Parathyroid Hormone-mediated Regulation of Na\(^+\)-K\(^+\)-ATPase Requires ERK-dependent Translocation of Protein Kinase Ca\(^+\)∗

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Parathyroid hormone (PTH) inhibits Na\(^+\)-K\(^+\)-ATPase activity by serine phosphorylation of the α\(_1\) subunit through protein kinase C (PKC)- and extracellular signal-regulated kinase (ERK)-dependent pathways. Based on previous studies we postulated that PTH regulates sodium pump activity through isoform-specific PKC-dependent activation of ERK. In the present work utilizing opossum kidney cells, a model of renal proximal tubule, PTH stimulated membrane translocation of PKC\(\alpha\) by 102 ± 16% and PKC\(\beta I\) by 41 ± 7% but had no effect on PKC\(\beta II\) and PKC\(\gamma\). Both PKC\(\alpha\) and PKC\(\beta I\) phosphorylated the Na\(^+\)-K\(^+\)-ATPase α\(_1\) subunit in vitro. PTH increased the activity of PKCs but not PKC\(\beta I\). Coimmunoprecipitation assays demonstrated that treatment with PTH enhanced the association between Na\(^+\)-K\(^+\)-ATPase α\(_1\) subunit and PKC\(\alpha\), whereas the association between Na\(^+\)-K\(^+\)-ATPase α\(_1\) subunit and PKC\(\beta I\) remained unchanged. A PKC\(\alpha\) inhibitory peptide blocked PTH-stimulated serine phosphorylation of the Na\(^+\)-K\(^+\)-ATPase α\(_1\) subunit and inhibition of Na\(^+\)-K\(^+\)-ATPase activity. Pharmacologic inhibition of MEK-1 blocked PTH-stimulated translocation of PKC\(\alpha\), whereas transfection of constitutively active MEK-1 cDNA induced translocation of PKC\(\alpha\) and increased phosphorylation of the Na\(^+\)-K\(^+\)-ATPase α\(_1\) subunit. In contrast, PTH-stimulated ERK activation was not inhibited by pretreatment with the PKC\(\alpha\) inhibitory peptide. Inhibition of PKC\(\alpha\) expression by siRNA did not inhibit PTH-mediated ERK activation but significantly reduced PTH-mediated phosphorylation of the Na\(^+\)-K\(^+\)-ATPase α\(_1\) subunit. Pharmacologic inhibition of phosphoinositide 3-kinase δ-kinase blocked PTH-stimulated ERK activation, translocation of PKC\(\alpha\), and phosphorylation of the Na\(^+\)-K\(^+\)-ATPase α\(_1\) subunit. We conclude that PTH stimulates Na\(^+\)-K\(^+\)-ATPase phosphorylation and decreases the activity of Na\(^+\)-K\(^+\)-ATPase by ERK-dependent activation of PKC\(\alpha\).

Na\(^+\)-K\(^+\)-ATPase is an energy-dependent enzyme responsible for the maintenance of intracellular sodium and potassium balance (1). In the proximal renal tubule, the activity of this basolateral membrane protein provides the driving force for the vential transport of various solutes and ions, including sodium, from the tubular lumen to the renal vasculature. Regulation of proximal renal tubule sodium reabsorption by Na\(^+\)-K\(^+\)-ATPase activity is a major determinant of total body sodium homeostasis, extracellular fluid volume status, and blood pressure control. Abnormalities in the regulation of Na\(^+\)-K\(^+\)-ATPase activity have been implicated in the pathogenesis of some forms of hypertension associated with impaired salt excretion (2, 3). Not surprisingly, Na\(^+\)-K\(^+\)-ATPase activity is highly regulated by many hormones through multiple signaling pathways, including those relying on protein kinase C (PKC) activity (4).

Multiple PKC isoforms have been identified, comprising a family of closely related serine/threonine kinases (5). Upon activation, PKC translocates from cytosol to membrane compartments (6–9). Three major classes of PKCs have been identified: conventional, Ca\(^++\)-dependent PKCs (α, βI, βII, and γ); novel, Ca\(^++\)-independent PKCs (δ, ε, η, and θ); and atypical PKCs (ζ and λ) (10–12). Several laboratories have demonstrated differential regulation of PKC isoforms in kidney by hormones such as angiotensin II (11), dopamine (13), and norepinephrine (14). Parathyroid hormone (PTH), through interaction with the G protein Ga, activates protein kinase C through a phospholipase C-dependent pathway (15).

Several laboratories, including our own, reported that PTH-stimulated PKC activation is critical for inhibition of Na\(^+\)-K\(^+\)-ATPase in proximal renal tubule cells (2, 16–18). Other investigators have shown that membrane translocation of PKC\(\alpha\) induced by nitric oxide and by phorbol 12-myristate 13-acetate is associated with inhibition of Na\(^+\)-K\(^+\)-ATPase activity in opossum kidney cells (OK), a model of renal proximal tubule (19). The PKC isoforms stimulated by PTH that regulate Na\(^+\)-K\(^+\)-ATPase have not been identified. We have previously demonstrated that PTH regulation of Na\(^+\)-K\(^+\)-ATPase activity is PKC- and ERK-dependent (17, 18). However, the mechanisms for PTH-mediated ERK and PKC activation involved in Na\(^+\)-K\(^+\)-ATPase regulation have not been determined. Based on our previous work showing that PKC activates the ERK pathway, we postulated that PTH regulates Na\(^+\)-K\(^+\)-ATPase in OK cells by activation of ERK through a PKC\(\alpha\)-dependent mechanism. To address this hypothesis, we examined the ability of PTH to stimulate activation of specific PKC isoforms, the ability of PTH to regulate Na\(^+\)-K\(^+\)-ATPase after inhibition of specific

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The abbreviations used are: PKC, protein kinase C; PTH, parathyroid hormone; ERK, extracellular signal-regulated kinase; OK, opossum kidney; siRNA, small inhibitory RNA; PBS, phosphate-buffered saline; EMEM, minimal essential medium with Earle’s salts; IF, immunoprecipitation; MOPS, 4-morpholinosoupropanesulfonic acid; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; GFP, green fluorescent protein; PI, phosphatidylinositol; pNPase, p-nitrophenylphosphatase.
PKC isoforms, and the role of PKC isoforms and ERK in the regulation of phosphorylation and activity of the sodium pump. In contrast to our original hypothesis, the results show that PTH regulates Na\(^+\)-K\(^+\)-ATPase in OK cells by ERK-dependent activation of PKCα.

**EXPERIMENTAL PROCEDURES**

**Materials**—PTH (1–34) was purchased from Bachem Biosciences Inc. (King of Prussia, PA). Polyclonal antibodies against the Na\(^+\)-K\(^+\)-ATPase α subunit (for immunoprecipitation), \(\alpha\)-tubulin active PKCα, PKCβ1, and MEK-1, MEK-1 cDNA, PKCα-specific small inhibitory RNA (siRNA), control nonspecific siRNA, and PKC activity kit were purchased from Upstate Biotechnology, Inc. (Waltham, MA). Monoclonal antibodies against Na\(^+\)-K\(^+\)-ATPase α subunit (for Western blots) were purchased from Sigma-RBI (Natick, MA). Antibodies against PKC α, β, γ, and δ were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Polyantily-phosphoserine antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA). Dr. D. Mochly-Rosen (Stanford University School of Medicine, Stanford, California) kindly provided PKC isoform-specific inhibitory peptides. The peptides were cross-linked via an N-terminal Cys-Cys bond to Drosophila Antennapedia homedomain-derailed carrier peptide (C-RDHPKCC-RDHPKCC-RDHPKCC-RDHPKCC) (20–24) from Pro- mega. Wortmannin and LY294002 were purchased from Calbiochem-EMD Biosciences Inc (San Diego, CA). Biopporter transfection, Geneporter transfection, and Genesilencer siRNA transfection reagents were purchased from Gene Therapy Systems Inc. (San Diego, CA). Phosphate-buffered saline (PBS) without calcium and magnesium, pH 7.4, was purchased from Invitrogen. All other chemicals were purchased from Sigma unless otherwise specified.

**Cell Culture**—The OK cells are a continuous cell line derived from Virginia opossum and a widely used model for mammalian renal proximal tubule. These cells exhibit several characteristics of mammalian renal proximal tubules including a polarized morphology, basolateral expression of Na\(^+\)-K\(^+\)-ATPase, and regulation of Na\(^+\)-K\(^+\)-ATPase by PTH, CAM-dependent protein kinase, and PKC (17). OK cells were maintained in 2% FBS in DMEM supplemented with 10% fetal calf serum and 1% penicillin/streptomycin maintained in minimal essential medium with Earl's salts (EMEM) supplemented with 1% Triton X-100 was added, homogenized, and left on a rotator for 2 h at 4°C. The samples were centrifuged at 100,000 rpm for 1 h at 4°C. The resultant supernatant (Triton-soluble fraction) was transferred to a separate tube, and a sample was taken for protein estimation, and an equal volume of 2× Laemmli sample buffer was added, boiled for 5 min, and stored at −20°C. Western blot was performed as described previously (18).

**Immunoprecipitation**—The whole cell lysate or crude membranes solubilized in immunoprecipitation (IP) buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM NaF, 1 mM EDTA, 1 mM EGTA, 5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 1 mM sodium pyrophosphate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 100 μM phosphatase inhibitor mixture, 1% Triton X-100, 0.5% Nonidet P-40, and 0.5% SDS were centrifuged at 70,000 × g for 1 h in a Beckman ultracentrifuge. 100 μg of protein from the supernatant was precleared with Protein A-Sepharose beads for 2 h at 4°C. The beads were separated by centrifugation at 14,000 rpm for 1 min in a tabletop centrifuge. An equal volume of 2× Laemmli sample buffer was added and boiled for 5 min. The beads were centrifuged as above, and the proteins in the supernatant were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes and probed with Na\(^+\)-K\(^+\)-ATPase α subunit or anti-PKC isoform-specific antibodies.

**Determination of PKC Activity**—The OK cells were incubated in the presence or absence of 10–7 M PTH for 15 min. The cells were washed twice with PBS, pH 7.4, and lysed in immunoprecipitation buffer. PKCα or β1 was immunoprecipitated from 100 μg of whole cell lysate proteins as described above. The beads were washed three times with IP buffer, and PKC activity was determined according to the manufacturer's protocol (Upstate Biotechnology, Inc.). Briefly 10 μl each of assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and 1 mM CaCl\(_2\)), substrate mixture (500 μM substrate), cAMP-dependent protein kinase inhibitor mixture (2 μM protein kinase inhibitor cocktail II (Sigma), 20 μM NaF, 1 mM EDTA, 0.5% SDS were centrifuged at 70,000 g of protein from the supernatant was precleared with Protein A-Sepharose beads containing the PKC antibody as described above. Protein A-Sepharose beads containing the immunoprecipitated Na\(^+\)-K\(^+\)-ATPase α subunit were incubated in 40 μl of PKC phosphorylation buffer containing 100 mM HEPES, pH 7.4, 6.5 mM CaCl\(_2\), 4 mM dithiothreitol, 50 mM MgCl\(_2\), 5 mM ATP, 1 mM Na\(^+\)-K\(^+\)-ATPase subunit were incubated for 1 h at 37°C in 95% air, 5% CO\(_2\). The cells were fed twice a week and split supplemented with 10% fetal calf serum and 1% penicillin/streptomycin maintained in minimal essential medium with Earl's salts (EMEM).
NaOH containing 0.1% Triton X-100. A standard curve was constructed by serial dilutions of p-nitrophenol, and the color was read at 410 nm. The samples and the standards were centrifuged at 1500 × g for 15 min before spectrophotometric analysis. Ouabain-sensitive pNPPase activity was determined by subtracting the activity measured in the presence of 10 mM ouabain from total activity measured in the absence of ouabain. The assay was run in triplicate, and activity is expressed as μmol of p-nitrophenol released/mg protein/h.

**86Rb Uptake—Ouabain-sensitive 86Rb uptake was measured as an index of Na+/K+-ATPase-mediated ion transport as described previously (18) following the method of Okafor et al. (20). OK cells were pretreated with 5 μM monensin for 30 min in the presence or absence of PKC inhibitory peptides. The cells were exposed to PTH for 5 min prior to adding trace amount of 86Rb (1 μCi/ml 86RbCl) in Dulbecco's modified Eagle's without serum. Half the cells received ouabain (final concentration, 1 mM) 15 min prior to the start of 86Rb uptake. 86Rb uptake was carried out for 10 min at room temperature after which the cells were washed five or six times with ice-cold PBS. 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 the 86Rb uptake measured in the presence of 1 mM ouabain and the absence of ouabain was used as a measure of Na+/K+-ATPase-mediated transport activity. The uptake data are expressed as nmol of rubidium (86Rb) accumulated/mg protein/min, and the results are plotted as percentages of control.

**PKCs siRNA Transfection—**200 pmol of SMART-Pool PKCs or nonspecific control small inhibitory RNA (Upstate Biotechnology, Inc.) was transfected into OK cells using Genesilencer Transfection reagent according to the manufacturer's protocol. Briefly, 5 μl of Genesilencer reagent was diluted with 25 μl of serum-free EMEM. In another tube 25 μl of siRNA diluent was diluted with 15 μl serum-free EMEM, and 200 pmol of siRNA was added. The siRNA containing mixture was mixed with diluted Genesilencer reagent and incubated at room temperature for 15 min. The siRNA, Genesilencer reagent mixture was added onto growing cells in serum-free EMEM and incubated at 37 °C in 95% air, 5% CO2 for 6 h, following which 1 ml of EMEM containing 20% serum was added and further incubated for 24 h at 37 °C in 95% air, 5% CO2.

**PKC cDNA Transfection—**A cDNA for constitutively active MEK-1 (Upstate Biotechnology, Inc.) was transfected into OK cells using Geneporter transfection reagent according to the manufacturer's protocol. Briefly, the cDNA and the Geneporter reagent were diluted separately in serum-free medium. The diluted cDNA was mixed with diluted Geneporter reagent and incubated at room temperature for 30 min. The culture medium from the cells was replaced with the mixture containing Geneporter and MEK-1 cDNA and incubated for 24 h at 37 °C in 95% air, 5% CO2, following which the cells were lysed, and the membrane and cytosolic fractions were separated and processed for Western blot analysis. The supernatant proteins were separated by 10% SDS-PAGE and analyzed by Western blot using phospho-ERK antibodies to document successful uptake of active recombinant MEK-1.

**Uptake of Constitutively Active Recombinant MEK-1—**Wild type OK cells were washed with Dulbecco's modified Eagle's medium without fetal calf serum 24 h prior to uptake of active recombinant MEK-1. 1 μg/ml GFP-linked constitutively active recombinant MEK-1 was mixed with 5 μl of dried Biopporter film in a 1.5-ml tube and incubated for 5 min at room temperature. The mixture was then transferred to OK cells and incubated for 4 h at 37 °C in a humidified 95% air, 5% CO2 incubator. After 4 h, the cells were washed twice with 1× PBS and then lysed in 50 mM mannitol, 5 mM Tris-HCl buffer, pH 7.4. The crude membranes were prepared as described above. The supernatant proteins were separated by 10% SDS-PAGE and analyzed by Western blot using phospho-ERK antibodies to document successful uptake of MEK-1. Protein concentration was measured by the BCA method (Sigma) using bovine serum albumin as standard.

**Densitometry**—The Western blots were scanned and analyzed using Personal densitometer (Molecular Probes) and expressed as arbitrary densitometric units.

**Statistics**—The data are shown as the means ± S.E. All of the experiments were repeated at least three times unless otherwise stated to document reproducibility. The p values are calculated using SigmaStat software utilizing a paired t test. A p value less than 0.05 was a priori considered statistically significant.

**RESULTS**

**Identification of PKC Isoforms Activated by PTH in OK Cells**—Consistent with previous reports (19, 30, 31) preliminary studies demonstrated the expression of PKCa, βI, βII, and γ but not of PKCδ, η, and ε in OK cells (data not shown). To determine which PKC isoforms were activated by PTH, OK cells were treated with PTH (10−7 M) for 15 min. Activation of specific PKC isoforms was determined by measuring the translocation to the membrane fraction by Western blot analysis (19) and by measuring the enzyme activity of immunoprecipitated PKC isoforms. As shown in Fig. 1 (top panel), PTH stimulated translocation of PKCa and PKCβ1 to the membrane fraction, whereas no translocation of PKCβII or γ was observed. The bar diagram shows the change in membrane expression as determined by densitometric analysis of Western blots from three separate experiments. PTH increased the membrane abundance of PKCa by 102 ± 16%, whereas PKCβ1 was increased by 41 ± 7%. To determine whether translocation of PKCa and PKCβ1 was associated with an increase in the activity, the activity of the PKC isoforms was measured. As shown in Fig. 2, PTH increased PKCa activity but had no effect on PKCβ1 activity.

**In Vitro Phosphorylation of Na+/K+-ATPase α1 Subunit by PKCa and PKCβ1**—To determine the ability of PKCa and PKCβ1 to phosphorylate the Na+/K+-ATPase α1 subunit, immunoprecipitated Na+/K+-ATPase α1 subunit was subjected to an in vitro phosphorylation assay using constitutively active recombinant PKCa or PKCβ1. Fig. 3 shows that both PKCa and PKCβ1 were capable of phosphorylating the Na+/K+-ATPase α1 subunit.
and PKCβ1 induced a 5–6-fold increase in Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit phosphorylation.

**Association of PKC Isoforms with the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) Subunit**—The ability to phosphorylate the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit suggests that specific PKC isoforms should be physically associated with this substrate. To determine whether the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit physically associates with either of the PKC isoforms, we immunoprecipitated Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit from vehicle- and PTH-treated OK cells and analyzed the immunoprecipitates for the presence of PKCα and PKCβ1 by Western blot analysis. As shown in Fig. 4A, immunoprecipitation with the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit antibody of untreated cells produced faint staining for the PKCα isoform and marked staining for the β1 isoform. PTH significantly enhanced the association between PKCα and the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit, whereas association between PKCβ1 and Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit did not change with PTH treatment. Reciprocal immunoprecipitation assays with anti-PKCα and anti-PKCβ1 antibodies and Western blot analysis for Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit confirmed the increased association with PKCα upon treatment with PTH (Fig. 4A). Fig. 4B shows densitometric data from three independent experiments, indicating a significant increase in the association of PKCα with Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit.

**Role of PKCα in PTH-mediated Phosphorylation of Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) Subunit and Inhibition of Na\(^+\)-K\(^+\)-ATPase Activity**—The preceding data strongly point toward PKCα as the isoform mediating PTH regulation of Na\(^+\)-K\(^+\)-ATPase. To determine whether PKCα is responsible for phosphorylation of the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit in intact cells, OK cells were pretreated with 10\(^{-7}\) M PTH for 15 min. Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit confirmed the increased association with PKCα upon treatment with PTH. **WB** Western blot.
subunit. Pretreatment with the PKCα inhibitory peptide blocked PTH-mediated phosphorylation of Na⁺⁻K⁺-ATPase α₁ subunit.

To determine whether PKCα played a role in PTH-mediated regulation of Na⁺⁻K⁺-ATPase activity, OK cells were treated with 10⁻⁷ M PTH for 15 min in the presence or absence of 100 nM PKCα inhibitory peptide. As shown in Fig. 6, PTH inhibited Na⁺⁻K⁺-ATPase activity by about 25%. Pretreatment with PKCα inhibitory peptide, but not with Antennapedia carrier peptide, blocked PTH-mediated inhibition of Na⁺⁻K⁺-ATPase activity, as measured by K⁺⁻dependent pNPPase activity. Each bar represents the mean (± S.E.) percentage of respective control from four independent experiments.

Role of ERK in PTH-mediated Phosphorylation of Na⁺⁻K⁺-ATPase α₁ Subunit—As described previously (18), inhibition of ERK by the MEK-1 inhibitor U0126 (final concentration, 10⁻⁵ M) inhibited PTH-mediated phosphorylation of Na⁺⁻K⁺-ATPase α₁ subunit (Fig. 5). To determine whether activation of PKCα was upstream or downstream of ERK, we measured the translocation of PKCα and activation of ERK in OK cells treated with PTH for 15 min following 30 min of pretreatment with vehicle, PKCα inhibitory peptide, or U0126. Inhibition of either PKCα or ERK blocked PTH-mediated translocation of PKCα to the membrane (Fig. 7A). Inhibition of PKCα did not alter PTH-mediated activation of ERK (Fig. 7B), suggesting that PKCα activation is downstream of ERK activation in the PTH-mediated signaling cascade. To confirm that PTH-stimulated ERK activation is not dependent upon PKCα activity, we reduced the expression of PKCα by transfection with PKCα siRNA and treated the cells with 10⁻⁷ M PTH for 15 min. As shown in Fig. 8A, PKCα siRNA reduced the expression of PKCα by about 70%, whereas it had no effect on the expression of PKCβ1. Western blot analysis of the Na⁺⁻K⁺-ATPase α₁ subunit phosphorylation showed that PTH stimulated phosphorylation in control and mock transfected cells. However, transfection with PKCα siRNA completely blocked PTH-stimulated Na⁺⁻K⁺-ATPase α₁ subunit phosphorylation (Fig. 8B). PTH increased ERK phosphorylation in all groups, including cells transfected with PKCα siRNA (Fig. 8C).

To investigate the involvement of ERK in the translocation of PKCα and phosphorylation of the Na⁺⁻K⁺-ATPase α₁ subunit, a cDNA for active MEK-1 was transfected into OK cells. Expression of active MEK-1 resulted in increased phosphorylation of endogenous ERK (Fig. 9A), translocation of PKCα to the membrane fraction (Fig. 10A), and increased phosphorylation of the Na⁺⁻K⁺-ATPase α₁ subunit phosphorylation. *, p < 0.05.
Fig. 7. Effect of PKCa inhibitory peptide and U0126 on PTH-mediated PKCa translocation and ERK activation. OK cells were treated with 10^{-7} M PTH for 15 min in the continued presence or absence of 100 nM PKCa inhibitory peptide or U0126. A presents a Western blot of membrane fractions for PKCa showing that both PKCa inhibitory peptide and ERK inhibition with U0126 block PTH-stimulated translocation of PKCa. The graph presents densitometric analysis of three independent experiments as mean ± S.E. B presents a representative Western blot of cytosolic proteins for phospho-ERK (upper panel) and ERK2 (lower panel) showing that PTH-stimulated ERK activation is blocked by U0126 but not PKCa inhibitory peptide. The graph shows densitometric quantitation of phospho-ERK in three independent experiments expressed as the means ± S.E. in arbitrary densitometry units, confirming the inhibition of ERK phosphorylation by U0126 but not by PKCa inhibitory peptide. *, p < 0.05. WB, Western blot.

Fig. 8. Effect of PKCa siRNA on PKCa expression, PTH-mediated ERK activation, and Na^+-K^+-ATPase α1 subunit phosphorylation. OK cells were transfected with PKCa siRNA or control siRNA or were cultivated with reagent or vehicle alone, followed by incubation with or without 10^{-7} M PTH for 15 min. A shows an immunoblot for PKCa and PKCβ1 of lysates from control cells and cells transfected with PKCa siRNA and control siRNA. The figure demonstrates that PKCa siRNA specifically reduces PKCa, but not PKCβ1, expression. B shows a representative Western blot of Na^+-K^+-ATPase α1 subunit immunoprecipitated from crude membrane fractions from the same groups of cells using anti-phosphoserine antibodies. The figure shows that PTH induces phosphorylation of Na^+-K^+-ATPase α1 subunit in control cells, whereas down-regulation of PKCa expression inhibits PTH-stimulated phosphorylation. C shows a representative Western blot of the same groups of cells from two independent experiments for phospho-ERK (upper panel) and ERK1 (lower panel). The results show that down-regulation of PKCa expression by siRNA has no effect of PTH-stimulated ERK phosphorylation. WB, Western blot.

Fig. 9. Effect of transient transfection of active MEK-1 on ERK activation, PKCa translocation, and Na^+-K^+-ATPase α1 subunit phosphorylation. OK cells were transiently transfected with constitutively active MEK-1 cDNA, transfected with plasmid alone, or exposed only to vehicle. A presents a Western blot for phosphorylated and total ERK representative of three independent experiments. The blots show that transfection with active MEK-1 induces phosphorylation of ERK. B shows a Western blot of membrane proteins from the same groups of cells for PKCa. The results show that PKCa translocated to the membrane in cells transfected with active MEK-1 but not in mock transfected cells. The blot is representative of three independent experiments. C is a Western blot of Na^+-K^+-ATPase α1 subunit immunoprecipitated from the crude membrane fractions from the same groups of cells using anti-phosphoserine antibodies (upper panel) or anti-Na^+-K^+-ATPase α1 subunit (lower panel). The results show that transfection with active MEK-1 stimulated phosphorylation of the Na^+-K^+-ATPase α1 subunit, and equal amounts of the subunit were immunoprecipitated. The blots are representative of three independent experiments. WB, Western blot.
MEK-1 transduction on ouabain-sensitive $K^+$ phosphorylation of $Na^+$ performed in triplicate. The data show that introduction of MEK-1 mean ($\mu$) wortmannin and LY294002 blocked PTH-mediated activation MEK-1 on ERK activation, PKC $\alpha$ subunit, and eventual phosphorylation of Na$^+$-K$^+$-ATPase. Although PKC$\beta$I communoprecipitated with the Na$^+$-K$^+$-ATPase $\alpha_1$ subunit and phosphorylated the $\alpha_1$ subunit in an in vitro phosphorylation assay, treat-
ment with PTH did not stimulate PKCβ1 activity or increase the association of PKCβ1 with Na+/K+-ATPase. These findings are also consistent with the report of Middleton et al. (31), which detected PKCβ1 in OK cells but failed to identify its activation with phorbol esters. Studies on regulation of the sodium pump through PKC activation in renal proximal tubules demonstrate variable involvement of other PKC isoforms. Budu et al. (13) showed that treatment of OK cells expressing rodent wild type Na+/K+-ATPase α1 subunit with a serotonin agonist increased sodium pump activity in a PKCβ-dependent manner. Efendiev et al. (33) demonstrated a role for PKCβ1 and PKCβ2 (I or II not specified) in the regulation of rat proximal tubule sodium pump activity by phorbol ester and dopamine.

The other novel finding emerging from this study is that the major role for PTH-stimulated ERK activity in regulation of Na+/K+-ATPase activity is to activate PKCa. Our data demonstrate that inhibition of ERK blocked PTH-mediated translocation of PKCa, suggesting that activation of PKCa is dependent upon ERK activation. Previous studies demonstrated that PKCa is phosphorylated at Thr(638), Thr(697) and Ser(687) residues (34). Computer-based phosphorylation motif screening of PKCa identified a potential ERK1/2 phosphorylation site at Thr(638) (scansite.mit.edu) (35). Therefore, it is likely that PKCa is regulated through direct phosphorylation by ERK1/2. This observation is consistent with the studies of Adadyev et al. (36), which showed that 8-hydroxy-2-(di-n-propylamino)tetralin-mediated activation of PKCa is dependent upon activation of ERK in a hippocampal cell model. Our present demonstration that PKCa activation is ERK-dependent is also consistent with our previously published findings that 1) the initial activation of ERK is PKC-independent (37) and 2) Na+/K+-ATPase regulation by PKC is ERK-dependent (17).

We and others recently demonstrated that ERK might directly phosphorylate the Na+/K+-ATPase α1 subunit (18, 38). In our studies, inhibition of MEK-1 by U0126 blocked PTH-mediated phosphorylation of the Na+/K+-ATPase α1 subunit and inhibition of Na+/K+-ATPase activity. Further, we showed that the Na+/K+-ATPase α1 subunit immunoprecipitated from OK cells transfected with exogenous rat α1 was phosphorylated when incubated with constitutively active recombinant GFP-ERK1 in an in vitro phosphorylation assay. Interestingly, phosphorylation occurred in wild type and Ser(11) to alanine mutant rat α1 but not in the Ser(11) to alanine mutant (18). Feschenko and Sweadner (32) demonstrated that Ser(11) is a PKC phospho-tyrosine site. Ser(11) is not followed by proline and therefore is not expected to be a substrate of mitogen-activated protein kinases, suggesting that in vitro phosphorylation by ERK may not be physiological. In the present work, we tested whether transfection with constitutively active MEK-1 into native OK cells could phosphorylate the Na+/K+-ATPase α1 subunit. The results showed that phosphorylation occurred in these cells but was completely blocked by an inhibitor of PKCa. Similar results were obtained when constitutively active recombinant GFP-MEK-1 was introduced into native OK cells. We cannot exclude the possibility that PTH-stimulated ERK can directly phosphorylate the Na+/K+-ATPase α1 subunit. However, the data presented here show that PTH-stimulated activation of PKCa by an ERK-dependent pathway is required for phosho-
rylation of the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit.

The present study showed that inhibition of PI 3-kinase blocked PTH regulation of sodium pump phosphorylation and activity. These data are consistent with dependence of ERK transduction pathway leading from PTH receptor ligation to inhibition of Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit in primary human skeletal muscle cells. Similarly, Isenovic et al. (39) also showed in vascular smooth muscle cells that angiotensin II increased sodium pump activity through activation of PI 3-kinase and ERK.

In conclusion, the results of this study define a novel signal transduction pathway leading from PTH receptor ligation to inhibition of Na\(^+\)-K\(^+\)-ATPase activity in one model of proximal tubular cells, the opossum kidney cell line. The results demonstrate that PTH stimulates ERK by a PI 3-kinase-dependent mechanism. ERK activation is required for activation and translocation of PKC\(\epsilon\), leading to enhanced association with the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit. This association facilitates subsequent phosphorylation of the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) subunit and inhibition of sodium pump activity.

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