sodium entry is less certain because the PKC inhibitor did not alter the ATP-induced decrease in $I_{sc}$ (Fig. 6) and the effect of calcium mobilization on sodium absorption can be explained by inhibition of basolateral Na+/K+-ATPase (Fig. 7). Recent studies by Eaton and coworkers (48) demonstrated that phosphatidylinositol 4,5-bisphosphate stimulates ENaC, and they speculated that local depletion of phosphatidylinositol 4,5-bisphosphate in response to purinergic stimulation of PLC could underlie the ATP-induced inhibition of ENaC activity. Our results do not address the mechanism of ATP-induced inhibition of sodium absorption, but they do support a role for the MAP kinase signaling cascade because exposure to extracellular ATP elicited rapid phosphorylation of ERK1/2 (p42/44), 2 ATP-induced phosphorylation of ERK1/2 was prevented by an inhibitor of ERK kinase, and 3) inhibition of ERK1/2 phosphorylation partially blocked (~50%) the inhibitory effects of ATP on sodium absorption. 

Mechanism of ERK1/2-mediated inhibition of amiloride-sensitive sodium absorption. Several recent studies bear directly on the mechanisms of MAP kinase-mediated regulation of ENaC. Garty and coworkers (33) showed that ERK2 could
amiloride-sensitive sodium absorption, but the rapid reversibility of PKC-mediated degradation of ENaC in A6 cells was recently proposed (3). The authors used immunodetection of ENaC subunits to argue that PKC caused enhanced degradation of β- and γ-ENaC, but not α-ENaC. The evidence for involvement of ERK1/2 activation in the process was based on partial inhibition of PKC induced loss of β- and γ-ENaC by MEK inhibitors. Unfortunately, the earliest time point at which inhibitors of MAP kinase signaling were tested was 6 h, so it is difficult to unequivocally assign a role for ERK1/2 in the early decrease in sodium transport after PKC activation (i.e., PKC causes ~90% inhibition of Isc within 60 min in A6 cells). They also showed that in the presence of cycloheximide, to block channel synthesis, proteosome inhibitors prevented the PMA-induced loss of γ-ENaC, but failed to prevent the PMA-induced decrease in Isc. Previous studies of PKC-induced inhibition of ENaC function have suggested that PKC rapidly decreases open probability with a slower decrease in the number of active channels; however, the role of ERK1/2 activation was not evaluated (14). Nicod et al. (27) reported that the vasopressin-induced transcript VIT32, when coexpressed with ENaC in oocytes, caused MAP kinase activation and ENaC downregulation (3). The authors used immunodetection of ENaC subunits to argue that PKC caused enhanced degradation of β- and γ-ENaC, but not α-ENaC. The evidence for involvement of ERK1/2 activation in the process was based on partial inhibition of PKC-induced loss of β- and γ-ENaC by MEK inhibitors. Unfortunately, the earliest time point at which inhibitors of MAP kinase signaling were tested was 6 h, so it is difficult to unequivocally assign a role for ERK1/2 in the early decrease in sodium transport after PKC activation. A wide range of physiological and pathological conditions influences MAP kinase signaling in epithelial cells (41). This is particularly relevant in polycystic kidney disease, in which abnormalities in the EGFR/EGFR axis have been implicated in disease progression (29). Because of the extent to which MAP kinase activation contributes to inhibition of sodium transport by other receptor agonists and signaling pathways is undetermined and because the acute inhibition of sodium transport by EGF is readily reversible, phosphatase activity may represent another potential step for regulation.

ACKNOWLEDGMENTS

The authors acknowledge helpful discussions with Cathy Carlin, Bill Sweeney, Stephanie Orellana, and Ellis Avner. We also thank Mike Haley, Elizabeth Carroll, and Mike Wilson for expert technical assistance.

GRANTS

This study was supported by Polycystic Kidney Research Foundation Grant 99013 and National Institute of Diabetes and Digestive and Kidney Diseases Grants P50-DK57306, P30-DK27651, and T32-DK007678 (to R. Falin).

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