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# Regulation of Na,K-ATPase Transport Activity by Protein Kinase C

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**Abstract.** Considerable evidence indicates that the renal $Na^+,K^+$-ATPase is regulated through phosphorylation/dephosphorylation reactions by kinases and phosphatases stimulated by hormones and second messengers. Recently, it has been reported that amino acids close to the $NH_2$-terminal end of the $Na^+,K^+$-ATPase α-subunit are phosphorylated by protein kinase C (PKC) without apparent effect of this phosphorylation on $Na^+,K^+$-ATPase activity. To determine whether the α-subunit $NH_2$-terminus is involved in the regulation of $Na^+,K^+$-ATPase activity by PKC, we have expressed the wild-type rodent $Na^+,K^+$-ATPase α-subunit and a mutant of this protein that lacks the first thirty-one amino acids at the $NH_2$-terminal end in opossum kidney (OK) cells. Transfected cells expressed the ouabain-resistant phenotype characteristic of rodent kidney cells. The presence of the α-subunit $NH_2$-terminal segment was not necessary to express the maximal $Na^+,K^+$-ATPase activity in cell membranes, and the sensitivity to ouabain and level of ouabain-sensitive $Rb^+$-transport in intact cells were the same in cells transfected with the wild-type rodent α1 and the $NH_2$-deletion mutant cDNAs. Activation of PKC by phorbol 12-myristate 13-acetate increased the $Na^+,K^+$-ATPase mediated $Rb^+$-uptake and reduced the intracellular $Na^+$ concentration of cells transfected with wild-type α1 cDNA. In contrast, these effects were not observed in cells expressing the $NH_2$-deletion mutant of the α-subunit. Treatment with phorbol ester appears to affect specifically the $Na^+,K^+$-ATPase activity and no evidence was observed that other proteins involved in $Na^+$-transport were affected. These results indicate that amino acid(s) located at the α-subunit $NH_2$-terminus participate in the regulation of the $Na^+,K^+$-ATPase activity by PKC.

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**Key words:** $Na^+,K^+$-ATPase — $Na^+$-pump — $Na^+$-transport

## Introduction

The $Na^+,K^+$-ATPase transports $Na^+$ and $K^+$ ions across the plasma membrane of most eukaryotic cells and plays a key role in cellular ionic homeostasis (Glynn & Karlish, 1975; Kaplan, 1983; Jorgensen & Andersen, 1988). In renal epithelial cells, which are responsible for urinary $Na^+$ reabsorption and extracellular fluid volume homeostasis (Simmons & Fuller, 1985; Garty & Benos, 1988), the $Na^+,K^+$-ATPase provides the driving force for vectorial $Na^+$ transport from the lumen of the tubule to the blood supply (Soltoff & Mandel, 1984; Katz, 1988; Stanton & Kaissling, 1989). The minimum functional $Na^+,K^+$-ATPase molecule is a heterodimer composed of an α-subunit (Mr ≈ 100 KD) and a heavily glycosylated β-subunit (Mr ≈ 50 KD) (Pedemonte & Kaplan, 1990).

In recent years, an increasing number of publications have suggested the short-term regulation of kidney $Na^+,K^+$-ATPase by hormones and intracellular second messengers (Bertorello et al., 1991; Aperia et al., 1992; Bertorello & Katz, 1993; Middleton et al., 1993; Aperia et al., 1994; Fisone et al., 1994). Several reports indicate that renal $Na^+,K^+$-ATPase activity may be regulated by phosphorylation/dephosphorylation processes (Bertorello & Katz, 1993). Both cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) appear to phosphorylate the $Na^+,K^+$-ATPase (Bertorello et al., 1991; Middleton et al., 1993; Fisone et al., 1994; Lowndes et al., 1990; Chilbalin et al., 1992). Depending on the tissue studied and the experimental conditions, stimulation of PKC leads to inhibition (Vasilets et al., 1990; Bertorello et al., 1991; Middleton et al., 1993; Satoh, Cohen & Katz, 1993) or activation (Schuster, Kokko & Jacobson, 1984; Lynch et al., 1986; Hootman,

Brown & Williams, 1987; Beach et al., 1987; Wang & Chan, 1990; Liu & Cogan, 1990; Middleton et al., 1993) of $Na^+,K^+$-ATPase activity.

Working independently, both Beguin et al. (1994) and Feschenko and Sweadner (1995) have reported that amino acids close to the $NH_2$-terminal end of the $Na^+,K^+$-ATPase $\alpha$1-subunit are phosphorylated by PKC. This result would suggest that the $\alpha$1-subunit $NH_2$-terminus is involved in regulation of the $Na^+,K^+$-ATPase activity. However, the fact that Feschenko and Sweadner (1994) did not observe any effect of phosphorylation on $Na^+,K^+$-ATPase activity raises the possibility that phosphorylation of the $NH_2$-terminus by PKC has no physiological significance. In this report, we show that stimulation of PKC by phorbol esters leads to activation of ouabain-sensitive $^{86}Rb^+$-transport and reduced intracellular $Na^+$ concentration ($[Na^+]_i$) of OK cells. The fact that these effects were not observed in cells expressing a $NaH_2$-deletion mutant of the $\alpha$-subunit supports the hypothesis that the $\alpha$-subunit $NH_2$-terminus plays a role in the regulation of $Na^+,K^+$-ATPase activity.

## Materials and Methods

### Materials

Cell culture supplies were purchased from Gibco and Hyclone. Molecular biology reagents were from New England Biolabs, Dupont, Promega and USB. Ouabain was purchased from Calbiochem. Phorbol esters, phorbol 12-myristate 13-acetate (PMA) and 4$\alpha$-phorbol 12,13-didecanoate (4$\alpha$-PDD) were obtained from Sigma Chemical. Other reagents were of the highest quality available.

### Cell Culture and Transfection

The expression vector pCMV containing the rodent $Na^+,K^+$-ATPase $\alpha$1-subunit cDNA was obtained from Pharmingen. The preparation of the expression vector (myc/1.32) that encodes a shortened mutant of the $\alpha$1-subunit was described by Shanbaky and Pressley (1994). This vector expresses a rodent $\alpha$-subunit in which the first 31 amino acids of the nascent polypeptide are replaced by an initiation methionine and a sequence of 10 amino acids (EQKLISEEDL) from the human c-myc oncogene product.

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). Plasmids containing the wild-type and mutant $\alpha$-subunits were transfected into OK cells using liposomes. Cationic liposomes were prepared by sonication with 1 mg dioleoyl-L-$\alpha$-phosphatidylethanol-amine and 0.4 mg dimethyl-dioctadecyl-ammonium bromide as indicated by Rose et al. (1991). The day before transfection, OK cells were seeded in the wells of a 96-well plate (3500 cells/well). The following day, the cells were transfected in 50 $\mu$l of Opti-MEM I containing 3 $\mu$g/ml of total DNA and 15 $\mu$l/ml of liposomes. The $Na^+,K^+$-ATPase of mock-transfected cells (vector alone, or vector plus liposomes, or liposomes alone) had the same activity and sensitivity to ouabain as non-treated host cells. Five hours after transfection, 200 $\mu$l/well of DMEM-10 was added. Two days later, cells were transferred to a medium containing 1 $\mu$M ouabain. Since the endogenous $Na^+,K^+$-ATPase of OK cells is sensitive to this level of ouabain, only OK cells that express the $Na^+,K^+$-ATPase containing the rodent $\alpha$-subunit would be able to survive. After 10 days, cells from the wells that have single colonies were transferred to a medium containing 10 $\mu$M ouabain to select for those cells expressing the highest level of rodent $\alpha$-subunit. Resistant colonies were expanded and maintained in DMEM-10 containing 10 $\mu$M ouabain.

### Preparation of Crude Plasma Membranes from OK Cells

OK cells were harvested by mild trypsinization and suspended in lysis buffer (10 mM imidazole, 1 mM EDTA, pH 7.5). The cells were probe-sonicated twice for 15 sec with a 15-sec interval in an Ultrasonic homogenizer 4710 (Cole Parmer) at 25 watts and 80% power output. Samples were maintained in an ice-water bath during sonication. The suspension was centrifuged for 4 min. at 1,500 × *g*. The resulting supernatant was collected and centrifuged at 513,000 × *g* for 15 min at 2°C (Beckman Optima TLX ultracentrifuge). The pellet was resuspended with a small volume of lysis buffer and used to determine protein and $Na^+,K^+$-ATPase activity. Protein was determined by the bicinchoninic acid method (Pierce Chemical) using BSA as standard.

### Determination of $Na^+,K^+$-ATPase

Protein aliquots (2 mg/ml) were treated with 0.7 mg/ml SDS in the presence of 3 mM ATP and 10 mM imidazole, 0.4 mM EDTA, pH 7.5 for 10 min at room temperature. Then, protein samples were put in an ice-water bath and BSA was added to a final concentration of 0.4 mg/ml. The SDS treatment was determined to be optimal for exposing latent $Na^+,K^+$-ATPase activity (Pedemonte, 1995). The $Na^+,K^+$-ATPase assay medium contained 0.05 mg/ml membrane protein; 0.3 mg/ml BSA and (mM): EGTA, 0.5; NaCl, 130; KCl, 20; $MgCl_2$, 4; ATP, 3; imidazole, 50; pH 7.3. Enzymatic activity was determined as previously described (Pedemonte & Kaplan, 1986) at 37°C for 30 min from the difference between the ATP hydrolysis measured in the absence and presence of ouabain. Concentrations of ouabain in the original dilutions were determined from the absorption at 221 nm by using a value of $15.5 \times 10^3$ for the molar extinction coefficient. Experiments were carried out in duplicate and repeated at least five times. Results are the average of at least five experiments ± SEM.

### Determination of $Rb^+$-Transport

The experiments were performed with cells seeded at about 60% confluence in 24-well plates. To facilitate access of introduced ligands to the $Na^+,K^+$-ATPase, cells grown on plastic were exposed to culture medium containing EGTA prior to the measurement of $Rb^+$-uptake (Contreras et al., 1989). No cell detachment from the plastic was observed during the 30-min incubation. EGTA was not present during treatment with phorbol esters and $Rb^+$-transport assay. However, we have determined that the stimulation of $Rb^+$-transport by phorbol 12-myristate 13-acetate (PMA) was not affected by the presence of EGTA (*data not shown*).

To measure $Rb^+$-transport, transfected cells ($1 \times 10^5$ cells/well of a 24-well plate) were transferred to serum-free DMEM containing 50 mM Hepes (DMEM-Hepes), 2 mM EGTA and 10 $\mu$M or 10 mM ouabain (incubation medium). Cells were incubated for 20 min at 37°C in an air atmosphere and 10 min at room temperature before addition of 1 $\mu$M phorbol ester. Five min later, a trace amount of [$^{86}Rb^+$]-RbCl was added. $Rb^+$-uptake was terminated after 20 min by washing the cells

four times with ice-cold saline. Cells were dissolved with SDS, and accumulated radioactivity was determined. $Na^+,K^+$-ATPase mediated $Rb^+$-transport was estimated from the difference in tracer uptake between samples incubated in 10 μM and 10 mM ouabain. For nontransfected OK cells, $Rb^+$-transport was measured in the absence and presence of 10 mM ouabain.

Since phorbol esters were 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 (*data not shown*). Each experiment was repeated at least four times.

### Determination of Intracellular $Na^+$ Concentration

Fluorescence measurements of $[Na^+]_i$ were performed using the membrane-permeant tetra(acetoxy-methyl) ester of the $Na^+$-binding dye benzofuran-isophthalate-acetoxymethylester (SBFI-AM, Molecular Probe) following standard protocols (Harootunian et al., 1989; Moore & Fay, 1993). Cells were loaded for 2 hr. with the dye at room temperature in a serum-free HEPES medium (DMEM-50 mM Hepes; pH 7.4) containing 2–5 μM SBFI-AM and 0.1% wt/vol Pluronic F-127 (Molecular Probe, Eugene, OR). After loading, the cells were washed several times in DMEM-50 mM Hepes and incubated 30 min in the same medium to allow de-esterification of SBFI-AM. The complete hydrolysis of SBFI-AM to SBFI was judged by changes in the excitation and emission spectra (Harootunian et al., 1989). Optical measurements were performed in serum-free HEPES buffered medium.

The optical setup consists of an upright epi-illumination microscope (Nikon Eiphot) with a video camera (MV-1070; Marshal Elect. CA) in the photographic port. $[Na^+]_i$ was calculated according to the equation described by Grynkiewicz et al. (1985) with a Kd of SBFI for $Na^+$ of 18 mM (Minta & Tsien, 1989). Other terms of the equation were assessed by *in situ* calibration. No significant level of autofluorescence was observed in the cells, and the concentration of reagents added to the cell medium did not affect the fluorescence levels as judged by determinations performed at an excitation wavelength corresponding to the isosbestic point of SBFI (370 ± 2.5 nm; Omega Optical, Brattleboro, VT). In the pictures, $[Na^+]_i$ changes are illustrated by pseudocolors resulting from subtracting the basal level of $[Na^+]_i$ from those obtained after addition of PMA. The basal $[Na^+]_i$ was the same in cells transfected with the wild-type and mutant α1 cDNA.

At the end of each experiment, *in situ* calibration of the excitation ratio of SBFI was performed to accurately assess $[Na^+]_i$. After permeabilization with gramicidin D (10 μM), cells were superfused with different $Na^+$ concentrations. Calibration curves of $[Na^+]_i$ were the same for cells transfected with both plasmids.

### Statistics

Comparisons between groups were performed by Student's *t*-test for unpaired data.

### Abbreviations

$Na^+,K^+$-ATPase and $Rb^+$-transport refers to the same protein activity; PKC, protein kinase C; OK, opossum kidney; PMA, phorbol 12-myristate 13-acetate; 4α-PDD, 4α-phorbol 12,13-didecanoate

## Results

### Expression of the $NH_2$-Deletion Mutant $Na^+,K^+$-ATPase in OK Cells

Many of the studies examining the regulation of the $Na^+,K^+$-ATPase by hormones and second messengers have been performed in isolated rat proximal tubule segments or dissociated rat proximal tubule cells (Bertorello & Katz, 1993). The OK cells used in our experiments are an established epithelial cell line which is often studied as a physiological model system of renal proximal tubule function (Malstrom, Stange & Murer, 1987; Nash, Godinot & Caron, 1993). $Rb^+$ was used as a $K^+$ congener to determine the transport activity of the $Na^+,K^+$-ATPase. We found that both the ouabain-sensitive and ouabain-insensitive $Rb^+$-uptake were linear with respect to time for at least 30 min. (Fig. 1). Although this experiment was done with wild-type OK cells, similar results were obtained with the transfected cells described below.

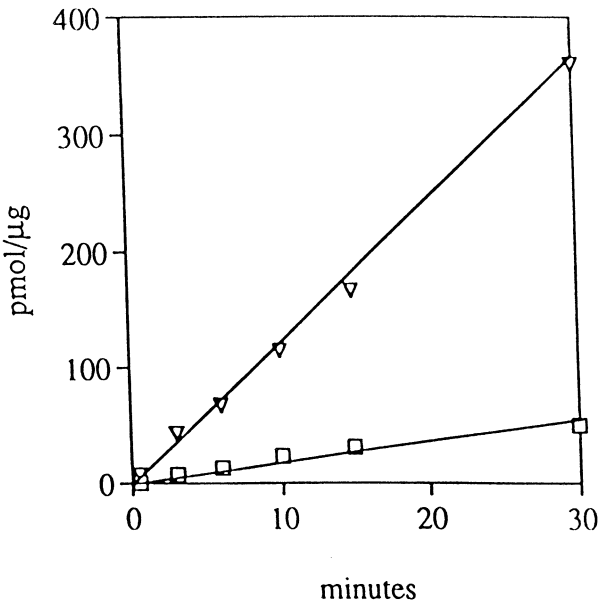


**Fig. 1.** Incorporation of $Rb^+$ into OK cells incubated at room temperature in the conditions described in Materials and Methods. $Rb^+$-transport was measured in the presence and absence of 10 mM ouabain. Ouabain-sensitive (▽) and ouabain-insensitive (□) transports are represented. Data presented are the average of two experiments carried out in triplicate.

To investigate the functional role of the α-subunit $NH_2$-terminus in regulation of enzymatic activity by PKC, we transfected OK cells with wild-type rodent kidney α1 cDNA or a mutant cDNA that encodes a shortened form of the α-subunit. Expression of rodent α1 cDNA would be expected to confer resistance to 10 μM ouabain, which would be otherwise lethal for nontransfected cells. As previously shown in COS-1 cells (Shanbaky & Pressley, 1994), OK cells expressing the wild-type rodent α1 or the $NH_2$-deletion mutant were resistant to 10 μM ouabain (Fig. 2). There was no difference in the growth rate between cells transfected with wild type α1 cDNA and the $NH_2$-deletion mutant α1 cDNA.

OK cells express an endogenous α-subunit with a high affinity for ouabain (Fig. 2). In contrast, cells ex-

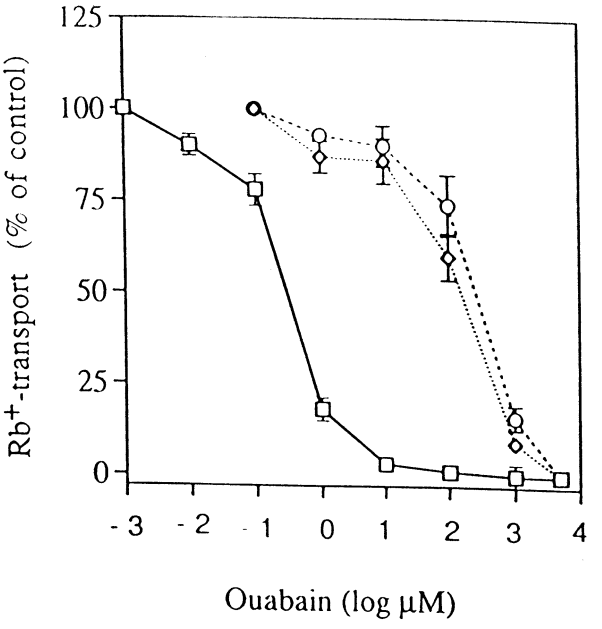


**Fig. 2.** Dependence of ouabain-sensitive $Rb^+$-transport on ouabain concentration. Data are presented as percentage of remaining activity with respect to noninhibited $Na^+,K^+$-ATPase. The $Rb^+$-transport of wild-type host OK cells (□), cells transfected with wild-type rodent α1 cDNA (○) and cells transfected with the $NH_2$-deletion mutant α1 cDNA (◇) was determined as indicated in Materials and Methods. Plotted are means ± SE ($n$ = 4).

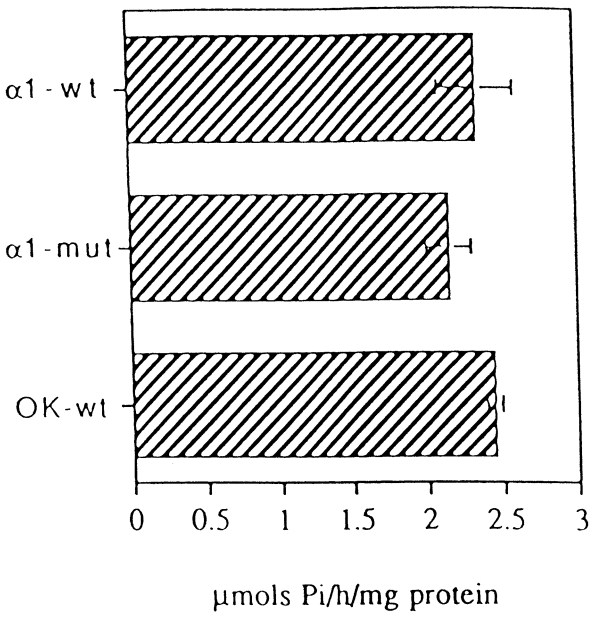


**Fig. 3.** $Na^+,K^+$-ATPase activity in nontransfected OK cells (OK-wt) and in cells transfected with the wild-type α1 cDNA (α1-wt) and the $NH_2$-deletion mutant α1 cDNA (α1-mut). Enzymatic activity was determined at saturating concentrations of all the enzyme ligands as indicated in Materials and Methods.

pressing wild-type or mutant rodent α1 were more resistant to the glycoside, consistent with the expression of a ouabain-resistant exogenous form (Price & Lingrel, 1988). There was no significant difference in the ouabain sensitivity between cells transfected with the α1 cDNA and the $NH_2$-deletion mutant α1 cDNA.

The same level of $Na^+,K^+$-ATPase activity was determined in transfected and nontransfected cell membranes (Fig. 3). Similarities in α-subunit abundance have been demonstrated by Shanbaky and Pressley (1994) in host and transfected COS-1 cells. These observations suggest that $Na^+,K^+$-ATPases containing the endogenous α-subunits have been replaced by $Na^+,K^+$-ATPases containing the ouabain-resistant wild-type or mutant rodent α1.

### Effect of PKC Activation on $Rb^+$-Transport

We next evaluated the regulation of the endogenous and exogenous $Na^+,K^+$-ATPase by PKC. Cells were transferred to serum-free DMEM-Hepes medium containing different amounts of ouabain, [$^{86}Rb^+$]-RbCl, and 1 μM phorbol 12-myristate 13-acetate (PMA). Note that changes in the medium were held to a minimum to minimize any alterations in cellular homeostasis. The ouabain-sensitive $Rb^+$-transport in nontransfected host OK cells was 11.6 ± 0.7 nmol/mg/min, which is almost equal to that measured by Middleton et al. (1993) in the same cells (49.8 ± 4.7 nmol/mg/4 min). Treatment of host cells with PMA increased $Rb^+$-transport by 24 ± 3% (Fig. 4).

Cells transfected with the rodent α1 cDNA displayed a $Rb^+$-transport of 9.5 ± 1.4 nmol/mg/min. This activity was measured in cells that were maintained in the presence of 10 μM ouabain during growth and assay. Under these conditions, endogenous $Na^+,K^+$-ATPase activity should have been negligible, and any ouabain-sensitive $Rb^+$-transport measured should have been attributable to $Na^+,K^+$-ATPase containing the introduced rodent α-subunit. The $Rb^+$-uptake of cells transfected with the rodent α1 cDNA (α1-wt) was increased 58 ± 5% by treatment with PMA (Fig. 4). On the contrary, 1 μM 4α-phorbol 12,13-didecanoate (4α-PDD), a phorbol ester that does not stimulate PKC, did not change the level of ouabain-sensitive $Rb^+$-transport. This suggests that the activation induced by PMA was specific and mediated by PKC.

Beguin et al. (1994) and Feschenko and Sweadner (1995) determined that amino acids close to the $NH_2$-terminus of the $Na^+,K^+$-ATPase α1-subunit are targets for PKC phosphorylation. It follows that the activation of $Rb^+$-transport we observed may have been produced by phosphorylation within this region. To test whether the α-subunit $NH_2$-terminus plays any role in the modulation of $Rb^+$-transport, the experiments described above

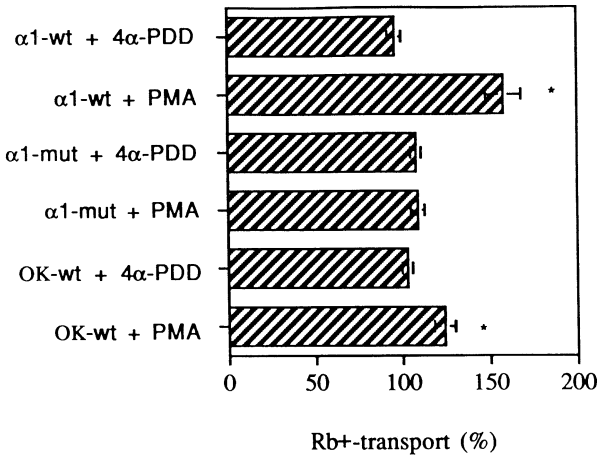


**Fig. 4.** Effect of phorbol ester treatment on $Rb^+$-transport mediated by the $Na^+,K^+$-ATPase of nontransfected OK cells (OK-wt), and cells transfected with the wild-type rodent α1 cDNA (α1-wt) and the $NH_2$-deletion mutant α1 cDNA (α1-mut). Data are presented as percentage of activity with respect to nontreated control. Treatments were performed as indicated in Materials and Methods and in the text. $*P < 0.001$.

were repeated using cells transfected with the $NH_2$-deletion mutant α1 cDNA (α1-mut). These cells displayed a $Rb^+$-transport of 11.2 ± 1.9 nmol/mg/min, comparable to the level of transport measured in both nontransfected and wild-type rodent α1-expressing cells. Thus, cells expressing the α-subunit $NH_2$-terminus deletion mutant have the same level of maximal $Na^+,K^+$-ATPase, ouabain-sensitive $Rb^+$-transport, and similar concentration dependency to ouabain as cells transfected with the wild-type rodent α1 cDNA. However, in sharp contrast to the endogenous and introduced wild-type enzymes, the activity of the mutant was not significantly modified by treatment with PMA (Fig. 4). The same result was observed with 4α-PDD, the phorbol ester that does not stimulate PKC (Fig. 4). Thus, an intact α-subunit $NH_2$-terminus was essential for activation of the $Na^+,K^+$-ATPase by PKC.

### *PMA Treatment Reduces the* $[Na^+]_i$

An increased $Na^+,K^+$-ATPase activity should result in reduced $[Na^+]_i$. To test this possibility, the $[Na^+]_i$ was determined *in situ* by changes in fluorescence produced by the $Na^+$ indicator SBFI (Minta & Tsien, 1989). Transfected cells were loaded with the membrane permeant derivative of SBFI (SBFI/AM) and the level of emitted fluorescence upon excitation at 340 and 385 nm was monitored using a video imaging system, as previously described (Cinelli et al., 1995). PMA treatment of wild-type rodent α1 transfected cells produced a reduction in $[Na^+]_i$ (Fig. 5). Images of SBFI/AM-loaded cells (displayed in pseudocolor) obtained before and 2 seconds after addition of PMA illustrates the rapid change in $[Na^+]_i$. *In situ* calibration of the excitation ratio of SBFI at various $[Na^+]_i$ indicated that PMA treatment reduced $[Na^+]_i$ from 19.7 ± 2.4 ($n = 42$) to 5.6 ± 0.6 ($n = 18$) mM. Washing out the PMA from the cell medium restored the initial steady-state level of $[Na^+]_i$, and phorbol esters that do not stimulate PKC had no effect on the $[Na^+]_i$.

As previously observed with $Rb^+$-uptake, the $[Na^+]_i$ of cells expressing the $NH_2$-deletion mutant α1 (17.2 ± 2.5 mM) was not affected by PMA (Fig. 5, bottom). This lack of response was not due to the cells being dead or damaged since a 15 mM reduction of $[Na^+]_i$ was observed when the cells were treated with 1 mM 8-Br-cAMP, which stimulates protein kinase A, or 8 μM 5-(N-methyl-N-isobutyl)-amiloride, which inhibits the $Na^+/H^+$-exchanger. These reagents had the same effect on cells transfected with the wild-type α1 cDNA. An increase in $[Na^+]_i$ was observed when $K^+$ was removed from the cell medium and following the application of ouabain (100 μM) in cells transfected with both plasmids (*data not shown*).

## Discussion

In this study, we have demonstrated that the $Na^+,K^+$-ATPase mediated $Rb^+$-transport is stimulated by activation of PKC and that the presence of the α1-subunit $NH_2$-terminus is essential to observe the modulation of activity. It is important to notice that the lack of response to PMA observed in cells transfected with the α-subunit $NH_2$-deletion mutant was not due to a lowered ouabain-sensitive $Rb^+$-transport activity. These cells have the same level of $Rb^+$-transport, measured under *in vivo* conditions (intact cells), and maximal $Na^+,K^+$-ATPase, measured at saturating concentration of the ligands, as cells transfected with the wild-type rodent α1 cDNA. This indicates that the total number of active $Na^+,K^+$-ATPase molecules is the same in both cell lines. In cells transfected with either the wild-type or mutant α-subunit cDNAs, the $Na^+,K^+$-ATPase activity was about 2.5 μmoles Pi/mg/h. This corresponds to 42 nmoles Pi/mg/min. In cells transfected with either the wild-type or mutant α-subunit cDNAs, the $Rb^+$ transport was about 10 nmoles/mg/min. Since the $Na^+,K^+$-ATPase transports 2 $Rb^+$ ions per ATP that is hydrolyzed, this level of $Rb^+$-transport corresponds to an ATPase activity of 5 nmoles Pi/mg/min. Thus, the maximal ATPase activity was about 8 times higher than the ATPase activity associated with the $Rb^+$-transport. Therefore, the $Na^+,K^+$-ATPase of intact cells transfected with the $NH_2$-deletion mutant cDNA was working at about 8 times lower velocity than Vmax and the lack of effect of phorbol ester treatment was not due to the pump working at Vmax.

Even though it has been shown that elimination of

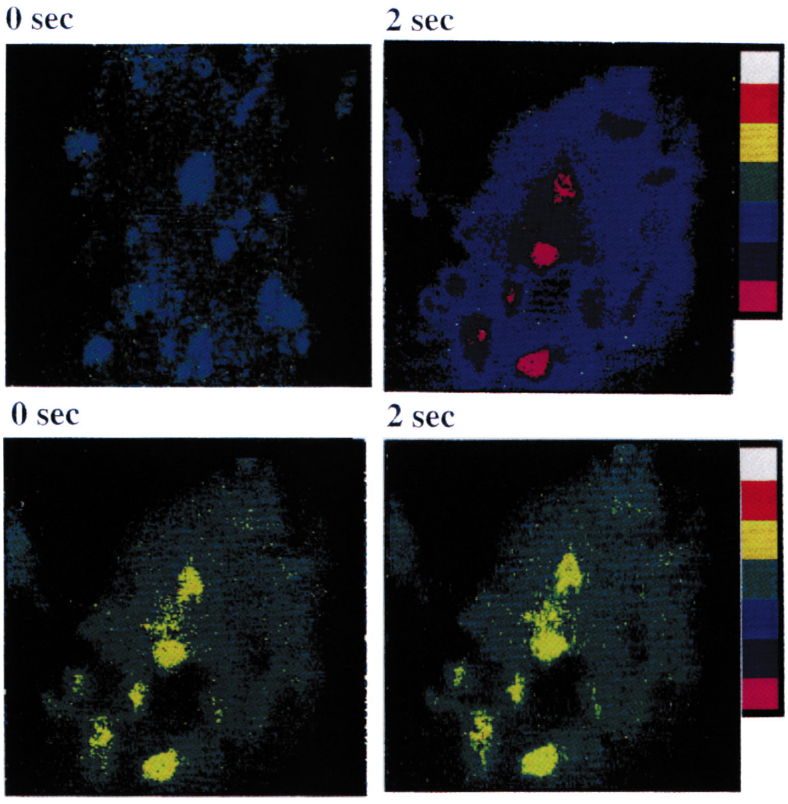


**Fig. 5.** *Top:* PMA-dependent reduction of $[Na^+]_i$ revealed by fluorescence microscopy from cells transfected with the wild-type α1 cDNA. Images correspond to 0 and 2 sec after the addition of 10 μM PMA. Pseudocolor calibration: dark pink, 0–5 mM; dark blue, 5–10 mM; light blue, 10–15 mM; green, 15–20 mM; yellow, 20–25 mM; red, 25–30 mM; white, above 30 mM. *Bottom:* Images from cells transfected with the $NH_2$-deletion mutant of the α1 cDNA before and 2 sec after the addition of 10 μM PMA. The same result was observed after 60 sec.

the α-subunit $NH_2$-terminus may produce a change in the equilibrium $E_1/E_2$ of the protein conformations (Jorgensen & Karlish, 1980) or affect the interaction between the cations and the enzyme (Wierzbicki & Blostein, 1993), these experiments were performed with purified protein or with enzyme ligand concentrations that were very far from an in vivo condition. It is therefore unclear if these findings can be extrapolated to the enzyme when operating in an intact cell. Our experiments, which were performed in an in vivo condition, suggest that the basic functioning of the $Na^+,K^+$-ATPase activity appears not to be altered by the elimination of the α-subunit $NH_2$-terminus. Therefore, this region may have no effect on the basal $Na^+,K^+$-ATPase activity but on the regulation of this activity. This paper is the first report showing in an in vivo condition that the $NH_2$-terminal end of the α-subunit is involved in the regulation of the $Na^+,K^+$-ATPase activity by PKC.

The same hormones that regulate $Na^+,K^+$-ATPase in proximal tubules (Bertorello & Katz, 1993; Aperia et al., 1994) appear to regulate the activity of the $Na^+/H^+$-exchanger which mediates $Na^+$ entry into epithelial cells (Mahninsmith & Aronson, 1985; Grinstein & Rothstein, 1986; Nord et al., 1987; Gesek & Schoolwerth, 1991). Therefore, it can be argued that the observed increase of $Rb^+$ uptake might be due not to regulatory modulation of the $Na^+,K^+$-ATPase, but to an increased $[Na^+]_i$ produced by PKC mediated stimulation of the $Na^+/H^+$-exchanger.

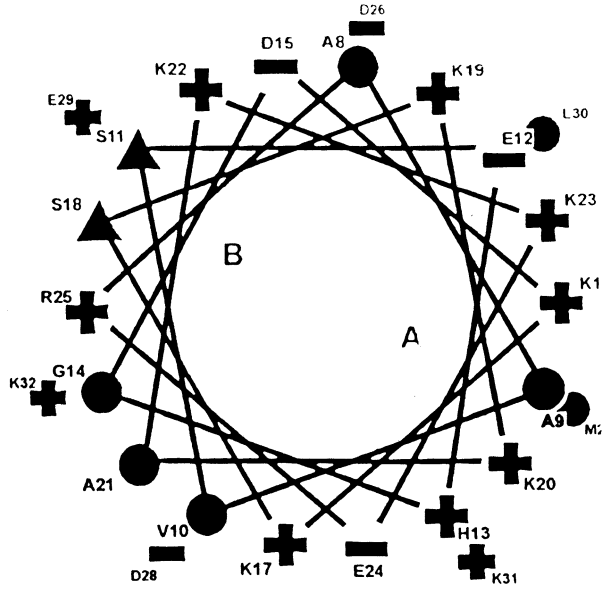


**Fig. 6.** Representation of the rodent α1 $NH_2$-terminal segment as an α-helical wheel. Polar (△), aliphatic (○), positively-charged (+) and negatively-charged (—) amino acids are included.

However, if this were true, $Rb^+$ transport would have been similarly affected in cells expressing both the wild-type and the $NH_2$-deletion mutant. Furthermore, we have determined that upon treatment with PMA the

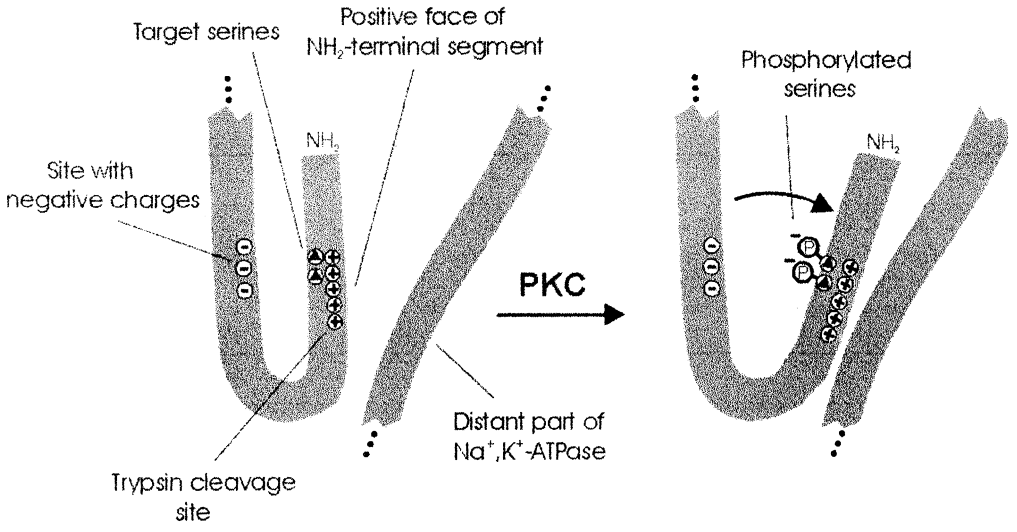


**Fig. 7.** Hypothetical scheme depicting the role of phosphorylation by PKC and the accumulation of positive charges on one face of the α-subunit $NH_2$-terminus.

$[Na^+]_i$ was reduced in cells transfected with the wild-type rodent α1 cDNA and not affected in cells expressing the $NH_2$-terminal deletion mutant α1. Taken together, these results indicate that PKC has specifically stimulated the $Na^+,K^+$-ATPase activity, and there is no evidence suggesting that PKC has affected other proteins mediating $Na^+$ transport. Our results are consistent with the observations that hormones with receptors coupled to stimulation of PKC increase $Na^+$ reabsorption in proximal convoluted tubules (Schuster et al., 1984; Beach et al., 1987; Wang & Chan, 1990; Liu & Cogan, 1990). Stimulation of $Na^+,K^+$-ATPase activity with phorbol esters has been observed in rat proximal tubule cells (Ferraile et al., 1995), rat hepatocytes (Lynch et al., 1986), human lymphocytes (Norby & Obel, 1993), pancreatic acinar cells (Hootman et al., 1987) and peripheral nerve from diabetic rabbits (Lattimer, Sima & Greene, 1989).

Previous reports have identified Ser-11 and Ser-18 of the $NH_2$-terminal end of the $Na^+,K^+$-ATPase α1-subunit as targets for phosphorylation by PKC (Beguin et al., 1994; Feschenko & Sweadner, 1995). Since Feschenko and Sweadner (1994) did not observe any effect of phosphorylation on $Na^+,K^+$-ATPase activity,[1] the relevance of the α-subunit $NH_2$-terminus in the regulation of $Na^+,K^+$-ATPase by PKC was an open question. Even though our results have not demonstrated explicitly phosphorylation of the α-subunit, such covalent modification seems a likely explanation for the increased $Rb^+$-transport that we observed. However, we cannot rule out the possibility that PKC may phosphorylate other amino acids of the $Na^+,K^+$-ATPase subunits or even another protein. Nevertheless, independent of which amino acids or protein is phosphorylated, our results clearly indicate that amino acids of the α-subunit $NH_2$-terminus are involved in the PKC modulation of the $Na^+,K^+$-ATPase activity.

Recent reports of biochemical and electrophysiological studies have suggested that the $NH_2$-terminal segment may affect the interaction between cations and the enzyme (Wierzbicki & Blostein, 1993; Vasilets et al., 1991, 1993; Horisberger et al., 1993; Daly, Lane & Blostein, 1994). Because it is a region of high sequence diversity among the α-subunits from various species and between the α-isoforms, the $NH_2$-terminus of the α-subunit has generated interest and speculation with respect to its role in $Na^+,K^+$-ATPase function. The most prominent feature of the $NH_2$-terminal segment is a high proportion of charged amino acids. The rodent α1 has 12 positively charged, 8 negatively charged and 3 polar amino acids in the first 32 residues of the mature subunit. Between Ser-11 and Lys-32, all amino acids but two are charged or polar. The amino acid sequence, as well as Raman spectroscopy, suggests that the secondary structure of the $NH_2$-terminal segment corresponds to an α-helix (Lupas, Van Dyke & Stock, 1991; Ovchinnikov et al., 1988).

Figure 6 illustrates amino acids 8–32 in an α-helical representation. A 26-residue region beginning just carboxy to Pro-7 is depicted. Pro-7 was excluded because it is not stable in the helical conformation and the maximal propensity to form α-helix was determined for amino acids 8 to 32 (Lupas et al., 1991). By examination of this putative α-helix, it is possible to distinguish some orga-

[1] The lack of effect may be due to the authors determination of Vmax. It is likely that PKC may affect the affinity for $Na^+$ rather than the Vmax (Feraille et al., 1995).

nization of the charged amino acids with respect to the serines that are the putative target of PKC (Fig. 6). There is a 90° face of the helix that contains only positive charges (sector A), and it is just opposite to the face containing Ser-11 and Ser-18 (sector B). The other two sectors of the helix contain an almost equal number of interdispersed positive and negative charges. This asymmetric distribution of charges may have functional relevance. This is represented schematically in Fig. 7, which may help to visualize a hypothesis that considers the distribution of charges and interprets some of the results previously obtained. This model assumes that serines 11 and 18 are located just in front of a group of negative charges in another arm of the α-subunit. Upon the addition of the bulky, negatively-charged phosphates by PKC, the $NH_2$-terminal segment may move by charge repulsion. Due to this movement, the positively charged face of the $NH_2$-terminal segment would then interact with other parts of the $Na^+$,$K^+$-ATPase molecule and lead to the modulation of enzymatic activity.

In conclusion, we have shown that stimulation of PKC activates $Na^+$,$K^+$-ATPase in a kidney cell line. This activation involves amino acid(s) located in the $NH_2$-terminus of the α-subunit. Even though the presence of the α-subunit $NH_2$-terminus is not required for expression of the basic $Na^+$,$K^+$-ATPase activity, the differential interaction of the $NH_2$-terminus with other intracellular domains of the α-subunit may be a mechanism by which hormones regulate $Na^+$,$K^+$-ATPase activity.

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## References

Aperia, A., Holtbakc, U., Syren, M.-L., Svensson, L.-B., Fryckstedt, J., Greengard, P. 1994. Activation/deactivation of renal Na,K-ATPase: a final common pathway for regulation of natriuresis. *FASEB J.* **8:**436–439

Aperia, A., Isarra, F., Svensson, L.B., Klee, C., Greengard, P. 1992. Calcineurin mediates α-adrenergic stimulation of Na,K-ATPase activity in renal tubule cells. *Proc. Natl. Acad. Sci. USA* **89:**7394–7397

Beach, R.E., Schwab, S.J., Brazy, P.C., Dennis, V.W. 1987.L Norepinephrine increases Na,K-ATPase and solute transport in rabbit proximal tubules. *Am. J. Physiol.* **252:**F215–F220

Beguin, P., Beggah, A.T., Chibalin, A.V., B.-Kairuz, P., Jaisser, F., Mathews, P.M., Rossier, B.C., Coteccia, S., Geering, K. 1994. Phosphorylation of the Na,K-ATPase α-subunit by protein kinase A and C in vitro and in intact cells. *J. Biol. Chem.* **269:**24437–24445

Bertorello, A.M., Aperia, A., Walaas, S.I., Nairn, A.C., Greengard, P. 1991. Phosphorylation of the catalytic subunit of Na,K-ATPase inhibits the activity of the enzyme. *Proc. Natl. Acad. Sci. USA* **88:**11359–11362

Bertorello, A.M., Katz, A.I. 1993. Short-term regulation of renal Na,K-ATPase activity: physiological relevance and cellular mechanisms. *Am. J. Physiol.* **265:**F743–F755

Chibalin, A.V., Vasilets, L.A., Hennekes, H., Pralong, D., Geering, K. 1992. Phosphorylation of Nα-ATPase α-subunit in microsomes and in homogenates of *Xenopus laevis* resulting from the stimulation of protein kinase A and protein kinase C. *J. Biol. Chem.* **267:**22378–22384

Cinelli, A.R., Neff, S.R., Kauer, J.S., Salamander, 1995. Olfactory bulb neuronal activity observed by video-rate, voltage-sensitive dye imaging. I. Characterization of the recording system. *J. Neurophysiol.* **73:**2053–2071

Contreras, R.G., Avila, G., Gutierrez, C., Bolivar, J.J., Gonzalez-Mariscal, L., Darzon, A., Beaty, G., Rodriguez-Boulan, E., Cereijido, M. 1989. Repolarization of Nα-K pumps during establishment of epithelial monolayers. *Am. J. Physiol.* **257:**C896–C905

Daly, S.E., Lane, L.K., Blostein, R. 1994. Functional consequences of amino-terminal diversity of the catalytic subunit of the Na,K-ATPase. *J. Biol. Chem.* **269:**23944–23948

Feraille, E., Carranza, M.-L., Buffin-Meyer, B., Rousselot, M., Doucet, A., Favre, H. 1995. Protein kinase C-dependent stimulation of Na,K-ATPase in rat proximal convoluted tubules. *Am. J. Physiol.* **268:**C1277–C1283

Feschenko, M.S., Sweadner, K.J. 1995. Structural basis for species-specific differences in the phosphorylation of Na,K-ATPase by protein kinase C. *J. Biol. Chem.* **270:**14072–14077

Feschenko, M.S., Sweadner, K.J. 1994. Conformation dependent phosphorylation of Na,K-ATPase by protein kinase A and protein kinase C. *J. Biol. Chem.* **269:**30436–30444

Fisone, G., Cheng, S.X.-H., Nairn, A.C., Czernik, A.H., Hemmings, H.C. Jr., Hoog, J.-O., Bertorello, A.M., Kaiser, R., Bergman, T., Jornvall, H., Aperia, A., Greengard, P. 1994. Identification of the phosphorylation site for cAMP-dependent protein kinase on Na,K-ATPase and effects of site-directed mutagenesis. *J. Biol. Chem.* **269:**9368–9373

Garty, H., Benos, D.J. 1988. Characteristics and regulatory mechanisms of the amiloride-blockage Na channel. *Physiol. Rev.* **68:**309–373

Gesek, F.A., Schoolwerth, A.C. 1991. Hormone responses of proximal Na/H-exchanger in spontaneously hypertensive rats. *Am. J. Physiol.* **258:**F514–F521

Glynn, I.M., Karlish, S.J.D. 1975. The sodium pump. *Annu. Rev. Physiol.* **37:**13–54

Grinstein, S., Rothstein, A. 1986. Mechanisms of regulation of the Na/H-exchanger. *J. Membrane Biol.* **90:**1–12

Grynkiewics, G., Poenie, M., Tsien, R.Y. 1985. A new generation of $Ca^{2+}$ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260:**3440–3450

Harootunian, A.T., Kao, J.P.Y., Eckert, B.K., Tsien, R.Y. 1989. Fluorescence ratio imaging of cytosolic free $Na^+$ in individual fibroblasts and lymphocytes. *J. Biol. Chem.* **264:**19458–19467

Hootman, S.R., Brown, M.E., Williams, J.A. 1987. Phorbol esters and A23187 regulate Na-K-pump activity in pancreatic acinar cells. *Am. J. Physiol.* **252:**G499–G505

Horisberger, J.D., Jaisser, F., Canessa, C., Rossier, B.C. 1993. Na/K-pump mutant with a slow charge translocating step. *In:* The Sodium Pump. E. Bamberg and W. Schoner, Editors. pp. 549–556. Springer, NY

Jorgensen, P.L., Andersen, J.P. 1988. Structural basis for E1–E2 conformational transitions in Na,K-pump and Ca-pump proteins. *J. Membrane Biol.* **103:**95–120

Jorgensen, P.L., Karlish, S.J.D. 1980. Defective conformational response in a selective trypsinized Na,K-ATPase studied with tryptophan fluorescence. *Biochim. Biophys. Acta* **597:**305–317

Kaplan, J.H. 1983. Sodium ions and the sodium pump: transport and enzymatic activity. *Am. J. Physiol.* **245:**G327–G333

Katz, A. 1988. Role of Na,K-ATPase in kidney function. *In:* The Na,K-pump. Part B (J. Skou, J. Norby, A. Mansbach, M. Esman, Editors) pp. 207–232. Liss, New York

Lattimer, S.A., Sima, A.A., Greene, D.A. 1989. In vitro correction of impaired Na,K-ATPase in diabetic nerve by protein kinase C agonists. *Am. J. Physiol.* **256:**E264–E269

Liu, F.Y., Cogan, M.G. 1990. Role of protein kinase C in proximal bicarbonate absorption and angiotensin signaling. *Am. J. Physiol.* **258:**F927–F933

Lowndes, J.M., Hokinnneaverson, M., Bertics, P.J. 1990. Kinetics of phosphorylation of Na/K-ATPase kinase C. *Biochim. Biophys. Acta* **1052:**143–151

Lupas, A., Van Dyke, M., Stock, J. 1991. Predicting coiled coils from protein sequences. *Science* **252:**1162–1164

Lynch, C.J., Wilson, P.B., Blackmore, P.F., Exton, J.H. 1986. The hormone-sensitive hepatic Na-pump: evidence for regulation by diacylglycerol and tumor promoters. *J. Biol. Chem.* **261:**14551–14556

Mahnensmith, R.L., Aronson, P.S. 1985. The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. *Circ. Res.* **56:**773–788

Malstrom, K., Stange, G., Murer, H. 1987. Identification of proximal tubular transport functions in the established kidney cell line, OK. *Biochim. Biophys. Acta* **902:**269–277

Middleton, J.P., Khan, W.A., Collinsworth, G., Hannun, Y.A., Medford, R.M. 1993. Heterogeneity of protein kinase C-mediated rapid regulation of Na/K-ATPase in kidney epithelial cells. *J. Biol. Chem.* **268:**15958–15964

Minta, A., Tsien, R.Y. 1989. Fluorescent indicators for cytosolic sodium. *J. Biol. Chem.* **15:**19449–19457

Moore, E.D.W., Fay, F.S. 1993. Isoproterenol stimulates rapid extrusion of sodium from isolated smooth muscle cells. *Proc. Natl. Acad. Sci. USA* **90:**8058–8062

Nash, S.R., Godinot, N., Caron, M.G. 1993. Cloning and characterization of the opossum kidney cell D1 dopamine receptor: expression of identical D1A and D1B dopamine receptor mRNAs in opossum kidney and brain. *Molec. Pharmacol.* **44:**918–925

Norby, J.G., Obel, N. 1993. Stimulation by phorbol 12-myristate 13-acetate (PMA) of Na,K-pump mediated $Rb^+$-influx into lymphocytes. *Biol. Chem. Hoppe-Seyler* **374:**601

Nord, E.P., Goldfarb, D., Mikhail, N., Moradeshagi, P., Hafezi, A., Vaystub, S., Insel, P.A. 1987. $\alpha$-2 adrenergic agonists stimulate Na/H-antiport activity in the rabbit renal proximal tubule. *J. Clin. Invest.* **80:**1755–1762

Ovchinnikov, Y.A., Aystarkhova, E.A., Arzamazova, N.M., Dzhandzhugazyan, K.N., Efremov, R.G., Nabiev, I.R., Modyanov, N.N. 1988. Detailed structural analysis of exposed domains of membrane-bound Na,K-ATPase. *FEBS Lett.* **227:**235–239

Pedemonte, C.H. 1995. Inhibition of N$\alpha$-pump expression by impairment of protein glycosylation is independent of the reduced sodium entry into the cell. *J. Membrane Biol.* **147:**223–231

Pedemonte, C.H., Kaplan, J.H. 1986. Carbodiimide inactivation of Na,K-ATPase via intramolecular cross-link formation is due to inhibition of phosphorylation. *J. Biol. Chem.* **261:**3632–3639

Pedemonte, C.H., Kaplan, J.H. 1990. Chemical modifications as an approach to elucidation of sodium pump structure-function relations. *Am. J. Physiol.* **258:**C1–C23

Price, E.M., Lingrel, J.B. 1988. Structure-function relationships in the Na,K-ATPase $\alpha$-subunit: site-directed mutagenesis of glutamine-111 to arginine and asparagine-122 to aspartic acid generates a ouabain-resistant enzyme. *Biochemistry* **27:**8400–8408

Rose, J.K., Buonocore, L., Whitt, M.A. 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. *BioTechniques* **10:**520–525

Satoh, T., Cohen, H.T., Katz, A.I. 1993. Cellular signalling in the regulation of renal Na,K-ATPase. I. Role of cyclic AMP and phospholipase A2. *Am. J. Physiol.* **265:**F399–F405

Schuster, V.L., Kokko, J.P., Jacobson, H.R. 1984. Angiotensin II directly stimulates sodium transport in rabbit proximal convoluted tubules. *J. Clin. Invest.* **73:**507–515

Shanbaky, N.M., Pressley, T.A. 1994. Mammalian $\alpha$1-subunit of Na,K-ATPase does not need its amino terminus to maintain cell viability. *Am. J. Physiol.* C590–C597

Simons, K., Fuller, S.D. 1985. Cell surface polarity in epithelia. *Ann. Rev. Cell Biol.* **1:**243–288

Soltoff, S.P., Mandel, L.J. 1984. Active ion transport in the renal proximal tubule. *J. Gen. Physiol.* **81:**601–622

Stanton, B.A., Kaissling, B. 1989. Regulation of renal ion transport and cell growth by sodium. *Am. J. Physiol.* **257:**F1–F10

Vasilets, L.A., Ohta, T., Noguchi, S., Kawamura, M., Schwarz, W. 1993. Voltage-dependent inhibition of the sodium pump by external sodium: species differences and possible role of the N-terminus of the $\alpha$-subunit. *Eur. Biophys. J.* **21:**433–443

Vasilets, L.A., Omay, H.S., Ohta, T., Noguchi, S., Kawamura, M., Schwarz, W. 1991. Stimulation of the Na/K pump by external $K^+$ is regulated by voltage-dependent gating. *J. Biol. Chem.* **266:**16285–16288

Vasilets, L.A., Schmalzing, G., Madefessel, K., Haase, W., Schwarz, W. 1990. Activation of protein kinase C by phorbol ester induces downregulation of the Na/K-ATPase in oocytes of *Xenopus laevis*. *J. Membrane Biol.* **118:**131–142

Wang, T., Chan, Y.L. 1990. Time- and dose-dependent effects of protein kinase C on proximal bicarbonate transport. *J. Membrane Biol.* **117:**131–139

Wierzbicki, W., Blostein, R. 1993. The amino-terminal segment of the catalytic subunit of kidney Na,K-ATPase regulates the potassium deocclusion pathway of the reaction cycle. *Proc. Natl. Acad. Sci. USA* **90:**70–74