# Simultaneous Phosphorylation of Ser11 and Ser18 in the α-Subunit Promotes the Recruitment of $Na^+$,$K^+$-ATPase Molecules to the Plasma Membrane†

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ABSTRACT: Renal sodium homeostasis is a major determinant of blood pressure and is regulated by several natriuretic and antinatriuretic hormones. These hormones, acting through intracellular second messengers, either activate or inhibit proximal tubule $Na^+$,$K^+$-ATPase. We have shown previously that phorbol ester (PMA) stimulation of endogenous PKC leads to activation of $Na^+$,$K^+$-ATPase in cultured proximal tubule cells (OK cells) expressing the rodent $Na^+$,$K^+$-ATPase α-subunit. We have now demonstrated that the treatment with PMA leads to an increased amount of $Na^+$,$K^+$-ATPase molecules in the plasmalemma, which is proportional to the increased enzyme activity. Colchicine, dinitrophenol, and potassium cyanide prevented the PMA-dependent stimulation of activity without affecting the increased level of phosphorylation of the $Na^+$,$K^+$-ATPase α-subunit. This suggests that phosphorylation does not directly stimulate $Na^+$,$K^+$-ATPase activity; instead, phosphorylation may be the triggering mechanism for recruitment of $Na^+$,$K^+$-ATPase molecules to the plasma membrane. Transfected cells expressing either an S11A or S18A mutant had the same basal $Na^+$,$K^+$-ATPase activity as cells expressing the wild-type rodent α-subunit, but PMA stimulation of $Na^+$,$K^+$-ATPase activity was completely abolished in either mutant. PMA treatment led to phosphorylation of the α-subunit by stimulation of PKC-*β*, and the extent of this phosphorylation was greatly reduced in the S11A and S18A mutants. These results indicate that both Ser11 and Ser18 of the α-subunit are essential for PMA stimulation of $Na^+$,$K^+$-ATPase activity, and that these amino acids are phosphorylated during this process. The results presented here support the hypothesis that PMA regulation of $Na^+$,$K^+$-ATPase is the result of an increased number of $Na^+$,$K^+$-ATPase molecules in the plasma membrane.

The $Na^+$,$K^+$-ATPase (EC 3.6.1.37) transports $Na^+$ and $K^+$ ions across the plasma membrane of eukaryotic cells and plays a key role in cellular ionic homeostasis (*1*, *2*). The minimal $Na^+$,$K^+$-ATPase functional molecule consists of two polypeptide chains, an α-subunit ($M \sim 100$ kDa) and a heavily glycosylated *β*-subunit ($M \sim 50$ kDa). In recent years, an increasing number of publications have presented evidence that hormonal short-term regulation of $Na^+$,$K^+$-ATPase contributes to the kidney's ability to adjust $Na^+$ reabsorption (*3*−*7*). Considerable evidence indicates that renal $Na^+$,$K^+$-ATPase is regulated through phosphorylation−dephosphorylation reactions catalyzed by kinases and phosphatases in response to hormones and second messengers. Both cAMP-dependent protein kinase (PKA) and protein kinase C (PKC)[1] phosphorylate the $Na^+$,$K^+$-ATPase α-subunit (*4*−*11*). A known target of PKC-mediated phosphorylation is the $NH_2$-terminus of the α-subunit (*12*, *13*). The fact that this region is involved in regulation of $Na^+$,$K^+$-ATPase activity by PKC was shown by experiments with $NH_2$-terminal deletions (*14*, *15*). Chibalin et al. (*16*, *17*) have demonstrated that dopamine-induced inhibition of $Na^+$,$K^+$-ATPase in proximal tubule cells is mediated by endocytosis of $Na^+$,$K^+$-ATPase molecules and that this process is triggered by phosphorylation of the α-subunit at Ser18. In contrast, phorbol ester-dependent activation and phosphorylation of $Na^+$,$K^+$-ATPase have also been described in rodent proximal tubule cells (*18*). We have shown that PMA stimulation of endogenous PKC in OK cells activates $Rb^+$-transport with a concomitant decrease in intracellular $Na^+$ concentration indicating that PMA primarily activates $Na^+$,$K^+$-ATPase and not $Na^+$ entry pathways (*15*, *19*). PMA-dependent stimulation of $Rb^+$-transport is abolished in cells expressing a mutant missing the first 26 amino acids of the rodent α-subunit, consistent with a role for the $NH_2$-terminus in regulation (*15*, *19*). In this study, we have examined the molecular mechanism by which PMA stimulates $Na^+$,$K^+$-

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[1] Abbreviations: PKC, protein kinase C; OK, opossum kidney; PMA, phorbol 12-myristate 13-acetate. $Na^+$,$K^+$-ATPase and $Rb^+$-transport refer to the same protein activity.

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ATPase activity. We found that both Ser11 and Ser18 in the α-subunit $NH_2$-terminus are phosphorylated and essential for this process, and that the PMA-dependent activation appears to be mediated by recruitment of $Na^+,K^+$-ATPase molecules to the plasma membrane.

## EXPERIMENTAL PROCEDURES

*Materials.* Cell culture supplies were purchased from Life Science Technologies (Grand Island, NY) and Hyclone Laboratories (Logan, UT). Molecular biology reagents were from New England Biolabs (Beverly, MA), Promega (Madison, WI), Stratagene (La Jolla, CA), and Sigma Chemical Co. (St. Louis, MO). Ouabain was purchased from Calbiochem (San Diego, CA). Phorbol 12-myristate 13-acetate (PMA) and protease inhibitors were obtained from Sigma Chemical Co. [$^{86}Rb^+$]RbCl was obtained from NEN Life Science Products, Inc. (Boston, MA); $^{32}P_i$ was from ICN Pharmaceuticals (Costa Mesa, CA), and [$^3$H]ouabain was from Amersham (Arlington Heights, IL). Other reagents were of the highest quality available.

*Cell Culture and Transfection.* Opossum kidney (OK) cells were maintained at 37 °C (10% $CO_2$) in Dulbecco's modified Eagle's medium with 10% calf serum and antibiotics (DMEM-10). The expression vector pCMV containing the rodent $Na^+$-pump α1-subunit cDNA was obtained from PharMingen. Mutants of α1 were prepared from a plasmid containing the wild-type α-subunit sequence and complementary oligonucleotides containing the desired change as described previously (*15*, *16*, *19*). Briefly, annealed plasmid and oligonucleotides were subjected to PCR amplification with *Pfu* polymerase, followed by restriction of the original wild-type template with *Dpn*I. After transformation of bacteria, the recovered mutant plasmids were evaluated by restriction analysis and direct sequencing of the altered region. Construction of one of the mutants, Δ5−26, was described in a previous report (*20*). Plasmids containing the wild-type and mutant α-subunit cDNAs were transfected into OK cells using liposomes, as previously described (*15*, *16*, *19*). Selection for cells expressing the highest level of rodent α-subunit was achieved by exposing them to a medium containing 3 μM ouabain. Since the endogenous $Na^+$-pump of OK cells is completely inhibited by this concentration of ouabain, only successful recipients of the transfected rodent α-subunit would be able to survive. Resistant colonies were expanded and maintained in DMEM-10 containing 3 μM ouabain. Experiments were performed with a mix of at least 20 independent clones for each cell line. The $Na^+,K^+$-ATPase of mock-transfected cells (vector alone, vector plus liposomes, or liposomes alone) had the same activity and sensitivity to ouabain as nontransfected host cells.

*Determination of Protein Concentrations.* Cells were solubilized with SDS, and aliquots were used for protein quantitation. Protein concentrations were determined by the bicinchoninic acid method (Pierce) using BSA as a standard.

*Quantitation of $Rb^+$-Transport.* Measurements of $Na^+,K^+$-ATPase-mediated transport by $Rb^+$-uptake were performed with attached cells. The conditions for assessing $Rb^+$-transport were established previously (*15*, *19*). To measure $Rb^+$-transport, transfected cells were transferred to serum-free DMEM containing 50 mM HEPES (pH 7.4), 2 mM EGTA, and 3 μM or 5 mM ouabain (incubation medium). Cells were incubated with these amounts of ouabain for 20 min at 37 °C in an air atmosphere and for 10 min at 25 °C before addition of 1 μM PMA. Ten minutes later, a trace amount of [$^{86}Rb^+$]RbCl was added. After 20 min, cells were washed four times with ice-cold saline and dissolved with SDS, and the amount of accumulated radioactivity was determined by scintillation counting. $Na^+,K^+$-ATPase-mediated $Rb^+$-transport was estimated from the difference in tracer uptake between samples incubated in 3 μM and 5 mM ouabain. For nontransfected cells, the level of $Rb^+$-transport was measured in the absence and presence of 5 mM ouabain. The level of ouabain-insensitive $Rb^+$-transport was 20−25% of the total level of $Rb^+$-transport measured. As PMA was dissolved in DMSO, the same amount of solvent was added to control samples. The amount of solvent used did not alter the $Rb^+$-transport of control samples. Each experiment was carried out in triplicate, and the results are the average of at least three experiments ± the standard deviation.

*Phosphorylation and Immunoprecipitation of the $Na^+,K^+$-ATPase.* In these experiments, the following steps were followed: (1) loading of the cells with radiolabeled inorganic phosphate, (2) treatment of the cells with PMA, (3) preparation of a crude microsomal fraction, (4) solubilization of the membranes with detergents, and (5) immunoprecipitation of the $Na^+,K^+$-ATPase. We prepared microsomes before the immunoprecipitation because we have determined that when cells are dissolved in a small volume DNA forms a gel with the detergents and this interferes with solubilization and immunoprecipitation. The experiments were performed with cells grown at about 80% confluence in six-well plates. Cells were washed and incubated for 2 h in phosphate-free serum-free medium [110 mM NaCl, 44 mM $NaHCO_3$, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM glucose, 2 mM HEPES, 4 mM L-glutamine, and 15 mg/L phenol red (pH 7.3)]. To load the cells with radioactive phosphate, they were incubated for 2 h in the same medium containing 0.5 mCi of $^{32}P_i$ per milliliter at 37 °C in a 10% $CO_2$ atmosphere.

After incubation with 1 μM PMA for 10 min at 25 °C, the cells were transferred to an ice−water bath and washed twice with ice-cold PBS. One milliliter of immunoprecipitation (IP) buffer containing 20 mM Tris, 2 mM EDTA, 2 mM EGTA, 30 mM sodium pyrophosphate (pH 7.3), and a cocktail of protease inhibitors was added per well. The cocktail of protease inhibitors contained 4-(2-aminoethyl)-benzenesulfonyl fluoride, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), bestatin, leupeptin, aprotinin, and sodium EDTA. The cells were scraped on ice, and the suspension was frozen in liquid nitrogen, thawed rapidly, and probe-sonicated three times for 15 s with 15 s intervals in an Utrasonic model 4710 homogenizer (Cole Parmer) at 25 W and 80% output. Samples were maintained in an ice−water bath during sonication. The cell suspension was frozen and thawed again, and then centrifuged at 1400*g* and 4 °C for 5 min. Under this condition, the supernatant should contain a crude plasma membrane preparation free of nucleus. Supernatants were transferred to clean tubes, and 0.2% SDS and 1% Triton X-100 were added. After incubation at 60 °C for 15 min, an anti-α-subunit monoclonal antibody (α1) was added. The antibody α1 was a kind gift from R. Mercer and G. Blanco (Washington University, St. Louis, MO).

Samples were incubated with α1 antibody at 4 °C for 1 h with end-over-end shaking. Protein A/G-PLUS Agarose beads (Santa Cruz Biotechnology, Inc.), prewashed three times with PBS and once with IP buffer containing 1% Triton X-100, were added to samples, and they were incubated at 4 °C overnight with end-over-end shaking. The immunobeads were separated by centrifugation and washed four times with IP buffer containing 1% Triton X-100 and 0.1% SDS and once with 50 mM Tris-HCl (pH 7.4) and finally resuspended in Laemmli (*21*) sample buffer [125 mM Tris-HCl (pH 7.3), 10% glycerol, 5% SDS, 2% 2-mercaptoethanol, and 0.001% bromophenol blue]. Samples were incubated at 60 °C for 15 min and then centrifuged to separate the beads. Aliquots from the supernatants were separated in an 8% Laemmli gel. The separated proteins were electrotransferred to a piece of PVDF-Plus membrane (MSI) which was dried and exposed to a Fuji BAS-III imaging plate at room temperature for 36 h. The imaging plate was analyzed in a Fuji BAS 1000 Bio Imaging Analyzer, using Fujifilm Image Reader 1.4E software. The detected radioactive bands were quantified using Image Gauge 3.0 software. After exposure to detect radioactivity, the pieces of PVDF membrane were used for Western blot analysis (as described below) to detect the $Na^+,K^+$-ATPase α-subunit, and the protein bands were quantified by densitometric analysis as indicated above. The ratio of radioactivity to protein was calculated, and the results of phosphorylation are expressed as the PMA-dependent percent of change. Each experiment was performed in triplicate. Results are the average of at least three independent experiments ± the standard deviation.

*Validation of the Use of the Antibody for Immunoprecipitation.* We have shown previously that expression of the rodent α-subunit in OK cells and selection with ouabain result in near total elimination of the endogenous $Na^+,K^+$-ATPase α-subunit. Several lines of evidence support this conclusion. (1) In cells expressing a mutant of the α-subunit lacking the first 28 amino acids, very little protein reacting with an antibody directed to the amino-terminal end of the α-subunit was observed (*16*). (2) Even though $Na^+,K^+$-ATPase containing the endogenous α-subunit is activated by PMA in the same way as $Na^+,K^+$-ATPase containing the rodent α-subunit, cells expressing the amino-terminal mutant of the rodent α-subunit did not display any PMA-dependent activation of $Na^+,K^+$-ATPase at all (*15*, *19*). (3) A similar result was observed when inhibition by dopamine was assessed (*16*, *17*). (4) PMA treatment of cells expressing the rodent α-subunit results in a reduced intracellular sodium level; however, cells expressing the amino-terminal mutant of the rodent α1 did not show any alteration of the intracellular sodium level (*15*). Therefore, all of these results support the conclusion that expression of the rodent wild type or mutant α1 greatly reduces the level of expression of the endogenous protein. Other authors have shown similar results in relation to the $Na^+,K^+$-ATPase α-subunit and other membrane proteins (*22*). Thus, the α1 antibody would immunoprecipitate the expressed rodent α1, and no significant interference of the endogenous protein should occur.

*Quantification of the $Na^+,K^+$-ATPase α-Subunit by Western Blot Analysis*. Pieces of PVDF membrane containing the separated proteins were blocked by incubation for 1 h in 5% w/v nonfat dry milk in Tris-buffer saline [150 mM NaCl and 10 mM Tris-HCl (pH 7.4)] at room temperature. Membranes were treated with α1 antibody (1:100 dilution) in blocking solution at room temperature for 1 h, and then washed three times for 5 min with Tris-buffer saline containing 0.1% Tween 20 and three times for 5 min with Tris-buffer saline alone. The membranes were then incubated for 1 h at room temperature with anti-mouse IgG (Fab-specific) peroxidase conjugate (Sigma, dilution of 1:1000), and then washed as before. Finally, the immunoreactivity was detected by enhanced chemiluminescence (SuperSignal Substrate, Pierce). Quantitation at nonsaturating levels of exposure of immunodetected α-subunit bands was performed by densitometric analysis as indicated above.

*Ouabain Binding*. Ouabain binding was assessed in entire cells attached to filter inserts (Costar). The ouabain assay medium contained 120 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM Tris-phosphate (pH 7.2), and 0.4 μM [$^3$H]ouabain (Amersham). Nonspecific binding was assessed in the presence of 1 mM ouabain. Cells were washed with nonradioactive assay medium. Filter inserts were kept upside down (the basolateral side of the cells was against the filter), and the assay medium was added on top of the filters. Some of the samples were treated with 1 μM PMA for 10 min before the assay of ouabain binding. This reaction was performed at room temperature, in a humidified chamber, for either 20 or 120 min. Cells were then scraped from the filters, washed five times with nonradioactive assay medium, and dissolved with SDS. Aliquots were used to determine protein and radioactivity contents. The level of specific ouabain binding was calculated from the difference between the level of ouabain binding to cells exposed to 0.4 M and 1 mM ouabain. These measurements were carried out in triplicate. Results are the average of at least three independent experiments ± the standard deviation.

*Biotin Labeling.* The experiments were performed with OK cells transfected with the rodent wild-type α1 subunit and grown to 60−80% confluence in six-well plates. Cells were incubated in serum-free cell culture medium containing 2 mM EGTA for 2 h at 25 °C, treated with 1 μM PMA for 10 min, and then transferred to a 4 °C environment. Sulfo-NHS-Biotin (Pierce) at a final concentration of 1.5 mg/mL was added. After 20 min at 4 °C, the medium was changed to 10 mM Tris-Cl (pH 7.5), 2 mM $CaCl_2$, 150 mM NaCl, and 1.5 mg/mL Sulfo-NHS-Biotin, and the cells were incubated for an additional 30 min at 4 °C. Following biotin labeling, the cells were scraped in IP buffer containing a protease inhibitor cocktail, frozen in liquid nitrogen, thawed rapidly, probe-sonicated twice in an ice−water bath, and frozen-thawed again. The cell suspension was centrifuged at 14000*g* and 4 °C for 5 min. After the supernatants were transferred to clean tubes, the pellets were solubilized with 1% Triton X-100 and 0.2% SDS. The solutions were maintained at 60 °C for 15 min. Streptavidin paramagnetic beads (Promega), prewashed three times with PBS and once with IP buffer containing 1% Triton X-100, were added to the supernatants. The suspensions were incubated for 2 h at 4 °C with end-over-end shaking. Immunomagnetic beads were separated with a magnet, washed four times with IP buffer containing 1% Triton X-100 and 0.1% SDS and once with 50 mM Tris-HCl (pH 7.4), and finally resuspended in Laemmli sample buffer. Electrophoresis, Western blot analysis with α1 antibody, and densitometric analysis were performed as described above.

*Coprecipitation of the $Na^+,K^+$-ATPase α-Subunit and Adaptor Protein 1 (AP-1)*. The experiments were performed with OK cells transfected with the wild-type α1 cDNA grown to 60−80% confluence in six-well plates. Cells were treated with PMA and disrupted in IP buffer as indicated above. The $Na^+,K^+$-ATPase was immunoprecipitated with α1 monoclonal antibody and A/G agarose beads, separated by PAGE, and transferred to PVDF membrane as described above. Then, Western blot analysis was performed with an antibody that specifically reacts with γ-adaptor protein 1 (AP-1) (Sigma). The reaction of the antibody was quantified by densitometric analysis.

*Other Cell Treatments.* Unless indicated, all cell treatments were performed with reagents dissolved in water at 25 °C. Cells were treated with either 1 mM KCN or 1 mM DNP for 5 min, and 100 μM colchicine for 30 min.

*Statistical Analysis*. Comparisons between groups were performed with the Student's *t* test for unpaired data. Each experiment was carried out in triplicate and repeated at least three times for each treatment.

## RESULTS

The wild-type rodent $Na^+,K^+$-ATPase α-subunit was expressed in OK cells, as previously described (*15*, *19*). Transfected cells displayed the ouabain-resistant phenotype characteristic of rodent-type cells, and selected transfected cell lines were maintained continuously in the presence of 3 μM ouabain to inhibit totally the activity of $Na^+,K^+$-ATPase molecules containing endogenous α-subunits. Under these conditions, OK cells transfected with wild-type rodent α1-subunit cDNA exhibited levels of $Rb^+$-transport and maximal $Na^+,K^+$-ATPase activity similar to those observed in non-transfected OK cells (*15*−*17*, *19*).

*PMA Treatment of OK Cells Results in a Larger Plasma Membrane Pool of $Na^+,K^+$-ATPase*. We have demonstrated previously that PMA treatment of OK cells expressing the rodent α1-subunit results in stimulation of $Na^+,K^+$-ATPase activity and that this effect is mediated by PKC (*15*, *19*). Whether this activation is due to an improved turnover of the enzyme or of the number of molecules of $Na^+,K^+$-ATPase at the plasma membrane has not been previously elucidated. To examine this matter, we determined whether stimulation of PKC activity by PMA was associated with an increased number of $Na^+,K^+$-ATPase molecules at the plasma membrane. To determine $Na^+,K^+$-ATPases in the plasmalemma, we used either ouabain or NHS-biotin binding. $Na^+,K^+$-ATPase molecules are the specific receptors for ouabain, which binds to a single site located on the extracellular surface of the α-subunit (*23*, *24*). It follows that measurement of ouabain binding is a direct indication of the number of $Na^+,K^+$-ATPase molecules present in the plasma membrane. However, this experiment cannot be performed in cells expressing the rodent α-subunit because $Na^+,K^+$-ATPases containing this subunit have a low affinity for ouabain (*23*, *24*). We have previously demonstrated that $Na^+,K^+$-ATPase of nontransfected OK cells is also activated by PMA and that this effect is mediated by PKC (*15*, *19*). We therefore determined the effect of PMA on ouabain binding in nontransfected OK cells, as previously described (*25*). Maximal ouabain binding was attained at about 2 h (Table 1), and PMA treatment increased the maximal level of binding of ouabain (Table 1), indicating an increased pump abundance in plasmalemma. The PMA-induced increment of $Rb^+$-transport was generally measured at 20 min, but the maximal level of ouabain binding was determined at 120 min. To be sure that the extended time is not increasing the level of ouabain binding by other means than PMA treatment, we determined ouabain binding at 20 min (Table 1) and observed the same proportional increment of $Na^+,K^+$-ATPase molecules as at 120 min.

Table 1: KCN Prevents PMA-Dependent Increases in the Level of Ouabain Binding to the $Na^+,K^+$-ATPase of OK Cells[a]

| | level of ouabain binding (pmol/mg of protein) | | |
|---|---|---|---|
| treatment | 20 min | 60 min | 120 min |
| control | 0.43 ± 0.06 | 1.03 ± 0.08 | 1.19 ± 0.10 |
| PMA | 0.69 ± 0.06 | nd | 1.79 ± 0.13 |
| KCN | 0.41 ± 0.02 | nd | 1.09 ± 0.11 |
| KCN/PMA | 0.44 ± 0.04 | nd | 1.19 ± 0.12 |

[a] Accumulation of $Na^+,K^+$-ATPase molecules at the plasma membrane of OK cells was assessed by the level of ouabain binding at 20 and 120 min: control, nontreated cells; PMA, cells treated with 1 μM PMA for 10 min before the ouabain binding assay; KCN, cells treated for 5 min with 1 mM KCN before the ouabain binding assay; and KCN/PMA, cells treated with 1 mM KCN for 5 min before addition of PMA.

Whether the number of $Na^+,K^+$-ATPase molecules at the plasma membrane is increased by PMA can also be determined by biotinylation of the proteins that are at the cell surface and subsequent immunoprecipitation of the biotinylated $Na^+,K^+$-ATPase. OK cells expressing the rodent α-subunit were grown at 80% confluence, and NHS-biotin was added to the cell culture medium to label plasma membrane proteins. This reagent reacts with primary amino groups and does not permeate across biological membranes; thus, protein side chains containing primary amines that are exposed to the extracellular medium can be biotinylated. After labeling had been carried out, cells were lysed and biotinylated proteins were immunoprecipitated with streptavidin bound to magnetic beads. The immunoprecipitated proteins were separated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and blotted onto PVDF membrane, and the α-subunit of the $Na^+,K^+$-ATPase was identified by Western blotting with an anti-α1 antibody. The results presented in Figure 1A indicate that treatment of the cells with PMA results in a significant increase in the level of biotinylated $Na^+,K^+$-ATPase that is consistent with an increased abundance of $Na^+,K^+$-ATPase at the plasma membrane.

The translocation of at least some proteins from intracellular storage compartments to the plasma membrane is inhibited by potassium cyanide (KCN) (*26*, *27*). We tested whether this reagent has any effect on the PMA-dependent increase in the level of ouabain binding. Table 1 shows that this increment was not observed in cells treated with KCN before PMA with both 20 and 120 min of ouabain binding, and that by itself KCN has no effect on the basal binding of the glycoside. Therefore, the results presented in Table 1 and Figure 1A are consistent with the idea that the PMA-dependent activation of $Na^+,K^+$-ATPase is mediated by an increased number of $Na^+,K^+$-ATPase molecules at the plasma membrane.

*$Na^+,K^+$-ATPase Interacts with AP-1*. Recruitment of integral membrane proteins is initiated by selective recogni-

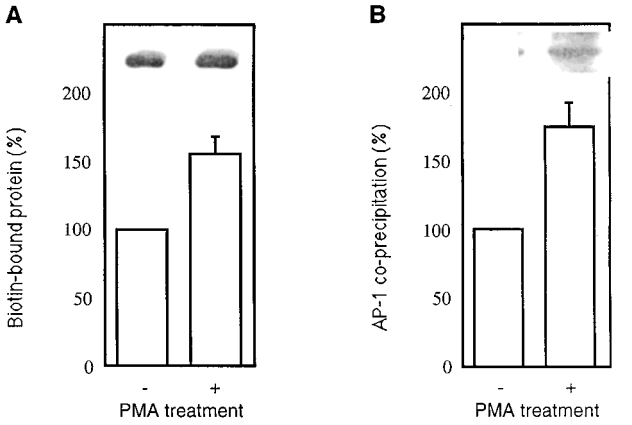


FIGURE 1: (A) Binding of biotin to the plasma membrane $Na^+,K^+$-ATPase of OK cells transfected with the rodent α-subunit. OK cells transfected with the rodent α-subunit were treated with NHS-biotin, and the level of specific labeling of the Na,K-ATPase molecules was determined after specific immunoprecipitation as indicated in Experimental Procedures. A representative experiment of the quantitation of biotin is illustrated in the upper panel. (B) PMA-dependent interaction between the $Na^+,K^+$-ATPase α-subunit and AP-1. OK cells expressing the rodent α1-subunit were treated with 1 μM PMA for 10 min. The cells were dissolved with IP buffer, and the α-subunit was immunoprecipitated with the α1 antibody and transferred to a piece of PVDF membrane. The presence of AP-1 was determined by Western blot analysis as indicated in Experimental Procedures. A representative Western blot is illustrated in the upper panel. The experiment was repeated three times, and the reaction of the AP-1 antibody was quantitated by scanning.

tion of the target protein, located in intracellular compartments, by interaction with adaptor protein 1 (AP-1) followed by the protein translocation into the membrane via clathrin-coated vesicles (*32*). To determine whether this mechanism is involved in the PMA-induced activation of $Na^+,K^+$-ATPase, we treated cells with PMA and then immunoprecipitated $Na^+,K^+$-ATPase molecules with an anti-α-subunit antibody. In the precipitated material, we tested for the presence of AP-1. Since the immunoprecipitation buffer contained detergents for dissolving the plasma membrane, only AP-1 molecules that are interacting with $Na^+,K^+$-ATPase molecules would be precipitated. As shown in Figure 1B, AP-1 coprecipitated with the $Na^+,K^+$-ATPase, and the interaction between these two proteins was enhanced significantly by treatment of the cells with PMA. This result lends further support to the hypothesis that PMA induces recruitment of $Na^+,K^+$-ATPase molecules to the plasma membrane.

*PMA-Dependent Stimulation of $Na^+,K^+$-ATPase Is Blocked by Colchicine, KCN, and Dinitrophenol (DNP).* Traffic of $Na^+,K^+$-ATPase molecules to the plasma membrane should depend on a dynamic cellular microtubule cytoskeleton. We examined the effect of PMA on $Rb^+$-transport in OK cells pretreated with the microtubule depolymerizing agent colchicine (*28*−*31*). Cells were incubated for 30 min with 100 μM colchicine at 25 °C before treatment with PMA. Under these conditions, colchicine blocked almost totally the PMA-dependent activation of $Rb^+$-transport (Figure 2A). Colchicine alone had no effect on basal $Na^+,K^+$-ATPase activity, and OK cells treated with colchicine displayed the same level of $Rb^+$-transport as nontreated control cells. Therefore, the PMA-dependent stimulation of $Rb^+$-transport observed may be due not to an increased catalytic turnover of $Na^+,K^+$-ATPase but to an increased number of $Na^+,K^+$-ATPase molecules at the plasma membrane.

If the above conclusion is correct and PMA-induced activation of $Na^+,K^+$-ATPase results from recruitment of molecules to the plasma membrane, we should expect that the activation would be prevented by KCN treatment. Indeed, treatment of OK cells with 1 mM KCN for 5 min at 25 °C before addition of PMA blocked the increase in the level of $Rb^+$-transport (Figure 2B, KCN/PMA), and the same effect was produced by dinitrophenol (DNP) (Figure 2B, DNP/

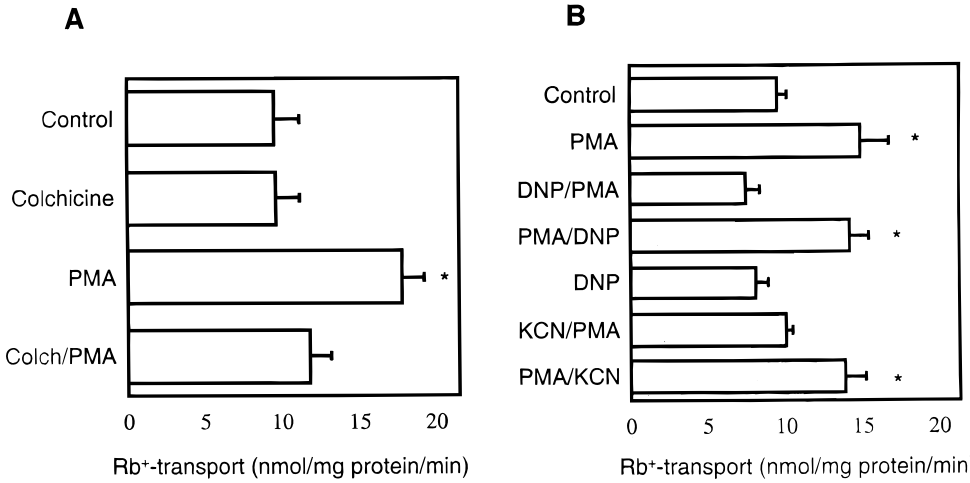


FIGURE 2: (A) Effect of colchicine on PMA-dependent activation of $Rb^+$-transport mediated by $Na^+,K^+$-ATPase of cells expressing the wild-type rodent α1-subunit: control, nontreated cells; colchicine, cells treated with 100 μM colchicine for 30 min before the $Rb^+$-transport assay; PMA, cells treated with 1 μM PMA for 10 min before the $Rb^+$-transport assay; and Colch/PMA, cells treated with 100 μM colchicine for 30 min before treatment with 1 μM PMA for 10 min. Each experiment was made in triplicate and repeated at least three times as indicated in Experimental Procedures. The asterisk indicates where $P < 0.02$ with respect to nontreated cells. (B) Effect of dinitrophenol (DNP) and potassium cyanide (KCN) on the PMA-dependent activation of $Rb^+$-transport mediated by the $Na^+,K^+$-ATPase of cells expressing the wild-type rodent α1-subunit: control, nontreated cells; PMA, cells treated with 1 μM PMA for 10 min before the $Rb^+$-transport assay; DNP/PMA, cells treated with 1 mM DNP for 5 min before addition of PMA; PMA/DNP, cells treated with 1 mM DNP for 5 min after addition of PMA; DNP, cells treated with 1 mM DNP for 5 min before the $Rb^+$-transport assay; KCN/PMA, cells treated with 1 mM KCN for 5 min before addition of PMA; and PMA/KCN, cells treated with 1 mM KCN for 5 min after addition of PMA. Treatments were performed as indicated in Experimental Procedures. The asterisk indicates that $P < 0.02$ with respect to nontreated cells.

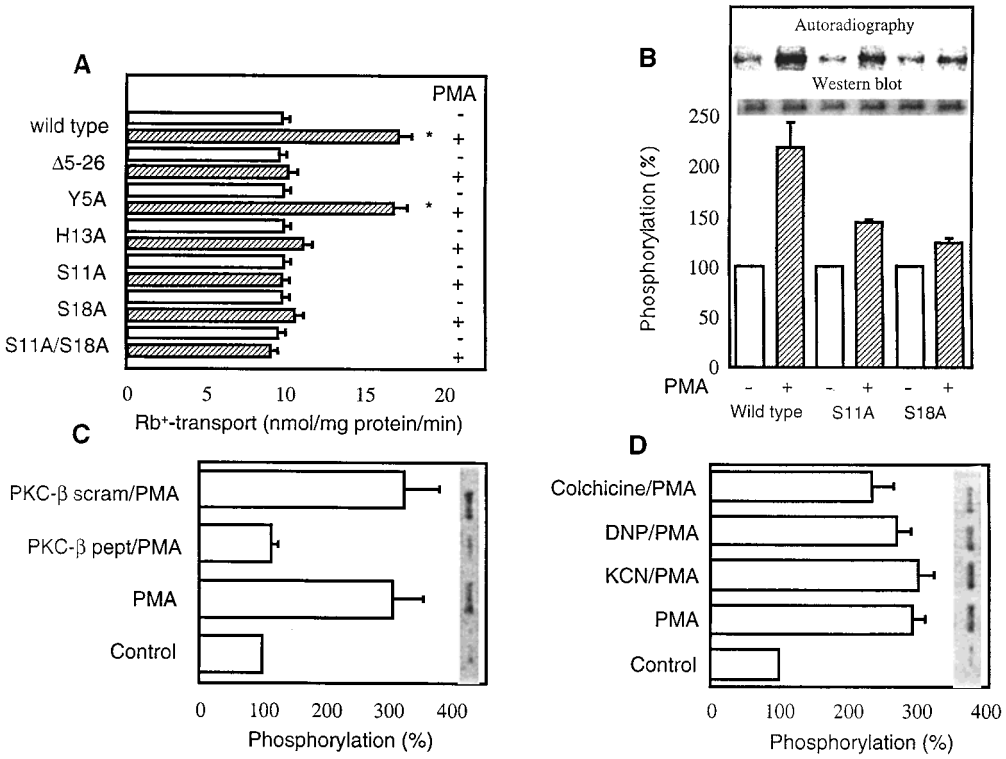


FIGURE 3: (A) Ser11 and Ser18 are essential for PMA-dependent activation of $Na^+,K^+$-ATPase. The level of $Rb^+$-transport mediated by the $Na^+,K^+$-ATPase of cells expressing the wild-type rodent α1-subunit (wild type) and several α-subunit mutants was determined. Mutants were prepared, and treatments were performed as indicated in Experimental Procedures. Each experiment was carried out in triplicate and repeated at least three times with each cell line. The asterisk indicates that $P < 0.02$ with respect to nontreated cells. (B) PMA-dependent phosphorylation of the $Na^+,K^+$-ATPase α-subunit. The effect of PMA treatment on the phosphorylation of the rodent α1-subunit (wild type) and α-subunit mutants was determined. Each cell line was treated with PMA, and $Na^+,K^+$-ATPase was immunoprecipitated as indicated in Experimental Procedures. After separation by PAGE, the ratio of radioactivity to protein was calculated for each α-subunit band. A representative autoradiogram and the corresponding Western blot are shown in the upper panel. No significant change in the basal level of phosphorylation between mutants and the wild-type α-subunit was observed. Data are presented as a percentage of PMA-dependent phosphorylation with respect to the corresponding nontreated controls. Phosphorylation levels were obtained from experiments repeated at least three times. Individual experiments were carried out in triplicate. (C) PKC-*β* is involved in the PMA-dependent phosphorylation of the $Na^+,K^+$-ATPase α-subunit. PMA-dependent phosphorylation of the α-subunit was inhibited by a peptide that specifically interacts with PKC-*β* (PKC-*β* pept) but not by a scrambled peptide (PKC-*β* scram). The experiment was performed as indicated in Experimental Procedures. A representative experiment of phosphorylation is shown at the right side of the figure. (D) Effect of colchicine, DNP, and KCN on the PMA-dependent phosphorylation of the $Na^+,K^+$-ATPase α-subunit. OK cells were pretreated with colchicine, DNP, or KCN as indicated in Experimental Procedures before determination of the level of PMA-dependent phosphorylation of the $Na^+,K^+$-ATPase α-subunit. A representative experiment of phosphorylation is shown at the right side.

PMA). It should be noted that neither KCN nor DNP affected the PMA-dependent activation of $Rb^+$-transport when added *after* the cells had been treated with phorbol ester (Figure 2B, PMA/KCN and PMA/DNP), and that DNP by itself had no effect on basal ouabain-sensitive $Rb^+$-transport (Figure 2B, DNP). Therefore, both KCN and DNP block specifically the PMA-dependent activation and had no effect on the intrinsic $Na^+,K^+$-ATPase activity.

*Mutations of Ser11 and Ser18 Impair PMA-Dependent Activation of $Na^+,K^+$-ATPase.* We have shown previously that activation of $Rb^+$-transport by PMA requires the integrity of the $Na^+,K^+$-ATPase α-subunit $NH_2$-terminus (*15*, *19*). To characterize further the amino acids that are involved in the PMA activation, we produced several mutants of the $Na^+,K^+$-ATPase α-subunit. As shown in Figure 3A, the basal level of ouabain-sensitive $Rb^+$-transport is the same in cells transfected with wild-type and mutant rodent α1-subunit cDNAs, and treatment with PMA resulted in an increased level of ouabain-sensitive $Rb^+$-transport in cells expressing the wild-type rodent α-subunit. As previously observed with an $NH_2$-terminal deletion mutant that eliminated the first 26 amino acids of the α-subunit (*15*, *19*), cells expressing a deletion mutant of the $Na^+,K^+$-ATPase α-subunit without amino acids 5−26 at the $NH_2$-terminal end (Δ5−26) did not display the PMA-dependent stimulation of $Na^+,K^+$-ATPase activity (Figure 3A). This result suggests that residues within amino acids 5−26 of the $NH_2$-terminus are necessary for PMA-dependent $Na^+,K^+$-ATPase activation. Two serine residues in the $NH_2$-terminus, Ser11 and Ser18, are obvious candidates since it has been shown that they are the only residues phosphorylated in vitro by PKC (*12*, *13*). Indeed, substitution of either Ser11 or Ser18 with an alanine residue (S11A and S18A) in the rodent α-subunit totally impaired the PMA-dependent stimulation of $Rb^+$-transport (Figure 3A). The same result was obtained when both Ser11 and Ser18 were simultaneously substituted with alanine residues (data not shown). None of the mutations altered the basal $Na^+,K^+$-ATPase activity measured in the absence of PMA, which suggests that these mutations have affected specifically the mechanism of PMA-dependent activation and not the intrinsic mechanism of $Na^+,K^+$-ATPase activity.

Substitution of His13 with an alanine residue (H13A) totally impaired the PMA response (Figure 3A), in agreement with Beguin et al. (*12*), who suggested that this histidine

residue is essential for PKC to phosphorylate Ser11, which is in an unusual nonconsensus PKC site. On the other hand, substitution of the α-subunit tyrosine residue at position 5 from the $NH_2$-terminal end with an alanine residue (Y5A) had no effect on basal or PMA-stimulated $Rb^+$-transport (Figure 3A).

*PMA-Dependent Phosphorylation of the $Na^+$,$K^+$-ATPase α-Subunit*. The fact that Ser11 and Ser18 are essential amino acids for PMA-dependent activation of $Na^+$,$K^+$-ATPase suggests that these residues are phosphorylated by PKC. To test this hypothesis, we determined the level of PMA-mediated $Na^+$,$K^+$-ATPase phosphorylation in cells expressing the rodent wild type and the S11A and S18A mutants of the $Na^+$,$K^+$-ATPase α-subunit. As previously observed (*17*), there was no significant change in the basal level (without PMA) of phosphorylation between the mutants and the wild-type α-subunit, but there was a significant increase in the level of phosphorylation of the wild-type α-subunit after treatment with PMA (Figure 3B). S11A and S18A mutants had reduced levels of phosphorylation compared to the wild type. Comparisons with the measurements of the level of $Rb^+$-transport illustrated in Figure 3A strongly suggest that PMA-dependent stimulation of $Na^+$,$K^+$-ATPase activity requires phosphorylation of both Ser11 and Ser18 in the α-subunit.

*PKC-β Is Involved in the PMA-Dependent Phosphorylation of the $Na^+$,$K^+$-ATPase α-Subunit*. We have demonstrated previously that while PKC-ζ is involved in the inhibition of $Na^+$,$K^+$-ATPase by dopamine, PKC-*β* is involved in the stimulation of this activity by PMA (*33*). However, our results did not establish whether PKC-*β* phosphorylates the $Na^+$,$K^+$-ATPase α-subunit or mediates another step of the mechanism that underlies PMA-dependent activation. We therefore assessed the effect of PKC inhibition on the PMA-dependent phosphorylation of the $Na^+$,$K^+$-ATPase α-subunit. PKC-*β* was inhibited specifically using a peptide inhibitor, as previously described (*33*). Figure 3C shows that blockade of PKC-*β* by the specific peptide prevented the PMA-dependent phosphorylation of the $Na^+$,$K^+$-ATPase α-subunit, while a scrambled, irrelevant peptide had no effect. These results suggest that the PKC-*β* isoform is indeed responsible for phosphorylation of the α-subunit.

*PMA-Dependent Activation of $Na^+$,$K^+$-ATPase Is Due to Recruitment and Not to Phosphorylation.* To determine the relative roles of α-subunit phosphorylation and membrane recruitment during the activation of $Na^+$,$K^+$-ATPase by PMA, we examined the effect of PMA on α-subunit phosphorylation under conditions where membrane recruitment was blocked. For this, we assessed the effect of PMA-induced phosphorylation in cells expressing the rodent α1-subunit that have been pretreated with KCN, DNP, or colchicine. Exposure of nontreated cells to PMA increased the level of α-subunit phosphorylation, and this increment was not affected by preincubation of the cells with KCN, DNP, or colchicine (Figure 3D). As KCN, DNP, and colchicine prevented the activation of $Na^+$,$K^+$-ATPase elicited by PMA (Figure 2A,B); the results illustrated in Figure 3D suggest that phosphorylation is necessary (a possible triggering signal), but not sufficient for PMA-dependent stimulation of $Na^+$,$K^+$-ATPase activity.

## DISCUSSION

In this report, we present several lines of evidence that PMA-dependent activation of $Na^+$,$K^+$-ATPase is due to an increased number of $Na^+$,$K^+$-ATPase molecules at the plasma membrane and not to an increased enzyme turnover rate. Using both cell surface biotin labeling and ouabain binding under conditions of maximal binding, we detected a significant increment in the plasmalemma pool of $Na^+$,$K^+$-ATPase induced by PMA, suggesting that the phorbol ester promoted the recruitment of $Na^+$,$K^+$-ATPase molecules to the plasma membrane. The level of ouabain binding was determined at maximal (120 min) and nonmaximal (20 min) binding conditions (Table 1). Although the level of ouabain binding at 20 min was lower than at 120 min, the PMA-induced increment of ouabain binding was proportionally similar at the two times at which it was measured. Under the conditions of these experiments, ouabain would bind exclusively to $Na^+$,$K^+$-ATPase molecules located in the plasma membrane, which are the only ones contributing to $Rb^+$-transport. Interestingly, PMA produced a similar proportional increase in the levels of both ouabain binding and $Rb^+$-transport. The fact that an increase in the level of ouabain binding was determined under conditions of maximal binding (120 min), and that the same proportional increase in the level of ouabain binding was observed at 20 and 120 min, suggests that the higher level of ouabain binding is produced by an increased number of pumps at the plasma membrane rather than conformational changes in the pump that might change its affinity for the glycoside. Moreover, the PMA-dependent increase in the level of labeling was also obtained with biotin (Figure 1A). The PMA-induced increment of ouabain binding is blocked by KCN (Figure 2), which is known to block the recruitment of glucose transporters to the plasma membrane (*26*, *34*). PMA also promoted the interaction of $Na^+$,$K^+$-ATPase with AP-1 (Figure 1B) 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 (*32*).

Treatment of the cells with colchicine, KCN, or DNP before incubation with PMA blocked the activation of $Na^+$,$K^+$-ATPase (Figure 2), but it did not affect the PMA-dependent phosphorylation of the $Na^+$,$K^+$-ATPase α-subunit. Therefore, phosphorylation is not enough to produce the activation of $Na^+$,$K^+$-ATPase. Instead, phosphorylation may be the triggering mechanism for producing the translocation of $Na^+$,$K^+$-ATPase molecules from intracellular compartments to the plasma membrane. This would explain previous reports that PKC-dependent phosphorylation of isolated rodent $Na^+$,$K^+$-ATPase activity is not associated with changes in $Na^+$,$K^+$-ATPase activity (*13*, *35*). It is important to take into account the fact that phosphorylation of $Na^+$,$K^+$-ATPase is not affected by KCN, DNP, and colchicine which indicates that the activities of the PKC and other enzymes that participate in the phosphorylation process were not affected by the treatment of the cells with those reagents. The fact that basal $Na^+$,$K^+$-ATPase activity was not affected by these treatments, and that DNP or KCN did not produce any effect on the stimulated $Na^+$,$K^+$-ATPase activity if applied after PMA (Figure 2), suggests that (i) these reagents did not influence the pool of $Na^+$,$K^+$-ATPase molecules

already present in the plasma membrane and (ii) the concentration of ATP was not rate-limiting during the length of the experiments. The details of the mechanism that underlies the actions of DNP and KCN were not the focus of our research. However, independent of the mechanism by which DNP and KCN may act, the results presented here indicate that these reagents interfere with the recruitment of pump molecules to the plasma membrane.

Recruitment of $Na^+,K^+$-ATPase molecules to the plasmalemma is likely dependent on the integrity of the cellular actin cytoskeleton and the microtubule system (*30*, *31*). Colchicine has been shown to produce depolimeryzation of the microtubule network and halt hormone-dependent recruitment of transport proteins to the plasma membrane, including the $Na^+,K^+$-ATPase (*28*−*31*, *37*, *38*). Taken together, the results of the experiments with KCN, DNP, colchicine, biotin, and ouabain binding suggest that PMA-dependent activation of $Na^+,K^+$-ATPase is mediated by translocation of pump molecules from intracellular compartments to the plasma membrane. Similar recruitment of $Na^+,K^+$-ATPase molecules to the plasma membrane has been described in response to insulin in muscle cells (*36*), cAMP in rat proximal tubule (*39*), and isoproterenol in lung epithelia (*30*, *31*). Insulin also regulates the plasma membrane pool of insulin-like growth factor II receptor (*34*) and Glut4 (*26*, *27*), and both translocations are inhibited by KCN (*27*, *40*). The conclusion that PMA treatment leads to an increase in the level of plasma membrane $Na^+,K^+$-ATPase molecules does not preclude the possibility of direct effects on the affinity of the pump for intracellular $Na^+$ (*41*).

Our results suggest that simultaneous phosphorylation of both Ser11 and Ser18 in the α-subunit is essential for PMA-dependent stimulation of $Na^+,K^+$-ATPase activity (Figure 3A). Determinations of the levels of $Rb^+$-transport and phosphorylation with S11A and S18A mutants suggest that PMA-dependent stimulation of $Rb^+$-transport is exclusively dependent on PKC-mediated phosphorylation of Ser11 and Ser18. The fact that the presence of both serines is essential and that they are phosphorylated by the PMA treatment suggests that phosphorylation is indeed involved in the mechanism of PMA-dependent activation of $Na^+,K^+$-ATPase. We have also shown that PKC-*β* appears to be the isozyme that directly phosphorylates Ser11 and Ser18 of the $Na^+,K^+$-ATPase α-subunit (Figure 3C). Recently, it was demonstrated that insulin-dependent stimulation of proximal tubule $Na^+,K^+$-ATPase activity is mediated by phosphorylation of the α-subunit Tyr5 (*42*). The results presented here indicate that neither phosphorylation of this residue nor phosphorylation of any residue of the α-subunit other than Ser11 or Ser18 is involved in the PMA-dependent activation of $Na^+,K^+$-ATPase.

There is an apparent lack of a correlation between the level of phosphorylation of the α-subunit and the activation of $Na^+,K^+$-ATPase produced by PMA treatment. Our results are consistent with the model wherein PMA produces phosphorylation of $Na^+,K^+$-ATPase molecules that are in the intracellular compartment, and these are translocated to the plasma membrane. Since activity is measured only 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, and the number of $Na^+,K^+$-ATPase molecules that are translocated from the intracellular compartment to the plasma membrane.

Dopamine treatment of proximal tubule cells results in inhibition of $Na^+,K^+$-ATPase activity, and this effect is abolished by removing the α-subunit $NH_2$-terminal segment (*17*). Dopamine also acts through PKC, and only phosphorylation of Ser18 appears to be essential (*16*). Unlike the results with PMA, however, the integrity of Ser11 or its phosphorylation is not required for the effect of dopamine. This is a clear difference between the mechanisms involved in PMA activation and dopamine inhibition of $Na^+,K^+$-ATPase. Also, we have determined that different PKC isoforms are involved. While the stimulatory effect of PMA is blocked specifically by inhibition of PKC-*β*, this does not affect the dopamine-dependent inhibition of $Na^+,K^+$-ATPase, which is mediated by PKC-*ζ* (*33*). Inhibition of $Na^+,K^+$-ATPase activity in response to dopamine occurs by removal of pumps from the plasma membrane (endocytosis), and phosphorylation at Ser18 of the α-subunit may trigger this process (*16*). In contrast, we have shown in this report that activation of $Na^+,K^+$-ATPase in response to PMA occurs by recruitment of pumps to the plasma membrane, and that phosphorylation of Ser11 and Ser18 may trigger the recruitment, but phosphorylation by itself does not produce any change in the intrinsic activity of the $Na^+,K^+$-ATPase. Several laboratories have reported either activation or inhibition of the $Na^+,K^+$-ATPase upon stimulation of PKC in various tissues and species (*15*). Our results from experiments with dopamine and PMA would suggest that this apparent contradiction is the product of distinct mechanisms in which different serine residues are phosphorylated and different PKC isoforms are involved. However, it should be stressed that the mechanism of hormonal regulation of the $Na^+,K^+$-ATPase may be very different in different cells and tissues.

The renal $Na^+,K^+$-ATPase can be either activated or inhibited by hormones (*7*). Dopamine inhibits the $Na^+,K^+$-ATPase, and several aspects of the mechanism that is involved in this process have been reported (*16*, *17*). In this report, we present for the first time evidence of some aspects of the mechanism involved in the hormonal activation of the renal $Na^+,K^+$-ATPase. Treatment of OK cells with PMA offers a convenient experimental model for pump regulation in the proximal tubule. PMA treatment may activate a signaling pathway that stimulates $Na^+,K^+$-ATPase and that it is normally utilized by hormones that increase the level of proximal tubule sodium reabsorption (angiotensin II or noradrenaline; *7*, *43*, *44*). Our results would be consistent with the following model; 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. In contrast, PMA stimulates a pathway that results in activation of $Na^+,K^+$-ATPase by translocation of pump molecules to the plasma membrane from intracellular compartments. This hypothesis offers 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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