Heterogeneity of Protein Kinase C-mediated Rapid Regulation of Na/K-ATPase in Kidney Epithelial Cells

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Na/K-ATPase in renal epithelium is expressed at the basolateral surface and thus is critical for vectorial solute transport. One potential mode of regulation of Na/K-ATPase involves the intracellular effector protein kinase C (PKC). In kidney cell lines, activation of PKC by the phorbol ester phorbol 12,13-dibutyrate (PDBu) (1 μM) inhibited Na/K-ATPase transport activity in OK cells (Vₘₐₓ decreased 42%; p < 0.02), but not in LLC-PK₁ cells. By immunoblot, both cell types express detectable levels of PKCα and PKCδ. In response to PDBu, PKCα translocated from the cytosol to the membrane fractions of both cell lines. Phorbol ester treatment increased incorporation of ³²PO₄ in multiple substrates in both cell types, but a ~109-kDa substrate with neutral pI was detected only in the OK cell. Anti-LEAVE, directed against a highly conserved sequence in the H₄-H₅ loop of all known α isoforms of Na/K-ATPase, recognized a ~109-kDa membrane protein from both cell lines. Anti-LEAVE also identified a protein that comigrated with the large phosphoprotein which was only present in OK cells. Following ³²PO₄ loading and PDBu treatment, anti-LEAVE immunoprecipitated a ~109-kDa phosphoprotein in OK but not LLC-PK₁ cells. These data suggest the protein capable of phosphorylating the α subunit and inhibiting Na/K-ATPase transport activity in intact renal cells. Furthermore, they suggest that some forms of Na/K-ATPase in the kidney are not susceptible to PKC phosphorylation and that this heterogeneity may contribute to response diversity.

Membrane Na/K-ATPase eliminates intracellular sodium, imports extracellular potassium, and establishes an electrochemical gradient across the cell membrane (Gick et al., 1988; Katz, 1988; Soltoff and Mandel, 1984). In epithelial cells of the renal tubule, this gradient provides a driving force for vectorial solute transport (Katz, 1988; Soltoff and Mandel, 1984). A broad range of transport processes, particularly in the proximal nephron, can undergo rapid changes as a result of altered Na/K-ATPase activity. Transport activity of the sodium pump in an intact cell depends upon amounts of the transporter expressed, concentrations of the cations sodium and potassium, and availability of intracellular ATP (Edelman et al., 1985; Gick et al., 1988; Lingrel et al., 1990). Each of these factors represents a potential site of physiologic regulation. Recent work suggests that rapid changes in sodium pump activity also derive from membrane receptors and signal transduction mechanisms. Protein kinase C (PKC) is commonly activated by receptors which are coupled to phospholipase C, including receptors for adrenergic agonists (Aperia et al., 1992: Berthon et al., 1985, Lynch et al., 1986; Middleton et al., 1990a) or for parathyroid hormone that may be expressed in the proximal nephron (Middleton et al., 1989). Since the sodium pump of the proximal nephron is important for transepithelial transport of a variety of different solutes, the relationship between protein kinase C and Na/K-ATPase has potential bearing on understanding states of disordered solute transport.

Regulation of Na/K-ATPase in the context of an intact organ could depend on coincident expression of a specific population of the transporter or a particular isoform of protein kinase C. Protein kinase C exists in at least eight different isoforms which vary in tissue distribution (Nishizuka, 1984; Wetsel et al., 1992), requirement for calcium (Kiley et al., 1990), and binding of phorbol esters (Olivier and Parker, 1992; Ryves et al., 1991). Similarly, considerable heterogeneity is described for Na/K-ATPase. A functional Na/K-ATPase transporter is comprised of a single α and β subunit, each of which demonstrates structural diversity (Shull et al., 1986a, 1986b). Isolated and purified Na/K-ATPase α subunit is phosphorylated by PKC (Lowndes et al., 1990; Bertorello et al., 1992; Aperia et al., 1992; Chibalin et al., 1992), but this action has not been observed in an intact cell. Furthermore, modification of the catalytic subunit of Na/K-ATPase by PKC decreases Na/K-ATPase enzyme activity in vitro (Bertorello et al., 1991), but a corresponding response by transport activity has not been demonstrated in an intact epithelial cell. These in vitro studies have been applied to the intact cell where PKC-directed modification of Na/K-ATPase is postulated to be a universal phenomenon (Aperia et al., 1992), but this notion has not been systematically examined in different species. In the kidney, molecular mechanisms for regulation of Na/K-ATPase would seem to be consistent across animal species since the renal sodium pump is primarily composed of a single isoform, the α₁ (Lingrel et al., 1990; Shull et al., 1986a; Svedner, 1989).

To clarify the relationship between PKC and Na/K-ATP-

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† The abbreviations used are: PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
ase in the proximal nephron, this study compares two renal cell lines that differ in phylogenetic backgrounds but share some morphological and functional features of proximal renal epithelia. The opossum kidney (OK) cell and the LLC-PK1, derived from porcine kidney, are established cell culture models of renal epithelia (Haggerty et al., 1988; Middleton et al., 1989; Pollock et al., 1986; Reshkin et al., 1990). They are suitable for these studies since they share features of polarity (Haggerty et al., 1988; Reshkin et al., 1990), sodium-dependent phosphate transport (Middleton et al., 1989), Na/H exchange (Pollock et al., 1986), and Na/K-ATPase. By examining specific activation of isoforms of protein kinase C, phosphorylation of substrates, and regulation of Na/K-ATPase in the two cell types, we gain insight into the interaction between PKC and the renal sodium pump. These studies confirm that PKC phosphorylates and rapidly inhibits renal Na/K-ATPase in an intact renal epithelial cell. Furthermore, these observations demonstrate that across animal species, the response to PKC in renal epithelia is not constant and may depend on heterogeneity within the Na/K-ATPase α1 isoform.

MATERIALS AND METHODS

Cell Culture—OK cells (passage 95-150) and LLC-PK1 cells were obtained and maintained in culture as described previously (Middleton et al., 1989). Cell monolayers were grown in a humidified 5% CO2 incubator with Dulbecco's modified Eagle's medium/F-12 medium containing 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Medium was changed to serum-free medium (0.1% globulin-free bovine serum albumin) prior to biochemical and transport studies. Cells were grown in plastic dishes or collagen-coated filters as noted (Costar Corp., Cambridge, MA). All culture reagents were obtained from Life Technologies, Inc.

Transport Studies—Previous studies validated the use of rubidium transport to estimate transport activity of Na/K-ATPase (Middleton et al., 1990a). To improve access to Na/K-ATPase, subconfluent monolayers were grown on porous membranes on supports. After incubation in Earle's solution (143 mM Na+, 5.4 mM K+, 1.2 mM MeCl2, 1.5 mM MgCl2, 1.8 mM Ca2+, 125 mM Cl, 15 mM HEPES, 25 mM glucose) was used to incubate each cell monolayer in 60-mm dishes with 32P04 (200 pCi/dish) for 2 h prior to treatment with phorbol Nonidet P-40, 2.5% 2-mercaptoethanol, and 1% ampholines and the phosphate transport (Middleton et al., 1990). Krebs buffer (143.4 mM Na+, 4.8 mM K+, 1.2 mM Mg2+, 1.4 mM Ca2+, 121.1 mM Cl, 1.2 mM SO42-, 10 mM HEPES, 25 mM glucose) was used to incubate each cell monolayer in 60-mm dishes with 32PO4 (200 μCi/dish) for 2 h prior to treatment with phorbol ester or dimethyl sulfoxide (MeSO4). The monolayer was washed and cells were scraped and suspended in 50 mM Tris/HCl, 1 mM EGTA, 1.5 mM MgCl2, 10 mM benzamidine, 100 mM NaF, 5 mM dithiotreitol, 0.25 mM sucrose, and 2% Triton X-100, pH 7.4, at 0°C. After centrifugation at 50,000 × g for 20 min, the supernatant proteins were precipitated with 20% trichloroacetic acid, washed with acetone (−20°C), and solubilized in a lysis buffer containing 9% urea, 1% Nonidet P-40, 2.5% 2-mercaptoethanol, and 1% ampholines and the total counts were equalized. Samples were prepared for two-dimensional gel electrophoresis by incubation at 37°C for 30 min and centrifugation at 5000 × g for 5 min. The samples were separated with isoelectric focusing on tube gels that ranged from 4.4 to 8.3 pH after a total of 7600 V h. The tube gels were expressed, incubated in SDS-sample buffer (2.3% SDS, 0.0625 mM Tris-HCl, 5% β-mercaptoethanol, 10% glycerol, 37°C) for 30 min, and stacked on 9% SDS-PAGE.

Western Blot—Cell monolayers were washed with ice-cold phosphate-buffered saline (PBS, 0°C) and 2 ml of extraction buffer (30 mM Tris-HCl, 10 mM EGTA, 2 mM EDTA, 50 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 0.02% leupeptin, pH 7.4) was added to each flask. Cells were scraped, homogenized for 5 s with a Polytron homogenizer (Kinematica, Switzerland), and pelleted by centrifugation at 50,000 × g for 1 h at 4°C. Membrane fractions were solubilized in homogenization buffer with 0.1% Triton X-100, and cytosolic fractions were taken from the supernatant. Samples were separated on one- or two-dimensional SDS-PAGE as above. Dithiothreitol (0.5 mM) was substituted for 2-mercaptoethanol during sample preparation for two-dimensional gels to avoid autoradiography artifacts. Proteins were transferred to Immobilon-P (Millipore, Bedford, MA) for 20 min with a semi-dry blotting system (2.5 mA/cm2). Blots were washed with PBS, 5% nonfat milk for 45 min, PBS, 1% BSA for 1 min, and incubated with the antibodies in PBS, 1% BSA at 22°C for 2 h. Anti-PKC antibodies are highly specific for each isoform (Wetsel et al., 1992), and the blots were incubated with 1:10,000 dilution. Anti-LiEAVE was a generous gift from Dr. Thomas Pressley and was incubated with blots at 1:250 dilution. The latter antibody is specific for a subunit of Na/K-ATPase (Pressley, 1992). The primary antibody was washed for 45 min with PBS/milk, and the secondary reaction was performed with 32P04 goat anti-rabbit IgG (~10 μCi/blot, Du Pont-New England Nuclear).

Immuno precipitation—Confluent 60-mm dishes of OK and LLC-PK1 cells were loaded with radioactive tracer under the same conditions indicated above for intact cell phosphorylation. Monolayers were treated with either PDBu (1 μM) or MeSO4 for 10 min followed by three washes of iced Krebs buffer. After adding 250 μl/dish of precipitating buffer (50 mM Tris-HCl, 1% Nonidet P-40, 5 mM EDTA, 0.15 μM NaCl, pH 8.3) cells were scraped and homogenized 20 strokes in a glass-on-glass homogenizer. The suspension was incubated on ice for 5 min and centrifuged at 30,000 × g for 5 min. The samples were separated on one- or two-dimensional SDS-PAGE as above. Dithiothreitol (0.5 mM) was substituted for 2-mercaptoethanol during sample preparation for two-dimensional gels to avoid autoradiography artifacts. The proteins were equalized based on trichloroacetic acid-precipitable counts and averaged ~35 × 106 counts/min/sample for OK cells and ~7 × 106 counts/min/sample for LLC-PK cells. Homogenates were cleared with rabbit serum (1:100) for 60 min at 4°C followed by 10% (v/v) washed Pansorbin (Calbiochem Corp., San Diego, CA) for 80 min. The cleared supernatant was incubated overnight with affinity-purified anti-LiEAVE (1:100). A secondary goat anti-rabbit IgG antibody (1:100, Pierce Immunotechnology, Rockford, IL) was added at 4°C for 60 min, and immune complexes were again precipitated with 10% washed Pansorbin. The pellet was washed twice, vortexed, and resuspended four times with wash buffer (50 mM Tris-HCl, 0.15 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, pH 8.3) at 4°C. After the final wash, the pellet was suspended in SDS sample buffer, boiled for 5 min, and cleared by low speed centrifugation. The proteins were separated on 9% SDS-PAGE and analyzed with autoradiography.

Statistical Methods—Kinetics were evaluated by the ENZFIT nonlinear regression program (Elsevier, BIOSOFT), and simple weighting was used to derive the kinetic values. Results were compared with paired two-tailed t test as indicated.

RESULTS

The primary observation was made in OK and LLC-PK1 cells, two established renal cell lines. We have previously demonstrated that ouabain-sensitive rubidium uptake by monolayers grown on permeable supports is a useful measure of Na/K-ATPase transport activity in intact epithelial cells (Middleton et al., 1990a). Using this technique, we found that OK cells decreased Na/K-ATPase transport activity as a specific response to activation of protein kinase C (Fig. 1). Sodium pump inhibition was observed after 10 min of treatment with the active phorbol ester phorbol 12,13-dibutyrate (PDBu, 1 μM) and phorbol 12-myristate, 13-acetate (1 μM; p < 0.01; n = 8) but not in response to 4a-phorbol, an analogue that does not significantly activate PKC (Nishizuka, 1984; Ryve et al., 1991). Increased cytosolic calcium by treatment with either a calcium ionophore (A23187) or thapsigargin, a calcium ATPase inhibitor (Middleton et al., 1990b), caused no significant change in Na/K-ATPase transport activity in OK cells. Efforts to extend this fundamental observation to another renal cell model met with contrasting results. Acute exposure to the active phorbol ester PDBu did not significantly alter Na/K-ATPase transport activity in the LLC-PK1 cell (Fig. 1). For the sake of comparison, the transport studies in Fig. 1 are shown after 10-min treatments and in the presence of 2 mM extracellular potassium. Similar observa-
Na/K-ATPase Regulation in Kidney Epithelial Cells

**TABLE I**

<table>
<thead>
<tr>
<th>Active PKC</th>
<th>Inactive PKC</th>
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<tr>
<td>Control</td>
<td>67.4 ± 6.2</td>
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<tr>
<td>1 μM PDBu</td>
<td>49.5 ± 4.8*</td>
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**FIG. 1.** Heterogeneous regulation of Na/K-ATPase activity in renal cells in culture. Uptake of rubidium by OK (open bars) and LLC-PK1 (hatched bars) cell monolayers was measured in the presence or absence of 10 mM ouabain. Na/K-ATPase transport activity was determined by the ouabain-sensitive component of NaRb uptake. Na/K-ATPase transport activity was determined after 10 min of treatment with A23187 (1 μM), thapsigargin (100 nM), 4α-phorbol 12-myristate 13-acetate (1 μM), phorbol 12-myristate 13-acetate (PMA, 1 μM), or phorbol 12,13-dibutyrate (PDBu, 1 μM). Control values were 49.8 nmol Rb/mg/4 min for OK and LLC-PK1 cells, respectively (*p < 0.01; n = 8).

**FIG. 2.** Time course relationships between Na/K-ATPase transport activity and PDBu treatment in OK (solid line, filled squares) and LLC-PK1 (dashed line, open squares) cells. Na/K-ATPase activity was measured as in Fig. 1 using ouabain-sensitive uptake of rubidium. Monolayers of cells were treated for 2–60 min with phorbol 12-myristate 13-acetate (PDBu, 1 μM; n = 6).

**FIG. 3.** Response by ouabain-sensitive uptake of rubidium to phorbol ester treatment of OK cells. A, dose-response relationship for OK cells after 10 min of treatment revealed an EC50 = 35 nM PDBu (n = 6). B, apparent kinetics of rubidium uptake in OK cells. Rubidium uptake was performed in the presence of increasing concentration of potassium. Total uptake values after 10 min of treatment with MeSO4 (solid squares) or PDBu (1 μM, open squares) were compared to ouabain-insensitive values (circles). There was no discernible difference for ouabain-insensitive values with or without phorbol ester treatment. Calculated kinetic values showed no significant change in either apparent K0.5 (control, 16.9 ± 9.4; PDBu, 10.7 ± 4.5 mM K+) or Hill coefficient (control, 1.3 ± 0.4; PDBu, 1.0 ± 0.3) but a decrease in apparent Vmax (43 ± 33.8 to 218.4 ± 73.2 nmol/mg/4 min; p < 0.02; n = 5) with phorbol ester treatment.

The effective OK cells, transport activity of Na/K-ATPase was decreased by PDBu in a dose-responsive manner (EC50, 59 nM; Fig. 3A). The apparent kinetics of this transport response in OK cells are depicted in Fig. 3B. As seen in other epithelial cells (Middleton et al., 1990a), ouabain-sensitive uptake of rubidium was a saturable process. Short term treatment with PDBu (1 μM) caused minor changes in K0.5 or Hill coefficient but decreased apparent Vmax by ~38%. By analogy to enzyme kinetics, rather than an alteration in affinity of Na/K-ATPase, this corresponded with a decrease in number of active transporters (Segal, 1987). To address the possibility that the observed inhibition of Na/K-ATPase was mediated...
by a decrease in intracellular sodium concentration, we also measured the response to phorbol esters in OK cells loaded with intracellular sodium. Short term loading was achieved by treating intact OK cells with the polyene antibiotic nystatin (40 μg/ml) for 10 min (Middleton et al., 1990a) and basal rates of Na/K-ATPase increased from 63.4 ± 6.7 to 75.6 ± 8.4 nmol/mg/4 min (p < 0.05). When nystatin-treated cells were treated with PDBu (1 μM), transport rates decreased to 57.3 ± 6.1 nmol/mg/4 min (p < 0.001; n = 12), comparable to the 22% inhibition seen in control cells. We conclude that the two kidney cell types, OK and LLC-PK1, display a fundamental difference in the response to Na/K-ATPase to activation of PKC.

The observed discordance between OK and LLC-PK1 cells could derive from a difference in expression or response of respective PKC isoforms. By using polyclonal antisera derived from isoenzyme-specific peptides, PKC was characterized in the two cell types (Fig. 4) (Wetsel et al., 1992). In immunoblots not shown, there was no detection of PKCβII, γ, δ, or ε in proteins derived from either cell type. Significant levels of PKCα and PKCζ were present in OK and LLC-PK1 cells (Fig. 4). Furthermore, treatment of either cell with PDBu caused equivalent responses by the resident protein kinase isoforms. Cells were treated with 1 μM PDBu, proteins were fractionated to cytosolic (s) and membrane (p) components, and samples were equalized for protein and separated on SDS-PAGE. In untreated cells, PKCα was predominantly found in the cytosolic fraction but translocated to the membrane fraction with exposure to PDBu. A similar response to PDBu was seen in LLC-PK1 cells. In contrast, PKCζ was primarily found in the membrane fraction, and no changes were observed following treatment with PDBu. These studies suggest that the difference in response by Na/K-ATPase to PKC in the two cell types is not due to divergent responses by PKC.

Since PKCα presumably acts by phosphorylation of cell substrates, we next evaluated if translocation of PKCα in OK and LLC-PK1 cells corresponded with detectable phosphorylation of substrates. Using intact cell phosphorylation, Fig. 5 shows results from two-dimensional SDS-PAGE separation of OK whole cell phosphoproteins. Several different phosphoproteins were identified which had increased incorporation of 32P after activation of PKC. Acidic proteins in the 45–75-kDa molecular mass range were noted (straight arrows). In addition, a ~109-kDa phosphoprotein was noted in the cluster of proteins in the neutral PI range (white arrow). In contrast, the LLC-PK1 cell demonstrated enhanced phosphorylation of low PI proteins with PDBu, but no identifiable phosphoprotein was observed in the ~110 kDa range. The autoradiogram was purposely overexposed to improve visualization of a protein of this size range that might have been minimally phosphorylated. Similar results were seen in six experiments measured under shorter and longer exposure times. These data suggest that phosphorylation of the α subunit in Na/K-ATPase occurs only in the OK cell.

To more directly assess phosphorylation of Na/K-ATPase, we used anti-LEAVE to immunoprecipitate the α subunit. Following uptake of 32P-PO4 by intact cells, LLC-PK, and OK cells were treated with 1 μM PDBu for 10 min, homogenized, and the α subunit was immunoprecipitated (Fig. 7). There was antibody-dependent precipitation of a ~109-kDa phosphoprotein only in OK cells. In experiments not shown, unrelated rabbit serum failed to precipitate a similar phosphoprotein. Presence of the larger phosphoprotein in LLC-PK1 cells was not dependent on anti-LEAVE and was assumed to be a phosphorylated protein that bound in a non-specific manner to Pansorbin. After the cells were treated with PDBu (1 μM), there was no detectable phosphorylation in LLC-PK1 cells, but there was increased incorporation of 32P in the α subunit of the OK cell. The phorbol ester increased detectable phosphorylation of the α subunit only in the kidney cells with PKC-responsive Na/K-ATPase transport.

**DISCUSSION**

This study explores the important relationship in kidney cells between a common intracellular signaling enzyme, protein kinase C, and activity of Na/K-ATPase. Membrane transport is examined in two epithelial cell lines that display distinct phylogenetic origins but similar phenotypic features. OK and LLC-PK1 cells both exhibit membrane transport processes of Na/H exchange (Haggerty et al., 1988; Pollock et al., 1986), sodium-dependent phosphate uptake (Middleton et al., 1989), and Na/K-ATPase within the context of a polarized epithelium (Haggerty et al., 1988; Reshkin et al., 1990). In
each of these cell types, we examine isoforms of PKC, response of PKC to active phorbol esters, phosphorylation of presumed PKC substrates, and rapid regulation of transport activity by Na/K-ATPase. These data support previous observations that PKC inhibits Na/K-ATPase activity by covalent modification of the α subunit in vitro (Bertorello et al., 1991; Chibalin et al., 1992; Lowndes et al., 1990). This notion

**FIG. 5.** Autoradiograms from two-dimensional electrophoretic separation of phosphorylated substrates in OK and LLC-PK1 cells. Intact cells were loaded with ³²P for 2 h, treated for 10 min with Me₂SO or PDBu (1 μM), and whole cell proteins were separated with isoelectric focusing followed by SDS-PAGE. Corresponding pH and M₆₀ standards are shown. Arrows identify the most prominent phosphorylated substrates following phorbol ester treatment. Similar results were found in six experiments.

**FIG. 6.** One- and two-dimensional localization of Na/K-ATPase α subunit using immunoblots with anti-LEAVE and membrane proteins derived from OK and LLC-PK1 cells. Migration of M₆₀ markers is shown. Similar results were seen with three separate experiments.

**FIG. 7.** Autoradiograms from immunoprecipitation of phosphorylated Na/K-ATPase α subunit in intact epithelial cells. Intact cells were loaded with ³²P and treated for 10 min with Me₂SO or PDBu (1 μM) as in Fig. 5. Proteins were precipitated in the presence or absence of anti-LEAVE as noted. Migration of M₆₀ markers and α subunit (arrow) are noted. Similar results were seen in three experiments.
is extended by two observations: first, that PKC is capable of phosphorylating the α subunit in intact renal cells; second, that the state of kinase-mediated phosphorylation of the α subunit correlates with Na/K-ATPase transport activity. By inference, these data suggest that PKC activation and sodium pump regulation are coupled in an intact kidney cell, but that this relationship among different animal species is discontinuous.

The present study examines whether the lack of continuity could be based on different expression or response of isoforms of PKC. However, OK and LLC-PK1 cells primarily express the same two isoforms, PKCa and PKCɛ. Even though PKCa exhibits a wide tissue distribution (Wetsel et al., 1992), its presence in these cell models is particularly important since PKCa determines 34% of total PKC activity in the intact rat kidney (Dong et al., 1991). More specifically, PKCa immunolocalizes to the S3 segment of the proximal neprhon (Dong et al., 1991), a segment important for sodium and sodium-coupled transport processes. Rodent kidney also expresses lesser amounts of β and γ isoforms of PKC (Wetsel et al., 1992), in accord with the present observation in the renal cell lines. Since phorbol ester treatment rapidly translocates the PKCa isoform to the membrane in either the OK or the LLC-PK1 cell type, these studies confirm the observation that phorbol esters bind significantly with PKCa, but not PKCɛ (Ryves et al., 1991; Ways et al., 1992). These data do not exclude the possibility that there is concurrent low expression of an undetected PKC enzyme. For example, subspecies of PKC might remain undetectable with the present technique (Wetsel et al., 1992). Since only the α, β1, and γ isoforms are known to bind phorbol esters (Ryves et al., 1992), the current observations would likely pertain to an enzyme more closely related to this subgroup. Alternatively, the observed responses to the active phorbol ester in either cell type could originate from indirect effects of PKC, such as “cross-talk” with other signaling enzymes (Nishizuka, 1984). The present studies are unable to eliminate this possibility, but by merging specific biochemical and functional studies these data from intact cells support PKC as an important element for regulation of Na/K-ATPase transport activity.

Recent studies suggest that PKC phosphorylates, among other substrates, the α subunit of Na/K-ATPase. For the first time, the present study identifies PKC-mediated modification of the α subunit within intact kidney cells. Numerous reports measure responses to various agonists by intact cells and imply that PKC regulates Na/K-ATPase activity (Bertorello, 1992; Middleton et al., 1990a; Oishi et al., 1990; Vasillets et al., 1990). In contrast, direct biochemical evidence has only been provided in dog kidney, duck salt gland (Chibalin et al., 1992; Lowndes et al., 1990), shark rectal gland (Bertorello et al., 1991), B. marinus kidney, and Xenopus oocytes (Chibalin et al., 1992) that PKC promotes phosphorylation of the α component of the transporter. Purified PKC inhibits enzyme activity of Na/K-ATPase derived from other tissues in vitro (Bertorello et al., 1991), suggesting direct interaction with the transporting protein or a closely related membrane component. Regulation of Na/K-ATPase by phosphorylation reactions gains further support by recent work involving serine-threonine phosphatases. The dopamine and cAMP-regulated phosphoprotein DARPP-32, present in renal tubule cells, potently inhibits protein phosphatase-1 (PP1) (Aperia et al., 1992). In turn, PP1 in purified systems dephosphorylates Na/K-ATPase (Hemmings et al., 1984). More recently, the calcium/calmodulin-dependent phosphatase calcineurin was identified to activate DARPP-32 in vitro, therefore proposing a complex interplay of factors that control the phosphorylation state of Na/K-ATPase (Aperia et al., 1992). The present studies do not address the full spectrum of elements that possibly enhance or diminish the level of phosphorylation, but they document that modification of the Na/K-ATPase α subunit occurs in intact cells.

The present observations also suggest that the α subunit of Na/K-ATPase from all kidney cells does not serve as an equivalent substrate for PKC. PKC and Na/K-ATPase from a broad range of epithelial cells demonstrate a similar inconsistent relationship. First, active phorbol esters can stimulate (Bertorello et al., 1992; Friedlander et al., 1989) or inhibit (Bertorello et al., 1991, 1992; Edelman et al., 1985; Gick et al., 1988) sodium-coupled transport in renal epithelial cells. Second, within different nephron segments of the same animal PKC responsiveness exhibits similar heterogeneity (Fukuda et al., 1991). In these and other studies in intact cells, electrophysiological gradients, intracellular sodium, intracellular potassium, and ATP concentration all represent potential modes of rapid Na/K-ATPase regulation. Alternatively, coupling between the sodium pump and distinct membrane proteins could serve as a means of regulation (Swedner, 1989). These factors could participate in the present studies, but part of the observed dissimilarity could also be based on differences in Na/K-ATPase primary structure. The LLC-PK1 cell expresses a catalytic subunit of Na/K-ATPase of the α1 class (Ovchinnikov et al., 1986). The sequence of the α subunit for the opossum kidney is not reported, but it is assumed to be homologous to the rodent kidney, namely the α1. Using the α1 peptide-specific antibody anti-NASE (Pressley, 1992), a ~110-kDa protein is recognized in membrane proteins derived from OK cells specifically, suggesting homology within the H4-H5 intracellular loop of rat α1.2 These studies confirm that the catalytic subunits of both types of kidney cells are members of the same isoform family. Furthermore, these observations suggest that if phosphorylation and response to PKC is determined by specific PKC recognition sequences, these sites are poorly conserved among α1 isoforms of Na/K-ATPase.

By addressing the relationship between PKC and Na/K-ATPase, the present study may apply to regulation of the sodium pump by membrane receptors. Some receptors, such as those for insulin (Hundal et al., 1992), regulate translocation of Na/K-ATPase components and thus affect transport activity over the long term. Other receptors can regulate Na/K-ATPase more rapidly, and part of this response may be mediated by protein kinases. In physiologic terms, the parathyroid hormone receptor is one candidate to activate this pathway since it couples with phospholipase C and regulates solute transport in the proximal nephron (Middleton et al., 1989). Since PKCa is activated by elevations of [Ca2+], and diacylglycerol (Nishizuka, 1984), these intracellular signals might also activate phosphorylation of Na/K-ATPase α subunit by receptor-independent perturbations. This mechanism may provide a biochemical explanation for clinical states of diminished sodium reabsorption. Although the present study only addresses regulation of Na/K-ATPase by pharmacologic manipulation, the mechanisms described may apply to physiologic or pathologic regulation of sodium transport in the intact kidney.

These studies employ two immortalized renal cell lines to associate activation of PKC, phosphorylation of the α subunit of Na/K-ATPase, and inhibition of transport activity in an intact renal cell. Furthermore, they demonstrate that PKC-mediated phosphorylation of Na/K-ATPase is not universal. Neither the nature nor the cause of the inconsistency is

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2 R. Medford, unpublished observations.
identified, but the results raise the possibility that heterogeneity within the α1 isofrom class is responsible. This complex relationship between Na/K-ATPase and PKC warrants examination in a physiologic setting.

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