Ouabain stimulates protein kinase B (Akt) phosphorylation in opossum kidney proximal tubule cells through an ERK-dependent pathway

Syed J. Khundmiri, Vishal Amin, Jeff Henson, John Lewis, Mohamed Ameen, Madhavi J. Rane, and Nicholas A. Delamere

1Department of Medicine, University of Louisville, Louisville, Kentucky; and 2Department of Physiology, University of Arizona, Tucson, Arizona

Submitted 18 October 2006; accepted in final form 12 July 2007

Ouabain stimulates protein kinase B (Akt) phosphorylation in opossum kidney proximal tubule cells through an ERK-dependent pathway. Am J Physiol Cell Physiol 293: C1171–C1180, 2007. First published July 18, 2007; doi:10.1152/ajpcell.00535.2006.—Endogenous cardiotonic glycosides bind to the inhibitory binding site of the plasma membrane sodium pump (Na+/K+-ATPase). Plasma levels of endogenous cardiotonic glycosides increase in several disease states, such as essential hypertension and uremia. Low concentrations of ouabain, which do not inhibit Na+/K+-ATPase, induce cell proliferation. The mechanisms of ouabain-mediated response remain unclear. Recently, we demonstrated that in opossum kidney (OK) proximal tubular cells, low concentrations of ouabain induce cell proliferation through phosphorylation of protein kinase B (Akt) in a calcium-dependent manner. In the present study, we identified ERK as an upstream kinase regulating Akt activation in ouabain-stimulated cells. Furthermore, we provide evidence that low concentrations of ouabain stimulate Na+/K+-ATPase-mediated 86Rb uptake in an Akt-, ERK-, and Src-dependent manner. Ouabain-mediated ERK phosphorylation was inhibited by blockade of intracellular calcium release, calcium entry, tyrosine kinases, and phospholipase C. Pharmacological inhibition of PDK2, which phosphorylates PDK1, the identity of PDK2, which phosphorylates activated protein kinase-2 (MK2; Ref. 35), double-stranded RNA-activated protein kinase-2 (MDK2; Ref. 35), double-stranded DNA-dependent protein kinase (8, 15), ataxia telangiectasia major determinant of total body sodium homeostasis, extracellular fluid volume status, and blood pressure control (46).

Cardiac glycosides such as ouabain are potent inhibitors of Na+/K+-ATPase-mediated ion transport (3). In some cells however, ouabain at low concentrations appears to act as a steroid hormone and not an ion transport inhibitor. Ouabain activates several signaling proteins including phosphoinositide-3 kinase (PI-3K), tyrosine kinases, the Ras-Raf MEK pathway, and protein kinase C (PKC) in cardiac myocytes. This occurs through the activation of epidermal growth factor receptor (EGFR) and appears to cause hypertrophy (23, 24, 27, 30, 31, 43–45). In proximal tubule cells, a 1 nM concentration of ouabain is sufficient to activate extracellular-regulated kinase (ERK) and stimulate cell proliferation (6). Ouabain is also known to trigger calcium oscillations in kidney proximal tubular cells (1). Significantly, plasma levels of endogenous cardiotonic glycosides (ouabain and marinobufagenin), normal products of mammalian adrenal glands, are increased in various disease states such as essential hypertension (29) and chronic renal failure (19), experimental uremia (14), and high salt intake-induced hypertension (28).

We recently demonstrated that 10 nM ouabain induced Akt-Ser473 phosphorylation and promoted cell proliferation (22). However, results from other laboratories point to a role for ERK in ouabain response that leads to cell proliferation (6). Thus several questions remain for better understanding of how these two proliferative pathways regulate cell proliferation. Akt and ERK pathways could independently promote cell proliferation. Alternatively, cross talk or interdependence between ERK and Akt signaling pathways could exist. The goal of the present study was to decipher the relationship, if any, between ERK and Akt pathways from the context of Na+/K+-ATPase-mediated ion transport. Akt is present in the cytosol of unstimulated cells. Phospholipids generated by activation of PI-3K bind to the pleckstrin homology (PH) domain of Akt, recruiting it to the plasma membrane. Once at the plasma membrane, Akt is activated by phosphorylation on two sites, namely, Thr308 and Ser473 (36). Although Thr308 is known to be phosphorylated by 3'-phosphoinositide-dependent kinases-1 (PDK1), the identity of PDK2, which phosphorylates Ser473, has been debated for several years (7). Several candidate kinases have been reported to function as PDK2, including integrin-linked kinase (4, 40), Akt itself (39), MAP kinase-activated protein kinase-2 (MK2; Ref. 35), double-stranded DNA-dependent protein kinase (8, 15), ataxia telangiectasia

Na+/K+-ATPase, or the Na+ pump, is a member of the P-type ATPases. The primary function of Na+/K+-ATPase is to establish and maintain normal Na+ and K+ gradients across the plasma membrane (18). In kidney proximal tubules, the activity of Na+/K+-ATPase localized to the basolateral membrane establishes ion gradients that provide driving force for vectorial transport of various solutes and ions from the tubular lumen to the renal vasculature. Na+/K+-ATPase-mediated regulation of proximal renal tubule sodium reabsorption is a

Address for reprint requests and other correspondence: S. J. Khundmiri, Kidney Disease Program, Univ. of Louisville, 570 S Presto St., South POD 102, Louisville, KY 40202 (e-mail: syed.khundmiri@louisville.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
mutated kinase (42), and, most recently, mammalian target of rapamycin (mTOR; Refs. 17, 38). In this study we present data suggesting that in ouabain-stimulated cells, ERK is required for Akt phosphorylation at Ser473. Consistent with this idea, we have demonstrated that ouabain-stimulated Akt phosphorylation in opossum kidney (OK) proximal tubular cells is dependent on ERK phosphorylation. Interestingly, the response was associated with stimulation of Na+/K+ transport in a manner dependent on Src kinase.

**EXPERIMENTAL PROCEDURES**

**Materials.** Ouabain, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), and SKF-96365 were purchased from Sigma (St. Louis, MO). Akt inhibitor [IL-6-hydroxymethyl-chino-inositol 2-(R)-2-0-methyl-3-O-octadecylcarbonate], genistein, LY-294002, and edelfosine (ET18-OCH₃), an inhibitor of phosphatidylinositol-dependent phospholipase C were purchased from Calbiochem-EMD Biosciences (San Diego, CA). Constitutively active, dominant negative, and wild-type MEK1 and Akt cDNAs in pUHE vector, empty vector (pUHE), recombinant constitutively active and inactive ERK2, Akt1, the NH₂-terminal pleckstrin homology domain of Akt (PH domain, amino acids 1–144), control small interference RNA (siRNA) plasmid, pKD-2, pKD-MEK1-v2 (catalog no. 62-001), and siRNA plasmid pKD-MEK1-v2 were obtained from Upstate Biotechnologies (Charlottesville, VA). GenePorter transfection reagent was purchased from Genlantis (Gene Therapy Systems, San Diego, CA). Cell proliferation assay kit and U0126 were purchased from Promega. Antibodies against phospho-ERK1/2, total ERK1 and ERK2, and horseradish peroxidase-linked secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-Ser⁴⁷³-Akt (antibody no. 9271), phospho-Thr³⁰⁸-Akt (antibody no. 9275), phospho-Thr³⁰⁸-Akt (antibody no. 9336), phospho-Thr³⁰⁸-Akt (antibody no. 9315), total GSK (antibody no. 9271), total GSK (antibody no. 9272), total GSK (antibody no. 9315), total mTOR (antibody no. 2971), Akt2 (antibody no. 7503), and Akt3 (antibody no. 7506) were purchased from Cell Signaling Technology (Danvers, MA). All other chemicals were purchased from Sigma unless otherwise specified.

**Cell culture.** Wild-type OK cells were a generous gift from Dr. Steven Scheinman (Health Sciences Center, Syracuse, NY). Cells were maintained in minimal essential medium with Earle’s salts supplemented with 10% fetal calf serum, and 1% penicillin-streptomycin. Cell culture and all other studies were carried out at 37°C in a humidified atmosphere of 95% air-5% CO₂. Cells were fed twice a week and split once a week at a 1:4 ratio. All experiments were carried out using cells at 90–95% confluence. Cells grown on six-well culture plates were washed with serum-free medium for 24 h before use.

**Western blot analysis.** OK cells were treated with 10 nM ouabain for 15 min. The cells were lysed in lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 20 mM NaF, 1 mM EGTA, 1 mM EDTA, 5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml phosphatase inhibitor cocktail 1, 0.5% Nonidet P-40, and 1% Triton X-100. The cell lysate was homogenized by passing through a 27.5-gauge needle and centrifuged at 20,000 g at 4°C. The supernatant proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The nitrocellulose membrane was incubated in 5% nonfat dry milk in 20 mM Tris, 150 mM NaCl, and 0.05% Tween 20 (TTBS) at room temperature for 1 h to inhibit nonspecific binding, followed by overnight incubation at 4°C with anti-phospho-ERK1/2, phospho-Ser⁴⁷³, phospho-Thr³⁰⁸-Akt, phospho-Ser⁴⁴⁴-mTOR, or phospho-Ser⁴⁴⁴-GSK3 antibodies in 5% milk in TTBS. Location of specific antibodies was detected by incubation with peroxidase-labeled secondary antibodies at 1:2,000 dilution in 5% milk in TTBS, followed by development with enhanced chemiluminescence (New England Biolabs). The bands imaged by chemiluminescence were analyzed by densitometry. The films were scanned using a Personal Densimeter SI (Molecular Dynamics).

**Transfection of MEK1, Akt, or MEK1 siRNA plasmid cDNA and Akt activity.** Constitutively active (CA-MEK), dominant negative (D-MEK), or wild-type MEK1 (WT-MEK1), constitutively active (CA-Akt), dominant negative (D-Akt), or wild-type Akt (WT-Akt) in mammalian expression vector pUHE, or empty pUHE vector was transiently transfected in OK cells using GenePorter transfection reagent according to the manufacturer’s protocol and as described previously (21). Akt activity was determined using Western blot analysis with phospho-Ser⁴⁴⁴-GSK3 antibodies. Plasmid siRNA for MEK1 (pKD-MEK1) or pKD negative control siRNA plasmid was transfected as described above.

**In vitro phosphorylation of recombinant inactive Akt by recombinant active ERK2.** In vitro kinase assay for Akt phosphorylation by recombinant active ERK2 was carried at 30°C for 1 h by adding 1 μl of active recombinant ERK2 (0.1 μg/μl) to 29 μl of kinase buffer containing 20 mM HEPES, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM dithiothreitol, either 1 μM ATP (cold) or 1 μl of (γ⁻⁴-P)ATP, and 1 μl of recombinant inactive Akt1 (0.1 μg/μl) or the pleckstrin homology (Akt-PH, amino acids 1–144) domain of Akt. Active recombinant Akt or MAP kinase-activated protein kinase-2 (MK2; a kinase known to phosphorylate Akt at Ser⁴⁷³; Ref. 35) was used as positive control to identify the phosphorylated Akt band. The reaction was terminated by the addition of 6 μl of 6X Laemmli buffer. The samples were boiled for 3 min, the products were resolved by 4–12% gradient SDS-PAGE, and Akt phosphorylation was detected using either autoradiography or Western blot analysis with phospho-Ser⁴⁴⁴-Akt antibodies.

**Ouabain-sensitive ⁸⁶Rb uptake.** Ouabain-sensitive ⁸⁶Rb uptake was measured as described previously (21, 22) as an index of Na⁺/K⁺ ATPase-mediated ion transport. OK cells were pretreated with 5 μM monensin for 30 min. The cells were exposed to 10 nM ouabain for 5 min before a trace amount of ⁸⁶RbCl (~1 μCi/ml ⁸⁶RbCl) was added in DMEM without serum. Uptake was carried out for 10 min such that total ouabain treatment time was 15 min, after which the cells were washed five to six times with ice-cold PBS. One-half of the cells received ouabain (final concentration 1 μM) added 15 min before the start of ⁸⁶Rb uptake. The cells were lysed overnight in 0.5 N NaOH containing 0.1% Triton X-100 at 37°C. An aliquot (100 μl) of the lysate was used to measure radioactivity. The difference between ⁸⁶Rb uptake measured in the presence of 1 mM and 10 nM of ouabain was used as a measure of Na⁺/K⁺-ATPase-mediated transport activity. Uptake data are expressed as nanomoles of ⁸⁶Rb accumulated per milligram of protein per minute.

**Measurement of cell proliferation.** The number of living cells was determined using the CellTiter 96 AQueous One solution reagent (catalog no. G3580; Promega) according to the manufacturer’s recommendations as reported previously (22). Briefly, cells were cultured in a 96-well plate (100 μl of culture medium per well) for 6 h and then exposed to 10 nM or 10 μM ouabain for 24 h in the continuous presence or absence of U0126. The growth of cells as reflected by metabolism of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was determined using CellTiter 96 AQueous One solution reagent. Titer reagent (20 μl) was added directly to culture wells and incubated for 3 h, and the absorbance at 490 nm was measured with a 96-well plate reader to quantify the amount of formazan product. Cells in one lane of the plate (6 wells) were washed twice with 1× PBS and lysed in 0.5% Triton X-100 to determine the amount of protein. The data are expressed as optical density (OD at 490 nm) per milligram of protein.

**Protein determination.** Protein concentration was determined using a bichinchoninic acid protein kit (Sigma) with BSA as standard.

**Statistics.** Data are means ± SE. The n values represent the number of separate experiments. Each experiment was done in triplicate. P value was calculated using SigmaStat software utilizing Student’s t test.
Results

Effect of ERK on ouabain-mediated Akt phosphorylation. We demonstrated previously that 10 nM ouabain increases Akt phosphorylation (22). To determine whether ERK plays a role in ouabain-mediated Akt phosphorylation, cells were treated for 15 min with 10 nM ouabain in the presence or absence of 10 μM U0126, an inhibitor of upstream activator of ERK (MEK1), or 50 μM Akt inhibitor, an Akt inhibitor that interacts in a complementary manner with the positively charged pocket formed by β1–β2 and β3–β4 loops of the PH domain of Akt, thereby preventing the binding of inositol 1,4,5-trisphosphate and translocation of Akt to membrane (17). As shown in Fig. 1A, ouabain-mediated Akt phosphorylation at both Ser473 (left) and Thr308 (right) was prevented by inhibition of both Akt and MEK1. Ouabain-mediated Akt phosphorylation resulted in phosphorylation of Akt substrate GSK3 at Ser9, which was also prevented by inhibition of Akt and ERK (Fig. 1B, left). Ouabain also promoted phosphorylation of mTOR at Ser2448 (Fig. 1B, right). However, mTOR phosphorylation was independent of both Akt and ERK. To confirm that inhibition of ERK prevents ouabain-stimulated Akt phosphorylation, cells were transfected with either control siRNA plasmid (pKD-negative control) or pKD-MEK1-siRNA plasmid 48 h before treatment with 10 nM ouabain. As shown in Fig. 1C, transfection with MEK1 siRNA plasmid abolished ouabain-stimulated Akt and ERK phosphorylation. In cells transfected with control siRNA plasmid, ouabain stimulated both Akt and ERK phosphorylation.

To confirm the dependence of Akt phosphorylation on ERK, OK cells were transiently transfected with cDNA for constitutively active (CA) or dominant negative (D) MEK1 or Akt. As shown in Fig. 2A, transfection with CA-MEK1 increased ERK phosphorylation. Transfection with D-MEK1, CA-Akt, or D-Akt had no discernible effect on ERK phosphorylation. Akt phosphorylation increased in cells transfected with CA-MEK1 compared with D-MEK1, CA-Akt, D-Akt, or empty vector (pUSE; Fig. 2B). Next we determined whether Akt is activated upon phosphorylation by ERK. Akt activity was assessed by measuring phosphorylation of the Akt substrate GSK3 at Ser9. As shown in Fig. 2C, left, transfection with CA-MEK1 and CA-Akt increased GSK3 phosphorylation compared with untransfected control cells or cells transfected with D-MEK1, D-Akt, or empty vector. To confirm that the effects of transfection with CA-MEK1 or CA-Akt were specific, in a separate experiment cells were transfected with vector (pUSE), WT-MEK1, or WT-Akt. Cells were lysed 24 h after transfection and analyzed using Western blot analysis with phospho-ERK1/2 or phospho-Ser473-Akt antibodies. As shown in Fig. 2C, right, transfection with WT-MEK1 or WT-Akt did not induce phosphorylation of ERK or Akt. To confirm the ability of ERK to directly phosphorylate Akt, an in vitro Akt kinase assay was performed. As shown in Fig. 2D, ERK caused marked increase in Akt phosphorylation as determined by 32P autoradiography (left) and Western blotting using phospho-Ser473-Akt antibodies (right).

To determine whether dependence of Akt on ERK in ouabain-mediated signaling cascade is specific to ouabain, cells were treated for 15 min with 10 μM forskolin (PKA activator) or 1 μM PMA (PKC activator). As shown in Fig. 3, both forskolin and PMA increased ERK phosphorylation. However, forskolin and PMA had no effect on Akt phosphorylation.

Effect of ouabain on ERK phosphorylation. OK cells were treated for 15 min with 10 nM ouabain, a concentration of ouabain previously found to phosphorylate Akt and stimulate 86Rb uptake and cell proliferation (22). ERK phosphorylation in ouabain-treated cells was examined using Western blot analysis. As shown in Fig. 4, ouabain markedly increased ERK phosphorylation.

Influence of Akt, ERK, and Src kinase on ouabain-mediated stimulation of 86Rb uptake. We have previously demonstrated that 10 nM ouabain increases 86Rb uptake in OK cells (22). To determine the influence of Akt, ERK, and Src kinase on stimulation of 86Rb uptake, cells were treated for 15 min in the continued presence and absence of 50 μM Akt inhibitor, 10 μM U0126, or 100 nM PP2 (a Src kinase inhibitor). Ouabain-sensitive 86Rb uptake was measured as described in Experimental Procedures. As shown in Fig. 5, 10 nM ouabain increased 86Rb uptake. The ouabain-mediated increase in 86Rb uptake was prevented by inhibitors of Akt, ERK, and Src kinase.

Role of tyrosine kinase and PI-3K on ouabain-mediated ERK phosphorylation. Askari and colleagues (8) have demonstrated that ouabain at low concentration activates ERK through a mechanism that involves the EGFR and Src tyrosine kinase (43). To examine the role of tyrosine kinase and PI-3K in ouabain-mediated ERK phosphorylation, OK cells were treated for 15 min with 10 nM ouabain in the continued presence or absence of 1 μM genistein (tyrosine kinase inhibitor), 100 nM PP2 (Src kinase inhibitor), 5 μM LY-294002 (PI-3K inhibitor), 50 μM Akt inhibitor, or 10 μM U0126 (MEK-1 inhibitor). As shown in Fig. 6, ouabain-mediated ERK phosphorylation was inhibited by genistein, PP2 (A), and U0126 (B). In contrast, Akt inhibitor and LY-294002 had no effect on ouabain-mediated ERK phosphorylation (Fig. 6B).

Role of calcium. We have recently demonstrated that Akt phosphorylation by 10 nM ouabain is dependent on calcium (22). To examine the role of calcium on ouabain-mediated ERK phosphorylation, OK cells were treated for 15 min with 10 nM ouabain in the continued presence or absence of 100 μM M BAPTA-AM (20 loops of the PH domain of Akt, thereby preventing the binding of inositol 1,4,5-trisphosphate and translocation of Akt to membrane (17). As shown in Fig. 1A, ouabain-mediated Akt phosphorylation at both Ser473 (left) and Thr308 (right) was prevented by inhibition of both Akt and MEK1. Ouabain-mediated Akt phosphorylation resulted in phosphorylation of Akt substrate GSK3 at Ser9, which was also prevented by inhibition of Akt and ERK (Fig. 1B, left). Ouabain also promoted phosphorylation of mTOR at Ser2448 (Fig. 1B, right). However, mTOR phosphorylation was independent of both Akt and ERK. To confirm that inhibition of ERK prevents ouabain-stimulated Akt phosphorylation, cells were transfected with either control siRNA plasmid (pKD-negative control) or pKD-MEK1-siRNA plasmid 48 h before treatment with 10 nM ouabain. As shown in Fig. 1C, transfection with MEK1 siRNA plasmid abolished ouabain-stimulated Akt and ERK phosphorylation. In cells transfected with control siRNA plasmid, ouabain stimulated both Akt and ERK phosphorylation.

To confirm the dependence of Akt phosphorylation on ERK, OK cells were transiently transfected with cDNA for constitutively active (CA) or dominant negative (D) MEK1 or Akt. As shown in Fig. 2A, transfection with CA-MEK1 increased ERK phosphorylation. Transfection with D-MEK1, CA-Akt, or D-Akt had no discernible effect on ERK phosphorylation. Akt phosphorylation increased in cells transfected with CA-MEK1 compared with D-MEK1, CA-Akt, D-Akt, or empty vector (pUSE; Fig. 2B). Next we determined whether Akt is activated upon phosphorylation by ERK. Akt activity was assessed by measuring phosphorylation of the Akt substrate GSK3 at Ser9. As shown in Fig. 2C, left, transfection with CA-MEK1 and CA-Akt increased GSK3 phosphorylation compared with untransfected control cells or cells transfected with D-MEK1, D-Akt, or empty vector. To confirm that the effects of transfection with CA-MEK1 or CA-Akt were specific, in a separate experiment cells were transfected with vector (pUSE), WT-MEK1, or WT-Akt. Cells were lysed 24 h after transfection and analyzed using Western blot analysis with phospho-ERK1/2 or phospho-Ser473-Akt antibodies. As shown in Fig. 2C, right, transfection with WT-MEK1 or WT-Akt did not induce phosphorylation of ERK or Akt. To confirm the ability of ERK to directly phosphorylate Akt, an in vitro Akt kinase assay was performed. As shown in Fig. 2D, ERK caused marked increase in Akt phosphorylation as determined by 32P autoradiography (left) and Western blotting using phospho-Ser473-Akt antibodies (right).

To determine whether dependence of Akt on ERK in ouabain-mediated signaling cascade is specific to ouabain,
Fig. 1. Effect of ERK on ouabain-mediated Akt phosphorylation. Opossum kidney (OK) cells were treated for 15 min with 10 nM ouabain in the presence or absence of Akt inhibitor or U0126. Cells were lysed, and the cell lysates were subjected to 10% SDS-PAGE, transferred, and probed with phospho (p)-Ser<sup>473</sup>-Akt antibodies (A, top left) or p-Thr<sup>308</sup>-Akt antibodies (A, top right). Akt antibodies (A, top right), p-Ser<sup>9</sup>-glycogen synthase kinase-3β (GSK3β) antibodies (B, top left), or p-Ser<sup>444</sup>:mammalian target of rapamycin (mTOR) antibodies (B, top right). The nitrocellulose membranes were stripped and reprobed for total proteins (A and B, bottom). Representative Western blots are shown. Phosphorylated (phospho) and total band densities were analyzed by densitometry, and the results (means ± SE from 3 independent experiments) are presented as the ratio of phosphorylated to total protein band density. *P < 0.05 by Student’s t-test.

C: OK cells were transfected with either negative control small interference RNA (siRNA; pKD-negative control siRNA) or pKD-MEK1 siRNA plasmid. Cells were treated for 15 min with 10 nM ouabain 48 h after transfection, and the lysates were subjected to 10% SDS-PAGE, transferred, and probed with p-Ser<sup>473</sup>-Akt (top left) or p-ERK1/2 antibodies (top right). The nitrocellulose membranes were stripped and reprobed with total Akt or total ERK antibodies (bottom). Representative Western blots are shown. Phosphorylated and total protein band densities were analyzed by densitometry, and the results (means ± SE from 3 independent experiments) are presented as the ratio of phosphorylated to total protein band density. *P < 0.05 by Student’s t-test.
Fig. 2. Effect of constitutively active MEK1 transfection on Akt phosphorylation. OK cells were transiently transfected with catalytically active (CA-MEK1) or dominant negative MEK1 (D-MEK1), CA- or D-Akt, or empty pUSE vector cDNA (V). Cells were lysed 6 h after transfection, and the lysates were subjected to 10% SDS-PAGE, transferred, and probed with p-ERK1/2 antibodies (A, top), p-Ser⁴⁷³-Akt (B, top), or p-Ser⁹-GSK3β antibodies (C, top left). The nitrocellulose membranes were stripped and reprobed with respective total proteins (A–C, bottom). Representative Western blots are shown. Phosphorylated and total protein band densities were analyzed by densitometry, and the results (means ± SE from 3 independent experiments) are presented as the ratio of phosphorylated to total protein band density. *P < 0.05 by Student’s t-test. Akt-T, transfected Akt; C, control. C, right: OK cells were transiently transfected with vector, wild-type (WT)-Akt, or WT-MEK1 and lysed 24 h after transfection. The lysates were subjected to 10% SDS-PAGE, transferred, and probed with p-Ser⁴⁷³-Akt or p-ERK1/2 antibodies. Representative Western blots from 2 independent experiments are shown. D: recombinant inactive Akt1 was in vitro phosphorylated by catalytically active recombinant ERK2 in the presence of radioactive (left) or nonlabeled ATP (right) as described in EXPERIMENTAL PROCEDURES. A representative autoradiogram of phosphorylated proteins (left) is shown. Solid arrows indicate the phosphorylated Akt band, whereas broken arrow shows autophosphorylation of the respective kinase. The gels were exposed for 1 h to detect autophosphorylation of active recombinant Akt (positive control, last lane). Samples that underwent phosphorylation in presence of nonlabeled ATP were transferred to nitrocellulose membranes and probed with p-Ser⁴⁷³-Akt antibodies. The blots were stripped and reprobed with total Akt antibodies. The blots were again stripped and reprobed with ERK2 antibodies. A representative Western blot from 2 independent experiments is shown.
in the continued presence or absence of 30 μM edelfosine, an inhibitor of phosphatidylinositol-dependent PLC. As shown in Fig. 8, edelfosine suppressed ouabain-mediated ERK phosphorylation. The data suggest that ERK phosphorylation by ouabain is dependent on PLC.

**Effect of ERK on ouabain-stimulated cell proliferation.** We and others have shown that ouabain at low concentration increases the rate of cell proliferation in an Akt-dependent (22) and calcium-dependent manner (22, 25). To examine the influence of ERK on ouabain-induced cell proliferation, cells were treated with 10 nM or 10 μM ouabain for 24 h in the presence or absence of U0126. As shown in Fig. 9, 10 nM ouabain increased proliferation by ~30%. U0126 abolished the increase in proliferation. It is noteworthy that treatment with 10 μM ouabain decreased cell number, an effect that was enhanced by treatment with U0126 together with 10 μM ouabain. Added alone, U0126 did not significantly alter cell proliferation. At a 10 μM concentration, ouabain decreased ERK phosphorylation (Fig. 9, right). This effect appears to be due to decreased ERK expression.

**DISCUSSION**

Previously, we identified a role for Akt phosphorylation in cell proliferation response to nanomolar concentrations of ouabain (22). Studies by Askari and coworkers (23, 26, 27, 30, 31, 43–45) suggest that Na+/K+-ATPase in cardiac myocytes acts as a signal transducer in the sense that interaction of Na+/K+-ATPase with extracellular ouabain leads to activation of the ERK/MAP kinase pathway. In the present study we report evidence that ouabain causes phosphorylation of Akt through an ERK-dependent pathway. Akt phosphorylation in ouabain-treated cells was prevented by inhibition of ERK. Transient transfection of constitutively active MEK1 (an upstream regulator of ERK) mimicked the action of ouabain on
Akt phosphorylation. Furthermore, we confirmed that ERK-mediated phosphorylation increases Akt activity as evidenced by increased phosphorylation of an Akt substrate, GSK3. A 10 nM concentration of ouabain also stimulated OK cell growth. Importantly, inhibition of upstream activator of ERK (MEK) by U0126 abolished the effect of 10 nM ouabain on cell growth.

Fig. 6. Role of Akt, phosphoinositide-3 kinase (PI-3K), and Src kinase on ouabain-mediated ERK phosphorylation. OK cells were treated for 15 min with 10 nM ouabain in the presence of PP2 (an inhibitor of Src kinase) or genistein (a tyrosine kinase inhibitor) or Akt inhibitor, LY-294002 (LY; an inhibitor of PI-3K), or U0196 (an inhibitor of MEK1). The cell lysates were subjected to 10% SDS-PAGE, transferred, and probed for p-ERK1/2. The blots were stripped and reprobed for total ERK. Representative Western blots from 3 independent experiments are shown. Phosphorylated and total protein band densities were analyzed by densitometry, and the results (means ± SE from 3 independent experiments) are presented as the ratio of phosphorylated to total protein band density. *P < 0.05 by Student’s t-test. G, genistein; Ou, ouabain.

Fig. 7. Role of calcium on ouabain-mediated ERK phosphorylation. OK cells were treated for 15 min with 10 nM ouabain in the presence of 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) or EGTA, BAPTA-AM, or SKF-96365. The cell lysates were subjected to 10% SDS-PAGE, transferred, and probed for p-ERK1/2. The blots were stripped and reprobed for total ERK. Representative Western blots from 3 independent experiments are shown. Phosphorylated and total protein band densities were analyzed by densitometry, and the results (means ± SE from 3 independent experiments) are presented as the ratio of phosphorylated to total protein band density. *P < 0.05 by Student’s t-test. B, BAPTA; SKF, SKF-96365; E, EGTA.
proliferation. An in vitro kinase assay with active recombinant ERK and recombinant Akt demonstrated the ability of ERK to directly phosphorylate Akt on Ser473. Western blot analysis suggests that OK cells express Akt1 and Akt2 but not Akt3 (data not shown). Whether ERK phosphorylates Akt1 or Akt2 cannot be determined from the data presented, because the phospho-Akt Ser473 antibodies we used were not isoform specific. This effect of ERK on Akt phosphorylation appears to be specific for ouabain-induced signaling cascade, since both forskolin and PMA stimulated ERK phosphorylation without any effect on Akt phosphorylation. The results presented do not allow us to speculate on the mechanism of forskolin- or PMA-induced ERK phosphorylation.

Several kinases have been proposed as a hydrophobic motif kinase that phosphorylates Akt on Ser473. These include integrin-linked kinase (4, 40), PKCα (33), PKCβII (20), double-stranded DNA-dependent protein kinase (8, 15), ataxia telangiectasia mutated kinase (41), and mTOR (16, 37). Akt has also been shown to autophosphorylate at Ser473 (39). The data presented suggest that in the ouabain-stimulated signaling cascade, ERK is required for Akt phosphorylation at Ser473. In this study, using RNA interference-mediated knockdown and pharmacological inhibitors, we have shown that inhibition of ERK phosphorylation prevents ouabain-mediated Akt phosphorylation at Ser473. Furthermore, we have demonstrated that overexpression of catalytically active MEK1, an upstream activator of ERK1/2, induces Akt phosphorylation. Our results are in agreement with studies by Delehedde et al. (5), who showed that hepatocyte growth factor/scatter factor (HGF/SF) activates Akt phosphorylation in an ERK-dependent manner, leading to cell migration in rat mammary fibroblasts. They demonstrated that HGF/SF-mediated Akt phosphorylation was prevented by PD-98059. Kotova et al. (24) demonstrated that in human skeletal muscle cells, ouabain induces glycogen synthesis through a pathway involving Src kinase, ERK1/2, and p90rsk1. They further showed that activation of ERK1/2 leads to activation of p90rsk1. The data presented do not allow us to speculate whether ERK directly or indirectly through p90rsk1 phosphorylates Akt at Ser473 in the ouabain-stimulated signaling cascade. However, studies on the in vitro kinase reaction suggest the ability of ERK to directly phosphorylate Akt. The data presented also rule out the dependence of Akt phosphorylation at Ser473 on mTOR in cells stimulated with ouabain, since both U0126 and Akt inhibitor had no effect on ouabain-induced mTOR phosphorylation.

We and others have demonstrated that low doses of ouabain increase Na+/K+-ATPase-mediated 86Rb uptake (9–11, 22). However, the mechanism underlying stimulation of 86Rb uptake is not well understood. Our data suggest that the observed 86Rb uptake stimulation is dependent on a signaling cascade initiated by 10 nM ouabain. The response was prevented by inhibitors of Akt, ERK, and Src kinase. This fits in with a
report by Al-Khalili et al. (2), who showed that ERK, Akt, and
tyro sine kinases contribute to insulin-dependent increase of
86Rb uptake in human skeletal muscle cells. In that study, the
stimulation of 86Rb uptake was due to increased expression of
Na+/K+-ATPase protein in the plasma membrane. Kotova et al. (24) recently demonstrated that cardiotonic glycosides
increase glycogen synthesis through an ERK- and Src-dependent
pathway in human skeletal muscle cells. Oweis et al. (32)
demonstrated that ouabain at a concentration of 100 nM
inhibits Na+/H+ exchanger (NHE3) activity, reduces NHE3
mRNA, and suppresses NHE3 promoter activity in LLC-PK1
cells. Furthermore, they showed that the decrease in NHE3
activity was mediated through Src kinase and tyrosine kinase-
dependent mechanisms. However, the authors did not measure
Na+/K+-ATPase activity at a 100 nM concentration. Whether
ouabain-stimulated increase in 86Rb uptake in our study is due
to an increase in phosphorylation of Na+/K+-ATPase α-sub
unit, increased abundance of active Na+/K+-ATPase mole-
cules at the plasma membrane, or indirectly due to stimulation
of NHE3 activity remains to be determined.

Several investigators have demonstrated that ouabain medi-
ates ERK phosphorylation through Src kinase and PLC acti-
vation (38, 45). Importantly, it has been demonstrated that Src
kinase associates with Na+/K+-ATPase when LLC-PK1 cells
are exposed to low concentrations of ouabain (38). We have
recently demonstrated that Akt phosphorylation is dependent
on PLC (22). Based on the observed ability of PP2 (Src kinase
inhibitor), genistein (tyrosine kinase inhibitor), and edelfosine
(PLC inhibitor) to suppress ERK phosphorylation by ouabain,
the results from the present study suggest that ERK phosphor-
ylation in OK cells is dependent on both Src kinase and PLC.
Studies from the laboratory of Askari and colleagues (23, 30,
31) suggest that ERK phosphorylation by ouabain may occur
through two independent pathways. one through Src-mediated
activation of Ras-Raf MEK pathway and the other through
PLC-dependent, PKC-mediated direct phosphorylation of
ERK. However, from the data presented in this report, we
cannot determine whether Src and PLC are linked or are two
independent pathways.

Unlike Akt phosphorylation, ouabain-mediated ERK phos-
phorylation was not prevented by inhibitors of PI-3K, suggest-
ing that ERK phosphorylation is independent of PI-3K. This
observation may be explained by the fact that Akt requires
binding of phosphoinositides to the NH2-termin al PH domain
for its translocation to the membrane, where Akt then under-
goes phosphorylation at Thr308 and Ser473 to become fully
active (36). Generation of phosphoinositides by PI-3K is thus
an important step in regulation of Akt activity. In contrast,
ERK phosphorylation may not require PI-3K as was suggested
by Kotova et al. (24).

Ouabain is known to alter cytoplasmic calcium dynamics
even at ouabain concentrations too low to inhibit Na+/K+
-ATPase pump activity. For example, Aizman et al. (1) dem-
onstrated that ouabain elicits calcium oscillations in proximal
tubule cells. Findings in the present study suggest that phos-
phorylation of ERK is dependent on both extras cellular and
intracellular calcium. The ouabain-mediated ERK phosphory-
lization was suppressed under conditions where the concentra-
tion of extracellular calcium was reduced and by TMB-8, a
nonspecific intracellular calcium inhibitor that can prevent
release of calcium from cytoplasmic stores. Chelation of intra-
cellular calcium by BAPTA-AM prevented ouabain-mediated
ERK phosphorylation, confirming the requirement of intracel-
lar calcium in the ouabain-initiated signaling cascade. Import-
antly, the ERK response was sensitive to SKF-96365, an
inhibitor of calcium entry via store-operated calcium channels.
In the presence of SKF-96365, ouabain did not phosphorylate
ERK. It is noteworthy that ouabain is reported to elicit activa-
tion of store-operated calcium channels in astrocytes (13), and
there is evidence pointing toward disturbance of calcium dy-
namics following interaction of ouabain with the α2-isof orm
of Na+/K+-ATPase (12, 13).

The role of ouabain in cell proliferation, growth, and apo-
tosis has been difficult to define. Some studies suggest that
ouabain causes cell proliferation and hypertrophy (6, 30),
whereas others have shown that ouabain treatment leads to
apoptosis (34, 42). Different response patterns may depend on
the concentration of ouabain. We and others have shown that
10 nM ouabain significantly stimulates cell growth. The effect
of ouabain on cell proliferation can be prevented by inhibition
of calcium mobilization (22, 25) or by Akt inhibition (22). In
the present study we confirmed that the effect of ouabain on
cell proliferation requires ERK phosphorylation and does not
require inhibition of Na+/K+-ATPase-mediated ion transport.
Indeed, a reduction in cell number and decreased ERK expres-
sion was seen following exposure of OK cells to ouabain at a
concentration of 10 μM, which is sufficient to cause ∼40% inhibition in Na+/K+-ATPase-mediated ion transport.

In summary, the findings demonstrate that ERK phos-
phorylation by ouabain is required for Akt phosphorylation and cell
proliferation in OK cells. The findings point to a link between
Akt and ERK phosphorylation and the effect of 10 nM ouabain
on Na+/K+-ATPase-mediated 86Rb uptake and cell prolifera-
tion. The findings support the notion that ouabain is able to
change the pattern of cell growth at concentrations significantly
lower than the concentration required to inhibit Na+/K+-
ATPase-mediated ion transport.

ACKNOWLEDGMENTS

We thank Dr. Eleanor Lederer, Professor of Medicine, University of
Louisville, for critical reading of the manuscript. We also thank Nina Lesousky
and Sudarshan Sengupta for expert technical assistance.

GRANTS

The work was supported by a Fellowship Grant from The American Heart
Association, Ohio Valley Affiliate (to S. J. Khundmiri) and a Scientist
Development Grant (to S. J. Khundmiri and M. J. Rane), National Institutes of
Health Grants EY040414 (to N. A. Delamere) and R56 AI059165 (to M. J.
Rane), and a Project Grant from the J. Graham Brown Cancer Center,
University of Louisville (to N. A. Delamere).

REFERENCES

1. Aizman O, Uhlen P, Lal M, Brismar H, Aperia A. Ouabain, a steroid
hormone that signals with slow calcium oscillations. Proc Natl Acad Sci
2. Al-Khalili L, Kotova O, Tsuchida H, Ehren I, Ferailie E, Krook A,
Chibalim AV. ERK1/2 mediates insulin stimulation of Na+, K+-ATPase
by phosphorylation of the α-subunit in human skeletal muscles. J Biol
3. Askari A, Kakar SS, Huang W. Ligand binding sites of the ouabain-
4. Delcrommenne M, Tan C, Gray V, Rue L, Woodgett J, Dedhar S.
Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase
kinase 3 and protein kinase B/Akt by integrin-linked kinase. Proc Natl


V0L 293 • SEPTEMBER 2007 • www.AJPcell.org


