J. Membrane Biol. 152, 235–243 (1996)

The Journal of
Membrane
Biology

Regulation of Na-K-ATPase Activity in the Proximal Tubule: Role of the Protein Kinase C Pathway and of Eicosanoids

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Received: 7 January 1996/Revised: 24 April 1996

Abstract. To evaluate further the signal transduction mechanisms involved in the short-term modulation of Na-K-ATPase activity in the mammalian kidney, we examined the role of phospholipase C-protein kinase C (PLC-PKC) pathway and of various eicosanoids in this process, using microdissected rat proximal convoluted tubules. Dopamine (DA) and parathyroid hormone (either synthetic $PTH_{1\text{-}34}$ or $PTH_{3\text{-}34}$) inhibited Na-K-ATPase activity in dose-dependent manner; this effect was reproduced by $PKC_{530\text{-}558}$ fragment and blocked by the specific PKC inhibitor calphostin C, as well as by the PLC inhibitors neomycin and U-73122. Pump inhibition by DA, PTH, or arachidonic acid, and by PKC activators phorbol dibutyrate (PDBu) or dioctanoyl glycerol (DiC8) was abolished by ethoxyresorufin, an inhibitor of the cytochrome P450-dependent monooxygenase pathway, but was unaffected by indomethacin or nordihydroguaiaretic acid, inhibitors of the cyclooxygenase and lipoxygenase pathways of the arachidonic acid cascade, respectively. Furthermore, each of the three monooxygenase products tested (20-HETE, 12(R)-HETE, or 11,12-DHT) caused a dose-dependent inhibition of the pump. The effect of DA, PTH, PDBu or DiC8, as well as that of 20-HETE was not altered when sodium entry was blocked with the amiloride analog ethylisopropyl amiloride or increased with nystatin.

We conclude that short-term regulation of proximal tubule Na-K-ATPase activity by dopamine and parathyroid hormone occurs via the PLC-PKC signal transduction pathway and is mediated by cytochrome P450-dependent monooxygenase products of arachidonic acid metabolism, which may interact with the pump rather than alter sodium access to it.

Key words: Na:K pump — Dopamine — Parathyroid hormone — Phospholipase C — Protein kinase C — Cytochrome P450-monooxygenase

Introduction

The rapid regulation of Na-K-ATPase activity in transporting epithelia has been attributed mainly to changes in cell sodium concentration, which under basal conditions is rate-limiting for the pump[1] in intact cells [20, 38]. There is now considerable evidence, however, that in addition to such changes short-term (e.g., within $\leqslant$30 min) alterations in pump activity are mediated by complex intracellular signal transduction networks whose effects are tissue- and cell-specific, and which can produce distinct—and sometimes even opposite—effects in a given tissue or cell line in response to different stimuli or prevailing conditions. Examples of such regulation include modulation of Na-K-ATPase activity by agents that alter cell cyclic AMP content and thus protein kinase A activity, the levels and distribution of protein kinase C, or the generation of various eicosanoids [6].

Activation of adenylyl cyclase or addition of exogenous cAMP inhibits Na-K-ATPase activity in distal nephron segments [3, 9, 39], as well as in nonrenal tissues (*reviewed in* [6]). We have reported that activation of the cyclic AMP-protein kinase A (cAMP-PKA) pathway, and consequently the stimulation of phospholipase A_2 and arachidonic acid (AA) release, mediate short-

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[1] ''Na-K-ATPase'' and ''Na:K pump'' are used interchangeably in this paper.

term Na:K pump regulation in cortical collecting ducts and medullary thick ascending limbs of Henle's loop [32, 34]. In these nephron segments mechanisms of pump regulation by various classes of eicosanoids differed, as products of the cytochrome P450-monooxygenase pathway seemed to interact with the pump, whereas cyclooxygenase metabolites probably altered its activity by inhibiting sodium entry into tubule cells [33].

In contrast to the distal nephron, however, activation of the cAMP-PKA pathway alone did not alter Na-K-ATPase activity in the proximal convoluted tubule (PCT) [34]. It has been proposed by us and others [4, 7, 34] that pump regulation in this region of the nephron is mediated by the phospholipase C-protein kinase C pathway, although the precise mechanisms involved are not fully understood. For example, Ribeiro et al. [29] have recently demonstrated a role for 20-hydroxyeicosatetraenoic acid (20-HETE) in the downregulation of PCT Na-K-ATPase activity by PTH, but it is unclear whether this mechanism is shared by dopamine and other agents, and whether modulation of pump activity by the various PKC agonists is a primary effect or is secondary to alterations in apical sodium entry. The purpose of this study was therefore to identify the arachidonic acid metabolic pathways involved in the PKC-dependent Na-K-ATPase regulation in the proximal tubule, and to explore the role of altered sodium availability in this phenomenon. Studies aimed at verifying the participation of the PLC-PKC signaling pathway in PCT pump regulation were also performed.

Materials and Methods

Microdissection

Kidneys were obtained from male Sprague-Dawley rats weighing 200–300 g which had free access to regular laboratory chow and tap water. The procedures for tubule microdissection have been reported previously in detail [22]. Briefly, the left kidney was perfused *in situ* through a catheter placed in the left renal artery with a modified Hanks' buffered salt solution (HBSS) containing (in mM): 137 NaCl, 5 KCl, 0.8 $MgSO_4$, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 1 $MgCl_2$, 10 TRIS-HCl, 1 $CaCl_2$ at pH 7.4, with 400 U/ml collagenase (Type I, Sigma Chemical, St. Louis, MO) and 0.1% bovine serum albumin. The kidney was removed and cut along the corticopapillary axis into pyramids, which were incubated in the same collagenase solution at 37°C for 5 min. After incubation the pyramids were rinsed with cold modified HBSS (0.25 mM $CaCl_2$), and PCT segments were dissected freehand in this medium at 4°C under stereomicroscopic observation. Isolated PCT segments were individually transferred to a concave bacteriological slide and photographed to determine their length.

To obtain tubules with patent lumina (for experiments using EIPA [ethylisopropylamiloride[) animals received by jugular vein infusion an agarose solution (1 ml/100 g body wt) containing (wt/vol) 0.45% NaCl, 10% mannitol, and 2% agarose (Type IX, Sigma), prewarmed to 37°C [8]. At the onset of osmotic diuresis the left ureter was ligated and the left kidney was perfused with prewarmed collagenase solution in 1% agarose. The renal pedicle was then ligated and the kidney was removed and immersed in cold HBSS for several minutes (thus converting the intraluminal agarose from liquid to gel phase) before processing the tubules as described above.

Determination of Na-K-ATPase Activity

Tubules were incubated at room temperature for 30 min in 1 μl HBSS (0.25 mM $CaCl_2$) supplemented with 5 mM glucose, 2 mM Na acetate, and 5 mM Na lactate, with or without agonists and modifiers of Na entry such as EIPA or nystatin [33]. In experiments using $PKC_{530\text{-}558}$, a 29-residue fragment of the kinase's catalytic domain, tubules were exposed to a small concentration (10^{-7} M) of digitonin to ensure its access to the cells' interior. Procedures for the Na-K-ATPase assay followed, with minor modifications, protocols described previously in detail [22]. Tubules were permeabilized in a hypotonic solution (10 mM TRIS, pH 7.4), followed by rapid freezing on dry ice. After thawing and washing, total ATPase activity was determined during 15 min incubation at 37°C in a 1 μl droplet containing (in mM); 50 NaCl, 5 KCl, 10 $MgCl_2$, 1 EGTA, 100 TRIS-HCl, 10 Na_2ATP (grade II, vanadate-free, Sigma) and [γ-^{32}P]ATP (Amersham, Arlington Heights, IL) in tracer amounts (~5 nCi/μl). Magnesium-dependent ATPase activity was determined in the same solution containing 4 mM ouabain. Phosphate liberated by the hydrolysis of [γ-^{32}P]ATP was separated by filtration after adsorption of the unhydrolyzed nucleotide on activated charcoal (Sigma), and the radioactivity was counted in a liquid scintillation spectrometer (Packard Instruments, United Technologies, Downers Grove, IL).

Total and Mg-dependent ATPase activity were each determined on four or five replicate samples from individual animals and calculated per millimeter tubule length. Ouabain-inhibitable ATPase was taken as the difference between the means of each group of measurements, and thus represents a single datum point in each animal. To minimize variability between experiments, Na-K-ATPase activity was always measured in experimental and control tubules simultaneously.

Materials

Eicosanoids were purchased from Cayman Chemical, Ann Arbor, MI, or from Sigma; NDGA (nordihydroguaiaretic acid) from Aldrich Chemical, Milwaukee, WI; and U-73122 from Biomol Research Laboratories, Plymouth Meeting, PA. EIPA was a kind gift from Merck Sharp & Dohme Research Laboratories, West Point, PA. All other chemicals, including dopamine, synthetic $PTH_{1\text{-}34}$ and $PTH_{3\text{-}34}$, and $PKC_{530\text{-}558}$ fragment were obtained from Sigma. Dimethyl sulfoxide (DMSO) was used as a solvent for PDBu, DiC8 and calphostin C, whereas eicosanoids and inhibitors of arachidonate metabolism were dissolved in ethanol. Final concentrations of DMSO and ethanol during incubation were <0.1% and <0.5%, respectively, and were shown not to affect the Na-K-ATPase activity in preliminary experiments using these solvents alone.

Statistics

Statistical analysis was done with one-way analysis of variance followed by the Bonferroni correction for multiple comparisons. Results in text and figures are means ± SE.

Results

Dopamine and $PTH_{1\text{-}34}$ produced a dose-dependent inhibition of the pump (Fig. 1, solid symbols). Because

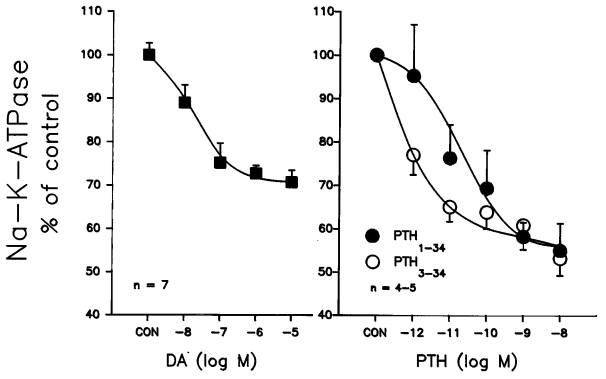

Fig. 1. Dose-dependent inhibition of Na-K-ATPase activity by dopamine and parathyroid hormone analogues. PTH_{3-34}, an analogue that stimulates protein kinase C but not adenylyl cyclase in this nephron segment, was at least as effective as PTH_{1-34}.

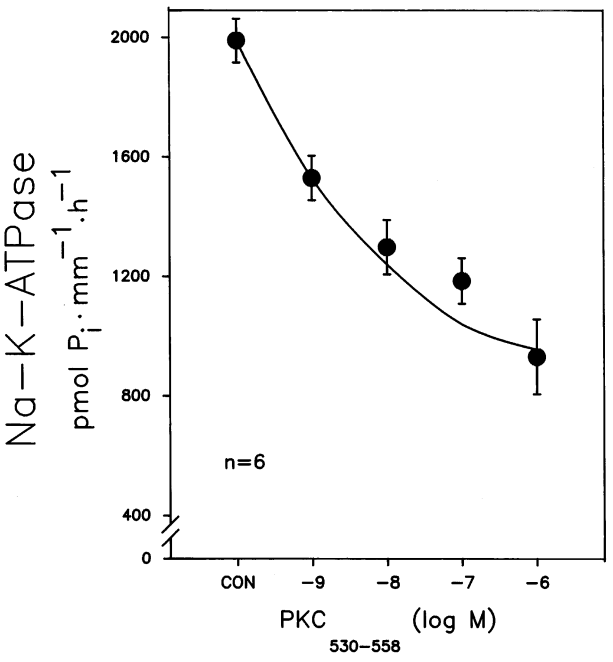

Fig. 2. Dose-dependent inhibition of Na-K-ATPase activity by $PKC_{530-558}$.

both hormones can activate either adenylyl cyclase or phospholipase C in the proximal tubule [10, 14], we also utilized PTH_{3-34}, an analogue that lacks the first two residues at the amino terminal required for stimulation of adenylyl cyclase [30], and consequently PKA, but does activate PKC [19]. That the effect of PTH is probably mediated through the PLC-PKC pathway is supported by observations that PTH_{3-34} also produced a dose-dependent inhibition of Na-K-ATPase (Fig. 1, open symbols), which in the physiologic range was more potent than that of PTH_{1-34}. A similar, dose-related inhibition of Na-K-ATPase activity was observed with the $PKC_{530-558}$ fragment (Fig. 2).

Further support for the hypothesis that the PLC-PKC cascade is involved in modulation of pump activity in the proximal tubule was obtained using inhibitors of this pathway (Fig. 3). The left panels show control experiments, and the right panels similar experiments with the inhibitor added. Neomycin and U-73122, two inhibitors of phospholipase C [36, 37], completely abolished the effect of dopamine and PTH[2] on the pump, as did calphostin C, a specific inhibitor of protein kinase C.

Protein kinase C stimulates phospholipase A_2 (PLA_2) [28, 41], which leads to increased AA release. Arachidonic acid is converted to active metabolites by three pathways that utilize, respectively, cytochrome P450-dependent monooxygenases, lipoxygenase, or cyclooxygenase. In these studies we used a selective inhibitor of each of these pathways (Fig. 4), and to avoid cross-inhibition we chose lower concentrations of each, shown previously to be effective [33]. The upper left panel again shows control experiments, without the addition of inhibitor. The effect of dopamine, PTH, phorbol dibutyrate, dioctanoyl glycerol or arachidonic acid on Na-K-ATPase activity was completely blocked by coincubation with ethoxyresorufin, an inhibitor of the cytochrome P450-monooxygenase pathway (upper right). In contrast, their effect was unaltered by inhibitors of the cyclooxygenase and lipoxygenase pathways [with indomethacin and NDGA, respectively (bottom panels)]. Pump inhibition by $PKC_{530-558}$ was also completely abolished by ethoxyresorufin (Fig. 5). These results suggest that P450-monooxygenase products are the eicosanoids that mediate the effects of dopamine, PTH, and other PKC agonists on Na-K-ATPase activity in the PCT.

Figure 6 shows the effect of various P450-monooxygenase-derived metabolites. All inhibited Na-K-ATPase (although in these experiments 5,6-EET [epoxyeicosatrienoic acid] did not quite reach statistical significance), and the largest effect was observed with 20-HETE, the major P450-monooxygenase metabolite in the proximal tubule [23]. Furthermore, all three cytochrome P450-monooxygenase products tested (20-HETE; 11,12-DHT [dihydroxyeicosatrienoic acid]; and 12(R)-HETE) caused a marked, dose-dependent inhibition of the pump (Fig. 7).

Finally, to evaluate further the mechanism(s) involved in pump inhibition by the signaling pathways described, we examined the effect of the various agonists in circumstances in which sodium availability was concurrently altered (i) by EIPA, which decreases sodium entry by inhibiting the sodium:proton exchanger, or (ii) by nystatin, an ionophore that renders the tubules per-

[2] When not indicated otherwise PTH denotes PTH_{1-34}.

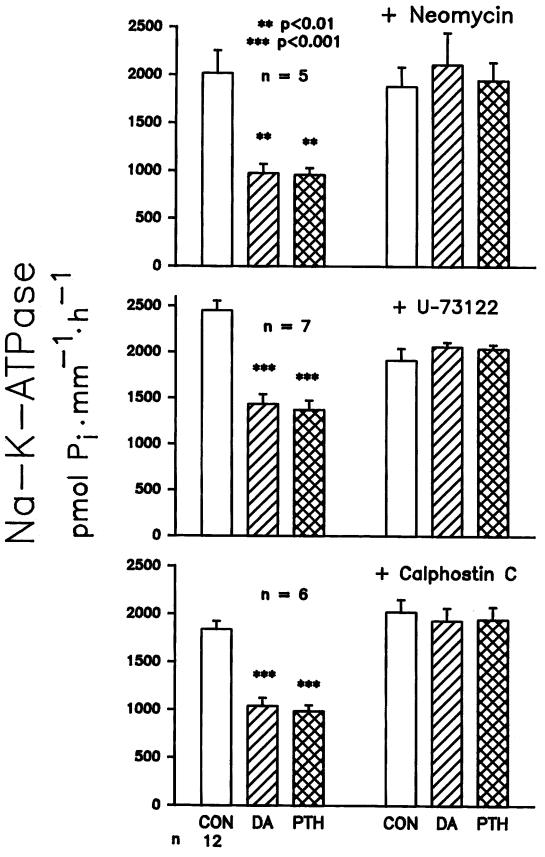

Fig. 3. Na:K pump inhibition by either dopamine (10^{-5} M) or PTH (10^{-8} M) is blocked by the phospholipase C antagonists neomycin (5×10^{-4} M) and U-73122 (10^{-5} M), and by the PKC antagonist calphostin C (10^{-7} M).

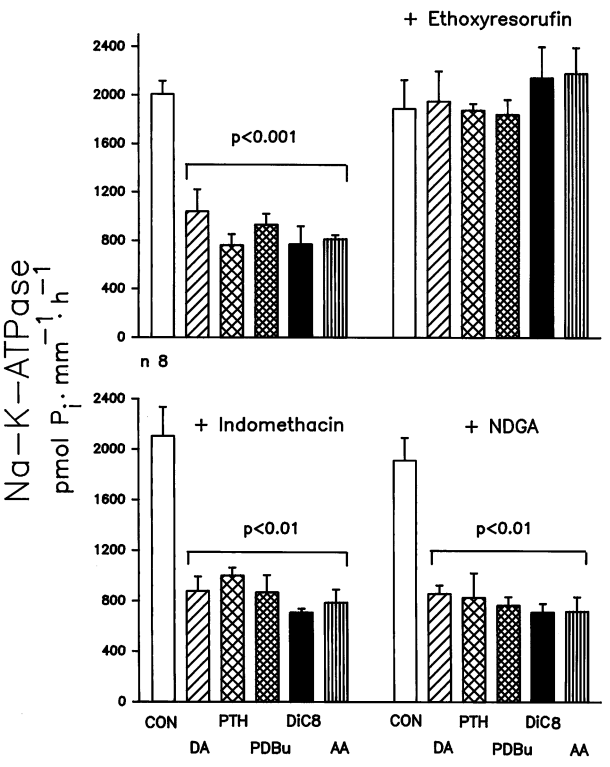

Fig. 4. Effect of inhibitors of the three main arachidonate metabolic pathways on the action of dopamine, PTH, PKC agonists (PDBu, 10^{-6} M, DiC8, 10^{-4} M), and arachidonic acid (AA, 10^{-5} M) on the Na:K pump (all $n = 4$, except as noted). Inhibition of the cytochrome P450-dependent monooxygenase (ethoxyresorufin, 10^{-7} M) abolished the effect of each PKC agonist, whereas inhibitors of cyclooxygenase (indomethacin, 10^{-7} M) or lipoxygenase (NDGA, 10^{-6} M) did not alter it.

meable to sodium so that it is no longer rate-limiting for the pump. Coincubation of dopamine, PTH, and PKC agonists with either EIPA or nystatin did not affect their action on pump activity (Fig. 8). Similarly, coincubation of either modifier of sodium entry with 20-HETE (Fig. 9)—as well as with other cytochrome P450 metabolites (*not shown*)—also did not alter their inhibition of the pump, suggesting that this effect occurs independently of alterations in intracellular sodium concentrations.

Discussion

This study describes intracellular signaling pathways involved in the modulation of proximal convoluted tubule Na-K-ATPase activity by two structurally and functionally dissimilar hormones, dopamine and PTH. The results suggest that these pathways may represent a general signal transduction mechanism responsible for the short-term regulation of Na:K pump activity in this nephron segment, which includes the sequential activation of phospholipase C, protein kinase C, and phospholipase A_2, and the ensuing stimulation of the arachidonic acid cascade. Products of cytochrome P450-dependent monooxygenase pathway of arachidonate metabolism appear to play a critical role in this regulation, which occurred independently of altered Na entry into the PCT cell. The proposed signaling mechanisms responsible for Na:K pump regulation by dopamine and PTH in the PCT are illustrated in Fig. 10.

Dopamine inhibits Na-K-ATPase activity in the proximal convoluted tubule [2, 4, 34], as does parathyroid hormone [29, 30, 34]. Both dopamine and PTH can utilize a dual signal transduction cascade, i.e., their receptors link to adenylyl cyclase as well as to phospholipase C in proximal tubule cells [10, 14]. Stimulation of adenylyl cyclase and cAMP-dependent protein kinase, by itself, does not bring about short-term Na:K pump regulation in the PCT [30, 34], and will not be discussed further. There is, on the other hand, growing evidence for activation of the phospholipase C-protein kinase C pathway by either dopamine or PTH in the proximal tubule. Thus dopamine, acting via its DA-1 receptor, stimulates PLC activity in rat renal cortical slices and

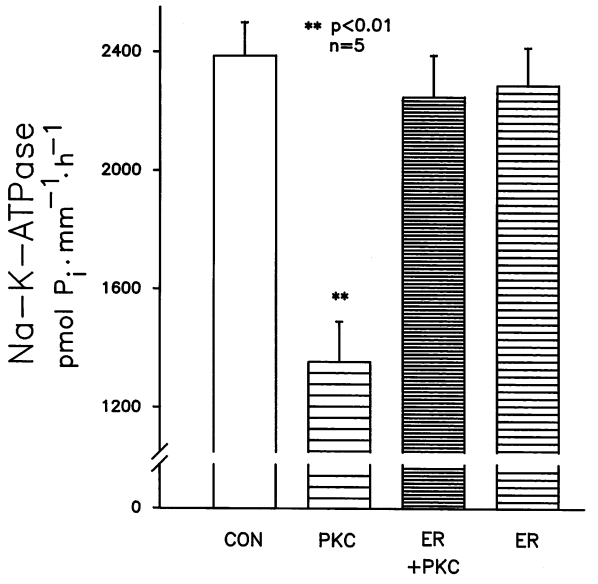

Fig. 5. Inhibition of Na-K-ATPase activity by $PKC_{530-558}$ is completely blocked by ethoxyresorufin (ER, 10^{-7} M). ER alone had no effect.

plasma membranes [12, 40], independently of adenylyl cyclase [14]. Likewise, PTH stimulates phospholipase C and consequently inositol triphosphate and diacylglycerol formation in renal tubular cells, a process that is not dependent on (and may be even inhibited by) cyclic nucleotides [10, 17]. The PLC-PKC pathway modulates important PTH effects in bone and kidney [10], including stimulation of 1,25-dihydroxyvitamin D_3 secretion by proximal tubule cells [18]. It has been postulated that a single receptor stimulates intracellular accumulation of both cAMP and inositol triphosphates, i.e., when coupled to PTH can initiate either of the two signal transduction cascades [1].

Several groups have suggested that signal transduction via the PKC may account for the modulation of Na-K-ATPase activity in the proximal tubule [4, 30, 34]. In the first part of this study we sought therefore to extend our previous observations [34] on the postulated role of the PLC-PKC pathway in pump regulation in this nephron segment. Although neomycin (and other aminoglycoside antibiotics) are effective inhibitors of phospholipase C in both brush border membranes and cytosol of proximal tubules [24, 36], we also examined the effect of U-73122, a potent novel inhibitor of phospholipase C that has no direct effect on PKC [7, 37]. Either neomycin or U-73122 effectively antagonized the inhibition of Na-K-ATPase activity induced by dopamine and parathyroid hormone, as did in a quantitatively similar fashion the specific PKC inhibitor calphostin C (Fig. 3). To confirm the role of protein kinase C, we also tested the effect of $PKC_{530-558}$ fragment, a peptide sequence corresponding to part of the catalytic domain of PKC that potently activates this kinase and mimics its effects [16]. The PKC fragment produced a comparable inhibition of Na-K-ATPase activity to that previously seen with phorbol esters and dioctanoyl glycerol [34], and the effect was dose-dependent (Fig. 2). Further, we demonstrated (Fig. 1) a strong concentration-dependent inhibition of the pump by PTH_{3-34}, an analogue that stimulates PKC activity but not cAMP formation or PKA activity [19], and indeed inhibits PTH_{1-34}-induced cAMP accumulation in PCT [30].

Although the foregoing analysis leaves little doubt about the role of the PLC-PKC pathway in the regulation of proximal nephron Na-K-ATPase activity, the manner whereby PKC activation translates into an effect on the pump remains unclear. The main purpose of this study was therefore to examine the events that *follow* stimulation of PKC activity, specifically the role of various eicosanoids in Na:K pump regulation in the proximal tubule. Protein kinase C stimulates phospholipase A_2 in cultured kidney cells [28, 41], and products of arachidonic acid metabolism mediate the inhibition of Na-K-ATPase activity by several hormones and autacoids in the distal nephron, albeit through mechanisms that involve stimulation of the cAMP-PKA pathway [9, 33]. Whereas earlier steps in signal transduction (the cell membrane effector and the protein kinase involved) differ in the PCT, work by us and others [23, 29, 34] suggests that eicosanoids participate in pump regulation in this segment as well.

To define the arachidonate products participating in Na-K-ATPase regulation we used inhibitors of each of its three main metabolic pathways. These experiments indicated that ethoxyresorufin, a relatively specific inhibitor of cytochrome P450-dependent monooxygenase [27], completely blocked the inhibition of Na-K-ATPase, whereas inhibitors of cyclooxygenase and lipoxygenase (indomethacin and NDGA, respectively) had no effect (Fig. 4). While these results suggest that only the monooxygenase pathway partakes in pump regulation, it is recognized that inhibitors of arachidonate metabolism are selective, but not entirely specific, for any given pathway (though this problem is generally manifest at concentrations higher than those utilized in this study). Thus while we can not exclude with certainty the possible participation of other arachidonate products in the signaling processes described, other experiments provide more direct evidence for a major role of cytochrome P450-monooxygenase pathway in pump regulation in the PCT: various metabolites of this pathway inhibited Na-K-ATPase significantly (Fig. 6), and in the case of those so tested, in dose-dependent manner (Fig. 7).

The cytochrome P450-dependent system metabolizes arachidonic acid to oxygenated compounds in the renal cortex [26, 27], and arachidonate products generated through this pathway (HETE isomers, EETs, or

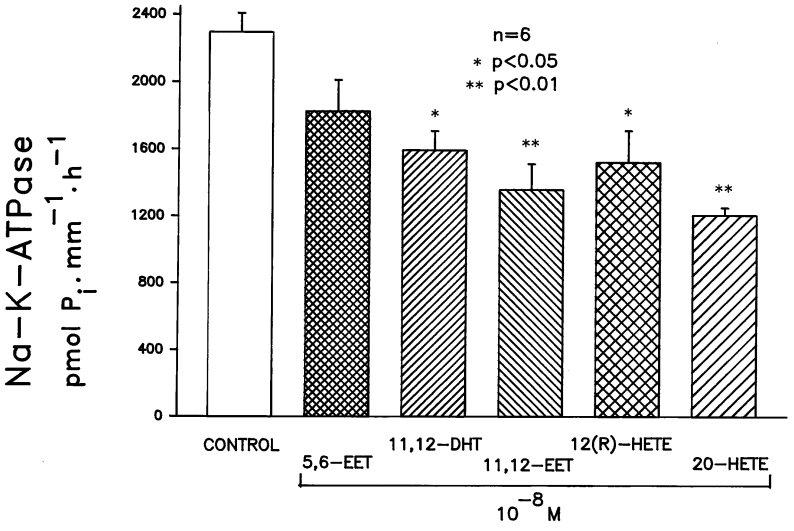

Fig. 6. Effect of various cytochrome P450-dependent monooxygenase products (all, 10^{-8} M) on Na-K-ATPase activity.

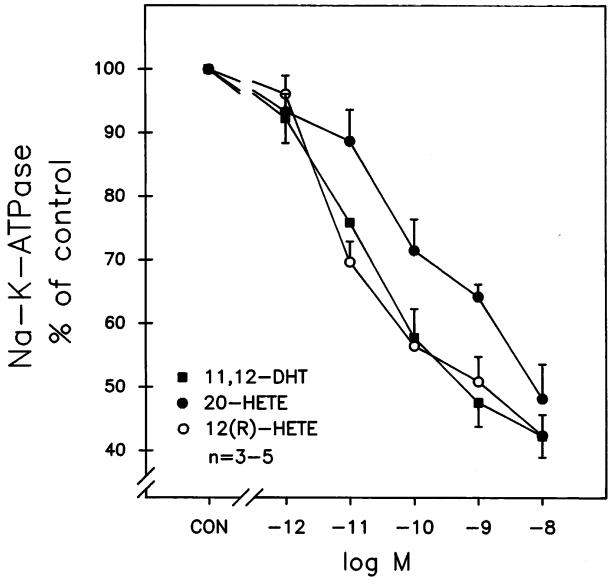

Fig. 7. Principal metabolites of the cytochrome P450-dependent monooxygenase pathway inhibit Na-K-ATPase activity in dose-dependent manner.

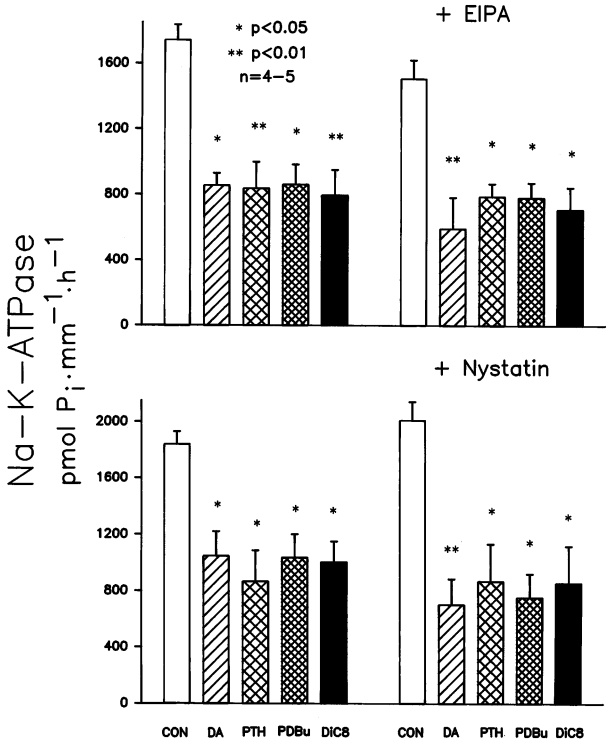

Fig. 8. Inhibition of Na-K-ATPase activity by dopamine, PTH, and PKC agonists is not altered when sodium entry is blocked by EIPA (10^{-6} M) or increased by nystatin (50 μg/ml).

DHTs) inhibit renal Na-K-ATPase activity [9, 11, 29, 31, 33, 35]. We and others have described earlier this effect in distal nephron segments [9, 11, 33, 35], where the arachidonic acid cascade is triggered by PKA activation. The present study demonstrates that dopamine and PTH modulate Na:K pump activity in the proximal nephron, where their effect is mediated through cytochrome P450-monooxygenase products and depends on activation of the PLC-PKC pathway. With respect to the effect of PTH our results are in agreement with those recently reported by Ribeiro et al. [29], who utilized a different PCT preparation and demonstrated that Na-K-ATPase inhibition by PTH_{3-34} is mediated via increased arachidonic acid release and production of the cytochrome P450 metabolite 20-HETE.

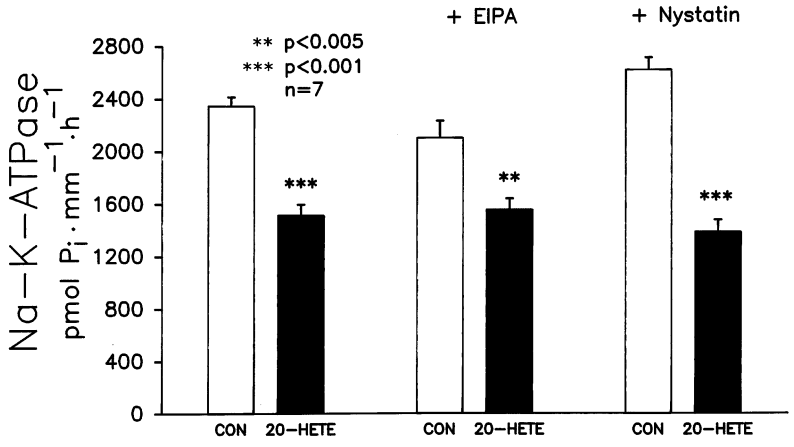

Fig. 9. Inhibition of Na-K-ATPase activity by 20-HETE (10^{-8} M), the principal cytochrome P450-dependent monooxygenase product in the PCT, is not affected when sodium entry is altered by EIPA or nystatin (concentrations as in Fig. 8).

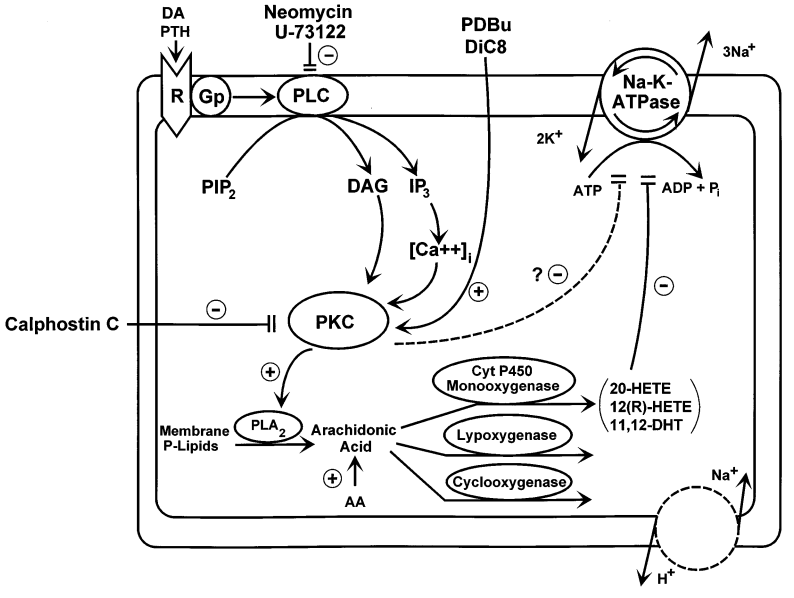

Fig. 10. Schematic representation of the proposed cellular mechanisms involved in Na-K-ATPase activity regulation by dopamine and PTH in the PCT. A possible direct interaction (?phosphorylation) with the pump by PKC, not examined in this study, is depicted as a broken line.

Because both dopamine and PTH can inhibit the proximal tubule Na:H exchanger [13, 21], we last addressed the question whether inhibition of Na-K-ATPase activity by these two agents is due to altered sodium entry into the PCT cell. The effect of either hormone was unchanged when the incubation was carried out in the presence of EIPA, a selective inhibitor of the Na:H antiporter [15] that probably limited Na entry, or of the Na ionophore nystatin, which by increasing sodium entry renders this cation no longer rate-limiting for the pump. These observations may reflect the fact that inhibition of the antiporter is mediated through activation of adenylyl cyclase and increased cell cyclic AMP [13, 21], a mechanism that seems not to be involved in the signaling events described in this work. Thus the arachidonic acid metabolites generated via the cytochrome P450-dependent monooxygenase pathway probably interact with the pump itself or through additional steps that remain elusive. Such regulation via eicosanoids does not exclude a role for pump phosphorylation by PKC [5, 25]—a possibility not examined in this study; indeed, the two mechanisms may operate in parallel in the intact cell [6].

We thank Bradley Hack for excellent technical assistance. This work

was supported in part by institutional grants from the University of Chicago and St. Marianna University, Kawasaki, Japan.

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