Ouabain stimulates Na-K-ATPase through a sodium/hydrogen exchanger-1 (NHE-1)-dependent mechanism in human kidney proximal tubule cells

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Departments of 1Medicine/Kidney Disease Program, 4Pathology, 5Physiology, and 6Veterans Administration Medical Center, University of Louisville, Louisville, Kentucky; 2Department of Physiology, University of Arizona, Tucson, Arizona; and 3Department of Medicine, Case Western Reserve University, Cleveland, Ohio

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Ouabain stimulates Na-K-ATPase through a sodium/hydrogen exchanger-1 (NHE-1)-dependent mechanism in human kidney proximal tubule cells. Am J Physiol Renal Physiol 299: F77–F90, 2010. First published April 28, 2010; doi:10.1152/ajprenal.00581.2009.—Recent investigations demonstrate increased Na/H exchanger-1 (NHE-1) activity and plasma levels of ouabain-like factor in spontaneously hypertensive rats. At nanomolar concentrations, ouabain increases Na-K-ATPase activity, induces cell proliferation, and activates complex signaling cascades. We hypothesize that the activity of NHE-1 and Na-K-ATPase are interdependent. To test whether treatment with picomolar ouabain regulates Na-K-ATPase through an NHE-1-dependent mechanism, we examined the role of NHE-1 in ouabain-mediated stimulation of Na-K-ATPase in kidney proximal tubule cell lines [opossum kidney (OK), HK-2, HKC-5, and HKC-11] and rat kidney basolateral membranes. Ouabain stimulated Na-K-ATPase activity and tyrosine phosphorylation in cells that express NHE-1 (OK, HKC-5, and HKC-11) but not in HK-2 cells that express very low levels of NHE-1. Inhibition of NHE-1 with 5 μM EIPA, a NHE-1-specific inhibitor, prevented ouabain-mediated stimulation of 86Rb uptake and Na-K-ATPase phosphorylation in OK, HKC-5, and HKC-11 cells. Expression of wild-type NHE-1 in HK2 cells restored regulation of Na-K-ATPase by picomolar ouabain. Treatment with picomolar ouabain increased membrane expression of Na-K-ATPase and enhanced NHE-1-Na-K-ATPase α1-subunit association. Treatment with ouabain (1 μg·kg body wt⁻¹·day⁻¹) increased Na-K-ATPase activity, expression, phosphorylation, and association with NHE-1 increased in rat kidney cortical basolateral membranes. Eight days’ treatment with ouabain (1 μg·kg body wt⁻¹·day⁻¹) resulted in increased blood pressure in these rats. These results suggest that the association of NHE-1 with Na-K-ATPase is critical for ouabain-mediated regulation of Na-K-ATPase and that these effects may play a role in cardioglycoside-stimulated hypertension.

cardioglycosides; phosphorylation

THE BASOLATERAL Na-K-ATPase- or the sodium pump-mediated regulation of renal proximal tubule sodium reabsorption is essential for whole-body sodium homeostasis, regulation of extracellular fluid volume, and blood pressure control (8, 24, 65). Plant-derived cardiac glycosides; including digitalis, digoxin, and ouabain, are well-established inhibitors of Na-K-ATPase activity that bind to an extracellular portion of the Na-K-ATPase α-subunit (34). Ouabain-like cardioglycosides, which are produced in the adrenal gland and hypothalamus, have been associated with salt-sensitive hypertension (19, 20, 36, 52, 55). Plant-derived cardiac glycosides have been exploited as drugs to treat congestive heart failure and atrial fibrillation (1). In cardiac cells, ouabain increases intracellular Ca²⁺ concentration and cardiac muscle contractility by inhibiting Na-K-ATPase activity, thus stimulating Na/Ca exchange (4). In some cells, however, ouabain at very low (pM to nM) concentrations increases Na-K-ATPase activity (23). Previously, we demonstrated that in opossum kidney (OK) cells, nanomolar concentrations of ouabain stimulate Na-K-ATPase-mediated ion transport through a Src kinase-, ERK1/2-, and Akt-mediated pathway (25, 26). Other investigators demonstrated that nanomolar ouabain also regulates the sodium pump by activating the Na-K-ATPase-associated Src, resulting in stimulation of protein tyrosine phosphorylation (32, 57). Multiple studies from numerous laboratories demonstrated that Na-K-ATPase complexes with membrane and cytoskeletal proteins in caveolae and converts ouabain-binding signaling into the activation of downstream signaling proteins, including phosphoinositide 3-kinase, tyrosine kinases, the Ras-Raf-MEK pathway, ERK, and protein kinase B (Akt) (20, 29, 30, 35, 36, 43, 52, 60, 62).

Like Na-K-ATPase, Na⁺/H⁺ exchanger-1 (NHE-1) is a ubiquitous integral membrane protein localized to the basolateral membrane (BLM) in polarized epithelial cells. NHE-1 regulates intracellular pH and cell volume via electroneutral exchange of intracellular H⁺ for extracellular Na⁺. It is also involved in cytoskeletal organization, cell growth, proliferation, and differentiation, heart disease, and cancer (9, 44, 61). NHE-1 is composed of an N-terminal membrane domain that functions in ion transport and a C-terminal cytoplasmic regulatory domain that regulates the activity and mediates cytoskeletal interactions (63, 64). Recent findings that link NHE-1 with the pathogenesis of hypertension (45) led us to investigate the possible role of NHE-1 in the mechanism of ouabain-stimulated Na-K-ATPase activation.

It has been well established that the activity of Na-K-ATPase can influence cardiac function and blood pressure because inhibition of Na-K-ATPase increases contractility of both cardiac and vascular smooth muscle cells (2, 5, 13). Additionally, several studies suggest that endogenous cardioglycosides directly interact with the Na-K-ATPase α-subunit and can play an important role in regulating cardiovascular function and blood pressure (2). Although the acute effects of nanomolar and micromolar concentrations of cardioglycosides on signal transduction pathways and Na-K-ATPase regulation in heart and vascular smooth muscle are well studied, the...
mechanisms of nanomolar or picomolar concentrations of cardioglycosides on Na-K-ATPase activity are largely unknown. We have previously demonstrated in OK cells that nanomolar ouabain increases Na-K-ATPase-mediated $^{86}$Rb uptake through a Src-ERK-Akt dependent mechanism. Based on this observation, we hypothesized that chronic stimulation with nanomolar ouabain will result in increased activity of Na-K-ATPase in kidney cortical BLMs through increased expression and tyrosine phosphorylation. To address this hypothesis, we treated rats with $1 \mu g/kg$ body wt ouabain for several days and measured blood pressure and renal cortical Na-K-ATPase expression, phosphorylation, and activity. Our data demonstrated an increase in Na-K-ATPase expression, phosphorylation, and activity in kidney cortical BLMs from ouabain-treated animals. Surprisingly, expression of NHE-1 was also increased in BLMs from ouabain-treated animals. This led us to hypothesize that NHE-1 may associate with Na-K-ATPase to increase its activity in ouabain-treated animals. The role of NHE-1 was investigated using human kidney proximal tubule cells. Our data demonstrate that treatment with picomolar ouabain increased association of the Na-K-ATPase $\alpha_1$-subunit with NHE-1. Inhibition of NHE-1 prevented the stimulation of Na-K-ATPase and expression and phosphorylation of Na-K-ATPase $\alpha_1$-subunit by picomolar ouabain.

**EXPERIMENTAL PROCEDURES**

**Materials**

Ouabain, 8-(diethyl amino) octy-3,4,5-trimethoxybenzoate (TMB-8), EIPA, and ammonium chloride were purchased from Sigma (St. Louis, MO). Polyclonal antibodies against phototyrosine were purchased from Zymed Laboratories (Carlsbad, CA), and monoclonal antibodies against phototyrosine (clone 4G10) were purchased from Millipore (Billerica, MA). Antibodies against caveolin were purchased from Novus Biological. The monoclonal antibody against the Na-K-ATPase $\alpha_1$-subunit (a6F) developed by Dr. D. M. Fambrough was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of NIH CD and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA). A rabbit polyclonal antibody against rat Na-K-ATPase $\alpha_1$ (RT-NKA) for immunoprecipitation was a gift from Dr. Thomas Pressley (Texas Tech University). Rabbit polyclonal antibodies against NHE-1 were previously characterized by J. R. Schelling (Case Western Reserve University, Cleveland, OH) (59), and monoclonal antibodies against NHE-1 were purchased from BD Biosciences. Horseradish peroxidase-linked secondary antibodies were purchased from Vector Laboratories. Streptavidin-agarose resins were purchased from Pierce Biotechnology (Rockford, IL). Phosphatase inhibitor cocktail-1 and protease inhibitor cocktail were purchased from Sigma. All other chemicals were purchased from Sigma, unless otherwise specified.

**Animal Model**

All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Louisville. Sprague-Dawley rats, weighing 200–250 g, were stabilized on standard rat chow and water ad libitum for 1 wk before the experiments. Rats ($n = 8$ in vehicle or ouabain treated) were intraperitoneally injected with $1 \mu g/kg$ body wt ouabain (dissolved in sterile PBS) once daily for 4 (BLM preparation and Na-K-ATPase activity) or 8 days (blood pressure measurement). Blood pressure was measured in ketamine-anesthetized rats after a 4-day treatment with ouabain by placing a catheter in the right carotid artery, and data were analyzed by using customized Micro-Med software as described by Sen et al. (53). Blood was collected, and serum was separated and analyzed for ouabain levels. The animals were killed, and kidneys were decapsulated and collected in ice-cold PBS. Kidneys were decapsulated for BLM preparation or for preparation of paraffin blocks for immunohistochemistry. Of note, blood pressure did not change significantly in animals treated with ouabain for 4 days. To detect changes in blood pressure, a separate group of animals was treated with either vehicle or ouabain (1 $\mu g/kg$ body wt $^{-1} \cdot$day $^{-1}$) for 8 days ($n = 8$ in each group), and blood pressure was measured as described above.

**Determination of Ouabain Levels in Serum**

Ouabain levels were measured in serum samples from rats treated with vehicle or ouabain (1 $\mu g/kg$ body wt $^{-1} \cdot$day $^{-1}$) for 4 or 8 days as described previously (16, 49). Briefly, ouabain concentration was measured by ELIs using antisera containing polyclonal antibodies to ouabain. Microtiter plate wells were coated for a minimum of 18 h at 4°C with 0.5 $\mu g$/well of BSA-conjugated ouabain diluted in carbonate-bicarbonate coating buffer containing 15 mM Na$_2$CO$_3$, 35 mM NaHCO$_3$, and 3.1 mM NaN$_3$ in water (pH 9.6). After coating, the plates were washed with 0.5 ml/l Tween 20 in PBS and then blocked with 10 g/l BSA solution in PBS for 1 h at 37°C. After washing, the standards and samples were added, followed by the addition of the appropriate antibody, and the plate was incubated at room temperature for 1 h. After another washing step, goat anti-rabbit horseradish peroxidase conjugate was added and allowed to bind to the primary antibody for an additional 2 h at room temperature. Finally, the plate was washed, and 100 $\mu l$ of 3,3',5,5'-tetramethylbenzidine (TMB) reagent as substrate was added to each well. Color development was monitored at 450 nm for a maximum of 30 min, after which the reaction was stopped with 100 $\mu l$ of TMB stop buffer and the plate was read at 450 nm. The readings were blanked and adjusted for nonspecific binding. We used the plant-derived ouabain as a standard in the immunoassays. Therefore, all concentrations and amounts of measured ouabain refer to the respective immunoequivalences to the plant-derived ouabain.

**BLM Isolation**

Kidney cortical BLMs were prepared from rats treated with or without ouabain for 4 days by the method of Sacktor et al. (50) with slight modifications. All steps were performed at 4°C unless otherwise stated. Briefly, 3-mm slices of kidney cortex were carefully separated and homogenized in 250 mM sucrose, 1 mM PMSF, and 10 mM Tris-HCl, pH 7.4, by 20 strokes in a glass teflon homogenizer. The homogenate was subjected to high-speed homogenization in a polytron-type homogenizer at maximum speed for three pulses of 30 s each with a 30-s interval. The homogenates were incubated with 15 mM MgCl$_2$ on ice with constant shaking for 20 min to precipitate other membrane organelles. The homogenate was centrifuged at...
2,500 g for 10 min in a Sorvall centrifuge using a SS-34 rotor. The supernatant was centrifuged at 24,000 g in a Sorvall centrifuge using a SS-34 rotor. The pellet was resuspended in 32.2 ml of homogenization buffer and mixed vigorously with 2.8 ml Percoll (final concentration 8.23%). The samples were centrifuged at 30,000 g for 35 min, and the middle layer (8 ml) containing BLMs was diluted with KCl-mannitol buffer containing 100 mM mannitol, 100 mM KCl, and 10 mM Tris-HEPES buffer, pH 7.1, and centrifuged at 34,000 g for 30 min as above. The white fluffy BLMs were resuspended in the KCl-mannitol buffer and centrifuged again at 38,000 g for 30 min, and the final pellet was resuspended in 300 mM mannitol, and 5 mM Tris-HEPES, pH 7.4, at 1 ml/g starting tissue. The BLMs showed seven- to eightfold enrichment of Na-K-ATPase activity compared with homogenates (data not shown).

ATP Hydrolysis Assay

BLM vesicles were quickly frozen in liquid nitrogen and slowly thawed on ice to make them permeable to ATP before measurement of Na-K-ATPase activity. Na-K-ATPase activity in BLMs was assayed as ouabain (1 mM)-sensitive ATP hydrolysis as previously described (27). The inorganic phosphate released was measured as described previously (27).

Immunohistochemistry

Kidney slices (3 μm) from rats treated with or without ouabain for 4 days were cut using a microtome from paraffin-embedded kidneys and fixed on glass slides. Paraffin was removed, and samples were rehydrated by passing slides through xylene (twice) followed by 100, 90, and 70% ethanol. Antigens were unmasked, and endogenous peroxidase was quenched using 3% hydrogen peroxide. Slides were blocked by 5% horse serum in Tris buffer, in a humidified chamber for 30 min at room temperature. Slides were incubated with anti-rabbit biotinylated secondary antibody for 30 min at room temperature. Slides were incubated with poly-L-lysine/HAP (566.5 nm).

Crude Membrane Isolation

Cells were washed twice with PBS and placed in ice-cold lysis buffer containing 50 mM mannitol, 5 mM Tris-HCl, pH 7.4, 10 μM IC50 phosphatase inhibitor cocktail, and 10 μM/ml protease inhibitor cocktail. The lysates were homogenized using 27.5-g needle syringes, followed by centrifugation at 2,500 g for 10 min to remove cell debris. The supernatant was centrifuged at 30,000 g for 45 min. The pellet was resuspended in buffer containing 300 mM mannitol, 5 mM Tris-HCl, pH 7.4, 10 μM/ml phosphatase inhibitor cocktail, and 10 μM/ml protease inhibitor cocktail.

Western Blot Analysis

Western blot analysis was performed exactly as described previously (26, 28).

Biotinylation

Surface biotinylation was performed as described previously (25). Briefly, cells were treated with ouabain for 15 min, washed with cold PBS, and incubated with N-hydroxysulfosuccinimidobiotin (10 μM/ml) in borate buffer, pH 9.0 (20 mM Tris, 150 mM NaCl, 10 mM boric acid, 7.2 mM KCl, 1.8 mM CaCl2) for 2 h at 4°C. Cells were then washed three times with cold PBS, quenched with 100 mM glycine in PBS for 15 min at 4°C, and then washed three times more. Next, crude membranes were isolated and incubated with streptavidin-agarose
resins overnight on a rotator at 4°C. Proteins bound to the resins were eluted and then resolved on 10% SDS-PAGE followed by immunoblotting using monoclonal antibodies against the Na-K-ATPase α1-subunit (α6F).

Immunoprecipitation

Crude membranes from cells or BLMs from kidneys solubilized in immunoprecipitation (IP) buffer (20 mM Tris·HCl, pH 7.4, 150 mM NaCl, 20 mM NaF, 1 mM EDTA, 1 mM EGTA, 100 μM/ml phosphatase inhibitor cocktail, 100 μM/ml protease inhibitor cocktail, 1% Triton X-100, 0.5% NP-40, and 0.5% SDS) were centrifuged at 70,000 g for 1 h in a Beckman ultracentrifuge. One hundred micrograms protein from the supernatant was precleared with protein A-Sepharose beads for 2 h at 4°C. The beads were separated by centrifugation at 14,000 rpm for 1 min in a tabletop centrifuge (Spectrafuge, 4°C). The beads were washed three times with IP buffer by centrifugation at 14,000 rpm for 1 min in a tabletop centrifuge, and an equal volume of 2X Laemml sample buffer was added and heated at 65°C for 10 min. The beads were centrifuged as above, and the proteins in the supernatant were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with the indicated antibodies.

Identification of Ouabain-Stimulated Phosphorylation Sites

Na-K-ATPase phosphorylation sites, in ouabain-treated HKC-5 and HKC-11 cells, were identified following the method described by Bodenmiller et al. (6). Briefly, Na-K-ATPase was immunoprecipitated as described above using monoclonal anti-Na-K-ATPase α1-antibody α6F from the Developmental Studies Hybridoma Bank (Univ. of Iowa), except that in place of Triton X-100 and SDS only 1% n-octylglucoside was used as a detergent. The immunoprecipitated proteins were eluted using 0.5 M glycine, pH 2.7, and collected in 10 μl 1 M triethylammonium bicarbonate, pH 8.5. The eluted proteins were reduced, alkylated, and digested with trypsin. The second step of enrichment was performed by affinity purification of the phosphorylated peptides using a TiO2-packed nanobore HPLC column. Bound peptides were eluted in a single step online to a one-dimensional reverse phase HPLC column. Putative phosphopeptides were eluted in a gradient fashion onto a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) target. A peptide mass list was compiled by serial MALDI-TOF-MS survey scans. A theoretical phosphopeptide mass list was established using a Na-K-ATPase FASTA file submitted to the Protein Prospector tool MS-DIGEST. Tandem MS data were acquired for all matching peptides in an attempt to confirm candidate phosphorylated Na-K-ATPase peptides.

Protein Determination

Protein concentration was determined using a bicinchoninic acid protein assay kit (Sigma) using a BSA standard.

Statistics

Data are shown as means ± SE. The n values represent the number of independent experiments. Each experiment was performed in triplicate. P values were calculated using SigmaStat software utilizing Student’s t-test or by one-way ANOVA, followed by Bonferroni analysis using GraphPad Prism software. A P value <0.05 was a priori considered statistically significant.

RESULTS

Role of Ouabain in Na-K-ATPase Regulation in Rat Kidney Cortical BLMs

Previous studies show that endogenous serum levels of cardiac glycosides like ouabain increase in animal models and in humans with salt-sensitive hypertension (4, 40, 55). We first confirmed that ouabain at nanomolar concentrations exerted hemodynamic effects in Sprague-Dawley rats (4). Circulating ouabain levels increased significantly in animals treated with ouabain (1 μg·kg body wt−1·day−1) for 4 days [baseline 0.52 ± 0.009 (~0.89 nM) vs. 0.99 ± 0.156 ng/ml (~1.69 nM) after ouabain treatment (P < 0.032 by t-test)]. The calculated ouabain values are slightly higher in both control and treated animals from the reported values (20). This may be due to the difference in the methods (RIA vs. ELA), the specificity of the anti-ouabain antibodies used, or the presence of endogenous circulating ouabain-like factors in serum (20). Sprague-Dawley rats treated with ouabain (1 μg·kg body wt−1·day−1) for 8 days showed a significant increase in blood pressure without a change in heart rate, as demonstrated in Table 1.

Effect of ouabain on Na-K-ATPase regulation in kidney cortical BLMs. To determine the effects of nanomolar ouabain on Na-K-ATPase regulation, Na-K-ATPase activity in kidney cortex BLM vesicles was assayed according to previously described methods (27). As shown in Fig. 1 (top), treatment with ouabain (1 μg·kg body wt−1·day−1) for 4 days significantly increased Na-K-ATPase activity in kidney BLM.

To determine the mechanism for the ouabain-stimulated increase in Na-K-ATPase activity, we examined Na-K-ATPase expression, NHE-1 expression, and tyrosine phosphorylation. As shown in Fig. 2, ouabain (1 μg·kg body wt−1·day−1) stimulated expression of the Na-K-ATPase α1-subunit (Fig. 2A) and NHE-1 (Fig. 2B) in kidney cortical BLM. To confirm the immunoblot data, we performed immunohistochemistry of kidney sections from the above animals. As shown in Fig. 2, C and D, expression of both the Na-K-ATPase α1-subunit (Fig. 2C) and NHE-1 (Fig. 2D) increased significantly in kidney sections from ouabain-treated rats relative to vehicle controls.

Table 1. Effect of ouabain on blood pressure

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Ouabain (1 μg·kg body wt for 8 days)</th>
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<tbody>
<tr>
<td>MAP, mmHg</td>
<td>75.93 ± 4.13</td>
<td>94.95 ± 2.66*</td>
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<tr>
<td>SBP, mmHg</td>
<td>92.27 ± 10.03</td>
<td>113.3 ± 2.59*</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>65.23 ± 3.86</td>
<td>86.1 ± 2.82*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>257.1 ± 20.13</td>
<td>253.5 ± 3.5</td>
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Values are means ± SE. MAP, mean arterial pressure; SBP and DBP, systolic and diastolic blood pressure, respectively. Sprague-Dawley rats were treated intraperitoneally for 8 days with ouabain (1 μg/kg body wt). After 8 days, blood pressure was determined by inserting a PE catheter in ketamine-anesthetized rats as described in EXPERIMENTAL PROCEDURES. *P < 0.05 by Student’s t-test.
The above suggested an increase in both the Na-K-ATPase α1-subunit and NHE-1 in kidney BLM. Therefore, we reasoned that the two proteins may associate with each other. Several investigators have demonstrated that treatment with ouabain induces association of Na-K-ATPase with caveolin-1 in heart and LLC-PK1 cells (20, 21, 60). To determine whether ouabain increases tyrosine phosphorylation of the Na-K-ATPase α1-subunit in kidney BLM, the Na-K-ATPase α1-subunit was immunoprecipitated from BLM using polyclonal antibodies against the Na-K-ATPase α1-subunit and analyzed by immunoblotting using polyclonal phosphorytrosine antibodies. As shown in Fig. 3A, treatment with ouabain significantly increased Na-K-ATPase α1-subunit tyrosine phosphorylation in kidney BLM. In contrast, treatment with ouabain did not change serine phosphorylation of the Na-K-ATPase α1-subunit (Fig. 3A, bar diagram).

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Role of NHE-1 in Picomolar Ouabain-Mediated Effects in Human Kidney Proximal Tubular Cells

Effect of picomolar to nanomolar concentrations of ouabain on Na-K-ATPase activity in human kidney proximal tubule cells. We first developed a dose-response relationship for the effect of ouabain on Na-K-ATPase-mediated ⁸⁶Rb uptake in human kidney proximal tubule cell culture models. All ⁸⁶Rb uptake experiments were carried out in the presence of 5 μM monensin to raise intracellular Na⁺ concentrations, and the assay is performed at Vₘₐₓ for sodium. Of note, all cell culture studies employed acute ouabain treatment (15 min) in contrast to animal studies, where rats were chronically treated with ouabain (4–8 days). As shown in Fig. 4A, 10 pM ouabain maximally increased ⁸⁶Rb uptake in HKC-11 cells, while higher concentrations of ouabain (1–1,000 nM) decreased Na-K-ATPase-mediated ⁸⁶Rb uptake. In HKC-5 cells, ouabain stimulated ⁸⁶Rb uptake maximally at 10 pM concentration, similar to HKC-11 cells (Supplemental Fig. 2A; all supplemental material for this article is available on the journal website). However, in OK cells ouabain increased ⁸⁶Rb uptake maximally at 10 nM concentration (Supplemental Fig. 2B). In contrast, picomolar to nanomolar ouabain had no effect on ⁸⁶Rb uptake in HK-2 cells. Higher concentrations of ouabain decreased ⁸⁶Rb uptake similarly in all human kidney cell lines. Of note, treatment with 5 μM monensin increased intracellular sodium concentrations in a time-dependent manner in both HKC-11 and HK-2 cells (Fig. 4B). To determine the reason for the lack of a stimulatory ouabain response in HK-2 cells, we compared Na-K-ATPase α1-subunit expression in HK-2 and HKC-11 cells and found no difference (Supplemental Fig. 3A). Because both NHE-1 and Na-K-ATPase show similar activation in common cell functions including cell proliferation and physiological functions like blood pressure, we compared NHE-1 expression in the human kidney cell lines and found that NHE-1 expression was significantly less in HK-2 cells compared with OK, HKC-11, and HKC-5 cells (Supplemental Fig. 3B). Treatment of HKC-11 and HK-2 cells with 10 pM ouabain increased NHE-1 expression in crude membranes but not in HK-2 cells (Fig. 4C). Expression of other major proximal tubule transporters NHE-3 and NBC-1 was not significantly different between the cell lines (Supplemental Fig. 3A).

To confirm that NHE-1 expression is required for Na-K-ATPase-mediated ⁸⁶Rb uptake by ouabain, we treated OK, HK-2, HKC-5, and HKC-11 cells with either 10 nM (OK cells) or 10 pM (HK cells) ouabain in the presence or absence of 5 μM EIPA, a concentration that specifically inhibits NHE-1 (41, 46, 59), with minimal effect on NHE-3. Figure 5 shows that pretreatment with EIPA prevented stimulation of ⁸⁶Rb uptake by ouabain in OK, HKC-5, and HKC-11 cells. EIPA alone had no effect on Na-K-ATPase-mediated ⁸⁶Rb uptake in any of the cell culture models studied. Neither ouabain (10 pM) nor EIPA had any effect on Na-K-ATPase-mediated ⁸⁶Rb uptake in NHE-1-deficient HK-2 cells.

Effect of ouabain on membrane expression and phosphorylation of Na-K-ATPase α1-subunit. To determine whether the increase in ⁸⁶Rb uptake is due to an increase in membrane expression of Na-K-ATPase, we examined the effect of 10 pM ouabain on plasma membrane expression of the Na-K-ATPase α1-subunit by surface biotinylation. As shown in Fig. 6A, 15-min treatment with 10 pM ouabain increased biotinylation of the Na-K-ATPase α1-subunit in HKC-11 cells compared with vehicle-treated cells but not in HK-2 cells. Ouabain increased surface expression of the Na-K-ATPase α1-subunit in OK cells similar to HKC-11 cells (Supplemental Fig. 4A). Tyrosine phosphorylation of the Na-K-ATPase α1-subunit has been identified as a mechanism for insulin-mediated stimulation of Na-K-ATPase and trafficking to the plasma mem-
brane (18). To determine whether ouabain induced tyrosine phosphorylation of the Na-K-ATPase α1-subunit, HK-2 and HKC-11 cells were treated with 10 pM ouabain for 15 min and the Na-K-ATPase α1-subunit was immunoprecipitated using polyclonal antibodies against the Na-K-ATPase α1-subunit and analyzed by Western blotting using polyclonal antibodies against phosphotyrosine. As shown in Fig. 6B, ouabain stimulated tyrosine phosphorylation of the Na-K-ATPase α1-sub-
unit in HKC-11 cells, but not in NHE-1-deficient HK-2 cells, suggesting a role for NHE-1 in tyrosine phosphorylation of the Na-K-ATPase α1-subunit. Similar to HKC-11 cells, ouabain also increased Na-K-ATPase α1-subunit phosphorylation in OK cells (Supplemental Fig. 4B). To confirm that ouabain induces tyrosine phosphorylation of the Na-K-ATPase α1-subunit, HK-2 and HKC-11 cells were treated with 10 pM ouabain for 15 min and the Na-K-ATPase α1-subunit was immunoprecipitated with monoclonal antibodies against Na-K-ATPase α1 (6F antibody) and analyzed by Western blotting using phosphotyrosine or phosphoserine (A), NHE-1 or caveolin-1 (B), or Na-K-ATPase α1-subunit (C) antibodies. The blots were stripped and analyzed by immunoblotting for Na-K-ATPase α1-subunit (A and B) or NHE-1 (C) for equal loading. Each bar represents data as AU (ratio of phosphotyrosine or phosphoserine to total Na-K-ATPase α1-subunit or Na-K-ATPase α1-subunit or NHE-1 to total NHE-1 or Na-K-ATPase α1-subunit immunoprecipitated); values are means ± SE from 8 BLM preparations from different animals (n = 8). *P < 0.05 by ANOVA followed by Bonferroni analysis.

Potential tyrosine kinase-mediated phosphorylation sites on the Na-K-ATPase α1-subunit were identified by utilizing MALDI-TOF-MS and MALDI-TOF/TOF analysis of HKC-5 and HKC-11 cells stimulated with ouabain. Five candidate phosphopeptides were identified within the list of putative peptides purified using a TiO2 affinity chromatography approach. As shown in Table 2, we identified a previously described Tyr-10 phosphorylation (peptide 4) (18) and a unique tyrosine phosphorylation at tyrosine 260 of the Na-K-ATPase α1-subunit. The two putative phosphorylation sites (Y-10 and Y-260) on Na-K-ATPase were predicted as Src tyrosine kinase (32, 57) and enhanced green fluorescent protein kinase phosphorylation sites by web-based phosphorylation prediction software available on the EXPASY website (http://www.cbs.dtu.dk/services/NetPhosK/).

To examine the role of NHE-1 in ouabain-mediated tyrosine phosphorylation of the Na-K-ATPase α1-subunit, HKC-11 cells were treated with 10 pM ouabain in the presence and absence of 5 μM EIPA. As shown in Fig. 6C, treatment with either ouabain or insulin increased tyrosine phosphorylation of the Na-K-ATPase α1-subunit, and these effects were prevented by preincubation with EIPA.

To confirm the role of NHE-1 in regulation of Na-K-ATPase by 10 pM ouabain in human kidney cells, we transfected HK-2 cells with either empty vector or human wild-type NHE-1. The cells were treated for 15 min with 10 pM ouabain. NHE-1 expression (Fig. 7A), Na-K-ATPase α1-subunit phosphorylation (Fig. 7B), and 86Rb uptake (Fig. 7C) were determined as described above. As shown in Fig. 7, ouabain increased Na-K-ATPase α1-subunit phosphorylation and 86Rb uptake in cells transfected with wild-type NHE-1, but not in vector-transfected cells.
Effect of ouabain on NHE-1 phosphorylation and association with Na-K-ATPase α1-subunit. Several studies suggest that NHE-1 activity is modulated by changes in its phosphorylation state (38, 39, 51, 56). To assess NHE-1 phosphorylation, we immunoprecipitated phosphorylated proteins from human kidney cell lysates treated with 10 nM ouabain using phosphoserine/threonine antibodies and analyzed by immunoblotting using NHE-1 antibodies. As shown in Fig. 8A, treatment with 10 nM ouabain significantly increased phosphorylation of NHE-1 in HKC-11 cells but not in HK-2 cells. Ouabain increased serine phosphorylation of NHE-1 in OK cells similar to HKC-11 cells (Supplemental Fig. 4C).

The above data suggest that 10 nM ouabain stimulated Na-K-ATPase regulation through NHE-1-dependent mechanisms. Both Na-K-ATPase and NHE-1 are localized to the BLMs in proximal tubular epithelial cells and may interact with each other. To determine whether the Na-K-ATPase α1-subunit associates with NHE-1, we treated HK-2 and HKC-11 cells with 10 nM ouabain. The Na-K-ATPase α1-subunit was immunoprecipitated and analyzed by immunoblotting. As shown in Fig. 8B, treatment with ouabain increased the association between the Na-K-ATPase α1-subunit and NHE-1 in HKC-11 cells but not in HK-2 cells. Reciprocal immunoprecipitation with NHE-1 and immunoblotting for the Na-K-ATPase α1-subunit (Fig. 8C) confirmed the ouabain-stimulated increase in association between NHE-1 and the Na-K-ATPase α1-subunit. Similar to HKC-11 cells, treatment with ouabain increased association between Na-K-ATPase and NHE-1 or caveolin-1 in OK cells (Supplemental Fig. 3D).

To confirm that ouabain induces association of the Na-K-ATPase α1-subunit with NHE-1, HK-2 and HKC-11 cells were treated with 10 nM ouabain for 15 min and the Na-K-ATPase α1-subunit was immunoprecipitated with monoclonal antibodies against Na-K-ATPase α1 (α6F antibody) and analyzed by Western blotting using monoclonal antibodies against NHE-1 or the Na-K-ATPase α1-subunit (α6F antibody). As shown in Supplemental Fig. 5, treatment with ouabain increased association of the Na-K-ATPase α1-subunit with NHE-1. Reciprocal immunoprecipitation of NHE-1 using monoclonal antibodies and Western blotting with the Na-K ATPase α1-subunit (α6F antibody) confirmed the association (Supplemental Fig. 5B).
DISCUSSION

The present studies demonstrate a novel interaction between Na-K-ATPase and NHE-1 that is stimulated by ouabain in the nanomolar or picomolar concentration range. Na-K-ATPase interaction with NHE-1 was observed in intact rat kidney cells as well as in cultured human renal tubule cells. The ability of picomolar concentrations of ouabain to stimulate interaction between the two ion transporters is interesting in light of recent evidence that ouabain at picomolar and nanomolar concentrations, depending on species and tissue, activates a signaling cascade through its interaction with Na-K-ATPase.

In several models of salt-sensitive hypertension, endogenous ouabain-like cardiac glycoside levels are increased. Infusion of exogenous ouabain has been demonstrated to increase blood pressure through action on both α1- and α2-subunits of Na-K-ATPase, which could reflect alterations in cardiac output, vascular tone, central nervous system stimulation, or renal salt handling (11–14, 17, 37). Ferrari and colleagues (22) demonstrated increased Na-K-ATPase activity in kidneys from rats treated with ouabain (15 μg/kg body wt for 15 days). In a recent paper, Loreaux et al. (37) demonstrated that mice expressing a genetically modified α1-subunit that was sensitive to ouabain experienced a significant natriuresis in response to salt loading, while animals expressing a ouabain-resistant α1-subunit did not. Interestingly, the animals expressing the ouabain-sensitive α1-subunit showed an increase in mean arterial blood pressure with ouabain infusion for 30 min while the animals expressing the ouabain-resistant α1-subunit did not. These findings suggested to the authors that the hypertensive effect of ouabain was mediated by the effects of ouabain on the Na-K-ATPase expressed on the vasculature and did not support a role for ouabain-stimulated Na-K-ATPase activity in the kidney. In the present study, we demonstrate that infusion of ouabain at 1 μg/kg body wt for 4 days in rats caused a significant increase in serum ouabain levels within the nanomolar range, which resulted in a significant rise in blood pressure at 8 days. Our studies demonstrate that stimulation of Na-K-ATPase by low-dose ouabain occurs before an increase in blood pressure, suggesting a potential role of increased renal Na-K-ATPase activity in the genesis of ouabain-induced hypertension. However, further studies are required to confirm that the effects of cardiac glycoside on Na-K-ATPase activity in the kidney are primary and causal to the increase in blood pressure.

Fig. 5. Effect of NHE-1 inhibition on stimulation of Na-K-ATPase-mediated 86Rb uptake in kidney proximal tubular cells by ouabain. Intact cells were treated for 15 min with vehicle, 10 nM (OK cells, A) or 10 pM (HK cells, B–D) ouabain in the presence or absence of NHE-1 inhibitor EIPA (5 μM). Na-K-ATPase-mediated 86Rb uptake was measured as described in EXPERIMENTAL PROCEDURES. Each bar represents means ± SE from 6 independent experiments performed in triplicate. Of note, all 86Rb experiments were performed in the presence of a sodium ionophore (monensin; 5 μM) such that the uptake was measured at Vmax for sodium. *P < 0.05 by 1-way ANOVA followed by Bonferroni analysis (GraphPad Prizm).
cortex Na-K-ATPase expression, phosphorylation, enzyme activity, and association with NHE-1 increased. Our data demonstrated that there was a \( \sim 47\% \) increase in activity; however, there was about a threefold increase in expression and phosphorylation of the Na-K-ATPase \( \alpha_1 \)-subunit. These findings are consistent with an increase in nonpumping Na-K-ATPase, as suggested by Liang et al. (33). Further experiments are required to confirm this conclusion. These findings are consistent with an increase in nonpumping Na-K-ATPase, as suggested by Liang et al. (33). Further experiments are required to confirm this conclusion.

Table 2. Identification of Na-K-ATPase phosphorylated peptides from ouabain-treated HKC-11 cells

<table>
<thead>
<tr>
<th>Peptide m/z</th>
<th>Putative Modifications</th>
<th>Start Sequence</th>
<th>End Sequence</th>
<th>Missed Cleavages</th>
<th>Peptide Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,554.157</td>
<td>1Phospho</td>
<td>662</td>
<td>683</td>
<td>1</td>
<td>(K)ACVVHOSDLKDMTSEQLDDILK(Y)</td>
</tr>
<tr>
<td>2,093.914</td>
<td>1Met-ox</td>
<td>608</td>
<td>625</td>
<td>1</td>
<td>(R)SAGIKVIMTGDPITAK(A)</td>
</tr>
<tr>
<td>1,139.453</td>
<td>2Phospho</td>
<td>256</td>
<td>264</td>
<td>0</td>
<td>(R)KIVVMMTGR(T)</td>
</tr>
<tr>
<td>1,453.528</td>
<td>2Phospho</td>
<td>10</td>
<td>21</td>
<td>0</td>
<td>(K)YEPAAVSEQDK(K)</td>
</tr>
<tr>
<td>2,537.037</td>
<td>3Phospho</td>
<td>75</td>
<td>94</td>
<td>1</td>
<td>(R)DPNALSPTTPMTPEWIKFCR(Q)</td>
</tr>
</tbody>
</table>

Table shows peptide mass, phosphorylation site, and putative peptide sequences from the immunoprecipitated 100-kDa band. The peptide sequences that showed homology with the Na-K-ATPase \( \alpha_1 \)-subunit are shown. Peptide 4 shows the known tyrosine phosphorylation of the Na-K-ATPase \( \alpha_1 \)-subunit (Tyr-10). The other 4 peptides show putative unique serine/tyrosine phosphorylation sites in ouabain-treated samples. These peptides were not identified in control samples.
consistent with a hypertensive effect of nanomolar concentrations of ouabain through activation of renal Na-K-ATPase activity and enhanced proximal tubule Na\(^+\) reabsorption (19). Our study differs from the Loreaux study in that their studies examined only the acute effect of ouabain infusion on blood pressure and electrolyte homeostasis. The present study shows a more chronic effect of mildly elevated serum ouabain concentrations and suggests that the mechanism of hypertension stimulated by ouabain may be twofold, first through inhibition of the Na-K-ATPase in vascular tissue, resulting in enhanced contractility, and second through stimulation of the Na-K-ATPase in renal tissue, resulting in enhanced renal sodium reabsorption, as has been suggested by Ferrari et al. (22). The contrasting action of low cardiac glycoside concentration on the sodium pump in these two tissues is a reflection of the differences in sensitivity of Na-K-ATPase \(\alpha\)-subunits in these two tissues. Specifically, in rodents, the \(\alpha_2\)-subunit which is expressed in the vascular smooth muscle and heart is relatively sensitive to the inhibitory effects of ouabain, while the \(\alpha_1\)-subunit which is expressed in the renal tubule cells is comparatively resistant to the inhibitory effects of ouabain. However, in humans, the different \(\alpha\)-subunits of Na-K-ATPase are similarly sensitive to cardioglycosides. Whether the effects of picomolar or nanomolar increases in ouabain-like substances produce similar effects remains to be elucidated.

In a cell culture model of proximal tubule cells (OK cells), we previously demonstrated that treatment with 10 nM ouabain stimulates Na-K-ATPase-mediated \(^{86}\)Rb uptake and cell proliferation through activation of a signaling cascade involving Src kinase, ERK, and Akt (26, 28). In a recent report, Mandal et al. (39) demonstrated an increase in NHE-1 activity and phosphorylation in response to 1 µM ouabain in rat astrocytes. These findings, plus the numerous commonalities between the sodium pump and NHE-1, which include regulation of cell survival and proliferation through ERK- and Akt-dependent pathways, restricted localization to BLMs in polarized epithelial cells, and functional roles and scaffold properties to assemble signaling proteins like Src, ERK, and Akt (3, 15, 32, 57, 48), led us to hypothesize that the activity of NHE-1 and Na-K-ATPase may be linked. Our data suggest that Na-K-ATPase stimulation by ouabain in the picomolar (human kidney cells) and nanomolar (OK cells) concentration range requires NHE-1. In human kidney proximal tubule cells (HKC-11 and HKC-5) and in OK cells, the ability of picomolar or nanomolar concentrations of ouabain to stimulate Na-K-ATPase-mediated \(^{86}\)Rb uptake was prevented by the NHE-1 inhibitor EIPA. Inclusion of monensin in the medium used for our \(^{86}\)Rb uptake experiments creates a sodium entry pathway (10, 15) that effectively short-circuits sodium entry via NHE-1 and other sodium-dependent exchangers or cotransporters. Thus the observed rate of Na-K-ATPase-mediated \(^{86}\)Rb uptake was close to the \(V_{\text{max}}\) for sodium and the increased rate in the presence of picomolar or nanomolar ouabain concentrations is not likely to be the result of an increase in NHE-1-mediated sodium entry. The results are consistent with an increase in Na-K-ATPase \(V_{\text{max}}\) in cells exposed to picomolar or nanomolar concentrations of ouabain (data not shown).

Src kinase-dependent tyrosine phosphorylation of the Na-K-ATPase \(\alpha_1\)-subunit at Y-10 has been demonstrated to be crucial for insulin-dependent stimulation of Na-K-ATPase activity and trafficking to the plasma membrane (18). Our data demonstrating tyrosine phosphorylation of the Na-K-ATPase \(\alpha_1\)-subunit by picomolar concentrations of ouabain suggest that phosphorylation is a crucial step in Na-K-ATPase regulation. We identified that Y-10 and a unique tyrosine (Y-260) residue were phosphorylated by picomolar ouabain concentrations. Whether these two tyrosines play a role in the association of the Na-K-ATPase \(\alpha_1\)-subunit and NHE-1, trafficking to the plasma membrane and increased activity remain to be determined. Our data further demonstrate that inhibition of NHE-1 by EIPA prevents Na-K-ATPase \(\alpha_1\)-subunit phosphorylation by picomolar ouabain and insulin, suggesting that NHE-1 plays a critical role in regulation of Na-K-ATPase.

Our findings suggest that NHE-1 plays a critical role in the mechanism that stimulates Na-K-ATPase stimulation in the presence of nanomolar or picomolar concentrations of ouabain. We considered whether treatment with nanomolar or picomolar concentrations of ouabain stimulate NHE-1 activity. NHE-1 stimulation requires phosphorylation at serine 770 and 771 (38). In a recent report, Meima et al. (42) demonstrated that NHE-1 is a substrate for Akt and that Akt phosphorylates Ser\(^{48}\) of NHE-1. We have previously demonstrated that treatment of OK cells with 10 nM ouabain increases Na-K-ATPase-mediated \(^{86}\)Rb uptake in an Akt-dependent manner (26, 28).
Taken together, it is possible that NHE-1 stimulation or NHE-1 phosphorylation through an Akt-mediated pathway is a required step in the chain of events leading to the increase in Na-K-ATPase activity. However, we cannot rule out the possibility that the scaffolding function of NHE-1 may be important for the Na-K-ATPase response (48).

In summary, the present studies demonstrate a novel interaction between NHE-1 and Na-K-ATPase that is triggered by ouabain in the nanomolar or picomolar range, depending on the species. The present studies also demonstrate that ouabain at nanomolar (OK cells and rat kidney BLMs) and at picomolar (HK cells) concentrations increase Na-K-ATPase activity and ion transport through a mechanism that involves tyrosine phosphorylation of the α1-subunit at positions 10 and 260 and association between NHE1 and the Na-K-ATPase α1-subunit. We speculate that an increase in endogenous ouabain-like factors may increase blood pressure through its cooperative effects on renal tubular NHE-1 and Na-K-ATPase activities.

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REFERENCES


DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.


