PKC-β and PKC-ζ mediate opposing effects on proximal tubule Na^+,K^+-ATPase activity

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Abstract Dopamine (DA) inhibits rodent proximal tubule Na^+,K^+-ATPase via stimulation of protein kinase C (PKC). However, direct stimulation of PKC by phorbol 12-myristate 13-acetate (PMA) results in increased Na^+,K^+-ATPase. LY333531, a specific inhibitor of the PKC-β isoform, prevents PMA-dependent activation of Na^+,K^+-ATPase, but has no effect on DA inhibition of this activity. A similar result was obtained with a PKC-β inhibitor peptide. Concentrations of staurosporine, that inhibits PKC-ζ, prevent DA-dependent inhibition of Na^+,K^+-ATPase and a similar effect was obtained with a PKC-ζ inhibitor peptide. Thus, PMA-dependent stimulation of Na^+,K^+-ATPase is mediated by activation of PKC-β, whereas inhibition by DA requires activation of PKC-ζ.

Key words: Na^+,K^+-ATPase; Na-pump; Sodium-pump; PKC isoform; Proximal tubule sodium transport

1. Introduction

The molecular mechanism by which hormone receptors coupled to stimulation of protein kinase C (PKC) regulate sodium re-absorption in renal proximal convoluted tubules is not well understood [1]. The Na^+,K^+-ATPase, located within the basolateral membrane of tubular epithelial cells, maintains a transmembrane concentration gradient for sodium, ensuring the net re-absorption of this cation [2,3]. It has been suggested that hormonal short term regulation of Na^+,K^+-ATPase activity may contribute to the ability of the kidney to adjust sodium re-absorption [1,4].

In opossum kidney (OK) cells, a cell culture model of proximal tubule epithelia [5,6], dopamine (DA) inhibits and phorbol esters stimulate Na^+,K^+-ATPase activity [7–10]. DA-dependent inhibition and phorbol 12-myristate 13-acetate (PMA)-dependent stimulation of Na^+,K^+-ATPase activity are mediated by PKC and phosphorylation of serine residues within the Na^+,K^+-ATPase α-subunit NH_2-terminus [7–11]. DA treatment of OK cells results in phosphorylation of α-subunit Ser-18 and this is essential for inhibition of Na^+,K^+-ATPase activity [9]. Upon phosphorylation, Na^+,K^+-ATPase molecules are internalized and this results in a lower Na^+,K^+-ATPase capacity at the cell membrane [9,10]. On the other hand, PMA treatment of OK cells results in phosphorylation of both Ser-11 and Ser-18 of the α-subunit and the presence of either of these amino acids is essential for PMA-dependent activation of Na^+,K^+-ATPase [11]. Increased Na^+,K^+-ATPase activity may be the consequence of translocation of Na^+,K^+-ATPase molecules from intracellular compartments to the plasma membrane as described in other transporting epithelia [12]. The fact that both activation and inhibition of Na^+,K^+-ATPase activity are mediated by stimulation of PKC suggests that two different isoforms of this enzyme may be responsible for these processes. In this report, we present evidence that PKC-β and PKC-ζ are the isoforms responsible for the opposing effects of DA and phorbol esters on proximal tubule Na^+,K^+-ATPase activity.

2. Materials and methods

2.1. Materials

Cell culture supplies were purchased from Life Sciences Technologies (Grand Island, NY, USA) and Hyclone Laboratories (Logan, UT, USA). Molecular biology reagents were from New England Biolabs (Beverly, MA, USA), Promega (Madison, WI, USA), Stratagene (La Jolla, CA, USA) and Sigma Chemical (St. Louis, MO, USA). DA (3-hydroxytyramine), monensin sodium salt, PMA and staurosporine (antibiotic AM-2282) were obtained from Sigma Chemical. LY333531 (mesylate monohydrate) was a kind gift from Eli Lilly (Indianapolis, IN, USA). [$^{86}Rb^+$]RbCl was obtained from DuPont NEN Life Science Products (Boston, MA, USA). Peptide inhibitors of PKC isoforms were a kind gift of Dr Daria Mochly-Rosen, Stanford University, School of Medicine.

2.2. Cell culture and transfection

OK cells were maintained at 37°C (10% CO_2) in Dulbecco's modified Eagle medium with 10% calf serum and antibiotics (DMEM-10). Transfection of OK cells with rodent α1-cDNA was performed as previously described [7]. Selection for cells expressing the highest level of rodent α-subunit was achieved by exposing them to a medium containing 3 μM ouabain. 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 non-transfected host cells. The endogenous Na^+,K^+-ATPase activity was inhibited by growing the cells and performing the experiments in the presence of 3 μM ouabain. Accordingly, any Na^+,K^+-ATPase activity that we observed must originate with the Na^+,K^+-ATPases containing the introduced rodent α-subunit.

2.3. Determination of Rb^+-transport

Measurements of Na^+,K^+-ATPase-mediated Rb^+-transport were performed as described [7]. To determine the effect of DA on Rb^+-transport, cells were incubated with 30 μM monensin for 10 min as described by Seri et al. [13]. Cells were incubated with 0.1 μM PMA (10 min) or 0.1 μM DA (5 min) before assaying.

2.4. Introduction of peptides into OK cells

To facilitate the delivery of peptides into cells without affecting the long-term cell viability, the permeabilization protocol described by Johnson et al. [14] was followed. Briefly, cells were slowly brought down in temperature with two sequential 2 min PBS incubations, first at room temperature and the second with chilled PBS. The cells were

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Abbreviations: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DA, dopamine; OK cells, opossum kidney cells

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then incubated on ice with freshly prepared permeabilization buffer (20 mM HEPES, 10 mM EGTA, 140 mM KCl, 50 μg/ml saponin, 5 mM NaN_3 and 5 mM oxalic acid dipotassium salt, pH 7.4) containing the desired peptide for 10 min. The cells were then gently washed for four times with chilled PBS and incubated for 20 min with chilled PBS on ice. The chilled PBS was discarded and the cells were incubated at room temperature for 2 min in room temperature PBS. Finally, the cells were transferred to assay medium.

2.5. Treatment with PKC inhibitors

The cells were incubated at room temperature with the inhibitors of PKC, LY333531 or staurosporine, for 30 min at different concentrations before the assay of Rb^+-transport. We used 1% of DMSO to solubilize LY333531 and found that the solvent alone had no effect on the Na^+,K^+-ATPase activity of the cells.

2.6. Statistical analysis

Comparison of the data between groups was performed by Student's *t*-test for unpaired data.

3. Result and discussion

In proximal tubules, angiotensin II and α1-adrenergic receptors stimulation increases sodium re-absorption in a process mediated by PKC [1,4,15–17]. On the contrary, DA stimulation of D1 receptor subtype leads to a reduced sodium re-absorption and this process also requires activation of PKC [18–21]. We have previously proposed that these effects could be mediated, at least in part, by regulation of renal tubules Na^+,K^+-ATPase activity [1,7]. Consistent with this hypothesis, the opposite effects of angiotensin II/epinephrine and DA in proximal tubule sodium re-absorption correlate with the activation (phorbol ester) and inhibition (DA) of Na^+,K^+-ATPase activity previously described in OK cells [7–10].

The PMA stimulation and DA inhibition of Na^+,K^+-ATPase-mediated Rb^+-transport are illustrated in Fig. 1. These effects are mediated by PKC-dependent phosphorylation of Ser residues located at the Na^+,K^+-ATPase α-subunit NH_2-terminus [7–10]. Therefore, it is likely that the differential effect of PMA and DA on sodium transport may be due to regulation of Na^+,K^+-ATPase activity by stimulation of different PKC isoforms. We have determined using immunoblot analysis that OK cells express all PKC isoforms except γ (data not shown). The appropriate experimental design to determine whether the opposite effects of PMA and DA are mediated by activation of different isoforms of PKC would be to use isoform-specific inhibitors of PKC. However, few of these inhibitors have been described and most of them are not commercially available [22,23]. Recently, the synthesis of a novel and

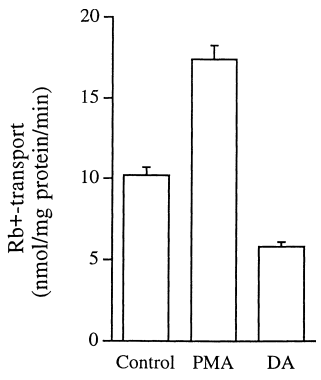

Fig. 1. Effect of DA and PMA on the Na^+,K^+-ATPase-mediated Rb^+-transport.

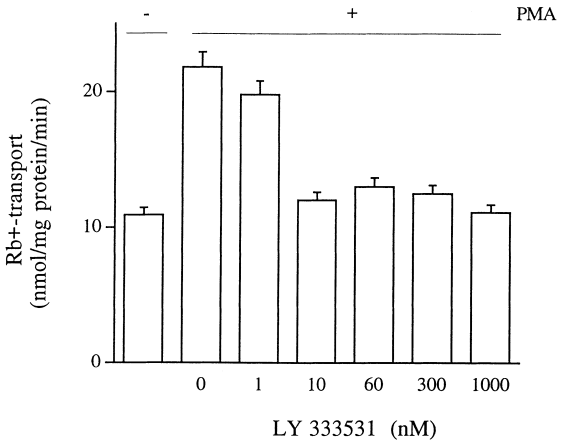

Fig. 2. Effect of various concentrations of LY333531 on PMA-dependent activation of Rb^+-transport.

highly specific inhibitor of PKC-β_1/β_2, LY333531, was reported [24]. LY333531 inhibits PKC-β_1/β_2 with a half-maximal inhibitory constant (IC_{50}) of approximately 5 nM. This concentration is 50-fold lower than the concentration required to inhibit other PKC isoenzymes [24]. Furthermore, it has been shown that LY333531 is highly specific and effective both in vitro and in vivo [24,25].

To study the involvement of different PKC isoforms, we determined the effect of PMA and DA on Rb^+-transport in cells that have been treated with LY333531 and other PKC isoform-specific inhibitors. To allow comparison of the results with those reported in our previous publications, the experiments were performed in OK cells transfected with the rodent Na^+,K^+-ATPase α1-cDNA. However, PMA-dependent activation and DA-dependent inhibition of Na^+,K^+-ATPase activity also occur in non-transfected OK cells. As illustrated in Fig. 2, 10 nM LY333531 almost totally impaired the stimulatory effect of PMA on Rb^+-transport. This result strongly suggests that PKC-β_1/β_2 isozymes may be responsible for the PMA effect. Low concentrations of staurosporine (60 nM) were sufficient to prevent the stimulatory effect of PMA on Na^+,K^+-ATPase activity (Fig. 3). However, LY333531 (even at a high concentration) could not prevent the inhibitory effect of DA on Na^+,K^+-ATPase (data not shown). It is possible that concentrations of LY333531 sufficient to inhibit PKC-ζ could not be accumulated into the cells.

The fact that PMA did not produce an inhibition of the Na^+,K^+-ATPase activity as DA did suggested that DA may be activating a member of the atypical PKC isozymes which are insensitive to phorbol esters [22,26,27]. It has previously been suggested that PKC-ζ may be involved in regulation of Na^+,K^+-ATPase activity [28,29]. To test whether PKC-ζ indeed mediates the effects of DA on Na^+,K^+-ATPase activity, we initially studied the effect of high concentrations of staurosporine. Unlike LY333531, staurosporine does not discriminate between the PKC isozymes [24]. Most PKC isoforms are inhibited by this compound in the nM range, except PKC-ζ which requires μmol concentrations. As anticipated, 10 μM staurosporine was necessary to prevent the inhibitory effect of DA on Na^+,K^+-ATPase activity (Fig. 4). Thus, results with PKC inhibitors LY333531 and staurosporine support the hypothesis that PMA-dependent activation and DA-dependent

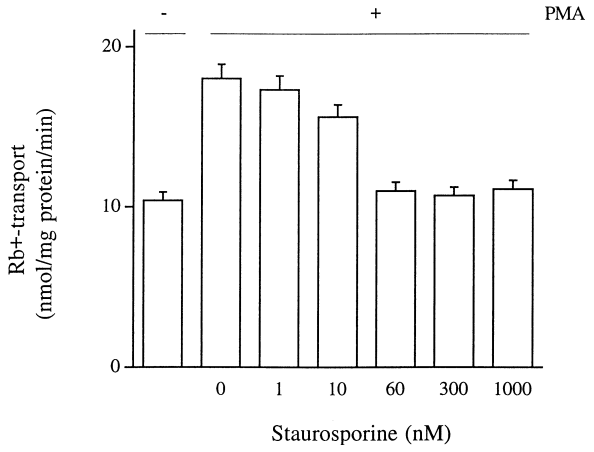

Fig. 3. Effect of various concentrations of staurosporine on PMA-dependent activation of Rb^+-transport.

inhibition of Na^+,K^+-ATPase activity are mediated by PKC-β and PKC-ζ, respectively.

It is known that when PKCs are activated by lipid-derived second messengers, they translocate from the cytosol to the cell membrane. Recently, Dr Mochly-Rosen and collaborators [26,30] have demonstrated that most inactive PKC isozymes are localized to subcellular structures and, upon activation, translocate to new distinct intracellular sites and this is due to their binding to specific anchoring molecules. The anchoring proteins for activated PKC isozymes were termed receptors for activated C-kinase (RACKs). It is then likely that the unique cellular functions of PKCs are determined by the binding of isozymes to specific anchoring molecules in close proximity to particular subsets of substrates and away from others [26,30]. Accordingly, peptides that mimic either the PKC binding site on RACKs or the RACK binding site on PKC are translocation inhibitors of PKC that block the function of the enzyme in vivo [26,30]. Using peptides kindly provided by Dr Mochly-Rosen to inhibit PKC-β and PKC-ζ, we employed the same strategy to investigate whether these isoforms indeed mediate the effects of PMA and DA. The peptides were internalized into OK cells following a protocol previously developed by these investigators [30]. As illustrated in Table 1, the PKC-β-derived inhibitor peptide C2-4 (SLNPEWNET)

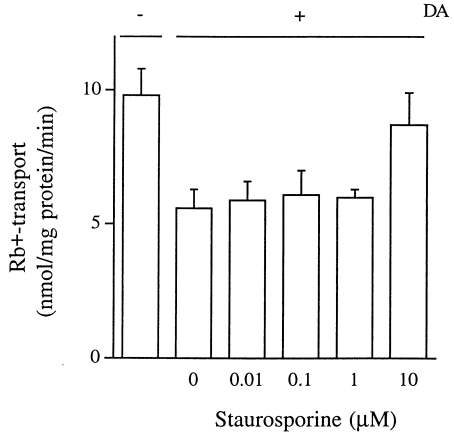

Fig. 4. Effect of various concentrations of staurosporine on DA-dependent inhibition of Rb^+-transport.

Table 1
Effect of PKC isozyme peptide inhibitors on PMA stimulation and DA inhibition of Rb^+-transport

Peptide inhibitors	Rb^+-transport (nmol/mg protein/min)		
	Control	PMA	DA
None	10.1 ± 1.3	17.4 ± 1.7	5.7 ± 0.6
βC2-4	9.9 ± 1.0	11.2 ± 1.5	5.9 ± 1.1
Scrambled	10.4 ± 1.1	18.3 ± 2.2	6.1 ± 0.9
PKC-ζ pseudo	9.7 ± 1.0	16.9 ± 1.3	9.1 ± 1.2

prevents the stimulatory effect of PMA on Na^+,K^+-ATPase activity and has no effect on the inhibitory action elicited by DA. A scrambled peptide (WNPESLNTE) has no effect on either the PMA or DA effect. On the contrary, a peptide derived from the pseudosubstrate region of PKC-ζ isozyme prevents the inhibitory effect of DA on Na^+,K^+-ATPase activity but does not affect the stimulatory action of PMA. These data correlate very well with the results obtained with LY333531 (Fig. 2) and staurosporine (Figs. 3 and 4). The fact that control cells treated with the permeabilizing protocol to introduce the scrambled peptide or none (mock-treated) presented the same level of Rb^+-transport as non-treated cells indicates that the permeabilizing protocol is not producing any deleterious effects that may affect the basal Na^+,K^+-ATPase or its regulation. Our novel finding that PKC-ζ is involved in DA-dependent inhibition of Na^+,K^+-ATPase activity is consistent with the previous published result that wortmannin and LY294009 also prevent this inhibition [9,10,31] since these are inhibitors of PI3K which has been described to be involved in the activation of PKC-ζ [32,33].

The present results help to understand the apparent contradiction in previously published reports concerning the different effects of PKC activation on Na^+,K^+-ATPase activity. Depending on the tissue studied and the experimental conditions, stimulation of PKC could lead to inhibition [20,34–37] or to activation [7,8,16,35,37–44] of Na^+,K^+-ATPase activity. It is therefore likely that these effects are due to activation of different PKC isoforms being able to phosphorylate Na^+,K^+-ATPase in different tissues. This conclusion is further supported by the following observation. Although stimulation of endogenous *Xenopus laevis* oocytes PKC produced inhibition of Na^+,K^+-ATPase-mediated Rb^+-transport, microinjection into oocytes of rodent PKC led to increased Rb^+-transport [37]. Furthermore, rat PKC inhibited the Na^+,K^+-ATPase-mediated Rb^+-transport of *X. laevis* oocytes transfected with rodent α1 but stimulated the endogenous activity [37]. Taken together, these data suggest that stimulation of hormone receptors or direct stimulation of PKC by phorbol esters may activate different PKC isoforms in different tissues. Thus, whether activation, inhibition or no effect on the activity of Na^+,K^+-ATPase is observed could depend on the coincident expression of a specific Na^+,K^+-ATPase α-isoform with a particular isoform of PKC.

In conclusion, phorbol ester and dopaminergic effects on proximal tubule Na^+,K^+-ATPase may be mediated by PKC-β and PKC-ζ, respectively. Although we have previously suggested this [7–9], the present findings are the first experimental evidence that activation and inhibition of the Na^+,K^+-ATPase produced by phorbol esters or DA are the consequence of different PKC isoforms.

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