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Bidirectional regulation of renal cortical Na^+,K^+-ATPase by protein kinase C✪

Jerzy Bełtowski✉, Andrzej Marciniak, Anna Jamroz-Wiśniewska, Ewelina Borkowska and Grażyna Wójcicka

Department of Pathophysiology, Medical University, Lublin, Poland

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We examined the role of protein kinase C (PKC) in the regulation of Na^+,K^+-ATPase activity in the renal cortex. Male Wistar rats were anaesthetized and the investigated reagents were infused into the abdominal aorta proximally to the renal arteries. A PKC-activating phorbol ester, phorbol 12,13-dibutyrate (PDBu), had a dose-dependent effect on cortical Na^+,K^+-ATPase activity. Low dose of PDBu (10^{-11} mol/kg per min) increased cortical Na^+,K^+-ATPase activity by 34.2%, whereas high doses (10^{-9} and 10^{-8} mol/kg per min) reduced this activity by 22.7% and 35.0%, respectively. PDBu administration caused changes in Na^+,K^+-ATPase V_{max} without affecting $K_{0.5}$ for Na^+, K^+ and ATP as well as K_i for ouabain. The effects of PDBu were abolished by PKC inhibitors, staurosporine, GF109203X, and Gö 6976. The inhibitory effect of PDBu was reversed by pretreatment with inhibitors of cytochrome P450-dependent arachidonate metabolism, ethoxyresorufin and 17-octadecynoic acid, inhibitors of phosphatidylinositol 3-kinase (PI3K), wortmannin and LY294002, and by actin depolymerizing agents, cytochalasin D and latrunculin B. These results suggest that PKC may either stimulate or inhibit renal cortical Na^+,K^+-ATPase. The inhibitory effect is mediated by cytochrome P450-dependent arachidonate metabolites and PI3K, and is caused by redistribution of the sodium pump from the plasma membrane to the inactive intracellular pool.

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✉Corresponding author: Jerzy Bełtowski, Department of Pathophysiology, Medical University, Jaczewskiego 8, 20-090 Lublin, Poland; phone: (48 81) 742 5837; fax: (48 81) 742 5828; e-mail: patfiz@asklepios.am.lublin.pl

Abbreviations: CCV, clathrin-coated vesicles; DAG, diacylglycerol; 20-HETE, 20-hydroxyeicosatetraenoic acid; OA, okadaic acid; 17-ODYA, 17-octadecynoic acid; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

Na^+,K^+-ATPase, located in the basolateral membranes of renal tubular cells, drives active Na^+ reabsorption throughout the nephron and is involved in the regulation of extracellular fluid volume and blood pressure. Na^+,K^+-ATPase is regulated by numerous hormones and neuromediators, including dopamine, aldosterone, angiotensin II, nitric oxide and atrial natriuretic peptide (Feraille & Doucet, 2001). These mediators trigger several signalling pathways including cyclic nucleotide-dependent protein kinases, protein kinase C and arachidonate cascade. Protein kinase C (PKC) is a family of at least 10 related serine/threonine kinases divided into three groups. Classical or conventional PKCs (cPKCs) including PKCα, βI, βII and γ, are activated by diacylglycerol (DAG), phorbol esters and calcium. Novel PKC isoforms (nPKCs), which include PKCδ, PKCε, PKCη and PKCθ, are also activated by DAG and phorbol esters but are Ca^{2+}-independent. Finally, atypical PKCs (aPKCs), which include PKCζ and PKCλ, are not activated by either DAG or phorbol esters but may be regulated by other lipid mediators (Nishizuka, 1995; Ron & Kazanietz, 1999). PKC is involved in the regulation of renal Na^+,K^+-ATPase by dopamine, angiotensin II, endothelin I and norepinephrine (Feraille & Doucet, 2001).

In vitro studies have revealed either stimulatory or inhibitory effect of PKC on renal Na^+,K^+-ATPase depending on the nephron segment, experimental conditions and the method of enzyme assay (Feraille & Doucet, 2001). Recent studies indicate that the mechanisms regulating renal sodium pump are affected by multiple features of the microenvironment such as oxygen tension, pH, osmolality, calcium and sodium concentrations; all of them changing along the nephron (Kiroytscheva *et al.*, 1999). It is often impossible to adequately reproduce these complex biophysical conditions *in vitro*. Recently, we have developed an experimental model which allows study of the effect of hormones administered *in vivo* on renal Na^+,K^+-ATPase. In this model, the investigated reagents are infused locally under anaesthesia and then the enzyme activity is assayed in the isolated microsomal fraction of the renal tissue. Using this model, we demonstrated that PKC-activating phorbol esters have a biphasic effect on renal cortical Na^+,K^+-ATPase: low doses stimulate, whereas higher doses inhibit the enzyme activity (Bełtowski *et al.*, 1998). However, that study had several important limitations. First, phorbol esters are nonspecific activators of all classical and new PKC isoforms. Second, these compounds may induce PKC-independent effects (Ron & Kazanietz, 1999). Although the observed changes were abolished by staurosporine, this inhibitor is not completely specific for PKC. Most importantly, however, the Na^+,K^+-ATPase assay used in that study is not specific because the measured activity includes also ouabain-sensitive H^+,K^+-ATPase (Bełtowski & Wójcicka, 2002). Thus, the dose-dependent effect of phorbol esters could be accounted for, at least in part, by opposite regulation of both ouabain-sensitive enzymes. More recently, we improved the specificity of our Na^+,K^+-ATPase assay by adding a specific inhibitor of H^+,K^+-ATPases, Sch 28080, to the incubation medium (Bełtowski & Wójcicka, 2002).

In the present study, we reinvestigated the effect of PKC on renal cortical Na^+,K^+-ATPase using this improved method. In addition, apart from phorbol esters, we used also PKC activators and inhibitors more specific toward different kinase isoforms. Finally, we wanted to get more insight into the mechanism of Na^+,K^+-ATPase regulation by PKC.

MATERIALS AND METHODS

Reagents. The specific inhibitor of PKCβ, LY 333531, was obtained from Eli-Lilly (Indianapolis, IN, U.S.A). Gö 6976 and thymeleatoxin were purchased from Calbiochem-Novabiochem (San Diego, CA, U.S.A). The

specific inhibitor of H^+,K^+-ATPases, 2-methyl 8-(phenylmethoxy)imidazol(1,2-α) pyridine-3-acetonitrile (Sch 28080) was kindly provided by the Schering-Plough Research Institute (Kenilworth, NJ, U.S.A). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A). Before infusion, phorbol esters, staurosporine, GF109203X, Gö 6976, LY333531, nocodazole, thymeleatoxin, okadaic acid, EIPA, amphotericin B, cytochalasin D, latrunculin B, wortmannin and LY294002 were dissolved in Me_2SO and then diluted with 0.9% NaCl. Ethoxyresorufin, brefeldin A and 17-ODYA were dissolved in ethanol. Stock solution of 20-hydroxyeicosatetraenoic acid (20-HETE) provided by the manufacturer was diluted with saline to the required concentration. Infusion of Me_2SO or ethanol alone at the respective concentrations had no effect on Na^+,K^+-ATPase activity.

Experimental protocol. All studies were performed on adult male Wistar rats weighing 250–300 g. They had free access to food and water before the experiments. The animals were anaesthetized with pentobarbital (50 mg/kg, i.p.) and a thin catheter was inserted through the femoral artery into the abdominal aorta proximally (< 0.5 cm) to the renal arteries for infusion of the investigated substances. The position of the catheter was verified at the end of experiment. The study protocol was approved by the Bioethics Committee of the Medical University of Lublin.

After the surgery, infusion with physiological saline was started at the rate of 66 μl/min for 30 min (stabilization period). All investigated substances were infused as saline solution at the rate of 66 μl/min (4 ml/h). The total time of infusion was 60 min. Animals from the control group received 0.9% NaCl during the whole experiment. Each investigated compound was administered for 30 min, between 1 and 30 min or between 31 and 60 min of infusion. Until otherwise stated, PKC activators were administered between 31 and 60 min of infusion, whereas the compounds expected to block their effect — between 1 and 30 min. After the infusion, the abdominal cavity was opened and the aorta was ligated proximally to the end of the catheter. Then, 5 ml of 0.9% NaCl was infused within 1–2 min through the catheter to remove erythrocytes from the kidneys. The kidneys were excised and the animals sacrificed by a lethal dose of pentobarbital. Na^+,K^+-ATPase activity was assayed in the microsomal fraction isolated from the renal cortex and medulla. The procedure of isolation was described in details previously (Bełtowski & Wójcicka, 2002).

Na^+,K^+-ATPase assay. Na^+,K^+-ATPase activity was assayed by measuring the amount of inorganic phosphate (P_i) liberated from ATP during the incubation of the microsomal fraction at 37°C. The assay medium (1 ml) contained: 100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, 40 mM Tris/HCl (pH 7.4), 0.2 mM Sch 28080, and 50 μg of microsomal protein. Preincubation was carried out for 10 min, and then 3 mM Na_2ATP was added. After 15 min the enzymatic reaction was terminated by adding 0.35 ml of ice-cold 1 M $HClO_4$. Then, P_i was assayed as previously described (Bełtowski & Wójcicka, 2002). To correct for spontaneous breakdown of ATP, the absorbance of a blank sample prepared as described above but without microsomes was subtracted from the absorbance of the test sample. The amount of phosphate was read from a standard curve constructed using known concentrations of KH_2PO_4. Na^+,K^+-ATPase activity (ouabain-sensitive fraction) was calculated as the difference between total ATPase (assayed in the absence of ouabain) and ouabain-resistant fraction, assayed in the presence of 2 mM ouabain, and was expressed in μmoles of P_i liberated by 1 mg of microsomal protein during 1 h (μmol/h per mg protein). Each sample was assayed in triplicate and the difference between averaged total ATPase and averaged ouabain-resistant ATPase was used in further calculations. Protein concentration in the microsomal fraction

was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Kinetic analysis of renal Na^+,K^+-ATPase. To get more insight into the function of renal Na^+,K^+-ATPase, we measured its activity not only under optimal conditions but also at varying concentrations of substrate, activating ions and specific inhibitor. To assess the effect of Na^+ concentration on Na^+,K^+-ATPase, we measured its activity in the presence of 10, 20, 30, 40, 60 and 100 mM Na^+, including Na^+ derived from the ATP salt. When Na^+ was varied, choline chloride was added in appropriate concentrations to maintain constant osmolality. The data were analyzed using a cooperative model based on the Hill equation:

$$v = V_{max} \times [Na^+]^n/([Na^+]^n + K_{0.5}^n),$$

where: v is actual enzyme activity, V_{max} is maximal activity at saturating sodium concentration, $[Na^+]$ is actual sodium concentration, $K_{0.5}$ is the dissociation constant for Na^+ measuring the affinity of Na^+,K^+-ATPase for sodium, and n is Hill coefficient. In this model V_{max}, $K_{0.5}$ and n were allowed to vary to obtain the best fit to the data.

The effect of potassium concentration on Na^+,K^+-ATPase was fitted to the following equation:

$$v = V_{max} \times [K^+]/([K^+] + K_{0.5})$$

where v is the activity measured at a given potassium concentration $[K^+]$, V_{max} is the maximal activity and $K_{0.5}$ is the dissociation constant for K^+. The following K^+ concentrations were used: 0.5, 1, 2, 5, 10 or 20 mM, supplemented to 20 mM with choline chloride. The effect of ATP on Na^+,K^+-ATPase activity was studied by performing the assay in the presence of 0.1, 0.2, 0.5, 1, 2 or 3 mM ATP, and $K_{0.5}$ was calculated from the formula analogous to that used for potassium.

The inhibition of Na^+,K^+-ATPase by ouabain was analyzed according to the following equation:

$$v = V_{max}/(1 + [I]/[K_i])$$

where v is the activity measured at a given concentration of the inhibitor [I], V_{max} is the maximal activity and K_i is the inhibition constant. Ouabain concentration was varied from 10^{-7} to 10^{-3} M in one-order steps. The kinetic parameters were calculated by nonlinear least squares regression separately for each animal and the values reported in Table 1 are means ± S.D. from 5 animals in each group.

Statistics. Data are presented as mean ± S.D. from 8 animals in each group. Statistical analysis was performed by Student's *t*-test or analysis of variance (ANOVA) followed by Duncan's multiple range test, for comparison of 2 or > 2 groups, respectively. A P value < 0.05 was considered significant.

RESULTS

Effect of phorbol esters on renal cortical Na^+,K^+-ATPase

Infusion of phorbol 12,13-dibutyrate (PDBu) exerted a dose-dependent effect on renal cortical Na^+,K^+-ATPase. PDBu administered at a dose of 10^{-11} mol/kg per min increased Na^+,K^+-ATPase activity by 34.2%. A higher dose of PDBu (10^{-10} mol/kg per min) tended to stimulate Na^+,K^+-ATPase, but the effect did not reach the level of significance. In contrast, 10^{-9} and 10^{-8} mol/kg per min PDBu decreased cortical Na^+,K^+-ATPase activity by 22.7% and 35.0%, respectively (Fig. 1). PDBu had no effect on Na^+,K^+-ATPase activity in the renal medulla (not shown).

Another PKC-activating phorbol ester, phorbol 12-myristate 13-acetate (PMA), also induced dose-dependent changes in cortical Na^+,K^+-ATPase activity. PMA infused at

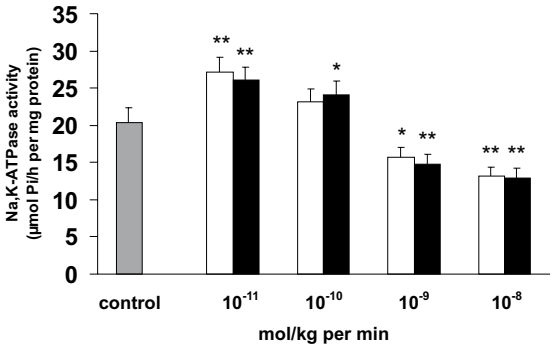

Figure 1. Dose-dependent effect of PDBu and PMA on Na^+,K^+-ATPase activity in the rat renal cortex.

Control group received infusion of 0.9% NaCl for 60 min, other groups were treated with increasing doses of PDBu (white bars) or PMA (black bars) between 31 and 60 min of infusion. Na^+,K^+-ATPase was assayed in isolated microsomal fraction. Enzyme activities are expressed in μmol of inorganic phosphate liberated by 1 mg of microsomal protein during 1 h. *$P < 0.05$, **$P < 0.01$; *compared to control by ANOVA and Duncan's test.*

doses of 10^{-11} and 10^{-10} mol/kg per min increased cortical Na^+,K^+-ATPase activity by 28.6% and 18.7%, respectively. Higher doses of PMA (10^{-9} mol/kg per min and 10^{-8} mol/kg per min) decreased cortical Na^+,K^+-ATPase activity by 27.1% and 36.5% (Fig. 1). Similarly to PDBu, PMA had no effect on medullary Na^+,K^+-ATPase (not shown).

In contrast to PDBu and PMA, 4α-phorbol didecanoate (4αPDD) which does not stimulate PKC, did not change cortical Na^+,K^+-ATPase activity (not shown).

Because both PKC-activating phorbol esters caused similar effects, only PDBu was used in further studies. In addition, in subsequent experiments we used this compound only at two doses, 10^{-11} and 10^{-8} mol/kg per min, which induced maximal stimulation and inhibition of Na^+,K^+-ATPase activity, and are therefore referred to as stimulatory and inhibitory doses, respectively.

Kinetic parameters of renal cortical Na^+,K^+-ATPase

Because the results described above were obtained under V_{max} conditions, in separate experiments we tested the effect of PDBu on kinetic parameters of this enzyme. As presented in Table 1, both the stimulatory and the inhibitory effects of PDBu were characterized by significant changes in enzyme V_{max}, whereas other kinetic parameters including $K_{0.5}$ for Na^+, Hill coefficient, $K_{0.5}$ for K^+, $K_{0.5}$ for ATP, and K_i for ouabain were not affected. Therefore, all further studies were performed under V_{max} conditions.

Table 1. The effect of the stimulatory and inhibitory doses of PDBu on kinetic parameters of renal cortical Na^+,K^+-ATPase

	Control	PDBu 10^{-11} mol/kg per min	PDBu 10^{-8} mol/kg per min
V_{max} (μmol P_i/h per mg protein)	21.1 ± 2.2	28.4 ± 2.7**	13.7 ± 1.4***
$K_{0.5}$ for Na^+ (mM)	21.2 ± 3.2	20.7 ± 1.9	21.7 ± 1.8
n	1.9 ± 0.1	1.8 ± 0.1	2.0 ± 0.1
$K_{0.5}$ for K^+ (mM)	1.8 ± 0.3	1.9 ± 0.2	2.1 ± 0.3
$K_{0.5}$ for ATP (μM)	425 ± 32	452 ± 41	417 ± 43
K_i (μM)	127 ± 14	131 ± 17	134 ± 14

**$P < 0.01$, ***$P < 0.001$ (compared to control by Student's *t*-test). n = 5 in each group. The reported V_{max} values were calculated in the analysis of Na^+ affinity.

Effect of PKC inhibitors

Both the stimulatory and the inhibitory effects of PDBu were abolished by pretreatment with PKC inhibitor, staurosporine (Fig. 2). Staurosporine administered at a dose

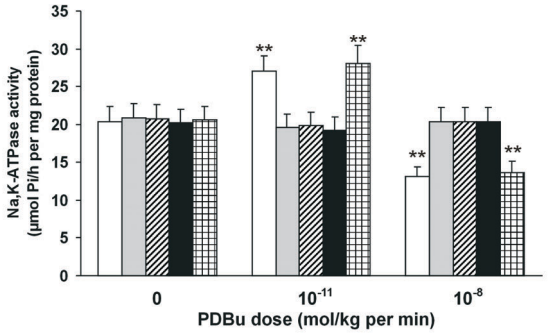

Figure 2. The effect of protein kinase C inhibitors on cortical Na^+,K^+-ATPase regulation by PDBu.

The animals received either 10^{-11} or 10^{-8} mol/kg per min PDBu between 31 and 60 min (white bars). In the separate groups, a non-specific PKC inhibitor, staurosporine (10^{-9} mol/kg per min, grey bars), a specific inhibitor of classical and novel PKC isoforms, GF109203X (10^{-8} mol/kg per min, batched bars), a specific inhibitor of classical PKC isoforms, Gö 6976 (10^{-9} mol/kg per min, black bars), or a specific inhibitor of PKCβ, LY333531 (10^{-8} mol/kg per min, checked bars) was infused before PDBu. In rats not treated with PDBu, the PKC inhibitors were infused between 31 and 60 min. $^{**}P < 0.01$; compared to control by ANOVA and Duncan's test.

of 10^{-9} mol/kg per min between 1 and 30 min of infusion had no effect on Na^+,K^+-ATPase activity in control animals, however, pretreatment with staurosporine abolished the stimulatory effect of PDBu administered at a dose of 10^{-11} mol/kg per min, as well as the inhibitory effect of 10^{-8} mol/kg per min of PDBu. Because staurosporine is a nonspecific inhibitor of all PKC isoforms, we investigated the effect of three additional more specific inhibitors. GF109203X, which inhibits all classical and novel but not the atypical PKC isoforms, infused at a dose of 10^{-8} mol/kg per min, prevented both the increase and the decrease of Na^+,K^+-ATPase activity induced by PDBu (Fig. 2). Gö 6976, which blocks all classical but not the novel or atypical PKC isoforms, administered at a dose of 10^{-9} mol/kg per min also completely abolished the stimulatory and the inhibitory effect of PDBu on renal cortical Na^+,K^+- ATPase. In contrast, LY333531, which is a specific inhibitor of PKCβ, infused at a dose of 10^{-8} mol/kg per min failed to block the effects of PDBu (Fig. 2). GF109203X, Gö 6976 and LY 333531 had no effect on cortical Na^+,K^+-ATPase if administered to animals which were not subsequently treated with PDBu.

Effect of thymeleatoxin on renal cortical Na^+,K^+-ATPase

In subsequent studies we investigated the effect of another PKC activator, thymeleatoxin (THX), on renal cortical Na^+,K^+-ATPase (Fig. 3). THX is a diterpene derivative of

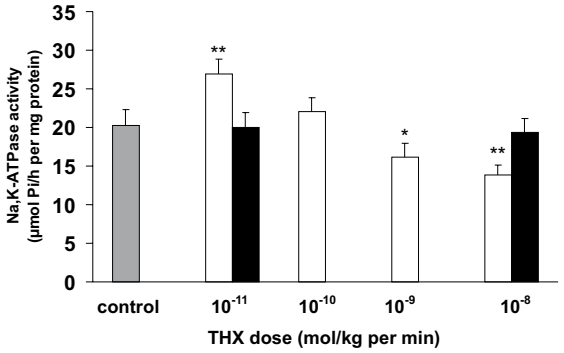

Figure 3. The effect of thymeleatoxin (THX) on renal cortical Na^+,K^+-ATPase activity.

THX (white bars) was administered in different doses between 31 and 60 min. Some animals received Gö 6976 (10^{-9} mol/kg per min, black bars) between 1 and 30 min of infusion. $^{*}P < 0.05$, $^{**}P < 0.01$; compared to control by ANOVA and Duncan's test.

mezerein which, unlike phorbol esters, stimulates only classical but not the novel PKC isoforms. THX administered at a dose of

10^{-11} mol/kg per min increased cortical Na^+,K^+-ATPase activity by 32.5%. Na^+,K^+-ATPase activity in animals receiving 10^{-10} mol/kg per min THX did not differ from control. Higher doses of THX, 10^{-9} and 10^{-8} mol/kg per min, decreased Na^+,K^+-ATPase activity by 20.2% and 31.5%, respectively. Thus, THX reproduced both the stimulatory and the inhibitory effect of phorbol esters. Gö 6976 administered at a dose of 10^{-9} mol/kg per min before the infusion of 10^{-11} or 10^{-8} mol/kg per min THX abolished the stimulation and the inhibition of Na^+,K^+-ATPase by this PKC activator, respectively (Fig. 3).

Effect of brefeldin A and nocodazole on Na^+,K^+-ATPase activation by PDBu

Recently, we (Bełtowski *et al.,* 2003a) and others (Gonin *et al.,* 2001; Vinciguerra *et al.,* 2003) have demonstrated that protein kinase A upregulates Na^+,K^+-ATPase in the renal cortex or isolated tubular cells by inducing the recruitment of latent pump molecules from intracellular stores to the plasma membrane; this process is inhibited by brefeldin A and requires integrity of the microtubule system. Therefore, we investigated whether PKC stimulates cortical Na^+,K^+-ATPase through a similar mechanism. Neither brefeldin A (100 μg/kg per min) nor the microtubule disrupting agent nocodazole (10^{-6} mol/kg per min) had any effect on Na^+,K^+-ATPase stimulation by 10^{-11} mol/kg per min PDBu (not shown). Brefeldin A and nocodazole had no effect on cortical Na^+,K^+-ATPase in animals which were not treated with PDBu.

Effect of okadaic acid on Na^+,K^+-ATPase regulation by PDBu

Na^+,K^+-ATPase may be phosphorylated by PKC, however, protein phosphatases are active *in vitro* and may partially dephosphorylate the sodium pump during tissue preparation. Addition of phosphatase inhibitors to the homogenization medium prevents enzyme dephosphorylation and augments the functional effects of PKC (Nowicki *et al.*, 1997; Kazanietz *et al.*, 2001). We found that the addition of okadaic acid (OA, 1 μM), an inhibitor of serine/threonine protein phosphatases, to the homogenization solution augmented the stimulatory effect of 10^{-11} mol/kg per min PDBu. Na^+,K^+-ATPase activity in samples treated with OA was 34.1 ± 2.7 μmol/kg per min, i.e. 25.8% higher than in samples not treated with OA ($P < 0.05$). Surprisingly, OA had no effect on the Na^+,K^+-ATPase inhibition by 10^{-8} mol/kg per min PDBu (not shown). OA added to samples obtained from control rats had no effect on cortical Na^+,K^+-ATPase activity. Thus, OA added during tissue preparation augmented the stimulatory but not the inhibitory effect of PDBu on renal cortical Na^+,K^+-ATPase.

Role of cytochrome P450-dependent arachidonate metabolites in Na^+,K^+-ATPase inhibition by PDBu

Our previous studies suggested that the inhibitory effect of high doses of PDBu on cortical Na^+,K^+-ATPase is mediated by cytochrome P450-dependent arachidonate metabolites (Bełtowski *et al.,* 1998). This was confirmed in the present study. Two distinct inhibitors of the cytochrome P450 pathway, ethoxyresorufin (ETX) and 17-ODYA, administered at a dose of 10^{-8} mol/kg per min, completely reversed the inhibitory effect of PDBu infused at a dose of 10^{-8} mol/kg per min (Fig. 4). In both ETX and 17-ODYA-pretreated animals, PDBu increased cortical Na^+,K^+-ATPase activity to a level indistinguishable from that observed following the low dose (10^{-11} mol/kg per min) of PDBu. These data indicate that PDBu is able to increase Na^+,K^+-ATPase activity also at the higher doses, however, this effect is masked by the activation of the cytochrome P450-dependent arachidonate cascade. Neither ETX nor 17-ODYA had any significant effect on Na^+,K^+-ATPase activity in animals which did

not receive PDBu. In addition, ETX and 17-ODYA had no effect on Na^+,K^+-ATPase stimulation by the low dose (10^{-11} mol/kg per min) of PDBu (Fig. 4). Consistently with our previous results (Bełtowski *et al.*, 2003a), a cytochrome P450-dependent arachidonate metabolite, 20-HETE, decreased cortical Na^+,K^+-ATPase activity. If 20-HETE was administered before the infusion of 10^{-8} mol/kg per min PDBu, the inhibitory effect of both compounds was not additive, i.e. Na^+,K^+-ATPase activity was not significantly different from that observed in animals receiving either 20-HETE or PDBu alone. However, when 20-HETE was infused before the stimulatory dose of PDBu (10^{-11} mol/kg per min), the Na^+,K^+-ATPase activity decreased to the level observed in animals receiving either 20-HETE alone or PDBu at the inhibitory dose (Fig. 4). Thus, in the presence of 20-HETE, PDBu was unable to stimulate Na^+,K^+-ATPase even at a low dose. These data indicate that if the synthesis of 20-HETE is inhibited, PDBu stimulates Na^+,K^+-ATPase independently of the dose administered. When PDBu is infused at the higher doses, the 20-HETE pathway is triggered and converts this stimulatory effect into inhibition.

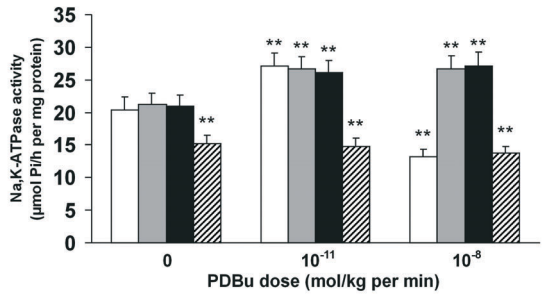

Figure 4. The effect of inhibitors of cytochrome P450-dependent arachidonate cascade (ethoxyresorufin and 17-ODYA) and 20-HETE on the regulation of renal cortical Na^+,K^+-ATPase by PDBu.

Control animals received 0.9% NaCl for 60 min. PDBu was administered at either 10^{-11} or 10^{-8} mol/kg per min between 31 and 60 min (white bars). Separate groups of animals received ethoxyresorufin (ETX, 10^{-8} mol/kg per min, grey bars), 17-ODYA (10^{-8} mol/kg per min, black bars), or 20-HETE (10^{-10} mol/kg per min, batched bars) for 30 min before PDBu. In animals not treated with PDBu, ETX, 17-ODYA or 20-HETE were administered between 31 and 60 min. $^{**}P < 0.01$; compared to control by ANOVA and Duncan's test.

Effect of sodium transport modulators on Na^+,K^+-ATPase regulation by PDBu

Recently, it has been demonstrated that the effect of PKC on renal Na^+,K^+-ATPase is affected by intracellular Na^+ concentration; PKC stimulates the sodium pump at low and inhibits at high intracellular Na^+ (Efendiev *et al.,* 2002). To investigate whether the dose dependent switch of the effect of PDBu may be associated with its modulating influence on intracellular Na^+, we examined interactions of this compound with reagents expected to affect apical sodium entry. Ethylisopropylamiloride (EIPA) selectively inhibits Na^+/H^+-exchanger – the major pathway of sodium entry into proximal tubule cells (Wang, 1997). EIPA infused at a dose of 10^{-6} mol/kg per min had no effect on Na^+,K^+-ATPase stimulation by 10^{-11} mol/kg per min PDBu. However, this dose of EIPA reversed the inhibitory effect of 10^{-8} mol/kg per min PDBu (Fig. 5). In contrast, two different Na^+ ionophores, amphotericin B (10^{-7} mol/kg per min) and monensin (10^{-6} mol/kg per min), infused before the administration of the stimulatory dose of PDBu, converted this stimulation to inhibition. However, administration of ethoxyresorufin together with monensin reversed this inhibitory effect (Fig. 5). Taken together, these data suggest that high dose of PDBu elicits Na^+,K^+-ATPase inhibition by increasing Na^+ entry into the tubular cells, which triggers the cytochrome P450-dependent arachidonate cascade. When

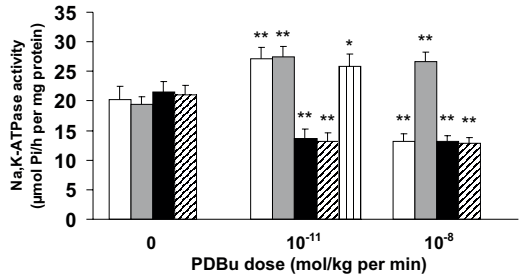

Figure 5. The effect of drugs modifying apical sodium entry into tubular cells on the regulation of renal cortical Na^+,K^+-ATPase by PDBu.

Control animals received 0.9% NaCl for 60 min. PDBu was administered at either 10^{-11} or 10^{-8} mol/kg per min between 31 and 60 min (white bars). In addition, separate groups of rats were treated with ethylisopropylamiloride (EIPA, 10^{-6} mol/kg per min, grey bars), amphotericin B (10^{-7} mol/kg per min, black bars), monensin (10^{-6} mol/kg per min, batched bars) or ethoxyresorufin (ETX, 10^{-8} mol/kg per min) + monensin (10^{-6} mol/kg per min, checked bar) before PDBu, i.e. between 1 and 30 min. In animals not treated with PDBu, EIPA, amphotericin B and monensin were administered between 31 and 60 min. $^{**}P < 0.01$; compared to control by ANOVA and Duncan's test.

Na^+ entry is blocked, PDBu stimulates Na^+,K^+-ATPase even at high doses.

Effect of reagents modulating endocytotic pathway and phosphatidylinositol-3-kinase activity on Na^+,K^+-ATPase regulation by PDBu

Cytochalazin D, an actin depolymerizing agent, infused at a dose of 10^{-7} mol/kg per min abolished Na^+,K^+-ATPase inhibition by PDBu (Table 2). A similar effect was observed following administration of latrunculin B (0.5 mg/kg per min) which sequesters actin monomers and impairs the formation of actin filaments (Lamaze *et al.*, 1997). In rats pretreated with cytochalasin D or latrunculin B, PDBu infused at a dose of 10^{-8} mol/kg per min stimulated cortical Na^+,K^+-ATPase activity (Table 2). In contrast, a microtubule-disrupting agent, nocodazole (10^{-6} mol/kg per min), did not prevent the Na^+,K^+-ATPase inhibition. Cytochalasin D, latrunculin B and nocodazole had no effect on Na^+,K^+-ATPase in animals not treated with PDBu. These data suggest that actin cytoskeleton but not the microtubule system is necessary for Na^+,K^+-ATPase inhibition by PKC.

Recent studies indicate that PKC and 20-HETE inhibit Na^+,K^+-ATPase in the proximal tubule by a phosphatidylinositol-3-kinase (PI3K)-dependent mechanism (Chibalin *et al.*, 1998b). To examine whether PI3K is involved in Na^+,K^+-ATPase inhibition in our experimental model, we investigated the effect of two structurally unrelated PI3K inhibitors. Wortmannin (10^{-8} mol/kg per min) and LY 294002 (10^{-6} mol/kg per min) abolished Na^+,K^+-ATPase inhibition by PDBu (Table 2). Neither of these PI3K inhibitors had any significant effect on cortical Na^+,K^+-ATPase in control animals (not shown). An inactive analogue of LY 284002, LY303511, had no effect on Na^+,K^+-ATPase inhibition by PDBu (Table 2).

DISCUSSION

The results of this study confirm our previous observation (Bełtowski *et al.*, 1998) that PKC-activating phorbol esters have dose-dependent effects on renal cortical Na^+,K^+-ATPase in the rat. Because in the present study Na^+,K^+-ATPase was assayed in the presence of Sch 28080, the observed effect must be specific for Na^+,K^+-ATPase and could not be accounted for by any changes in ouabain-sensitive H^+,K^+-ATPase activity.

It is likely that phorbol esters elicit both effects by activating classical PKC isoforms, because their effect was blocked by Gö 6976 — a specific inhibitor of Ca^{2+}-dependent PKC isoenzymes, and was mimicked by THX which is a specific activator of classical PKC

isoforms. However, the involvement of PKCβ should rather be excluded since LY 333531, a very selective inhibitor of PKCβI and βII (Ishi *et al.*, 1996; Kowluru *et al.*, 1998), failed to prevent both the stimulatory and the inhibitory effect of PDBu. This is consistent with the results of numerous studies demonstrating that PKCβI and PKCβII are either absent or expressed at a very low level in the whole renal cortex and in tubule segments isolated from this region of the kidney (Ostlund *et al.*, 1995; Karim *et al.*, 1995; Wilborn & Schafer, 1996; Yao *et al.*, 1998; Pfaff *et al.*, 1999; Padanilam, 2001). We cannot exclude the possibility that the stimulatory and the inhibitory effects of PDBu are mediated by distinct cPKC isoforms, e.g. α and γ. However, it seems unlikely for several reasons. First, PDBu and PMA have similar affinity for these isoforms (Hofmann, 1997) and would be expected to stimulate both of them simultaneously rather than at so very different doses. Second, it is unlikely that all three activators (PMA, PDBu and THX) used by us have so similar dose-dependent effects on both PKC isoforms. Most importantly, PKCγ is expressed in the kidney at a level much lower than PKCα. Activators and inhibitors with sufficient specificity toward PKCα and PKCγ are not currently available and it was not possible to examine the individual involvement of these isoforms. However, the data presented above strongly suggest that the bidirectional effect of phorbol esters on cortical Na^+,K^+-ATPase cannot be accounted for by differential effects of specific PKC isoforms, but is rather associated with the level of stimulation of a single isoform, presumably PKCα.

Table 2. The effect of cytoskeleton-disrupting agents and phosphatidylinositol-3-kinase inhibitors on renal cortical Na^+,K^+-ATPase inhibition by PDBu.

Treatment schedule		Na^+,K^+-ATPase activity
0–30 min	31–60 min	(μmol P_i/h per mg protein)
0.9% NaCl	0.9% NaCl	20.3 ± 2.1
0.9% NaCl	PDBu (10^{-8} mol/kg per min)	13.2 ± 1.2**
Cytochalasin D (10^{-7} mol/kg per min)	PDBu (10^{-8} mol/kg per min)	28.7 ± 2.5**
Latrunculin B (0.5 mg/kg per min)	PDBu (10^{-8} mol/kg per min)	25.7 ± 2.1*
Nocodazole (10^{-6} mol/kg per min)	PDBu (10^{-8} mol/kg per min)	13.7 ± 1.3**
Wortmannin (10^{-8} mol/kg per min)	PDBu (10^{-8} mol/kg per min)	24.3 ± 1.7*
LY 294002 (10^{-6} mol/kg per min)	PDBu (10^{-8} mol/kg per min)	26.2 ± 1.8**
LY 303511 (10^{-6} mol/kg per min)	PDBu (10^{-8} mol/kg per min)	13.8 ± 1.2**

*$P < 0.05$, **$P < 0.01$ (compared to control by ANOVA and Duncan's test).

Stimulatory effect of PKC

Recently, we have demonstrated that protein kinase A stimulates cortical Na^+,K^+-ATPase in a brefeldin A-sensitive manner, suggesting that this transduction pathway in-

creases the incorporation of pump molecules to the plasma membrane (Bełtowski *et al.*, 2003a). In contrast, the stimulatory effect of PKC was not affected by brefeldin A, indicating that redistribution of Na^+,K^+-ATPase from the intracellular to the plasma membrane pool was not involved. The α_1 subunit of rat Na^+,K^+-ATPase contains PKC phosphorylation sites at Ser16 and Ser21 (Feraille & Doucet, 2001) and may be phosphorylated by this kinase *in vitro* and in intact cells (Bertorello *et al.*, 1991; Feschenko & Sweadner, 1995; Logvinenko *et al.*, 1996; Kazanietz *et al.*, 2001). Although some studies reported no change (Feschenko & Sweadner, 1995; 1997) or even a decrease (Bertorello & Aperia, 1989; Middleton *et al.*, 1993; Belusa *et al.*, 1997; Blanco *et al.*, 1998; Kazanietz *et al.*, 2001) in Na^+,K^+-ATPase activity following PKC-dependent phosphorylation, this was observed mainly in an isolated enzyme preparation or in cells transfected with heterologous Na^+,K^+-ATPase α_1-subunit. In intact renal tubular cells, PKC-mediated phosphorylation correlates with an increase in Na^+,K^+-ATPase activity (Feraille *et al.*, 1995; Carranza *et al.*, 1996; Pedemonte *et al.*, 1997a; 1997b; Tsimaratos *et al.*, 2003). The α_1 subunit of Na^+,K^+- ATPase may be dephosphorylated by protein phosphatases during tissue homogenization and protein phosphatase inhibitors added during preparation procedures increase the rate of enzyme phosphorylation (Nowicki *et al.*, 1997). In our experiments, okadaic acid added to the homogenization medium augmented the stimulatory effect of PKC on cortical Na^+,K^+-ATPase. In contrast, cAMP-mediated stimulation was not affected by OA (unpublished observation), consistently with our hypothesis that cAMP induces redistribution of pump molecules to the plasma membrane (Bełtowski *et al.*, 2003a); the process which is completed *in vivo* and could not be modified by the inhibition of phosphatases during tissue preparation. Also, the inhibitory effect of phorbol esters probably mediated by the internalization of Na^+,K^+-ATPase (see below) was not affected by OA in the present study. Taken together, these data suggest that PKC stimulates cortical Na^+,K^+-ATPase by phosphorylating its catalytic subunit and modifying its catalytic properties. However, we can not exclude the possibility that PKC phosphorylates another protein which stimulates Na^+,K^+-ATPase, such as the recently described regulatory γ-subunit of this enzyme (Mahmmoud *et al.*, 2000).

Recently, Tsimaratos *et al.* (2003) have demonstrated that PKCα phosphorylates Na^+,K^+-ATPase α_1-subunit and increases its V_{max} without affecting Na^+ affinity or cell surface expression in rat medullary thick ascending limb. Although we studied the cortical enzyme and could not identify the PKC isoform involved, our results are consistent with their findings. In contrast, Feraille *et al.* (1995) have observed that PDBu increases Na^+ affinity of Na^+,K^+-ATPase but has no effect on V_{max} in isolated rat proximal tubule (PT) cells. The discrepancy between their and our results may be accounted for by several methodological differences. We administered PDBu *in vivo* and measured the Na^+,K^+-ATPase activity in the whole cortex, which apart from PT contains also cortical thick ascending limbs, distal convoluted tubules and cortical collecting ducts. PKC could stimulate Na^+,K^+-ATPase in these segments through different mechanisms. In addition, Feraille *et al.* (1995) were using smaller rats (130–150 g) than we did. Renal expression of specific PKC isoforms changes with age (Serlachius *et al.*, 1997), and different isoforms could have divergent effects on Na^+,K^+-ATPase activity. In opossum kidney cells, phorbol esters increase Na^+,K^+-ATPase activity by augmenting enzyme incorporation to the plasma membrane without affecting its turnover rate; this effect is mediated by PKCβ (Efendiev *et al.*, 1999; 2000; Budu *et al.*, 2002). As discussed earlier, it is unlikely that PKCβ mediated the stimulatory effect of phorbol esters in our study. In addition, the recruitment of

Na^+,K^+-ATPase to the plasma membrane is augmented by Na^+ entry blockade with amiloride (Budu *et al.*, 2002), whereas in our study EIPA did not enhance the stimulatory effect of PDBu. Thus, the mechanism of Na^+,K^+-ATPase stimulation by PKC differs depending on the experimental model, animal species, tubule segment studied and the PKC isoform involved.

Inhibitory effect of PKC

Several studies have demonstrated that PKC decreases Na^+,K^+-ATPase activity in renal cells through a mechanism involving cytochrome P450-dependent arachidonate metabolites, in particular 20-HETE (Satoh *et al.*, 1993; Ominato *et al.*, 1996). Subsequently, it has been suggested that this effect is observed only in hypooxygenated cells and is not physiologically relevant (Feraille *et al.*, 1995; Kiroytscheva *et al.*, 1999). We have observed that protein kinase A (Bełtowski *et al.*, 2003a) and G (Bełtowski *et al.*, 2003b) decrease Na^+,K^+-ATPase activity in the renal medulla in a 20-HETE dependent manner; the effect most likely accounted for by low oxygen tension in the medullary tissue. However, renal cortex has an adequate oxygen supply and it is unlikely that the inhibitory effect of PKC in the present study could be ascribed to hypoxic conditions. Efendiev *et al.* (2002) have demonstrated that the effect of PKC is determined by intracellular Na^+ concentration; PKC stimulates Na^+,K^+-ATPase at low and inhibits at high intracellular Na^+, the latter effect is mediated by 20-HETE. Sodium concentration in tubular cells is determined by apical passive entry and basolateral active transport by Na^+,K^+-ATPase. PKC increases the activity of Na^+/H^+-exchanger – the major pathway of Na^+ entry into PT cells (Karim *et al.*, 1999). We hypothesized that higher doses of phorbol esters could stimulate apical sodium entry and increase intracellular Na^+ triggering cytochrome P450-dependent Na^+,K^+-ATPase inhibition. Indeed, the inhibitory effect of PDBu was reversed by EIPA, whereas administration of Na^+ ionophores triggered Na^+,K^+-ATPase inhibition also in animals treated with a low dose of PDBu. Our results suggest that high doses of PDBu trigger both a stimulatory and an inhibitory effect on Na^+,K^+-ATPase, however, the latter effect dominates. At present we have no adequate explanation why higher doses of PDBu are needed to produce Na^+,K^+-ATPase inhibition. It is possible that because PDBu is delivered *via* the vascular route, i.e. it reaches tubular cells from the basolateral side, a higher concentration of this PKC activator is required to exert an effect on Na^+/H^+-exchanger, which is located within the opposite apical membrane. Alternatively, the effects on Na^+,K^+-ATPase and Na^+/H^+-exchanger could have different dose-response curves, with the former being saturable i.e. reaching plateau at a higher concentration, and the latter being not saturable. Consequently, low dose of PDBu could have no effect on intracellular Na^+ by causing coordinate stimulation of apical Na^+/H^+-exchanger and basolateral Na^+,K^+-ATPase, whereas higher doses could cause a more marked stimulation of Na^+/H^+-exchanger leading to an increase in intracellular Na^+ and thus triggering the Na^+,K^+-ATPase inhibitory pathway. This possibility is supported by our observation that, following the inhibition of 20-HETE formation, a high dose of PDBu stimulates Na^+,K^+-ATPase but this stimulation is not greater than after lower doses. It should be noted that the infusion of Na^+ ionophores without PDBu had no effect on Na^+,K^+-ATPase activity, suggesting that increased intracellular Na^+ without PKC stimulation is not sufficient to induce Na^+,K^+-ATPase inhibition.

In vitro studies suggest that PKC and 20-HETE downregulate Na^+,K^+-ATPase by causing its removal from the plasma membrane through clathrin-coated vesicles (CCV) to early and late endosomes (Chibalin *et al.*, 1997; 1998a; 1998b; 1999; Feraille *et al.*,

2000). We observed that actin-depolymerizing agents, cytochalasin D and latrunculin B, but not the microtubule-disrupting nocodazole, prevented the inhibitory effect of PDBu. This is consistent with the i*n vitro* observations that actin cytoskeleton is sufficient for Na^+,K^+-ATPase transport to clathrin-coated vesicles whereas the microtubule system is required for its transfer to endosomes, and that sodium pump is already inactivated in CCV (Chibalin *et al.*, 1997; Gomes & Soares-da-Silva, 2002). Whether the inhibitory effect of phorbol esters is specific for Na^+,K^+-ATPase or is associated with non-specific stimulation of fluid-phase endocytosis (Beron *et al.*, 1997) remains to be established.

Recent studies suggest that phosphatidylinositol-3-kinase (PI3K) is involved in the regulation of vesicular traffic and endocytosis (Li *et al.*, 1995; De Camilli *et al.*, 1996). In isolated rat PT cells, PKC and 20-HETE activate PI3K and induce endocytosis of Na^+,K^+-ATPase in a PI3K-dependent manner (Chibalin *et al.*, 1998b). Recently, it has been demonstrated that the interaction of PI3K with the α-subunit of the sodium pump initiates its endocytosis in opossum kidney cells (Yudowski *et al.*, 2000). The results of our study confirm the involvement of PI3K in the downregulation of Na^+,K^+-ATPase by phorbol esters.

In conclusion, PKC-activating phorbol esters have a dose-dependent effect on renal cortical Na^+,K^+-ATPase activity. Low doses of phorbol esters stimulate, whereas higher doses inhibit cortical Na^+,K^+-ATPase. Both effects are probably mediated by classical PKC isoform(s) except PKCβ. The stimulatory effect cannot be accounted for by the recruitment of inactive pumps to the plasma membrane but rather by phosphorylation of Na^+,K^+-ATPase or a regulatory protein, which increases the enzyme's V_{max}. The inhibitory effect is triggered by an increase in Na^+ entry, is mediated by cytochrome P450-dependent arachidonate metabolites and phosphatidylinositol-3-kinase, and is probably caused by endocytosis of Na^+,K^+-ATPase. The mechanisms of Na^+,K^+-ATPase regulation by PKC suggested by the results of this study are summarized in Fig. 6.

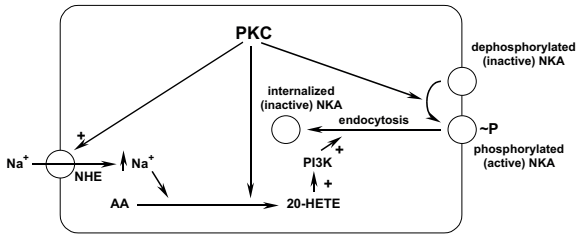

Figure 6. Suggested mechanisms of Na^+,K^+-ATPase (NKA) regulation by PKC in the renal cortex.

Low-level PKC activation increases the activity of Na^+,K^+-ATPase molecules existing in the basolateral membrane by phosphorylating the pump or a regulatory protein. If PKC activators are administered at high doses, this kinase increases Na^+ entry into the cell by activating Na^+/H^+-exchanger (NHE) localized in the apical membrane. Increased intracellular Na^+ stimulates conversion of arachidonic acid (AA) to 20-HETE, which activates phosphatidylinositol 3-kinase (PI3K) leading to redistribution of Na^+,K^+-ATPase from the basolateral membrane to the inactive intracellular pool.

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