Phosphorylation of the Catalytic α-Subunit Constitutes a Triggering Signal for Na\textsuperscript{+},K\textsuperscript{+}-ATPase Endocytosis*

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Inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity by dopamine is an important mechanism by which renal tubules modulate urine sodium excretion during a high salt diet. However, the molecular mechanisms of this regulation are not clearly understood. Inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in response to dopamine is associated with endocytosis of its α- and β-subunits, an effect that is protein kinase C-dependent. In this study we used isolated proximal tubule cells and a cell line derived from opossum kidney and demonstrate that dopamine-induced endocytosis of Na\textsuperscript{+},K\textsuperscript{+}-ATPase and inhibition of its activity were accompanied by phosphorylation of the α-subunit. Inhibition of both the enzyme activity and its phosphorylation were blocked by the protein kinase C inhibitor bisindolylmaleimide. The early time dependence of these processes suggests a causal link between phosphorylation and inhibition of enzyme activity. However, after 10 min of dopamine incubation, the α-subunit was no longer phosphorylated, whereas enzyme activity remained inhibited due to its removal from the plasma membrane. Dephosphorylation occurred in the late endosomal compartment. To further examine whether phosphorylation was a prerequisite for subunit endocytosis, we used the opossum kidney cell line transfected with the rodent α-subunit cDNA. Treatment of this cell line with dopamine resulted in phosphorylation and endocytosis of the α-subunit with a concomitant decrease in Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity. In contrast, none of these effects were observed in cells transfected with the rodent α-subunit that lacks the putative protein kinase C-phosphorylation sites (Ser\textsuperscript{11} and Ser\textsuperscript{18}). Our results support the hypothesis that protein kinase C-dependent phosphorylation of the α-subunit is essential for Na\textsuperscript{+},K\textsuperscript{+}-ATPase endocytosis and that both events are responsible for the decreased enzyme activity in response to dopamine.

The natriuretic effect of dopamine (DA)\textsuperscript{3} depends on its ability to increase the glomerular filtration rate and/or to modulate directly tubular sodium reabsorption (1–3). Changes in vectorial transport of sodium induced by DA in renal tubules are largely mediated by inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase (4, 5) and Na\textsuperscript{+}/H\textsuperscript{+}-exchanger activity (6). At the cellular level, DA triggers a specific signaling cascade that ultimately activates protein kinase C (PKC) (7), a process postulated to be responsible for the decreased Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity.

Activators of PKC, such as phorbol esters and diacylglycerol analogs, decrease Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in isolated rat renal PCT segments (7, 8) as well as the vectorial transport of sodium by isolated perfused PCTs (9). In isolated renal PCT cells, another activator of PKC, 1-oleoyl-2-acetoyl-sn-glycerol, decreased Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity determined as the rate of ouabain-sensitive oxygen consumption (10). In a renal cell line derived from opossum kidney (OK cells), but not from pig kidney (LLC-PK\textsubscript{1} cells), incubation with phorbol esters resulted in phosphorylation of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase α-subunit and inhibition of its activity (11). However, stimulation of Na\textsuperscript{+},K\textsuperscript{+}-ATPase by phorbol esters has also been reported (12, 13). Although phosphorylation of the α-subunit by PKC in a cell-free preparation was associated with a decrease in enzymatic activity (14–16), it is not clear whether this effect occurs in intact cells in response to phorbol esters (11, 17).

DA is produced locally in renal PCT cells (18–20) where its synthesis is regulated physiologically during ingestion of a high salt diet (21). Contrary to the diverse effects of PKC stimulation by phorbol esters and diacylglycerols on Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity, there is a consensus on the inhibitory action of DA on the enzyme. Moreover, we have recently demonstrated that inhibition of PCT Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity by DA is associated with endocytosis of its α- and β-subunits into early (EE) and late (LE) endosomes via a clathrin-coated vesicle (CCV)-dependent mechanism (22). Nevertheless, despite the information gained during the last few years on the regulation of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity, it is not known whether inhibition of enzyme activity in intact cells depends on the phosphorylation of the catalytic subunit, or whether such phosphorylation is necessary for subunit endocytosis in response to a physiologic agonist such as DA.

In the present study, using intact renal PCT cells metabolically labeled with [\textsuperscript{32}P]orthophosphate, we have examined whether dopamine phosphorylates the Na\textsuperscript{+},K\textsuperscript{+}-ATPase α-subunit and whether this effect is responsible for the decreased enzymatic activity and subunit endocytosis.

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‡ The abbreviations used are: DA, dopamine; PKC, protein kinase C; PCT, proximal convoluted tubules; EE, early endosomes; LE, late endosomes; BLM, basolateral plasma membrane; CCV, clathrin-coated vesicles; OKD, okadaic acid; 20-HETE, 20-hydroxyeicosatetraenoic acid; OK cells, opossum kidney cells.

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EXPERIMENTAL PROCEDURES

Materials—The Camp analog Rp-cAMPS was obtained from BioLog, Bremen, Germany. Bisindolylmaleimide was purchased from Calbiochem, San Diego, CA. All other chemicals were from Sigma. A monoclonal antibody, kindly provided by Dr. M. Caplan (Yale University), was used against the Na+,K+-ATPase α-subunit (antibody A, which recognizes only the N-terminal first five residues of the α-subunit). Immunoprecipitation of the Na+,K+-ATPase in the phosphorylation experiments and Western blots were performed using a polyclonal antibody (B) raised against the rat Na+,K+-ATPase α-subunit (23). The identity of EE was determined with a polyclonal antibody raised against a rat β synthetic peptide (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The late endosome fraction was identified with a mammose 6-phosphate receptor antibody (courtesy of Dr. B. Hollack, EMBL, Heidelberg, Germany).

Preparation of PCT Cells—PCT cells were prepared as described before (10, 24). Briefly, male Sprague-Dawley rats (BK Universal, Stockholm, Sweden) weighing between 150–200 g were used. After the kidneys were removed and the cortex isolated, the tissue was minced on ice to a paste-like consistency. The cortical mincette was incubated continuously oxygenated during its preparation and incubation with the PKI analog Rp-cAMPS for 2.5 h at 37 °C. All incubations with different agonists were performed as described previously (26).

Preparation of Basolateral Plasma Membranes—Preparation of basolateral plasma membranes (BLM) were further purified according to Hammond et al. (31), using a Percoll gradient. Briefly, the collected material was diluted by adding 500 μl of imidazole (3 mM, pH 7.4) buffer containing protease inhibitors (final sucrose concentration 25–26% w/w), and spun at 20,000 × g for 20 min. The yellow layer was resuspended again in the supernatant (carefully removed from the brown pellet containing mitochondria and cell ghosts) and centrifuged at 48,000 × g for 30 min. The supernatant was discarded, and the pellet was resuspended in 1 ml of buffer (300 mM mannitol and 12 mM HEPES, pH 7.6, adjusted with Tris) by gentle pipetting. To form a Percoll gradient, 0.19 g of undiluted Percoll (Pharmacia Biotech Inc.) was added to a 1-ml suspension (0.2–1 mg of protein). The suspension was gently mixed and centrifuged at 48,000 × g for 30 min, and the ring of BLM was collected.

Miscellaneous—Protein content was determined according to Bradford (32). Western blots were developed with an ECL (Amersham, UK) detection kit. Scans were performed using a ScanJet IIIc scanner (Hewlett Packard, Palo Alto, CA). Quantitation of the phosphorylated Na+,K+-ATPase α-subunit was performed using a Fuji Bas 1000 Bioimaging analyzer (Fuji, Japan), and the data (arbitrary units) were analyzed using TINA 2.07 ray test software (Isotopenmesstechnik GmbH, Staulenhardt, Germany).

Statistics—Comparison between two experimental groups were made by the unpaired Student’s t test. For multiple comparisons, one-way ANOVA with Sheffe’s correction was used. p < 0.05 was considered significant.

RESULTS

In this study we sought to determine whether inhibition of Na+,K+-ATPase activity and endocytosis was associated with phosphorylation of the α-subunit. In isolated renal PCT cells, incubation with DA decreased Na+,K+-ATPase activity (nmol P/mg prot/min, vehicle: 112 ± 8 versus DA, 1 μM: 60 ± 2, n = 4, p < 0.05), and this effect was blocked by PKI inhibitors (7, 8). Intact renal PCT cells were metabolically labeled with 32P or thereafter incubated for 25 min at room temperature with or without DA (Fig. 1A). The α-subunit was immunoprecipitated, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. In every experiment the amount of radioactivity (autoradiography or phosphoimager) incorporated into the α-subunit was corrected for the amount of protein present (Western blot), and the quantitative data are shown as percent of control at the bottom of each panel. DA increased the state of phosphorylation (to ~165% of control) of the α-subunit, as illustrated in Fig. 1A. This increased phosphorylation was inhibited by bisindolylmaleimide, a specific PKC inhibitor, but not by a cAMP-dependent protein kinase (PKA) inhibitor, suggesting that phosphorylation of the α-subunit induced by DA is mediated by PKC. Neither inhibitor affected the state of α-subunit phosphorylation in non-stimulated PCT cells.

Phosphorylation of the Na+,K+-ATPase α-subunit by DA was time-dependent (Fig. 1B). It increased significantly after 1 min and was maximal at 2.5 min (178% of control), whereas it was no longer evident at 10 min. However, while the initial (1 and 2.5 min) increase in α-subunit phosphorylation corresponded to the decrease in enzyme activity, this correlation was no longer present at 10 min, i.e. enzyme activity remained inhibited (percent of control, 60 ± 3, p < 0.05), whereas phosphorylation was similar to that of control cells.
To determine whether the Na\(^+\),K\(^+\)-ATPase has been dephosphorylated, we examined the effect of DA in the presence of a phosphatase inhibitor, 1 \(\mu\)M okadaic acid (OKD) (Fig. 1C). Basal phosphorylation (resting condition = control, C) was moderately higher (~1.5-fold) in the OKD-treated cells. As hypothesized, in OKD-treated cells, DA (10 min) did increase the state of \(\alpha\)-subunit phosphorylation, suggesting that at this time period it had been dephosphorylated by the action of protein phosphatases.

Because after 10 min the \(\alpha\)-subunit has been dephosphorylated yet the decreased enzymatic activity persisted, it is possible that the dephosphorylated \(\alpha\)-subunits no longer reside in the plasma membrane. To test this hypothesis, we evaluated the state of phosphorylation of the \(\alpha\)-subunit in BLM and in LE. In BLM prepared from cells that have been preincubated with DA for 10 min, the state of phosphorylation of the immunoprecipitated \(\alpha\)-subunit remained unchanged regardless of whether the PCT cells were previously treated with 1 \(\mu\)M OKD or not, whereas it increased significantly in LE. This was lower panel  

In BLM prepared from cells that have been preincubated with DA for 2.5 min (Fig. 2A, left panel) the state of phosphorylation of the immunoprecipitated \(\alpha\)-subunit was significantly increased, regardless of whether the PCT cells were previously treated with 1 \(\mu\)M OKD or not (phosphorylation (percent of control): 130 ± 2.0 without OKD, 131 ± 1.2 with OKD). These results suggest that the phosphorylated subunits (2.5 min) in the BLM are not affected by protein phosphatases.

Although the results described above support the concept that in response to DA the Na\(^+\),K\(^+\)-ATPase \(\alpha\)-subunits are phosphorylated in the plasma membrane and then internalized and dephosphorylated in LE, the link between these two processes (i.e., whether phosphorylation is a requisite for endocytosis) is not clear. Therefore, we next used an epithelial cell line from OK transfected with the rat Na\(^+\),K\(^+\)-ATPase \(\alpha\)-subunit cDNA carrying a deletion in the nascent 28 amino acids in which Ser\(^{11}\) and Ser\(^{18}\), the putative phosphorylation sites for PKC (33, 34), are absent. OK cells (non-transfected) behaved similarly to native PCT cells in their response to DA: DA decreased the Na\(^+\),K\(^+\)-ATPase activity, and this inhibition was similar to native PCT cells in their response to DA: DA decreased the Na\(^+\),K\(^+\)-ATPase activity, and this inhibition was not significant. B, time-dependent phosphorylation of the Na\(^+\),K\(^+\)-ATPase \(\alpha\)-subunit (filled circles) and inhibition of the catalytic activity (open circles). Isolated PCT cells were incubated with 1 \(\mu\)M DA at room temperature for different periods. A representative autoradiogram and the corresponding Western blot are shown in the upper panel and the quantitative data from four experiments (mean ± S.E.) performed in duplicate. *p < 0.05. C, isolated PCT cells were preincubated with 1 \(\mu\)M OKD or vehicle for 15 min at room temperature. Thereafter, they were incubated with or without 1 \(\mu\)M DA for 10 min at room temperature. The upper panel is a representative autoradiogram, and the quantitative data of four experiments (mean ± S.E.) are shown below. *p < 0.05.
response to DA in OKαrat and OKαrat-t (Fig. 3B). While basal Na⁺,K⁺-ATPase activity was similar in both groups of cells and comparable with that in earlier reports (13, 26), incubation with DA resulted in a significant decrease in Na⁺,K⁺-ATPase activity from OKαrat (p < 0.01), but not from OKαrat-t (p = 0.567). The inhibitory effect of DA in OKαrat was abolished by coincubation with a PKC inhibitor, bisindolylmaleimide (percent of control: 99.3 ± 7, n = 3). We further examined whether this inhibition was associated with phosphorylation of the α-subunit (Fig. 3C). 1 μM DA (3 min; room temperature) increased the state of phosphorylation of the α-subunit in OKαrat but not in OKαrat-t cells.

Last, to determine whether phosphorylation of the α-subunit was necessary for endocytosis, early and late endosomes were prepared from OKαrat and OKαrat-t cells incubated with DA (Fig. 4). DA stimulated the incorporation of α-subunits into EE and LE from OKαrat, and this effect was blocked by bisindolylmaleimide (percent of control, EE: 105 ± 12, n = 3; and LE: 96 ± 17, n = 3) or calphostin C (percent of control, EE: 98 ± 13, n = 3; and LE: 86 ± 11, n = 3). However, DA did not increase the incorporation of α-subunits from OKαrat-t.

**DISCUSSION**

In this report we have demonstrated that DA treatment of both isolated proximal tubule cells and OK cells transfected with the rodent α-subunit leads to inhibition of Na⁺,K⁺-ATPase activity and phosphorylation and endocytosis of the α-subunit. In contrast, when the DA effect was examined in OK cells expressing the Na⁺,K⁺-ATPase α-subunit isoform in which the putative PKC-phosphorylation sites were removed, DA-treatment neither inhibited the enzyme activity nor induced any significant phosphorylation or endocytosis of the α-subunit. These observations strongly suggest a causal link between PKC-dependent phosphorylation of amino acids at the α-subunit N terminus and Na⁺,K⁺-ATPase inhibition and endocytosis in response to a physiological agonist.

Inhibition of Na⁺,K⁺-ATPase activity by DA in renal PCT involves the sequential activation of arachidonic acid, 20-HETE, and PKC (35). Although cAMP stimulation has been suggested to contribute to the action of DA (36, 37), it is unlikely that it would be directly involved in Na⁺,K⁺-ATPase regulation (phosphorylation of the α-subunit in renal PCT cells) because increased cAMP in this segment does not inhibit (7) but is rather associated with stimulation of Na⁺,K⁺-ATPase activity (38). Accordingly, in this study phosphorylation of Na⁺,K⁺-ATPase α-subunits was blocked by PKC-, but not cAMP-K, inhibition. Our observation differs from that reported by Beguin et al. (39), perhaps reflecting differences in the preparations used. We examined isolated PCT cells, where DA is synthetized and physiologically regulates Na⁺,K⁺-ATPase activity, whereas Beguin et al. (39) used a reconstituted system in which the receptor (human dopaminergic DA1A) and the target (Bufo marinus Na⁺,K⁺-ATPase α-subunit) were expressed in a cell line (COS-7) that normally does not express this regulatory system.

The present results suggest that inhibition of total cell Na⁺,K⁺-ATPase activity is initially accomplished by phosphorylation of the α-subunit and that the activity remains decreased because the inhibited units no longer reside in the plasma membrane. Once the α-subunits become phosphoryl-
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It has also been reported that phorbol esters stimulate Na\(^+\),K\(^+\)-ATPase activity (12, 13, 26) and that this effect is accompanied by phosphorylation of the \(\alpha\)-subunit (12). Thus, although both effects (that of DA and of phorbol esters) share a common target, PKC, they are clearly different. For example, stimulation by phorbol esters of Na\(^+\),K\(^+\)-ATPase activity and phosphorylation of the \(\alpha\)-subunit were significant after 15 min of incubation (12). In contrast, the effect of DA occurs already at 1 min, and after 10 min, the \(\alpha\)-subunits are no longer phosphorylated and, in addition, they no longer reside in the plasma membrane. Finally, another reason why the effects of phorbol esters and DA are different in nature may be that the effect of DA on Na\(^+\),K\(^+\)-ATPase activity is mediated via a PKC isof orm that can be activated by arachidonic acid metabolism and generation (in the PCT) of the cytochrome P-450 metabolite, 20-HETE, an eicosanoid that activates PKC (41). The action of DA might therefore involve an atypical PKC isof orm that is not responsive to phorbol esters (42) but whose activation is rather dependent on membrane lipids.

In conclusion, while in intact cells the use of phorbol esters has not been proved to be an efficient probe to demonstrate the relationship between phosphorylation of the Na\(^+\),K\(^+\)-ATPase \(\alpha\)-subunit and inhibition of its activity (17), by using an agonist such as dopamine in cells where it is produced and exerts its physiologic action it was possible to demonstrate that phosphorylation of the \(\alpha\)-subunit is associated with inhibition of Na\(^+\),K\(^+\)-ATPase activity and that this step is required for subunit endocytosis.

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