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Short-Term Regulation of the Proximal Tubule Na^+,K^+-ATPase: Increased/Decreased Na^+,K^+-ATPase Activity Mediated by Protein Kinase C Isoforms

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In different species and tissues, a great variety of hormones modulate Na^+,K^+-ATPase activity in a short-term fashion. Such regulation involves the activation of distinct intracellular signaling networks that are often hormone- and tissue-specific. This minireview focuses on our own experimental observations obtained by studying the regulation of the rodent proximal tubule Na^+,K^+-ATPase. We discuss evidence that hormones responsible for regulating kidney proximal tubule sodium reabsorption may not affect the intrinsic catalytic activity of the Na^+,K^+-ATPase, but rather the number of active units within the plasma membrane due to shuttling Na^+,K^+-ATPase molecules between intracellular compartments and the plasma membrane. These processes are mediated by different isoforms of protein kinase C and depend largely on variations in intracellular sodium concentrations.

KEY WORDS: Na^+,K^+-ATPase; Na pump; sodium, potassium-ATPase; sodium pump; sodium transport regulation; protein kinase C; PKC; phosphoinositide-3 kinase; polyproline motif; serine phosphorylation.

INTRODUCTION

Renal sodium homeostasis, a major determinant of blood pressure, is regulated by a variety of endocrine, autocrine, and neuronal factors. The molecular mechanism by which these factors act involves regulation of the rate of tubular sodium reabsorption (Guyton, 1992). Renal epithelial cells are responsible for urinary sodium reabsorption and extracellular fluid volume homeostasis (Simons and Fuller, 1985; Soltoff and Mandel, 1984). Since the intracellular sodium concentration is relatively low compared to tubular ultrafiltrate, sodium movement from the lumen of the tubules into epithelial cells proceeds down an electrochemical potential gradient. In proximal tubules, the Na^+,K^+-ATPase provides the driving force for vectorial sodium transport from the lumen of the tubule to the blood supply (Katz, 1988). In recent years, evidence has accumulated that hormones that regulate kidney sodium metabolism, acting through intracellular second messengers, modulate the activity of the Na^+,K^+-ATPase (Bertorello and Katz, 1993; Aperia, 1995).

Considerable evidence indicates that the renal Na^+,K^+-ATPase is regulated through phosphorylation/dephosphorylation reactions by kinases and phosphatases stimulated by hormones and intracellular second messengers (Bertorello and Katz, 1993; Aperia, 1995). Apparent contradictory results suggest that, depending on the tissue studied and the experimental conditions, stimulation of protein kinase C (PKC) leads to either activation or inhibition of the Na^+,K^+-ATPase (for reference, see Pedemonte *et al*., 1997a). These differences are due to the fact that the regulation of the Na^+,K^+-ATPase has characteristics that are inherent to the cell and tissue studied and these characteristics vary in different cells, tissues, and species. Thus, it is well known that dopamine reduces sodium reabsorption and inhibits Na^+,K^+-ATPase activity in proximal tubule cells (Bertorello and Katz, 1993; Aperia, 1995). However, the same hormone increases sodium transport

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and stimulates Na^+,K^+-ATPase in pulmonary epithelial cells (Bertorello *et al.*, 1999; Barnard *et al.*, 1999). Interestingly, both activation and inhibition are mediated by PKC. Moreover, it has been observed that even in the same cell, opposing regulatory effects may be produced by stimulation of different isoforms of the molecules that are involved (Vasilets, 1997). Furthermore, we have observed that, depending on the concentration of intracellular sodium, phorbol esters stimulate different isoforms of PKC in proximal tubule cells and this results in either activation or inhibition of the Na^+,K^+-ATPase (Efendiev *et al.*, 2000a). Thus, the response hormones produce on the Na^+,K^+-ATPase depends on the cell type and it is important to relate the regulatory effect to a specific physiological condition.

In proximal tubules, different hormones produce different effects on the epithelial cell Na^+,K^+-ATPase (Aperia, 1995). Thus, hormones that inhibit urinary sodium reabsorption (diuretic hormones), like dopamine and atrial natriuretic peptide, inhibit the Na^+,K^+-ATPase. In contrast, hormones that stimulate urinary sodium reabsorption (antidiuretic hormones), like norepinephrine and angiotensin II, stimulate the Na^+,K^+-ATPase. Therefore, both activation and inhibition should be observed when studying the regulation of the Na^+,K^+-ATPase in proximal tubule cells. In this minireview, we describe some aspects of the molecular mechanism that are involved in the inhibition by dopamine and stimulation by phorbol ester of proximal tubule Na^+,K^+-ATPase activity. These results have been obtained in cells of rodent proximal tubules and in a cell culture line of opossum kidney (OK cells). We have chosen this cell line for heterologous expression of the rodent Na^+,K^+-ATPase $\alpha 1$ subunit because it is considered to be a good culture model of proximal tubule function (Malstron *et al.*, 1987; Nash *et al.*, 1993; Pedemonte *et al.*, 1997a,b).

ACTIVATION OF Na^+,K^+-ATPase BY PHORBOL ESTERS

PMA Activates the Na^+,K^+-ATPase Activity by Recruitment of Na^+,K^+-ATPase Molecules to the Plasma Membrane

Other researchers and ourselves have observed that direct stimulation of PKC by the phorbol ester PMA (phorbol 12-myristate 13-acetate)-produced activation of Na^+,K^+-ATPase (Pedemonte *et al.*, 1997a,b; Feraille *et al.*, 1995). Several lines of evidence indicate that this is a specific effect: (1) inhibition of PKC prevented the PMA-dependent inhibition of Na^+,K^+-ATPase, and phorbol esters that do not activate PKC did not have any effect on the Na^+,K^+-ATPase activity; (2) phorbol ester-dependent activation was not observed in cells expressing NH_2-terminal deletion mutants of the Na^+,K^+-ATPase α subunit in which either the first 26 amino acids or amino acids 5 to 26 have been eliminated; (3) experiments with α subunit mutants indicated that both Ser11 and Ser18 were essential for PMA-dependent activation of Na^+,K^+-ATPase (Efendiev *et al.*, 2000b); and (4) both Ser11 and Ser18 were the only residues of the α subunit that were phosphorylated by PMA treatment of OK cells. Insulin also activates the proximal tubule Na^+,K^+-ATPase (Feraille *et al.*, 1999); however, this stimulation is mediated by phosphorylation of the α subunit Tyr-5 and mutation of this residue to either Ala or Glu had no effect on the PMA-dependent activation of Na^+,K^+-ATPase. Our results indicate that neither phosphorylation of Tyr5 nor phosphorylation of any residue of the α subunit other than Ser11 and Ser18 is involved in the PMA-dependent activation of Na^+,K^+-ATPase (Efendiev *et al.*, 2000b).

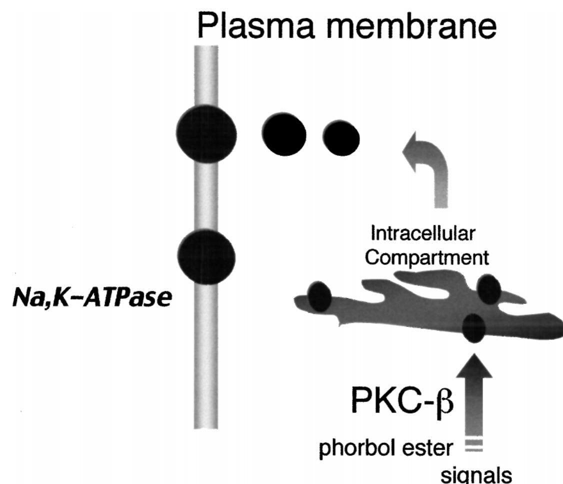

Fig. 1. Scheme of the PMA-dependent activation of Na^+,K^+-ATPase by recruitment of pump molecules from intracellular compartments to the plasma membrane. PKC, protein kinase C.

To determine whether the PMA-dependent activation of Na^+,K^+-ATPase is due to increased turnover rate or increased number of Na^+,K^+-ATPase molecules at the plasma membrane, the size of the pool of Na^+,K^+-ATPase at the plasma membrane was determined by either ouabain-binding or biotin-labeling of cell membrane proteins. Both assays indicated that the Na^+,K^+-ATPase pool at the plasma membrane was increased by PMA treatment, which suggests that Na^+,K^+-ATPase molecules have been translocated from intracellular compartments to the plasma membrane. The recruitment of Na^+,K^+-ATPase to the plasma membrane appears to be mediated

by clathrin-coated vesicles. Thus, we observed that PMA promoted the interaction of Na^+,K^+-ATPase with adaptor protein-1 (AP-1) which is a protein involved in the selection of the cargo and recruitment of clathrin during the translocation of proteins from intracellular compartments to the plasma membrane (Ohno *et al*., 1995).

Phosphorylation of Na^+,K^+-ATPase May Be the Triggering Mechanism for Recruitment to the Plasma Membrane, but Phosphorylation by Itself Does Not Affect the Intrinsic Activity of the Na^+,K^+-ATPase

Translocation of Na^+,K^+-ATPase molecules appears to be dependent on the cellular content of ATP since pretreatment of the cells with either potassium cyanide or dinitrophenol prevented both the PMA-dependent activation of Na^+,K^+-ATPase and increased membrane pool of Na^+,K^+-ATPase. The effect of potassium cyanide and dinitrophenol appears to be specific, since addition of these reagents after treatment with PMA did not prevent the stimulation of pump activity and translocation of Na^+,K^+-ATPase to the plasma membrane. Neither basal Na^+,K^+-ATPase nor activation of PKC was affected by potassium cyanide treatment. This suggested that the concentration of ATP was not rate limiting during the length of the experiment and it is likely that pump molecules have access to the remaining pool of ATP. The translocation of Na^+,K^+-ATPase is dependent on the integrity of the cellular microtubule network, since colchicine, which promotes disassembly of microtubules, prevented the PMA-dependent activation of Na^+,K^+-ATPase and increased the membrane pool of Na^+,K^+-ATPase. Potassium cyanide, dinitrophenol, and colchicine prevented the Na^+,K^+-ATPase translocation to the plasma membrane, but not the PMA-dependent phosphorylation of the Na^+,K^+-ATPase α subunit. This indicates that phosphorylation is necessary (a possible triggering signal), but not sufficient for PMA-dependent stimulation of Na^+,K^+-ATPase activity. This would explain the observation by other researchers that *in vitro* phosphorylation of purified rodent Na^+,K^+-ATPase with PKC does not affect the Na^+,K^+-ATPase activity (Feschenko and Sweadner, 1997).

The presence of Na^+,K^+-ATPase subunits in internal compartments has always posed the question of whether they represent an intracellular pool from where they can be recruited to the plasma membrane upon demand, either in response to situations of cellular stress (sudden rise in intracellular sodium) or in response to G-protein-coupled receptor signals. Our results indicate that PMA produces activation of Na^+,K^+-ATPase by recruitment of pump molecules to the plasma membrane (Efendiev *et al*., 2000b). Similar recruitment of Na^+,K^+-ATPase molecules to the plasma membrane has been described in response to insulin in muscle cells (Hundal *et al*., 1992), cAMP in rat proximal tubule (Carranza *et al*., 1996), and isoproterenol in lung epithelia (Saldias *et al*., 1998). Insulin also regulates other membrane proteins by translocation, including insulin-like growth factor II receptor (Wardzala *et al*., 1984) and Glut4 (Cushman and Wardzala, 1980; Kono *et al*., 1981), and both translocations are inhibited by potassium cyanide (Kono *et al*., 1981; Cong *et al*., 1997). The conclusion that PMA treatment leads to an increased pool of plasma membrane Na^+,K^+-ATPase molecules does not preclude the possibility of direct effects on the affinity of the pump for intrcellular sodium (Feraille *et al.*, 1995).

Activation of Na^+,K^+-ATPase by Phorbol Esters Results in a Reduced Intracellular Sodium Concentration

Treatment of OK cells with PMA leads to a rapid reduction of intracellular sodium concentration from ∼15 to ∼5 mM (Pedemonte *et al*., 1997b). In OK cells, sodium enters the cell through the apical Na^+/H^+-exchanger, and it is also cotransported with glucose, amino acids, and phosphate (Malstron *et al*., 1997). Therefore, a reduction in intracellular sodium concentration may be the result of a reduced entry, an increased exit, or a combination of both. In conditions in which the sodium exit is blocked by ouabain treatment, we observed that the total entry of sodium is not affected by PMA treatment. On the other hand, phorbol esters have no effect on the intracellular sodium concentration of cells expressing an NH_2-terminal deletion mutant of the rodent Na^+,K^+-ATPase α subunit. As indicated above, this mutant is insensitive to PMA since it lacks the serine residues that are phosphorylated by PKC. Therefore, in OK cells, the reduced intracellular sodium concentration is produced exclusively by PMA activation of the Na^+,K^+-ATPase. This effect is mediated by PKC, since it is blocked by PKC inhibitors and is not observed with phorbol esters that do not activate PKC.

INHIBITION OF Na^+,K^+-ATPase BY DOPAMINE

Dopamine Inhibits the Na^+,K^+-ATPase Activity by Endocytosis of Na^+,K^+-ATPase Molecules

Inhibition of Na^+,K^+-ATPase activity by dopamine is an important mechanism by which renal tubules

modulate urine sodium excretion during high salt diet (Bertorello *et al.*, 1988; Hedge *et al.*, 1989; Hussain and Lokhandwala, 1998). We determined that in both isolated renal proximal tubule cells and OK cells dopamine decreased Na^+,K^+-ATPase activity and this effect was blocked by inhibitors of PKC, but not by inhibitors of cAMP-dependent protein kinase (PKA) (Chibalin *et al.*, 1997, 1999). The inhibitory effect of dopamine was accompanied by increased phosphorylation of the Na^+,K^+-ATPase α subunit. Maximal phosphorylation was achieved at 2.5 min and it was restricted to Na^+,K^+-ATPase molecules that were at the plasma membrane. Subcellular fractionation did not show any increased phosphorylation of Na^+,K^+-ATPase in intracellular compartments (Chibalin *et al.*, 1998a). Because after 10 min the α subunit has been dephosphorylated, yet the decreased enzymc activity persisted, we hypothesized that the dephosphorylated α subunits no longer resided in the plasma membrane. Indeed, it was observed that dephosphorylation of the α subunit is accompanied by an increased amount of Na^+,K^+-ATPase in endosomes. Treating the cells with okadaic acid, an inhibitor of protein phosphatases, prevented the dephosphorylation of Na^+,K^+-ATPase α subunits. Furthermore, it was determined that phosphorylated Na^+,K^+-ATPase α subunits at the plasma membrane are not affected by protein phosphatases, which suggested that α subunits must be internalized first to be dephosphorylated (Chibalin *et al.*, 1998a). Na^+,K^+-ATPase α subunits that are phosphorylated in the plasma membrane are internalized by sequential translocation into clathrin-coated vesicles, early and late endosomes, where they are dephosphorylated. However, OK cells expressing a Na^+,K^+-ATPase mutant in which the first 28 NH_2-terminal amino acids were deleted did not show any of these effects: dopamine did not produce inhibition of Na^+,K^+-ATPase activity and the α subunit was not phosphorylated. Because the putative sites for PKC phosphorylation (Ser11 and Ser18) are within the deleted amino acids (Béguin *et al.*, 1994), these observations suggested a causal link between PKC-dependent phosphorylation of amino acids at the α subunit NH_2-terminus, and both Na^+,K^+-ATPase inhibition and endocytosis in response to the physiological agonist, dopamine.

To identify the phosphorylation site that mediates endocytosis, we repeated the above experiments in cells expressing Na^+,K^+-ATPase mutants in which either Ser11 or Ser18 was substituted for alanine residues (Chibalin *et al.*, 1999). Dopamine inhibited Na^+,K^+-ATPase activity and increased α subunit phosphorylation and clathrin-dependent endocytosis into endosomes in cells expressing the wild-type $\alpha 1$ subunit or the S11A mutant and both effects were blocked by PKC inhibitors. In contrast, dopamine did not elicit any of these effects in cells expressing the S18A $\alpha 1$ mutant.

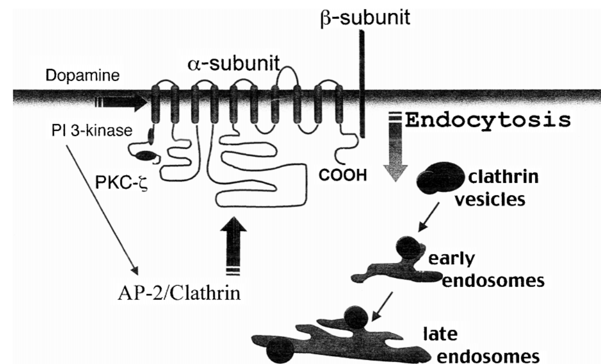

Fig. 2. Scheme of the dopamine-dependent inhibition of Na^+,K^+-ATPase by endocytosis. PI3-kinase, phosphoinositide-3 kinase; AP-2, adaptor protein-2; PKC, protein kinase C.

Dopamine-Dependent Phosphorylation of Na^+,K^+-ATPase Is the Triggering Mechanism for Endocytosis but Phosphorylation by Itself Does Not Affect the Intrinsic Activity of the Na^+,K^+-ATPase

The relative role of phosphorylation and endocytosis for the reduction of total cell Na^+,K^+-ATPase activity was examined in intact cells by (1) blocking endocytosis by mechanically stabilizing the cortical actin cytoskeleton, or (2) disrupting the microtubule organization, or (3) affecting vesicle traffic by blocking phosphatidylinositol 3-kinase activity. Stabilizing the cortical actin cytoskeleton with phallacidin prevented the incorporation of Na^+,K^+-ATPase units in clathrin vesicles while phosphorylation of the α subunit in the basolateral membrane was increased without any change in Na^+,K^+-ATPase activity. Disrupting the microtubule network with nocodazole did not affect the incorporation of α subunits into clathrin vesicles nor the decrease in Na^+,K^+-ATPase activity, however, this treatment affected the incorporation of α subunits into early and late endosomes. Similarly, inhibiting phosphatidylinositol 3-kinase activity prevented both the decrease in Na^+,K^+-ATPase activity and endocytosis into clathrin vesicles, but did not affect the increase in α subunit phosphorylation induced by dopamine. These results clearly indicated that phosphorylation of the Na^+,K^+-ATPase α subunit is not sufficient to reduce the catalytic activity in intact cells, and that the removal of active units from the plasma membrane is directly responsible for the decrease in total cell Na^+,K^+-ATPase activity.

Phosphatidylinositol 3-kinase Is Activated by Binding to a Polyproline-Rich Domain in the Na^+,K^+-ATPase α Subunit

Membrane traffic relies on the ability of lipid structures to accommodate changes in time and space, particularly adjacent to the site of motion. Generation of inositol lipids phosphorylated in the 3D position has been demonstrated to be critical in this process. Phosphorylation within this position is catalyzed by the action of phosphatidylinositol 3-kinase (PI3-K). We observed that PI3-K is critical for Na^+,K^+-ATPase endocytosis in response to dopamine (Chibalin *et al*., 1998b, 1999). The metabolic products of PI3-K may participate in recruitment of clathrin; binding to Na^+,K^+-ATPase and formation of coated pit vesicles; facilitating endosome movement by targeting the endosomes themselves; or involvement in the process that ultimately leads to actin reorganization. Whereas the consensus is that activation of PI3-K requires its binding and association with the target protein in the membrane via a SH_2-phosphotyrosine interaction, the activation by dopamine during Na^+,K^+-ATPase endocytosis does not require tyrosine phosphorylation but instead, serine/threonine phosphorylation (Yudowski *et al*., 2000). Because the action of dopamine was not associated with phosphorylation of PI3-K, we hypothesized that serine phosphorylation of the Na^+,K^+-ATPase α subunit may serve as an anchor signal for binding and recruitment of PI3-K to the site of endocytosis and this results in activation of PI3-K. This hypothesis was supported by the observation that in OK cells expressing the rat Na^+,K^+-ATPase α subunit bearing the mutation S18A, which is the PKC phosphorylation site, dopamine not only failed to increase PI3-K activity but also did not promote the interaction between PI3-K and Na^+,K^+-ATPase (Yudowski *et al*., 2000). Thus, stimulation of PI3-K activity in response to dopamine appears to be controlled by the state of phosphorylation of the Na^+,K^+-ATPase α subunit.

Activation of intracellular signaling networks by binding of agonists to G-protein-coupled receptors require a high degree of organization to avoid unspecific cross-talk and to ensure proper timing and spatial organization. This could be accomplished by specific protein–protein interactions through domains such as the Src homology (SH_2 and SH_3 domains), pleckstrin homology, phosphotyrosine binding, and PDZ domains, which are present in signaling and/or target molecules (Pawson and Scott, 1999). The IA-isoform of PI3-K bears a SH_3 domain in its p85α subunit (Yu *et al*., 1994; Pisabarro and Serrano, 1996), which may represent a site for self-association. However, it has been suggested that proline rich motifs present in other proteins may interact with the PI3-K SH_3 domain (Harpur *et al*., 1999). We identified within the Na^+,K^+-ATPase α subunit, a polyproline region (81-TPPPTTP-87) as a putative PI3-K binding domain. To establish whether this amino acid sequence represents a critical interaction motif, we used peptide competition assays in which the effect of dopamine on Na^+,K^+-ATPase activity was examined in the presence or absence of peptides that resembled either the proline-rich domain of the Na^+,K^+-ATPase α subunit or the PI3-K SH_3 domain. Both of these peptides greatly reduced the inhibitory action of dopamine on Na^+,K^+-ATPase activity. The relevance of the proline-rich domain within the α subunit was further supported by experiments performed with a Na^+,K^+-ATPase mutant. In OK cells expressing Na^+,K^+-ATPase α subunits bearing a mutation in the polyproline region (P83R), dopamine neither inhibited the Na^+,K^+-ATPase nor increased PI3-K activity and endocytosis of the plasma membrane Na^+,K^+-ATPase molecules. These effects were specific as dopamine still was able to phosphorylate the α subunit and phorbol ester-dependent stimulation of Na^+,K^+-ATPase activity remained unaffected in cells expressing the P83R Na^+,K^+-ATPase mutant. Therefore, binding of PI3-K to the Na^+,K^+-ATPase α subunit polyproline motif may not only activate PI3-K, but also localize this protein in a protein complex close to the site where the PI3-K phosphoinositide products can stimulate the endocytosis of the Na^+,K^+-ATPase. Since PI3-K only binds to Na^+,K^+-ATPase molecules with α subunits phosphorylated at Ser18, this interaction may recruit the components of the endocytic machinery exclusively around the Na^+,K^+-ATPase molecules that have been phosphorylated by PKC.

Because phosphatidylinositol 3-phosphate is known to stimulate the association of adaptor proteins with molecules to be translocated either from the plasma to intracellular compartments or vice versa (Ohno *et al*., 1995; Kirchausen *et al*., 1997; Bonifacino and Dell'Angelica, 1999), we examined whether colocalization of adaptor protein-2 (an isoform that recruits cargo exclusively from the plasma membrane) and the Na^+,K^+-ATPase α subunit was impaired in cells expressing the Na^+,K^+-ATPase with a mutation on the proline-rich domain (P83R) and thereby incapable of activating PI3-K in response to dopamine. In these cells, dopamine failed to promote the binding of AP-2 to the Na^+,K^+-ATPase α subunit and to increase the abundance of α subunits within clathrin vesicles, and early and late endosomes. These results suggest that activation of PI3-K and the local production of phosphatidylinositol 3-phosphate may be responsible for cargo recognition by stimulating the binding of AP-2 to Na^+,K^+-ATPase, and clathrin recruitment (Fig. 2).

Besides demonstrating the way PI3-K may be involved in the dopamine inhibition of Na^+,K^+-ATPase, these studies showed an alternative pathway by which class IA PI3-K could be activated in response to G-protein-coupled receptor stimulation. Unlike the conventional process of activation involving SH-domain interaction with phosphorylated tyrosine residues, dopamine stimulation of PI3-K appears to be controlled by serine phosphorylation of the protein to be internalized. A similar mechanism has been recently described for integrin receptors (Guthridge *et al.*, 2000).

STOICHIOMETRY OF PHOSPHORYLATION

Covalent phosphorylation of proteins could produce substantial changes in the structure–dynamic conformation and function of the target protein. As described above, Ser18 of the Na^+,K^+-ATPase α subunit is phosphorylated in response to dopamine. Under basal conditions, the Na^+,K^+-ATPase appears to be phosphorylated with a stoichiometry of $\sim$0.20 mol of phosphate/pmol of Na^+,K^+-ATPase. Dopamine increases Na^+,K^+-ATPase α subunit phosphorylation by approximately 40% to a stoichiometry of 0.35 mol/mol. Therefore, we estimated that dopamine phosphorylates the Na^+,K^+-ATPase to a stoichiometry of $\sim$0.15 mol of phosphate/pmol of Na^+,K^+-ATPase α subunit.

During PMA-dependent activation of Na^+,K^+-ATPase, Ser11 and Ser18 of the α subunit are phosphorylated; the phosphorylation of both serine residues appears to be essential for the stimulation of activity and recruitment of Na^+,K^+-ATPase molecules from intracellular compartments to the plasma membrane. Assuming that the ratio of PMA-elicited phosphorylation between Ser11 and Ser18 in the rodent wild-type α subunit is the same as that observed for mutants S11A and S18A, the 119% increase in phosphorylation produced by PMA treatment would correspond to a 77 and 42% increase in phosphorylation of Ser18 and Ser11, respectively. Since, under basal conditions, the wild-type rodent α subunit is phosphorylated with a stoichiometry of $\sim$0.20 moles of phosphate per mole of α subunit, the above values yield a predicted stoichiometry of 0.44 moles of phosphate/per α subunit and 0.34 and 0.18 moles of phosphate per mole of α subunit for Ser18 and Ser11, respectively.

There is an apparent lack of correlation between the level of phosphorylation of the α subunit and the PMA-dependent activation or dopamine-dependent inhibition of Na^+,K^+-ATPase activity. As described above, PMA produces phosphorylation of Na^+,K^+-ATPase molecules that are in an intracellular compartment and these are translocated to the plasma membrane. Since activity is measured exclusively in the Na^+,K^+-ATPase molecules that are at the plasma membrane, phosphorylation and increased activity correspond to molecules that are in two different compartments. Therefore, no linear correlation between the level of phosphorylation and the increase in Na^+,K^+-ATPase activity should be expected. The correlation would depend on the size of the initial pool of Na^+,K^+-ATPase molecules at the plasma membrane, on the percentage of phosphorylated molecules that are translocated, and on the total number of Na^+,K^+-ATPase molecules that are translocated from the intracellular compartment to the plasma membrane.

On the other hand, an increase of $\sim$0.15 moles of dopamine dependent phosphorylated Na^+,K^+-ATPase α subunit at the plasma membrane per total amount of cellular Na^+,K^+-ATPase α subunit translates in a $\sim$30% inhibition of Na^+,K^+-ATPase activity. We do not know what role, if any, is played by the basal phosphorylation, and it is possible that this may participate in some way in the identification of molecules to be removed from the plasma membrane. Furthermore, it has been described that aquaporyn molecules are translocated to the plasma membrane forming tetramer complexes and that only three of the monomers need to be phosphorylated for translocation (Kamsteeg *et al.*, 2000). Thus, it is possible that Na^+,K^+-ATPase molecules may be translocated to and removed from the plasma membrane in dimer or tetramer complexes and that not all of the Na^+,K^+-ATPase molecules in a complex need to be phosphorylated for translocation. Actually, we have observed that the PMA-dependent activation of Na^+,K^+-ATPase is very fast (Pedemonte *et al.*, 1997b) and translocation of complexes of various Na^+,K^+-ATPase molecules may be a way to accomplish this rapid activation.

PKC-β AND PKC-ζ MEDIATE OPPOSING EFFECTS ON PROXIMAL TUBULE Na^+,K^+-ATPase TRAFFIC

In renal epithelial cells, Na^+,K^+-ATPase molecules are localized within the basolateral plasma membrane, and in early and late endosomes (Chibalin *et al.*, 1998b). These organelles may represent intracellular storage compartments where Na^+,K^+-ATPase units reside either as a consequence of plasma membrane endocytosis or as newly synthesized units derived from the endoplasmic–Golgi network. The existence of Na^+,K^+-ATPase molecules in such structures revealed the possibility that it could recycle between those compartments during regulation by membrane receptor signals. Indeed, a decrease in renal Na^+,K^+-ATPase activity in response to dopamine is associated with a reduction in the number of active molecules

in the plasma membrane (Chibalin *et al*., 1998, 1999) and stimulation of Na^+,K^+-ATPase in renal proximal tubule cells by phorbol esters is associated with increased units at the plasma membrane (Pedemonte *et al*., 1997a; Efendiev *et al*., 2000b). As described above, both phorbol ester-dependent activation and dopamine-dependent inhibition of Na^+,K^+-ATPase are mediated by stimulation of PKC. This clearly indicated that different isoforms of PKC are involved in both processes. We identify these PKC isoforms using specific inhibitors (Efendiev *et al*., 1999). LY333531, a specific inhibitor of the PKC-β isoform (Ishii *et al*., 1996; Zimmerman *et al*., 1996), and low concentrations of staurosporine that inhibits PKC-β (Zimmermann *et al*., 1996) prevented the PMA-dependent activation of Na^+,K^+-ATPase, but had no effect on the dopamine inhibition of this activity.

The fact that PMA did not produce an inhibition of the Na^+,K^+-ATPase activity in cultured OK cells as dopamine did, suggested that dopamine was activating a member of the atypical PKC isozymes that are insensitive to phorbol esters (Hofmann, 1997; Zimmermann *et al*., 1996; Ishii *et al*., 1996). Indeed, other researchers have previously suggested that PKC-ζ may be involved in regulation of Na^+,K^+-ATPase activity (Sweeney *et al*., 1998; Yao *et al*., 1998). We determined that 10 μM staurosporine was necessary to prevent the inhibitory effect of dopamine on Na^+,K^+-ATPase activity (Efendiev *et al*., 1999). Since most of the PKC isoforms are inhibited by staurosporine in the nanomolar range, except PKC-ζ that requires micromolar concentrations, our results suggested that PKC-ζ was involved in the Na^+,K^+-ATPase inhibition. The results obtained with chemical reagent inhibitors were confirmed using specific peptide inhibitors. Dr. Mochly-Rosen and collaborators (Mochly-Rosen and Gordon, 1998; Johnson *et al*., 1996) have demonstrated that most inactive PKC isoforms are localized to subcellular structures and, upon activation, translocate to new distinct intracellular sites, which is due to their binding to specific anchoring molecules. The anchoring proteins for activated PKC isoforms were termed receptors for activated C-kinase (RACKs). It is then likely that the unique cellular functions of PKC isoforms are determined by the binding of isozymes to specific anchoring molecules in close proximity to particular subsets of substrate and away from others (Mochly-Rosen and Gordon, 1998; Johnson *et al*., 1996). Accordingly, peptides that mimic either the PKC binding site on RACKs or the RACK binding site on PKC are translocation inhibitors of PKC that block the function of the enzyme *in vivo* (Mochly-Rosen and Gordon, 1998; Johnson *et al*., 1996). Using peptides to inhibit PCK-β and PKC-ζ, we observed that the PMA-dependent activation of Na^+,K^+-ATPase is prevented by the PKC-β peptide inhibitor but not by the PKC-ζ peptide inhibitor and that the DA-dependent inhibition of Na^+,K^+-ATPase was prevented by the PKC-ζ peptide inhibitor, but not by the PKC-β peptide inhibitor. To further support the conclusions of these experiments, we studied the effects of these peptide inhibitors on the phosphorylation of the Na^+,K^+-ATPase α subunit and demonstrated that inhibition of PKC-β or PKC-ζ prevented the increased level of phosphorylation of the α subunit produced by either PMA or dopamine treatments, respectively (Efendiev *et al*., 1999). Therefore, it is likely that PKC-β and PKC-ζ isoforms are directly involved in the phosphorylation of the Na^+,K^+-ATPase α subunit stimulated by PMA or dopamine, respectively.

INTRACELLULAR SODIUM MAY ACTIVATE AN INTRACELLULAR SWITCH(ES) TO MODULATE THE HORMONAL REGULATION OF Na^+,K^+-ATPase

Both PMA (as previously described by Middleton *et al*., 1993) and dopamine inhibited the Na^+,K^+-ATPase activity in proximal tubule and OK cells when incubation with agonists was performed in Hank's medium (Bertorello and Aperia, 1989). The same result was observed in suspended OK cells independently of the assay medium (Efendiev *et al*., 2000a). However, dopamine failed to decrease Na^+,K^+-ATPase activity from OK cells, and PMA stimulated this activity if experiments were performed with attached OK cells in serum-free cell culture medium (Efendiev *et al*., 2000a). Since dopamine inhibits proximal tubule sodium reabsorption in conditions of sodium overload, we hypothesized that the excess extracellular sodium may translate in a transiently elevated intracellular sodium concentration and this may trigger sodium-sensitive intracellular switches that respond to the elevated intracellular sodium with inhibition in sodium reabsorption. To test this hypothesis, intracellular sodium was increased with monensin. The experiments were performed with attached OK cells in a serum-free medium and a concentration of ionophore that produced an elevation of intracellular sodium from ~10 to ~20 mM (R. Efendiev, A. M. Bertorello, A. R. Cinelli, and C. H. Pedemonte, unpublished result). While at basal intracellular sodium, dopamine inhibited and PMA activated the Na^+,K^+-ATPase, in cells treated with monensin, we observed that both dopamine and PMA inhibited the Na^+,K^+-ATPase activity (Efendiev *et al*., 2000a). These effects are not artifacts produced by monensin, since the ionophore by itself produced a small increase in Na^+,K^+-ATPase activity that is consistent with an elevation in intracellular sodium. The PMA-dependent inhibition of

Na^+,K^+-ATPase appears to occur via the intracellular messenger pathway usually stimulated by dopamine, since (1) the inhibitory effects of PMA and dopamine are not additive; (2) PKC-ζ is involved in both processes; (3) inhibition of the synthesis of 20-HETE (an arachidonic acid metabolite) by ethoxyresorufine prevented the inhibition of the Na^+,K^+-ATPase by both dopamine and PMA; and (4) PKC-β that is involved in PMA activation of the Na^+,K^+-ATPase does not participate in the PMA inhibition of the same activity in the presence of monensin. These results indicate the existence of two distinct signaling pathways that can be stimulated by PMA for either inhibition or activation of the Na^+,K^+-ATPase activity, depending on the intracellular sodium concentration. Furthermore, these results present evidence that the proximal tubule epithelial cells contain a sodium-sensor mechanism that activates or inhibits enzyme(s) involved in the process of sodium reabsorption.

CONCLUDING REMARKS

The renal Na^+,K^+-ATPase can be either activated or inhibited by hormones (Bertorello and Katz, 1993; Aperia, 1995). Consistent with this, several laboratories have reported either activation or inhibition of the Na^+,K^+-ATPase upon stimulation of PKC in various tissues and species (for references, see Pedemonte *et al*., 1997a). Our results from experiments with dopamine and PMA suggest that this apparent contradiction is the product of distinct mechanisms in which different serine residues of the Na^+,K^+-ATPase α subunit are phosphorylated and different PKC isoforms are involved.

Our results would be consistent with the following model: PMA stimulates a signaling pathway that results in activation of Na^+,K^+-ATPase by translocation of pump molecules to the plasma membrane from intracellular compartments. PMA treatment may activate a signaling pathway that is normally utilized by hormones that increase proximal tubule sodium reabsorption (Aperia *et al*., 1994; Schuster *et al*., 1984; Gopalakrishnan *et al*., 1995). Upon stimulation of membrane hormonal receptors, activation of an specific intracellular messenger pathway leads to phosphorylation of α subunit Ser11/Ser18 residues of the Na^+,K^+-ATPase molecules that are localized in a recycling intracellular compartment. Phosphorylated Na^+,K^+-ATPases are recognized by the clathrin/AP-1 mechanism that translocates these molecules to the plasma membrane, resulting in an enhanced capacity of the cell to translocate sodium ions.

In contrast, stimulation of proximal tubule dopaminergic receptors results in inhibition of Na^+,K^+-ATPase activity, a process that is mediated by removal of plasma membrane pumps to intracellular compartments. Upon stimulation of dopaminergic receptors, the G-protein coupled receptor intracellular pathway is activated and this leads to phosphorylation of α subunit Ser18 residues of Na^+,K^+-ATPase molecules that are localized exclusively in the plasma membrane. The SH_3 domain of PI3-K/p85α interacts with the Na^+,K^+-ATPase α subunit polyproline domain, which may become accessible after dopamine-dependent PKC phosphorylation of α subunit Ser18. Activation of PI3-K produces phosphatidylinositol-lipid phosphorylation, which stimulates the binding of AP-2 to phosphorylated Na^+,K^+-ATPase molecules. After recruitment and association of the clathrin vesicle machinery, Na^+,K^+-ATPase molecules are translocated from the plasma membrane to intracellular recycling compartments. This results in a reduced capacity of the cell to translocate sodium ions. These hypotheses offer an attractive explanation for how hormones involved in regulation of kidney Na^+ reabsorption may do so by regulating the activity of proximal tubule Na^+,K^+-ATPase.

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