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Regulation of renal Na^+,K^+-ATPase and ouabain-sensitive H^+,K^+-ATPase by the cyclic AMP-protein kinase A signal transduction pathway✪

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We investigated the effect of the cyclic AMP-protein kinase A (PKA) signalling pathway on renal Na^+,K^+-ATPase and ouabain-sensitive H^+,K^+-ATPase. Male Wistar rats were anaesthetized and catheter was inserted through the femoral artery into the abdominal aorta proximally to the renal arteries for infusion of the investigated substances. Na^+,K^+-ATPase activity was measured in the presence of Sch 28080 to block ouabain-sensitive H^+,K^+-ATPase and improve specificity of the assay. Dibutyryl-cyclic AMP (db-cAMP) administered at a dose of 10^{-7} mol/kg per min and 10^{-6} mol/kg per min increased Na^+,K^+-ATPase activity in the renal cortex by 34% and 42%, respectively, and decreased it in the renal medulla by 30% and 44%, respectively. db-cAMP infused at 10^{-6} mol/kg per min increased the activity of cortical ouabain-sensitive H^+,K^+-ATPase by 33%, and medullary ouabain-sensitive H^+,K^+-ATPase by 30%. All the effects of db-cAMP were abolished by a specific inhibitor of protein kinase A, KT 5720. The stimulatory effect on ouabain-sensitive H^+,K^+-ATPase and on cortical Na^+,K^+-ATPase was also abolished by brefeldin A which inhibits the insertion of proteins into the plasma membranes, whereas the inhibitory effect on medullary Na^+,K^+-ATPase was partially attenuated by 17-octadecynoic acid, an inhibitor of cytochrome P450-dependent arachidonate metabolism. We conclude that the cAMP-PKA pathway stimulates Na^+,K^+-ATPase in the renal cortex as well as ouabain-sensitive H^+,K^+-ATPase in the cortex and medulla by a mechanism requiring insertion of proteins into the plasma membrane. In contrast, medullary Na^+,K^+-ATPase is inhibited by cAMP through a mechanism involving cytochrome P450-dependent arachidonate metabolites.

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Abbreviations: db-cAMP, dibutyryl-cyclic AMP; 11,12-EET, 11,12-epoxyeicosatrienoic acid; 20-HETE, 20-hydroxyeicosatetraenoic acid; IBMX, 3-isobutyl-1-methylxanthine; 17-ODYA, 17-octadecynoic acid; PKA, protein kinase A.

Na^+,K^+-ATPase, present in 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 multiple mediators, including dopamine, aldosterone, angiotensin II, nitric oxide and atrial natriuretic peptide (Feraille & Doucet, 2001). The hormones regulating renal sodium pump trigger different intracellular signalling mechanisms, among which cyclic AMP-protein kinase A is one of the most important. In renal tubular cells, the cAMP-PKA pathway is activated by dopamine through D1-like receptors, β-adrenergic agonists, parathyroid hormone, vasopressin, and by prostaglandins through EP_2 receptors. In contrast, dopamine acting on D2-like receptors, neuropeptide Y, angiotensin II, and prostaglandins through EP_1 receptors inhibit adenylate cyclase (Feraille & Doucet, 2001). The effect of the cAMP-PKA pathway on renal Na^+,K^+-ATPase is extremely controversial; both stimulation (Charlton & Baylis 1990; Breton *et al.,* 1994; Beck *et al.,* 1995; Carranza *et al.,* 1996) and inhibition (Fryckstedt & Aperia, 1992; Aperia *et al.,* 1994; Cheng *et al.,* 1997; Hussain *et al.,* 1997; Anderson *et al.,* 1998) of renal sodium pump by cAMP have been reported. These discrepancies may be partially attributed to different nephron segments studied, experimental conditions, and different methods of Na^+,K^+-ATPase assay. In particular, *in vitro* studies may not adequately reproduce physiological conditions, because the regulation of Na^+,K^+-ATPase by protein kinases is 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; Cheng *et al.,* 1999). Recently, we have demonstrated that activators of the cAMP-PKA pathway administered *in vivo* by local infusion stimulate Na^+,K^+-ATPase in the renal cortex and inhibit it in the renal medulla, suggesting that the same signalling pathway may have opposite effects in different nephron segments depending on local microenvironment (Bełtowski *et al.,* 2002). Whereas this experimental approach bypasses many disadvantages of *in vitro* studies, it shares with them another drawback, i.e. nonspecificity of Na^+,K^+-ATPase assay. In most studies, including that performed by us, Na^+,K^+-ATPase was measured as ouabain-sensitive activity. However, ouabain is not a specific inhibitor of Na^+,K^+-ATPase, but inhibits also some H^+,K^+-ATPases (Younes-Ibrahin *et al.,* 1995; Buffin-Meyer *et al.,* 1997). H^+,K^+-ATPase, present in the apical membrane of tubular cells, is involved in potassium reabsorption and urine acidification. The enzyme exists in at least three isoforms: type I expressed in intercalated cells of the collecting duct, type II contained in proximal tubule and medullary thick ascending limb, and type III, which is not expressed under physiological conditions but appears in principal cells of the collecting duct in potassium-depleted animals. Types II and III are inhibited by ouabain (Doucet, 1997). Thus, "Na^+,K^+-ATPase" assayed as ouabain-sensitive activity consists in fact not only of true Na^+,K^+-ATPase but also of ouabain-sensitive H^+,K^+-ATPase. The α-subunit of H^+,K^+-ATPases contains putative PKA phosphorylation site (Kone & Higham, 1998), which raises the possibility that changes of ouabain-sensitive H^+,K^+-ATPase activity could contribute to the effect of cAMP on "Na^+,K^+-ATPase" in some experiments.

Recently, we have developed a method for the measurement of renal ouabain-sensitive H^+,K^+-ATPase activity and improved the specificity of our Na^+,K^+-ATPase assay by adding an inhibitor of all H^+,K^+-ATPases, Sch 28080, to the incubation mixture (Bełtowski & Wójcicka, 2002). Therefore, in the present study we reinvestigated the effect of cAMP on renal Na^+,K^+-ATPase using this modified method. We also studied whether this signalling pathway is involved in the regulation of renal ouabain-sensitive H^+,K^+-ATPase.

MATERIALS AND METHODS

Reagents. The following reagents were obtained from Sigma-Aldrich (St. Louis MO, U.S.A.): N^G,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate (db-cAMP), forskolin, 3-isobutyl-1-methylxanthine (IBMX), 17-octadecynoic acid (17-ODYA), miconazole, 20-hydroxyeicosatetraenoic acid (20-HETE), 11,12-epoxyeicosatrienoic acid (11,12-EET), adenosine triphosphate disodium salt (Na_2ATP), adenosine triphosphate Tris salt (TrisATP), and ouabain. The specific inhibitors of protein kinases A and G, KT 5720 and KT 5823, respectively, were purchased from Kamiya Biomedical Co. (Thousand Oaks, 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.). Other reagents were of the highest research grade available.

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 the 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 the 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 or between 31 and 60 min of infusion. In general, activators of the cAMP-PKA pathway 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. After this procedure the kidneys became pale and contained virtually no erythrocytes as confirmed by light microscopy. The kidneys were excised and the animals sacrificed by a lethal dose of pentobarbital. ATPase activities were 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. 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 N $HClO_4$. 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 microsomal protein 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 (Mg^{2+}-ATPase), assayed in the presence of 2 mM ouabain, and was expressed in moles of P_i liberated by 1 mg of microsomal protein during

1 h (mol/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.

H*$^+$,*K*$^+$-*ATPase assay. For H^+,K^+-ATPase assay, 50 μg of microsomal protein was added to the incubation mixture (1 ml) containing 5 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, and 25 mM Tris/HCl (pH 7.4). After 10 min, 5 mM TrisATP was added and the sample was incubated for 30 min at 37°C. Then, P_i was assayed as described above. The activity of ouabain-sensitive H^+,K^+-ATPase was calculated as the difference between the activities assayed in the absence and in the presence of 1 mM ouabain (Bełtowski & Wójcicka, 2002).

Statistics. Data are presented as mean ± S.E.M. 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

Infusion of a membrane-permeable, hydrolysis-resistant cAMP analogue, dibutyryl-cAMP, had dose dependent, opposite effects on Na^+,K^+-ATPase activity in the renal cortex and medulla (Fig. 1). db-cAMP administered at a dose of 10^{-7} mol/kg per min and 10^{-6} mol/kg per min increased cortical Na^+,K^+-ATPase activity by 34% and 42%, respectively. A lower dose of db-cAMP (10^{-8} mol/kg per min) tended to stimulate cortical Na^+,K^+-ATPase but the effect was not significant. In contrast, db-cAMP at doses of 10^{-7} mol/kg per min and 10^{-6} mol/kg per min decreased Na^+,K^+-ATPase activity in the renal medulla by 30% and 44%, respectively. The activity of ouabain-resistant fraction (Mg^{2+}-ATPase) did not change following db-cAMP administration either in the cortex or in the medulla.

Ouabain-sensitive H^+,K^+-ATPase activity increased in response to db-cAMP infusion in both regions of the kidney (Fig. 1). In the renal cortex, 10^{-6} mol/kg per min stimulated ouabain-sensitive H^+,K^+-ATPase activity by 33%, whereas in the renal medulla this dose of the cAMP analogue caused 30% stimulation. Lower doses of db-cAMP (10^{-8} and 10^{-7} mol/

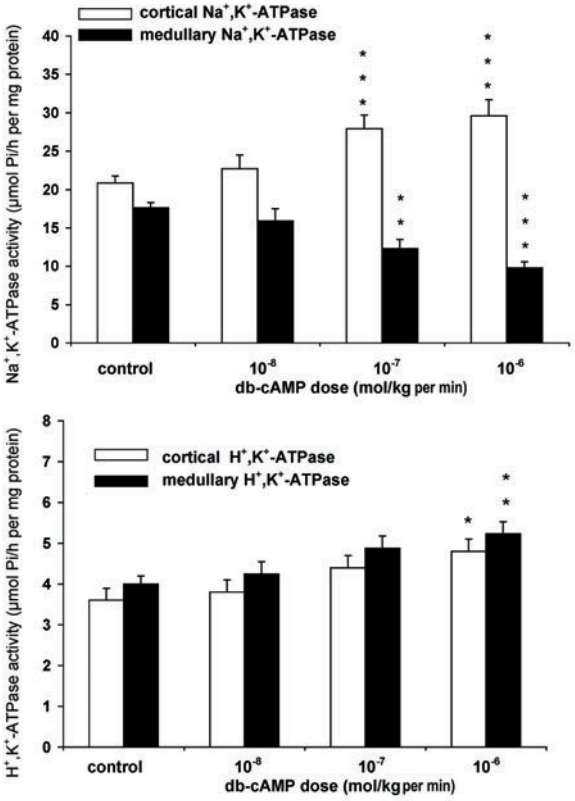

Figure 1. The effect of dibutyryl-cAMP (db-cAMP) on Na^+,K^+-ATPase (top panel) and ouabain-sensitive H^+,K^+-ATPase (bottom panel) activities in the renal cortex and medulla.

Control group received infusion of 0.9% NaCl for 60 min, other groups were treated with different doses of db-cAMP between 31 and 60 min of infusion. Then, ATPases were assayed in isolated microsomal fraction. Enzyme activities are expressed in μmol of inorganic phosphate liberated by 1 mg of microsomal protein during 1 h (μmol P_i/h per mg protein); n = 8 in each group. *P<0.05, **P < 0.01, ***P < 0.001, compared to control by ANOVA and Duncan's test.

kg per min) tended to stimulate cortical and medullary ouabain-sensitive H^+,K^+-ATPase, but the effect was not significant. Thus, db-cAMP has similar stimulatory effect on both ouabain-sensitive ATPases in the renal cortex but changes their activities in the opposite directions in the renal medulla.

To test whether db-cAMP has a direct effect on the renal ATPases, we examined the effect of this compound on enzyme activities *in vitro*. db-cAMP added to the incubation medium at a concentration of 10^{-3} M had no effect on either cortical or medullary Na^+,K^+-ATPase and on ouabain-sensitive H^+,K^+-ATPase activity. These data indicate that the action of db-cAMP requires intact intracellular signal transduction mechanisms.

Next, we investigated whether stimulation of endogenous adenylate cyclase causes similar changes in the activities of renal ATPases. Infusion of forskolin, an activator of adenylate cyclase, mimicked the effect of db-cAMP in the renal medulla. Forskolin at a dose of 10^{-7} mol/kg per min decreased medullary Na^+,K^+-ATPase activity by 47% and increased medullary ouabain-sensitive H^+,K^+-ATPase activity by 27% (Fig. 2). Previously we have demonstrated that forskolin alone has no effect on cortical ATPases (Bełtowski *et al.,* 2002), most likely due to low activity of adenylate cyclase and/or high rate of cAMP inactivation by phosphodiesterases. Therefore, we studied cortical ATPases after infusion of forskolin preceded by 30-min administration of a phosphodiesterase inhibitor, IBMX, expected to increase the effectiveness of forskolin by blocking cAMP breakdown. Although IBMX is a nonspecific inhibitor of cAMP-degrading phosphodiesterases and inhibits also the metabolism of cGMP, we have observed that cGMP has no effect on cortical Na^+,K^+-ATPase and ouabain-sensitive H^+,K^+-ATPase in the renal cortex (unpublished observation). Both Na^+,K^+-ATPase and ouabain-sensitive H^+,K^+-ATPase activity in the renal cortex increased following IBMX+ forskolin administration by 38% and 30%, respectively (Fig. 2).

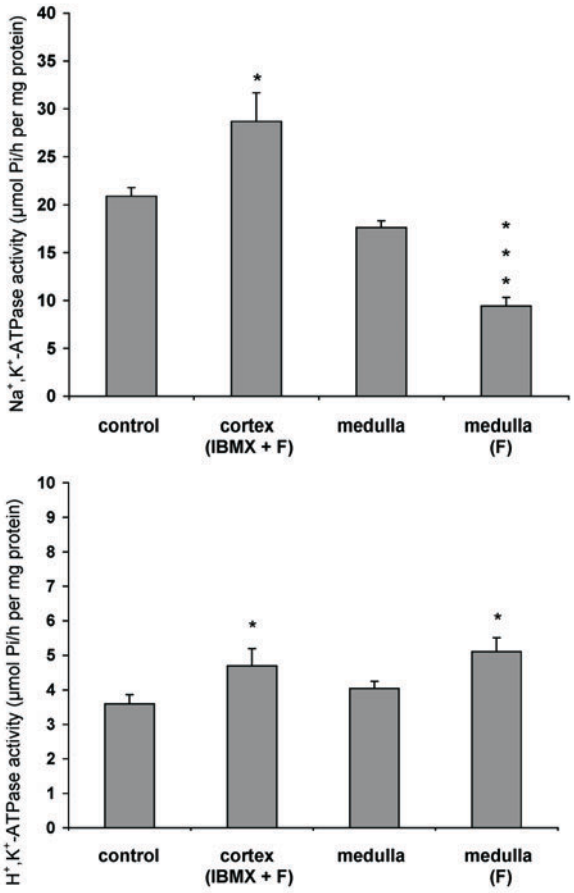

Figure 2. The effect of forskolin and IBMX on renal Na^+,K^+-ATPase (top panel) and ouabain-sensitive H^+,K^+-ATPase (bottom panel).

Control group received 0.9% NaCl for 60 min. In one experimental group (for studying cortical enzymes) IBMX (10^{-6} mol/kg per min) was infused for 30 min, followed by forskolin (10^{-7} mol/kg per min) for another 30 min (IBMX+F). Another group (for studying medullary enzymes) received 0.9% NaCl for 30 min and forskolin (10^{-7} mol/kg per min) for another 30 min (F). $^*P < 0.05$, $^{***}P < 0.001$, compared to control by unpaired Student's *t*-test.

Subsequently, we investigated whether the effect of cAMP on renal ATPases was mediated by protein kinase A. Infusion of specific protein kinase A inhibitor, KT 5720 (10^{-8} mol/kg per min) before the administration of db-cAMP (10^{-6} mol/kg per min) abolished the stimulatory effect of db-cAMP on cortical Na^+,K^+-ATPase, cortical and medullary ouabain-sensitive H^+,K^+-ATPase, as well as the inhibitory effect on medullary Na^+,K^+-ATPase (Fig. 3). In contrast, a specific inhibi-

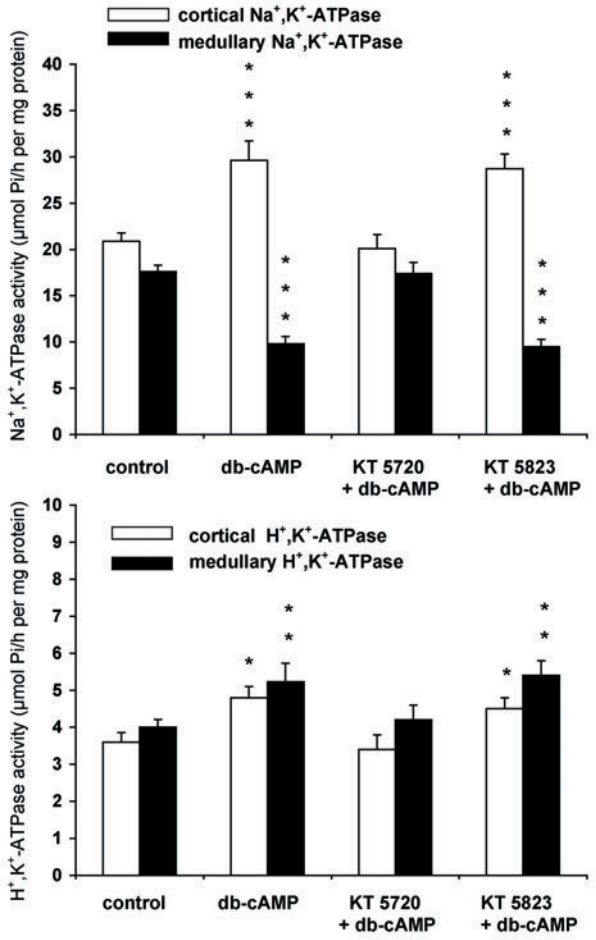

Figure 3. The effect of specific inhibitors of protein kinase A (KT 5720) and protein kinase G (KT 5823) on the regulation of renal ATPases by db-cAMP.

KT 5720 or KT 5823 (10^{-8} mol/kg per min) was administered between 1 and 30 min of infusion and db-cAMP (10^{-6} mol/kg per min) between 31 and 60 min. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control by ANOVA and Duncan's test.

tor of protein kinase G, KT 5823, had no effect on the db-cAMP-induced changes in both ATPases either in the cortex or in the medulla. Thus, db-cAMP altered the activity of renal Na^+,K^+-ATPase and ouabain-sensitive H^+,K^+-ATPase by stimulating protein kinase A. KT 5720 did not change the activity of renal ouabain-sensitive ATPases if administered to animals which were not treated with db-cAMP.

Short-term regulation of renal Na^+,K^+-ATPase is partially accounted for by the distribution of the enzyme between the plasma membrane (active pool) and intracellular stores (inactive pool) (Feraille & Doucet, 2001). To get more insight into the mechanism of regulation of renal ATPases by cAMP, we performed additional experiments using brefeldin A, which inhibits translocation of proteins from Golgi apparatus to the plasma membrane and has been demonstrated to attenuate incorporation of Na^+,K^+-ATPase into the basolateral membrane of tubular cells (Bertorello *et al.,* 1999; Gonin *et al.,* 2001). Administration of brefeldin A alone had no effect on Na^+,K^+-ATPase and ouabain-sensitive H^+,K^+-ATPase activities (Fig. 4). When brefeldin A was infused before the administration of db-cAMP, it abolished the stimulatory effect of this com-

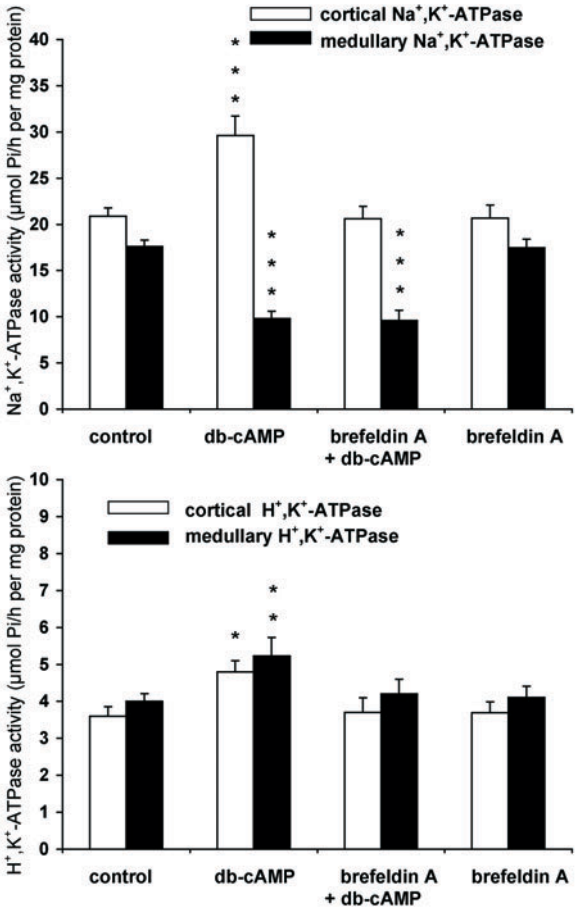

Figure 4. The effect of brefeldin A on the regulation of renal ATPases by db-cAMP.

Brefeldin A (100 μg/kg per min) was infused between 1 and 30 min of the experiment and db-cAMP (10^{-6} mol/kg per min) between 31 and 60 min. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control by ANOVA and Duncan's test.

pound on Na^+,K^+-ATPase in the renal cortex and the effect on ouabain-sensitive H^+,K^+-ATPase in both cortex and medulla. These data suggest that cAMP stimulates cortical Na^+,K^+-ATPase as well as cortical and medullary ouabain-sensitive H^+,K^+-ATPase by activating the recruitment of pumps (or other regulatory proteins) into the plasma membrane. In contrast, brefeldin A had no effect on the inhibition of medullary Na^+,K^+-ATPase by cAMP (Fig. 4).

Previous studies suggest that cytochrome P450-dependent arachidonate metabolites decrease renal Na^+,K^+-ATPase activity and mediate the inhibitory effect of some mediators such as dopamine and parathyroid hormone (Schwartzman *et al.,* 1985; Nowicki *et al.,* 1997). In the kidney arachidonic acid is metabolized by cytochrome P450 through the ω/ω-1 hydroxylation pathway to 19- and 20-hydroxyeicosatetraenoic acids (19- and 20-HETE) and by the epoxygenase pathway to different isomers of epoxyeicosatrienoic acid (EET). We observed that administration of 17-ODYA, which inhibits both pathways of cytochrome P450-dependent arachidonate metabolism, partially attenuated the inhibitory effect of db-cAMP on Na^+,K^+-ATPase in the renal medulla. Medullary Na^+,K^+-ATPase activity in animals treated with 17-ODYA (10^{-8} mol/kg per min) before the administration of db-cAMP (10^{-6} mol/kg per min) was 34% higher than in rats treated with db-cAMP alone, but still significantly lower (by 25%) than in control animals, which did not receive db-cAMP. In contrast to 17-ODYA, a specific inhibitor of the epoxygenase pathway, miconazole, has no effect on the inhibition of medullary Na^+,K^+- ATPase by db-cAMP. Neither 17-ODYA nor miconazole had any effect on the stimulation of cortical Na^+,K^+-ATPase or of cortical and medullary ouabain-sensitive H^+,K^+-ATPase by db-cAMP, suggesting that the cytochrome P450-dependent arachidonate metabolites are not involved in the stimulatory effect of the cAMP-PKA pathway on renal ouabain-sensitive ATPases. Also, 17-ODYA and miconazole had no effect on Na^+,K^+-ATPase and on ouabain-sensitive H^+,K^+-ATPase in animals which were not treated with db-cAMP.

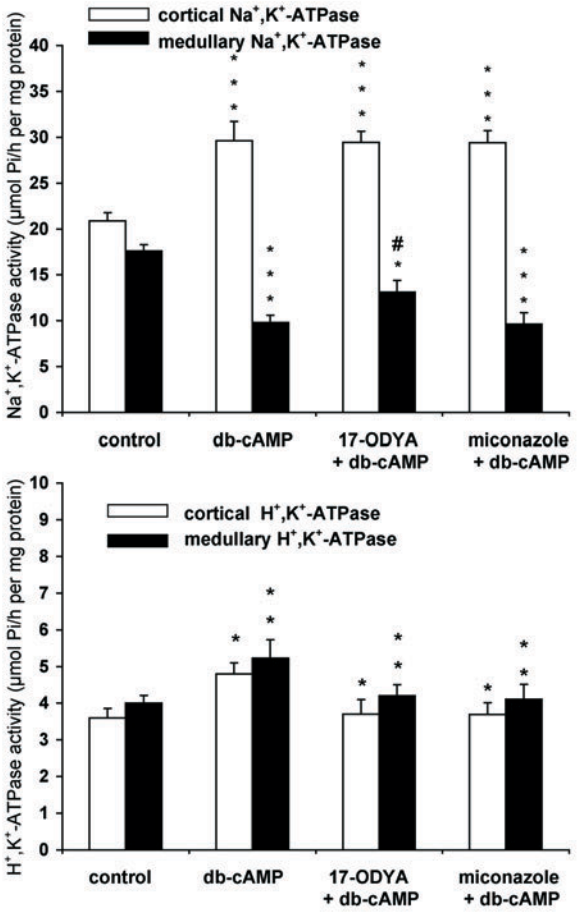

Figure 5. The effect of inhibitors of cytochrome P450-dependent arachidonate metabolism, 17-ODYA and miconazole, on the regulation of renal ATPases by db-cAMP.

17-ODYA or miconazole (each at a dose of 10^{-8} mol/kg per min) was infused between 1 and 30 min of the experiment and db-cAMP (10^{-6} mol/kg per min) between 31 and 60 min. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control by ANOVA and Duncan's test; $^{\#}P < 0.05$, compared by Duncan's test to medullary Na^+,K^+-ATPase in db-cAMP-treated rats which did not receive 17-ODYA.

Finally, we tested the effect of exogenous cytochrome P450-dependent arachidonate metabolites on renal ATPases (Fig. 6). 20-HETE administered at doses of 10^{-11} and 10^{-10} mol/kg per min decreased Na^+,K^+-ATPase activity in the renal cortex by 25% and

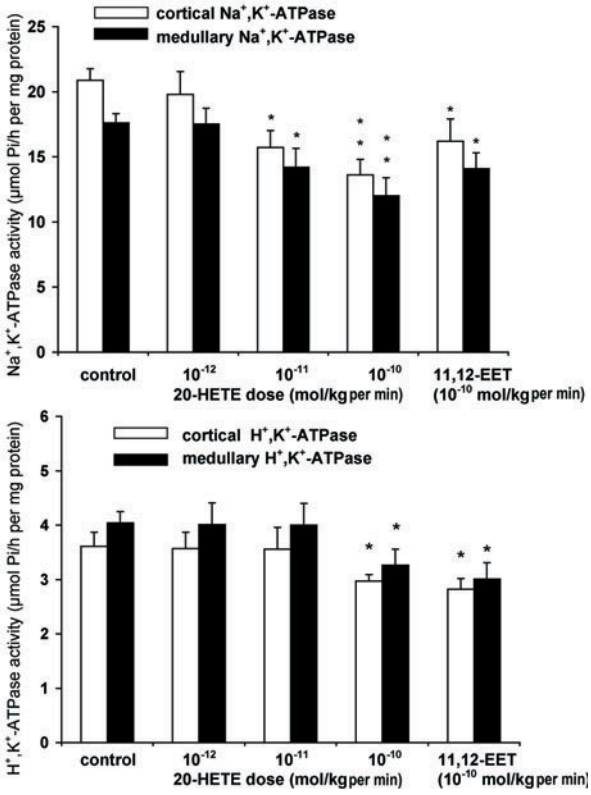

Figure 6. The effect of cytochrome P450-dependent arachidonate metabolites, 20-HETE and 11,12-EET, on renal Na^+,K^+-ATPase and ouabain-sensitive H^+,K^+-ATPase activities.

20-HETE or 11,12-EET was administered between 31 and 60 min of infusion. $^*P < 0.05$, $^{**}P < 0.01$, compared to control by ANOVA and Duncan's test.

35%, respectively, and in the renal medulla by 19% and 32%, respectively. Lower dose of 20-HETE (10^{-12} mol/kg per min) did not alter Na^+,K^+-ATPase activity in either the cortex or the medulla. 20-HETE infused at doses of 10^{-12} and 10^{-11} mol/kg per min had no effect on cortical and medullary ouabain-sensitive H^+,K^+-ATPase, but administered at a dose of 10^{-10} mol/kg per min decreased its activity in the cortex and medulla by 18% and 19%, respectively. Thus, although 20-HETE inhibits both ouabain-sensitive ATPases, Na^+,K^+-ATPase is more sensitive to this inhibition than ouabain-sensitive H^+,K^+-ATPase. A product of the epoxygenase pathway, 11,12-EET, infused at a dose of 10^{-10} mol/kg per min decreased the activity of cortical Na^+,K^+-ATPase, medullary Na^+,K^+-ATPase, cortical ouabain-sensitive H^+,K^+-ATPase and medullary ouabain-sensitive H^+,K^+-ATPase by 22%, 20%, 22%, and 26%, respectively. These data indicate that, in contrast to 20-HETE, 11,12-EET is an equally potent inhibitor of both renal Na^+,K^+-ATPase and ouabain-sensitive H^+,K^+-ATPase.

DISCUSSION

The results presented here confirm our recent observation that stimulation of the cAMP-PKA pathway increases Na^+,K^+-ATPase activity in the renal cortex and decreases it in the renal medulla (Bełtowski *et al.,* 2002). However, in the present study Na^+,K^+-ATPase activity was assayed in the presence of Sch 28080 to inhibit ouabain-sensitive H^+,K^+-ATPase, so the observed effects are accounted for specifically by Na^+,K^+-ATPase. In contrast to Na^+,K^+-ATPase, ouabain-sensitive H^+,K^+-ATPase is activated by cAMP through a PKA-dependent mechanism in both regions of the kidney.

We observed that the stimulation of Na^+,K^+-ATPase by cAMP was abolished by brefeldin A, which suggests that the cAMP-PKA pathway induces recruitment of the enzyme from an intracellular compartment to the plasma membrane. This is consistent with *in vitro* studies which demonstrated an increase in cell surface expression of Na^+,K^+-ATPase by cAMP in proximal convoluted tubule (Carranza *et al.,* 1998), cortical collecting duct (Gonin *et al.,* 2001), and in lung epithelial cells (Bertorello *et al.,* 1999). The method used by us for isolation of microsomal fraction does not separate plasma membranes from endoplasmatic reticulum, thus, both intracellular and cell surface pools of Na^+,K^+-ATPase are present during enzyme assay. The effect of brefeldin A suggests that the intracellular pool of Na^+,K^+-ATPase is inactive until transferred to the plasma membrane, even if its activity is assayed in a broken-cell preparation. However, an alternative explanation should be considered, i.e. that cAMP does not induce

the recruitment of Na^+,K^+-ATPase itself but rather of some regulatory protein(s) which activates a sodium pump preexisting in the plasma membrane. Because brefeldin A also inhibits secretion of proteins into the extracellular space, it is possible that cAMP induces the release of some mediator which activates Na^+,K^+-ATPase and ouabain-sensitive H^+,K^+-ATPase. Further studies are needed to discriminate between these possibilities.

Rat α_1 subunit of Na^+,K^+-ATPase contains a putative PKA phosphorylation site at Ser^{943} (Fisone *et al.,* 1994) and can be phosphorylated at this site *in vitro* (Feschenko & Sweadner, 1994; Beguin *et al.,* 1994; Kurihara *et al.,* 2000) and when transfected into cultured cells (Beguin *et al.,* 1994; Cornelius & Logvinenko, 1996). Some studies have demonstrated an increased level of phosphorylation of Na^+,K^+-ATPase following stimulation of PKA in intact renal cells (Cheng *et al.,* 1997; Kiroytscheva *et al.,* 1999). However, the localization of the incorporated phosphate was not defined. In addition, recently the possibility of Na^+,K^+-ATPase phosphorylation by PKA in intact cells has been questioned (Feschenko *et al.,* 2000; Sweadner & Feschenko, 2001). Thus, it is still not clear whether the regulation of Na^+,K^+-ATPase by cAMP includes phosphorylation of the pump by PKA itself. Alternatively, PKA may inhibit dephosphorylation of Na^+,K^+-ATPase (previously phosphorylated by other kinases) by phosphorylating and activating endogenous inhibitors of protein phosphatases, such as the dopamine- and cyclic AMP-regulated phosphoprotein 32 kDa (DARPP-32) or inhibitor-1 (I-1) (Meister *et al.,* 1989; Higuchi *et al.,* 2000). Finally, PKA can indirectly stimulate other kinases to phosphorylate the sodium pump.

The results of this study suggest that the inhibitory effect of cAMP on Na^+,K^+-ATPase activity in the renal medulla is mediated, at least in part, by cytochrome P450-dependent arachidonate metabolites. It seems that products of the ω/ω-1 hydroxylation pathway rather than of the epoxygenation pathway are involved, because the effect of cAMP was attenuated by a nonspecific inhibitor of the P450-dependent arachidonate cascade, 17-ODYA, but not by a specific inhibitor of epoxygenase, miconazole. Moreover, 20-HETE decreased Na^+,K^+-ATPase activity more effectively than the activity of ouabain-sensitive H^+,K^+-ATPase, whereas 11,12-EET inhibited both enzymes to a similar degree. If cAMP stimulated the epoxygenase pathway, it would inhibit both ouabain-sensitive ATPases rather than only the Na^+,K^+-ATPase.

In vitro studies demonstrated that cytochrome P450-dependent arachidonate metabolites mediate the inhibitory effect of cAMP on Na^+,K^+-ATPase in medullary thick ascending limb (Satoh *et al.,* 1993a) and cortical collecting duct (Satoh *et al.,* 1992, 1993b). More recently, it has been suggested that this is an artefact resulting from inadequate oxygen supply because cAMP inhibited Na^+,K^+-ATPase only under hypoxic conditions (Kiroytscheva *et al.,* 1999). Our study indicates that this mechanism may operate *in vivo* in the renal medulla. The renal medulla is characterized by low oxygen tension and the difference in oxygen availability between the medulla and cortex can account for the opposite effects of cAMP on Na^+,K^+-ATPase in these regions. The observation that while 20-HETE and 11,12-EET decreased both cortical and medullary Na^+,K^+-ATPase, the inhibitory effect db-cAMP was restricted to the renal medulla is consistent with the explanation that cAMP triggers the cytochrome P450-dependent arachidonate cascade specifically in the latter region.

The mechanisms regulating renal H^+,K^+-ATPases are poorly understood. Herein we demonstrate that cAMP stimulates ouabain-sensitive (type II) H^+,K^+-ATPase in both regions of the kidney through a PKA-dependent mechanism. The effect is sensitive to brefeldin A suggesting that PKA stimulates the incorporation of H^+,K^+-ATPase or another protein regulating its activity to the plasma

membrane. Notably, although ouabain-sensitive H^+,K^+-ATPase was inhibited by 20-HETE, it was less sensitive to this inhibition than Na^+,K^+-ATPase. The lower sensitivity of ouabain-sensitive H^+,K^+-ATPase to 20-HETE-induced inhibition can explain why this enzyme, in contrast to Na^+,K^+-ATPase, is unidirectionally regulated by cAMP in both parts of the kidney.

While this study was in progress, two papers concerning short-term regulation of renal H^+,K^+-ATPases were published. In one of them (Laroche-Joubert *et al.,* 2000) the stimulation of type I H^+,K^+-ATPase in intercalated cells of the collecting duct in normokalemic rats and stimulation of type III H^+,K^+-ATPase in principal cells of this nephron segment of potassium-depleted rats by cAMP were reported. Interestingly, another study revealed that whereas the effect of cAMP on type I H^+,K^+-ATPase in β-intercalated cells was mediated by PKA, the stimulation of this enzyme in α-intercalated cells was PKA-independent (Laroche-Joubert *et al.,* 2002). The involvement of PKA in the regulation of type III H^+,K^+-ATPase by cAMP was not studied. Together with the present study, those data indicate that all renal H^+,K^+-ATPases are stimulated by cAMP, although the signalling pathway distally to cAMP may differ depending on the cell type and H^+,K^+-ATPase isoform.

In conclusion, this study demonstrates that the cAMP-PKA pathway stimulates Na^+,K^+-ATPase in the renal cortex and inhibits it in the renal medulla. The inhibitory effect is partially mediated by cytochrome P450-dependent arachidonate metabolites. In contrast, cAMP increases the activity of ouabain-sensitive (type II) H^+,K^+-ATPase in both regions of the kidney. These data indicate that a single signalling mechanism can regulate the two renal ouabain-sensitive ATPases in opposite directions in a given nephron segment, as well as can mediate either stimulation or inhibition of Na^+,K^+-ATPase in different parts of the kidney.

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