Role of 20-HETE in D1/D2 dopamine receptor synergism resulting in the inhibition of Na\(^+\)-K\(^+\)-ATPase activity in the proximal tubule

Carolina Kirchheimer,1 Carlos F. Mendez,2 Andrea Acquier,2 and Susana Nowicki1

1Centro de Investigaciones Endocrinológicas (CEDIE-CONICET) and 2Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

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Address for reprint requests and other correspondence: S. Nowicki, CEDIE-CONICET, Gallo 1330, C1425EFD Buenos Aires, Argentina (e-mail: snowicki@cedie.org.ar).

Am J Physiol Renal Physiol 292: F1435–F1442, 2007. First published January 30, 2007; doi:10.1152/ajprenal.00176.2006.—Previous studies propose 20-hydroxyeicosatetraenoic acid (20-HETE), a major arachidonic acid metabolite of cytochrome P-450 (CYP), as a possible mediator of Na\(^+\)-K\(^+\)-ATPase inhibition by dopamine (DA). The aim of this study was to investigate the intracellular mechanisms involved in this effect and to elucidate the DA receptor associated with the 20-HETE pathway in the rat kidney. DA (10\(^{-5}\) M) inhibited Na\(^+\)-K\(^+\)-ATPase activity in microdissected tubular segments to 59.4 ± 3.8% of control activity. This response was suppressed by the CYP4A inhibitor 17-octadecynoic acid (10\(^{-6}\) M), which had no effect per se, thus confirming the participation of CYP arachidonic acid metabolites in DA-induced Na\(^+\)-K\(^+\)-ATPase inhibition. We next examined whether 20-HETE is involved in the signaling pathways triggered by either D\(_1\) or D\(_2\) receptors. Neither fenoldopam nor quinpirole (D\(_1\) agonists, respectively, both 10\(^{-5}\) M) modified Na\(^+\)-K\(^+\)-ATPase activity when tried alone. However, coincubation of a threshold concentration of 20-HETE (10\(^{-9}\) M) with fenoldopam resulted in a synergistic inhibition of Na\(^+\)-K\(^+\)-ATPase activity (66 ± 2% of control activity), while 20-HETE plus quinpirole had no effect. Furthermore, 20-HETE (10\(^{-9}\) M) synergized with forskolin (10\(^{-8}\) M) with the diacylglycerol analog 1-oleoyl-2-acetoyl-glycerol (OAG; 10\(^{-11}\) M; 62.0 ± 5.3 and 69.9 ± 2.0% of control activity, respectively), indicating a cooperative role of 20-HETE with the D\(_1\)-triggered pathways. In line with these results, no additive effect was observed when OAG and 20-HETE were combined at concentrations which per se produced maximal inhibition (10\(^{-6}\) M). These results demonstrate that the inhibition of Na\(^+\)-K\(^+\)-ATPase activity by DA in the proximal tubule may be the result of the synergism between 20-HETE and the D\(_1\) signaling pathway.

Na\(^+\)-K\(^+\)-ATPase provides the gradient for Na\(^+\) transport and thus for the activity of several Na\(^+\)-coupled transporters across the cell membrane. The extremely delicate mechanisms implicated in the regulation of the pump involve a variety of signaling molecules (16). Although the cellular mechanisms responsible for the regulation of Na\(^+\)-K\(^+\)-ATPase activity are not completely understood, it is clear that most intracellular pathways converge in the activation of protein kinases (11, 38).

Locally formed dopamine (DA) is a physiological inhibitor of renal Na\(^+\)-K\(^+\)-ATPase. In fact, numerous studies have demonstrated that DA induces a large increase in urinary sodium excretion that is dependent on the inhibition of tubular sodium reabsorption (1).

In the proximal tubule, stimulation of D\(_1\) receptors is linked to both activation of adenyl cyclase and of phospholipase C. The activation of adenyl cyclase leads to the formation of cAMP and subsequent activation of protein kinase A, whereas activation of phospholipase C leads to the generation of inositol phosphate and diacylglycerol (DAG), a physiological PKC activator (19). Additionally, it has been reported that D\(_1\) receptor-mediated activation of PKC stimulates phospholipase A\(_2\), which in turn releases arachidonic acid from membrane phospholipids (36).

The stimulation of D\(_2\)-like receptors causes a decrease in cAMP via Gi proteins. Recent studies have also revealed the involvement of a tyrosine kinase-p44/42 MAPK pathway following D\(_2\)-like receptor stimulation in renal proximal tubules (25). It has also been reported that stimulation of D\(_2\) receptors augments signaling involving arachidonic acid (7). In the inner medulla, a DA receptor termed DA\(_2K\) has been linked to the stimulation of phospholipase A\(_2\), leading to the release of arachidonic acid from membrane phospholipids (21, 22).

In the rat kidney, arachidonic acid is mainly metabolized to 20-hydroxyeicosatetraenoic acid (20-HETE), a ω-hydroxylated product of the cytochrome P-450 (CYP) pathway. The CYP isoforms responsible for the synthesis of 20-HETE are CYP4A1, 4A2, 4A3, and 4A8 which are localized in different segments of the rat nephron, including the proximal tubule (27). The heterogeneous effects of 20-HETE at different sites within the kidney are becoming increasingly apparent. On the one hand, 20-HETE formed in renal arterioles has a vasconstrictor role, supporting a prohypertensive action. On the other hand, 20-HETE formed in renal tubules inhibits Na\(^+\)-K\(^+\)-ATPase, the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, and HCO\(_3\) reabsorption, promoting salt excretion and diuresis in favor of an antihypertensive role (40).

Similarly as for DA (38), the inhibition of Na\(^+\)-K\(^+\)-ATPase activity by 20-HETE is dependent on a PKC-regulated pathway (34). Moreover, previous studies suggest 20-HETE as a possible mediator of Na\(^+\)-K\(^+\)-ATPase inhibition in response to DA (30, 36). In the present paper, the intracellular mechanisms involved in DA/20-HETE interaction for the inhibition of Na\(^+\)-K\(^+\)-ATPase are investigated. Our results show that in the proximal tubule, 20-HETE synergizes with the intracellular pathways triggered by D\(_1\) receptor stimulation for the inhibition of Na\(^+\)-K\(^+\)-ATPase activity.
MATERIALS AND METHODS

Animals. Male Wistar rats, aged 40–45 days with body weight of 150–180 g, were used for the experiments. They were kept at 22°C on a 12:12-h dark-light cycle and given free access to normal rat diet and tap water. The experiments were performed according to the guidelines recommended by the National Institute of Health and approved by the Institutional Ethical Committee.

Microdissection of single tubular segments. Proximal tubule segments (length 0.5–1 mm) were dissected from collagenase (0.05%)-perfused kidneys as described elsewhere (4). Each tubule segment was incubated at room temperature in oxygenated modified Hanks’ solution (MHS) containing (in mM) 137 NaCl, 5 KCl, 0.8 MgSO4, 0.33 Na2HPO4, 0.44 KH2PO4, 0.25 CaCl2, 1 MgCl2, 10 Tris-HCl, pH 7.4. Butyrate (10−5 M) was added to the solution to optimize mitochondrial respiration. Tubules were preincubated for 30 min in a final volume of 1 μl MHS alone (control tubules) or MHS plus 20-HETE, DA, 17-octadecynoic acid (17-ODYA), fenoldopam, quinpirole, forskolin, H-89, or 1-oleyl-2-acetoyl-sn-glycerol (OAG), at the indicated concentrations.

Determination of Na+/K+-ATPase activity. Na+/K+-ATPase activity was measured as ouabain-sensitive ATP hydrolysis (4). Segments were made permeable by a rapid hypotonic shock plus freezing and thawing. This procedure allows ATP and sodium free access to the interior of the cell. All Na+/K+-ATPase activity assays were performed in the presence of saturating concentrations of major substrates (70 mM Na+, 5 mM K+, 10 mM ATP). For the determination of total Na+/K+-ATPase activity, segments were incubated for 15 min at 37°C in a medium containing the following (in mM): 50 NaCl, 5 KCl, 10 MgCl2, 1 EGTA, 100 Tris-HCl, 10 Na2ATP, and 2–5 Ci/mmol [γ-32P]ATP in tracer amounts (5 nCi/μl), pH 7.4. For determination of ouabain-insensitive ATPase activity, NaCl and KCl were omitted and Tris-HCl (150 mM) and ouabain (1 mM) were added. The phosphate released by the hydrolysis of [γ-32P]ATP was separated by filtration through a Millipore filter after adsorption of the unhydrolyzed nucleotide on activated charcoal. Na+/K+-ATPase activity was noted as the difference between the total and the ouabain-insensitive ATPase activity and was expressed as picomoles of 32P hydrolyzed per millimetre tubule per hour. Results are given as the percentage of control.

Analysis of Na+/K+-ATPase phosphorylation state. Proximal tubule cells were prepared as previously described (6). Freshly isolated cells in suspension were preincubated for 20 min in a shaking bath at 37°C in Krebs buffer with or without (control) the addition of the CYP4A inhibitor 1-amino-2-naphthoxypropene (ABT; 5 × 10−4 M) (42), and further incubated for 3 min in the presence or absence of DA (10−5 M) or 20-HETE (10−6 M). In another set of experiments, cells were incubated for the same period of time in the absence (control) or the presence of 10−11 M OAG, 10−9 M 20-HETE, or in the combination of both.

Following incubations, cells were lysed as described in Analysis of Na+/K+-ATPase phosphorylation state with the exception that FK506 was excluded from the lysis buffer. Cell lysates were first centrifuged for 5 min at 8,000 g and the supernatants were further centrifuged at 100,000 g for 1 h at 4°C. The supernatant was designated as the cytosolic fraction. An aliquot of each fraction was collected for protein determination using the method of Bradford. Equal amounts of protein from each fraction (5 μg) were separated by SDS-PAGE (7.5%), transferred to PVDF membranes, and immunostained with rabbit polyclonal anti PKC α or PKC β (1:500, Santa Cruz Biotechnology). Bands were detected by chemiluminescence and quantified as described under Analysis of Na+/K+-ATPase phosphorylation state.

Statistics. Data are shown as means ± SE. All data were analyzed by one-way ANOVA. Unless specified, Dunnett’s test was used as post hoc test. A P value <0.05 was considered a priori as statistically significant. Calculations of EC50 were performed using a sigmoidal nonlinear regression program (GraphPad Prism 4 software, San Diego, CA).

RESULTS

Na+/K+-ATPase activity in proximal tubules. The dose dependence of 20-HETE effect on Na+/K+-ATPase activity in isolated proximal tubule segments was first analyzed. In our experimental conditions, 20-HETE (10−9 to 10−5 M, 30 min) inhibited Na+/K+-ATPase activity in a dose-dependent manner, with a threshold concentration of 10−9 M, a maximal effect at 10−7 M (Fig. 1) and an IC50 of 4.45 × 10−9 M.

According to previous results (2), treatment of proximal tubules with 10−5 M DA resulted in a maximal inhibition of Na+/K+-ATPase activity of ∼41%. Inhibition of CYP4A-catalyzed arachidonic acid metabolism by 10−6 M 17-ODYA (32), a suicide CYP inhibitor, abolished the effect of DA, although it did not modify Na+/K+-ATPase activity per se (Fig. 2).

It has been previously shown that DA inhibits Na+/K+-ATPase activity via the synergism resulting from D1 and D2 receptor stimulation (3–5). To examine whether 20-HETE is involved in the intracellular signaling pathways triggered by the stimulation of either type of these receptors, fenoldopam or quinpirole (D2 agonist) and fenoldopam or quinpirole (D2 agonist) were tried alone or in the presence of 20-HETE. As previously reported (3), the activity of Na+/K+-ATPase was not changed by fenoldopam 10−5 M. However, in the presence of a threshold concentration of 20-HETE (10−9 M), fenoldopam inhibited Na+/K+-ATPase activity by 34% (Fig. 3A). Conversely, quinpirole 10−5 M did not modify Na+/K+-ATPase activity.
Fig. 1. Dose-dependent inhibition of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity by 20-HETE in isolated proximal tubules. Individual tubular segments were incubated at room temperature for 30 min in the absence (control) or in the presence of increasing concentrations of 20-HETE. The activity of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in control condition was 2,566 ± 133 pmol Pi mm\textsuperscript{-1} h\textsuperscript{-1}. Each experiment was performed in sextuplicate, averaged, and considered as a single data point. Values are means ± SE and are expressed as percentage of control; n = 3 separate experiments. *P < 0.05 vs. control.

Fig. 2. Effect of CYP blockade on the inhibition of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity by dopamine (DA) in isolated proximal tubules. Individual tubular segments were incubated at room temperature for 30 min in the absence (control) or presence of DA (10\textsuperscript{-5} M), the CYP inhibitor 17-ODYA (10\textsuperscript{-6} M) was present 30 min before and throughout the incubation with DA. Control values for Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity were 3.094 ± 362 pmol Pi mm\textsuperscript{-1} h\textsuperscript{-1}. Each experiment was performed in sextuplicate, averaged, and considered as a single data point. Values are means ± SE expressed as percentage of control; n = 3 separate experiments. *P < 0.05 vs. control.

Fig. 3. Effect of selective dopaminergic agonists on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in isolated proximal tubules. Individual tubular segments were incubated at room temperature for 30 min in the absence (control) or in the presence of fenoldopam (FNP; 10\textsuperscript{-5} M; A) or quinpirole (QPR; 10\textsuperscript{-5} M; B), alone or in the presence of 20-HETE (10\textsuperscript{-9} M). The activity of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in control condition was in pmol Pi mm\textsuperscript{-1} h\textsuperscript{-1}; 2,823 ± 244 and 3,175 ± 267 for A and B, respectively. Each experiment was performed in sextuplicate, averaged, and considered as a single data point. Values are means ± SE expressed as percentage of control; n = 3–4 separate experiments. *P < 0.05 vs. control.

Activation of D\textsubscript{1} receptors activates adenyl cyclase, leading to the formation of cAMP and activation of PKA (19). In line with previous results (4), the adenyl cyclase activator forskolin (10\textsuperscript{-5} M) did not modify Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity. However, when tubules were incubated with forskolin in the presence of 20-HETE 10\textsuperscript{-9} M Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity was significantly inhibited by 38% (Fig. 4A). To examine a potential role of PKA in 20-HETE-elicited Na\textsuperscript{+}-K\textsuperscript{+}-ATPase inhibition, we compared the effect of 20-HETE in the absence or presence of the PKA inhibitor H-89 (3 \texttimes 10\textsuperscript{-7} M) (10). For these experiments, we used 10\textsuperscript{-6} M 20-HETE, a concentration which had proven to produce a maximal inhibition of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity. 20-HETE (10\textsuperscript{-6} M) decreased Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity by 25%, and this effect was completely abolished by H-89, which did not modify the activity of the pump by itself (Fig. 4B).

Activation of D\textsubscript{1} receptors is also coupled to PLC and subsequent DAG release (19). To test the possibility that 20-HETE may also synergize with the PLC pathway for the inhibition of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity, we used OAG, a cell-permeable DAG analog. OAG (10\textsuperscript{-11} to 10\textsuperscript{-5} M) inhibited Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in a dose-dependent manner, with a threshold concentration of 10\textsuperscript{-9} M, an IC\textsubscript{50} of 1.34 \times 10\textsuperscript{-9} M, and a maximal effect at 10\textsuperscript{-6} M (Fig. 5A). OAG (10\textsuperscript{-11} M) did not inhibit Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity by itself. However, when 10\textsuperscript{-11} M OAG was used in combination with 10\textsuperscript{-9} M 20-HETE, a concentration which had no effect per se (Fig. 1), a 30% decrease in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity was achieved (Fig. 5B). On the contrary, the maximal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase inhibition elicited by 10\textsuperscript{-6} M OAG was no further potentiated by 20-HETE, even when concentrations as high as 10\textsuperscript{-6} M were used (Fig. 5C).

Inhibition of rat Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity is dependent on phosphorylation of this pump by PKC (13, 26). To further examine the synergism between 20-HETE and OAG, we analyzed the effect of both drugs on the phosphorylation state of the pump. Lysates from cells incubated with 20-HETE (10\textsuperscript{-9} M), OAG (10\textsuperscript{-11} M), or the combination of both were probed with the McK1 antibody that selectively reacts with the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase \alpha\textsubscript{1}-subunit when it is not phosphorylated by PKC.
Both 20-HETE and OAG had little effect on PKC-induced phosphorylation when tried separately. In contrast, cells incubated with a combination of both drugs had a significantly lower immunosignal than vehicle-incubated cells, which is consistent with a higher phosphorylation state of the Na\(^+\)/H\(^+\)-ATPase\(\alpha1\)-subunit (Fig. 6).

PKC-\(\alpha\) subcellular distribution in proximal tubule cells.

Figure 7 depicts the effect of DA and 20-HETE on PKC-\(\alpha\) subcellular distribution. Under basal conditions, PKC-\(\alpha\) was detected mainly in the cytosolic fraction in both control cells and in cells pretreated with the CYP4A inhibitor ABT (5 \(\times\) 10\(^{-4}\) M). After incubation of control cells with either 10\(^{-5}\) M DA or 10\(^{-6}\) M 20-HETE a significant increase in the ratio of membrane to cytosol signal for PKC-\(\alpha\) was observed. Preincubation of cells with ABT precluded DA-induced PKC-\(\alpha\) subcellular redistribution but did not modify the effect of exogenously added 20-HETE. Finally, the possible synergism between 20-HETE and OAG on PKC-\(\alpha\) translocation was investigated. As depicted in Fig. 8, neither 20-HETE (10\(^{-8}\) M) nor OAG (10\(^{-11}\) M) modified the intracellular distribution of PKC-\(\alpha\). However, when these drugs were used in combination, a significant increase in the ratio of membrane to cytosol signal for PKC-\(\alpha\) was observed.

DISCUSSION

The results of this investigation confirm 20-HETE as a second messenger in the intracellular signaling triggered by DA receptor stimulation in proximal tubule cells. Also, this work provides evidence of a synergism between 20-HETE and the intracellular pathways triggered by the stimulation of D\(_1\) receptors, resulting in the inhibition of Na\(^+\)/K\(^+\)-ATPase activity in proximal tubule cells. These conclusions are supported by the following observations. First, the inhibition of Na\(^+\)/K\(^+\)-ATPase activity by DA in proximal tubules was blunted by...
blocking CYP4A activity. Second, the inhibition of CYP4A activity also precluded DA-induced PKC-α activation. Finally, threshold concentrations of 20-HETE had a synergistic effect not only with the D1 agonist fenoldopam, but also with the stimulators of its intracellular pathways forskolin and OAG.

Previous studies analyzed the role of DA receptors in the regulation of Na⁺-K⁺-ATPase activity. It has been suggested that activation of D1 receptors is sufficient to evoke the inhibition of the pump (19, 25), while stimulation of D2 receptors has been associated with an increase in pump activity (25, 33). In our hands, neither fenoldopam nor forskolin or quinpirole modified Na⁺-K⁺-ATPase activity when tried alone. This observation is in line with previous reports from other authors who concluded that inhibition of Na⁺-K⁺-ATPase activity by DA requires the simultaneous activation of D1 and D2 receptors. Neither selective agonists nor their known intracellular messengers alone had any effect on pump activity, while combined treatment with D1 and D2 agonists mimicked DA effect (3–5, 30). Furthermore, studies performed in vivo have also shown that the natriuretic effect of DA depends on the activation of both D1 and D2 receptors (15).

The participation of phospholipase A2 and arachidonic acid in DA-induced inhibition of Na⁺-K⁺-ATPase activity in proximal and distal tubules has been previously described (23, 30, 36). Our observation that inhibition of Na⁺-K⁺-ATPase activity by DA was suppressed by 17-ODYA is in line with these data and further suggests that arachidonic acid would not act by itself but rather through a product from CYP metabolism.

Current evidence suggests an increase in phospholipase A2 activity as a result of DA receptor stimulation in different tissues. It has been suggested that stimulation of D1-like receptors may activate the phospholipase A2 pathway through a PLC/PKC-dependent mechanism, whereas stimulation of D2 receptors has been reported to augment the release of arachidonic acid in vivo and in vitro (7, 25, 36, 43). However, in primary striatal neuron cultures D2 agonists increased, whereas D1 agonists caused inhibition of calcium-evoked arachidonic acid release (41). In addition, a DA receptor termed DA2K has been identified in the renal medulla and linked to the activation of phospholipase A2 (21, 22). In our hands, threshold concentrations of 20-HETE had a synergistic effect with a D1 agonist but not with a D2 agonist. Thus, while we cannot rule out the potential regulation of phospholipase A2 through the D1 receptor, our experiments point to a major role of the D2 receptor in the activation of the PLA2-CYP pathway in DA-mediated Na⁺-K⁺-ATPase activity inhibition. However, the complete cascade of events occurring between D2 receptor activation and Na⁺-K⁺-ATPase inhibition remains to be elucidated.

Most of the intracellular pathways involved in short-term regulation of the proximal tubule Na⁺-K⁺-ATPase activity converge in phosphorylation of the pump by PKA and PKC. Investigations on the effect of PKA/PKC phosphorylation on Na⁺-K⁺-ATPase activity have led to contradictory results (10, 16, 37). This controversy was partially cleared by the observation that activation of PKA/PKC inhibits Na⁺-K⁺-ATPase activity in intact cells at low intracellular calcium levels, whereas this inhibitory effect is abolished at high intracellular calcium concentrations (9).

It is reported here that Na⁺-K⁺-ATPase activity inhibition by 20-HETE was abolished by the PKA inhibitor H-89. This observation is in line with previous reports describing that regulation of rat Na⁺-K⁺-ATPase activity by PKC is modulated by PKA activity. Such modulation may be accomplished through a simultaneous PKA-dependent phosphorylation of Ser943 leading to an enhanced response to PDBu (11). Alternatively, PKA phosphorylation may account for inhibition of protein phosphatase-1 and subsequent reduction of dephosphorylation of Na⁺-K⁺-ATPase at the PKC phosphorylation site (18).

The synergistic effect of threshold concentrations of 20-HETE and OAG on Na⁺-K⁺-ATPase phosphorylation and inhibition, together with the lack of an additive effect when maximal concentrations of both drugs were used in combination, strongly suggests that both pathways converge in a unique intracellular pathway. Previous reports performed in vitro have shown a synergism among 20-HETE, OAG, and phosphatidylserine for activation of PKC (34). These data have now been confirmed in intact cells by the present observation of a synergism between 20-HETE and OAG for Na⁺-K⁺-ATPase phosphorylation and inhibition, and for PKC-α activation (measured through its translocation from the cytosol to the membrane). Different PKC isoforms, PKC-α, -βI, -βII, -ε, and -ζ, have been localized in rat proximal tubules by immunochecmical and immunoblotting studies (28, 39). It has been shown that stimulation of different PKC isoforms may lead
to either stimulation (PKC-β) or inhibition (PKC-ζ) of Na\(^+-\)K\(^+\)-ATPase activity (38). DA-induced inhibition of Na\(^+-\)K\(^+\)-ATPase activity is associated with the removal of active units from the basolateral plasma membrane and their transport into endosomes. DA-dependent endocytosis of Na\(^+-\)K\(^+\)-ATPase molecules is a complex process in which PKC isoforms may participate by phosphorylating either the α\(_1\)-subunit of the pump (12, 13) or other intermediate regulatory proteins (14). In previous reports, we demonstrated that dopamine selectively translocates PKC-α and -ε to the plasma membrane (35) and that only classical PKCs (α, βI, and γ) efficiently phosphorylate Na\(^+-\)K\(^+\)-ATPase α\(_1\)-subunit (29). In the present study, DA-induced activation of PKC-α was abolished by the inhibition of the synthesis of 20-HETE. Moreover, threshold concentrations of 20-HETE and OAG had a synergistic effect for PKC-α translocation. Taken together, these observations strongly support a physiological role for intracel-

Fig. 7. Immunoblot analysis of DA- or 20-HETE-stimulated cytosol to membrane translocation of PKC-α. Isolated proximal tubule cells were preincubated for 20 min either with 5 × 10\(^{-4}\) M 1-aminobenzotriazole (ABT) or vehicle (control) and further incubated for 3 min in the presence of 10\(^{-5}\) M DA, 10\(^{-6}\) M 20HT, or vehicle (V). Denatured proteins from cytosolic (cytosol) and membrane (membrane) fractions were separated in 7.5% SDS-PAGE, transferred to PVDF membranes, and immunoblotted for PKC-α. A: representative blot of 4 different experiments. B: densitometric analysis of the immunoreactivity of PKC-α is expressed as membrane bound-to-cytosol ratio. Results are expressed as means ± SE of 4 different experiments. *P < 0.05 vs. control.

Fig. 8. Immunoblot analysis of 20-HETE- and OAG-stimulated PKC-α redistribution in isolated proximal tubule cells. Isolated proximal tubule cells were incubated for 3 min in the presence of 10\(^{-9}\) M 20HT, 10\(^{-11}\) M OAG, or V. Denatured proteins from cytosolic (cytosol) and membrane (membrane) fractions were separated in 7.5% SDS-PAGE, transferred to PVDF membranes, and immunoblotted for PKC-α. A: immunoblot representative of 4 different experiments. B: densitometric analysis of the immunoreactivity of PKC-α expressed as membrane bound-to-cytosol ratio. Results are expressed as mean ± SE of 4 different experiments. *P < 0.05 vs. control.

Fig. 9. Proposed model for the role of 20-HETE in the intracellular mechanisms by which dopamine inhibits Na\(^+-\)K\(^+\)-ATPase in proximal tubules. AA, arachidonic acid; AC, adenylate cyclase; CYP, cytochrome P-450 monooxygenase; DAG, diacylglycerol; D\(_1\), D\(_2\) dopamine receptor; D\(_3\), D\(_4\) dopamine receptor; F\(_{1440}\), CAMP-dependent protein kinase; PKC, protein kinase C; PLA\(_2\), phospholipase A\(_2\); PLC, phospholipase C; +, stimulation; −, inhibition. For explanation, see text.
lular 20-HETE in DA-induced Na\(^{+}\)-K\(^{+}\)-ATPase inhibition, acting in synergism with the intracellular messengers from the D\(_{1}\) pathway.

These results confirm and extend previous reports on DA-mediated PKC activation in renal epithelial cells (35). PKC-\(\alpha\) may phosphorylate either an intermediate protein or Na\(^{+}\)-K\(^{+}\)-ATPase itself. The first possibility points to the phosphorylation of an intermediate protein which may be involved in Na\(^{+}\)-K\(^{+}\)-ATPase internalization (12). The second speculation is based on previous reports describing a direct interaction between PKC-\(\alpha\) and Na\(^{+}\)-K\(^{+}\)-ATPase (29).

The schematic diagram shown in Fig. 9 illustrates a hypothetical model of the role of 20-HETE for the inhibition of Na\(^{+}\)-K\(^{+}\)-ATPase activity by DA in proximal tubules. According to this hypothesis, which is based on data from the present and previous studies, an integrated network of intracellular signals triggered by the stimulation of both D\(_{1}\) and D\(_{2}\) receptors participates in the regulation of Na\(^{+}\)-K\(^{+}\)-ATPase activity by DA. 20-HETE, the main metabolite of arachidonic acid in kidney, inhibits Na\(^{+}\)-K\(^{+}\)-ATPase by activating PKC, which phosphorylates the Na\(^{+}\)-K\(^{+}\)-ATPase endocytosis pathway. These findings also propose that the renal dopaminergic system (1, 8, 25) as well as an altered synthesis of 20-HETE (20, 31, 40) have been proposed as pathophysiological states that contribute to the failure of DA to inhibit Na\(^{+}\)-K\(^{+}\)-ATPase activity in DA-induced Na\(^{+}\)-K\(^{+}\)-ATPase inhibition, acting in synergism with the intracellular messengers from the D\(_{1}\) pathway.

The lack of function of the renal dopaminergic system (1, 8, 25) as well as an altered synthesis of 20-HETE (20, 31, 40) have been proposed as pathophysiological states that contribute to the failure of DA to inhibit Na\(^{+}\)-K\(^{+}\)-ATPase activity in kidneys from spontaneously hypertensive rats (23, 24).

Thus the identification of the signaling pathways originated from arachidonic acid which are involved in sodium handling by renal tubules may not only have important implications for the understanding of the pathogenesis of salt-sensitive hypertension but also for a prospective gene therapy for this disease.

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