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Ouabain-Stimulated Trafficking Regulation of the Na/K-ATPase and NHE3 In Renal Proximal Tubule Cells

Yanling Yan^{1,3}, Steven Haller¹, Anna Shapiro¹, Nathan Malhotra¹, Jiang Tian¹, Zijian Xie^{2,1}, Deepak Malhotra¹, Joseph I. Shapiro^{1,2}, and Jiang Liu¹

¹Department of Medicine, University of Toledo College of Medicine, Toledo, OH

²Department of Physiology and Pharmacology, University of Toledo College of Medicine, Toledo, OH

³Institute of Biomedical Engineering, Yanshan University, China

Abstract

We have demonstrated that ouabain regulates protein trafficking of the Na/K-ATPase $\alpha 1$ subunit and NHE3 (Na/H exchanger, isoform 3) via ouabain-activated Na/K-ATPase signaling in porcine LLC-PK1 cells. To investigate whether this mechanism is species-specific, ouabain-induced regulation of the $\alpha 1$ subunit and NHE3 as well as transcellular $^{22}\text{Na}^+$ transport were compared in three renal proximal tubular cell lines (human HK-2, porcine LLC-PK1, and AAC-19 originated from LLC-PK1 in which the pig $\alpha 1$ was replaced by ouabain-resistant rat $\alpha 1$). Ouabain inhibited transcellular $^{22}\text{Na}^+$ transport due to an ouabain-induced redistribution of the $\alpha 1$ subunit and NHE3. In LLC-PK1 cells, ouabain also inhibited the endocytic recycling of internalized NHE3, but has no significant effect on recycling of endocytosed $\alpha 1$ subunit. These data indicated that the ouabain-induced redistribution of the $\alpha 1$ subunit and NHE3 is not a species-specific phenomenon, and ouabain-activated Na/K-ATPase signaling influences NHE3 regulation.

Keywords

Ouabain; Na/K-ATPase signaling; Na/K-ATPase; NHE3; redistribution

Introduction

Renal sodium handling is a key determinant of long term regulation of blood pressure (1–4). In the kidney, the renal proximal tubules (RPTs) are responsible for more than 60% of the net tubular Na^+ reabsorption, mainly through basolateral Na/K-ATPase and apical NHE3. Endogenous CTS (cardiotonic steroids, also known as digitalis-like substances), which were initially classified as specific inhibitors of the Na/K-ATPase and now classified as a new family of steroid hormones, are involved in regulation of blood pressure and renal sodium handling (5–7). Circulating CTS are markedly increased under certain conditions such as salt loading, volume expansion, renal insufficiency, and congestive heart failure (8–10).

The pathophysiological significance of endogenous CTS has been a subject of debate since it was first proposed (11–13). In essence, the Na/K-ATPase inhibitor (endogenous CTS) will rise in response to either a defect in renal Na^+ excretion or high salt intake. This increase, while returning Na^+ balance toward normal by increasing renal Na^+ excretion, also cause

increases in blood pressure through acting on vascular Na/K-ATPase (14). Increases in endogenous CTS regulate both renal Na⁺ excretion and blood pressure through the Na/K-ATPase (7, 14–17).

In porcine RPT LLC-PK1 cells, we have shown that ouabain inhibits active transepithelial ²²Na⁺ transport (from apical to basolateral aspect) via protein trafficking regulation of the Na/K-ATPase and NHE3 (18–20), a process requiring ouabain-activated Na/K-ATPase signaling. This novel regulatory mechanism may contribute to CTS-induced natriuresis, especially in rodents expressing ouabain-resistant Na/K-ATPase α 1 subunit (17). In the present study, we investigated if this regulatory mechanism is species-specific by characterizing the effect of ouabain on transcellular ²²Na⁺ flux and redistribution of the Na/K-ATPase and NHE3. Furthermore, we also investigate the endocytic recycling (reinsertion of endocytosed protein back to plasma membrane) of internalized Na/K-ATPase and NHE3 in LLC-PK1 cells.

Experimental Methods

Chemicals and Antibodies

All reagents, unless otherwise mentioned, were obtained from Sigma (St. Louis, MO). Src kinase inhibitor PP2 was from CalBiochem (San Diego, CA). EZ-Kink sulfo-NHS-ss-Biotin and ImmunoPure immobilized streptavidin-agarose beads were obtained from Pierce Biotechnology (Rockford, IL). A rabbit polyclonal antibody against a mixture of peptides from porcine NHE3 was prepared and affinity purified (21). Antibodies against Rab7, integrin- β 1 and human NHE3 were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against the Na/K-ATPase α 1-subunit (clone α 6F) was from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Monoclonal antibodies against NHE3 (clone 4F5) and early endosome antigen-1 (EEA-1) were from Millipore Chemicon (Temecula, CA). Radioactive rubidium (⁸⁶Rb⁺) and sodium (²²Na⁺) were from DuPont NEN Life Science (Boston, MA).

Cell cultures

Human HK-2 cells and pig LLC-PK1 cells were obtained from the American Type Culture Collection (Manassas, VA). AAC-19 cells were generated from LLC-PK1 cells as we described previously (22). Briefly, the ouabain-sensitive pig α 1 in LLC-PK1 cells was knock-down by siRNA method. To rescue the α 1 knock-down cell with ouabain-resistant rat α 1 (AAC-19 cells), the α 1 siRNA targeted sequence was silently mutated to introduce rat α 1 with rat α 1 pRc/CMV- α 1AAC expression vector. The expression of ouabain-insensitive rat α 1 was selected with 3 μ M ouabain in culture medium since untransfected LLC-PK1 cells are very sensitive to ouabain. Cells were cultured in DMEM (Dulbecco's modified Eagle's medium)/F-12 mixed medium (1:1, *vol/vol*) for HK-2 or DMEM for LLC-PK1 and AAC-19, with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin in a 5% CO₂-humidified incubator. Culture medium was changed daily until confluency. LLC-PK1 cells and AAC-19 cells were serum-starved (in serum-free DMEM medium) for 16–18 h before treatment, and HK-2 cells were changed to medium containing 1% FBS for 16–18 h before treatment. In assays for active transcellular ²²Na⁺ flux, cells were grown on Transwell membrane support to form monolayer, and then treated with ouabain either in the basolateral or apical compartment. Both LLC-PK1 and AAC-19 cells can be easily grown to monolayers in DMEM medium with 10% FBS. While HK-2 cells are hard to form monolayer with ATCC-recommended Keratinocyte Serum Free Medium, we found that HK-2 cells could be easily grown to monolayer in DMEM/F-12 medium (with 10% FBS) without losing its ouabain sensitivity.

Isolation of early endosome (EE) and late endosome (LE) fractions

EE and LE fractions were fractionated by a sucrose flotation gradient technique as we previously described (18, 19). EE and LE fractions were identified with antibodies against EE marker protein EEA-1 and LE marker protein Rab7, respectively (18, 19). In comparison with whole cell lysates, more than a 10-fold enrichment of these marker proteins was observed in representative endosome fractions as we have previously shown (18).

Cell surface biotinylation

Cell surface biotinylation was conducted as we described before (18, 19). Biotinylated proteins were pulled down with streptavidin-agarose beads, eluted with 2x Laemmli buffer (125 mM Tris-HCl, 20% Glycerol, 4% SDS, 0.025% Bromophenol Blue, 10% 2-mercaptoethanol, pH 6.8) at 55°C waterbath for 30 minutes, resolved by 10% SDS-PAGE, and then immunoblotted for the Na/K-ATPase α 1 and NHE3. The same membrane was also immunoblotted with antibody against integrin- β 1 to serve as loading control as described previously (17).

Ouabain-sensitive Na/K-ATPase activity assay ($^{86}\text{Rb}^+$ uptake)

For $^{86}\text{Rb}^+$ uptake assay, cells were cultured in 12-well plates and treated with or without different concentrations of ouabain for 15 min. Monensin (20 μM), a Na^+ -clamping agent, was added to the medium prior to the assay to assure that the maximal capacity of active uptake was measured (23). $^{86}\text{Rb}^+$ uptake was initiated by the addition of 1 μCi of $^{86}\text{Rb}^+$ as tracer of K^+ to each well, and the reaction was stopped after 15 minutes by washing four times with ice-cold 0.1 M MgCl_2 . Trichloroacetic acid (TCA)-soluble $^{86}\text{Rb}^+$ was extracted with 10% TCA and counted. TCA-precipitated cellular protein content was determined and used to calibrate the $^{86}\text{Rb}^+$ uptake. Data were expressed as the percentage of control $^{86}\text{Rb}^+$ uptake.

NHE3 mediated active transepithelial $^{22}\text{Na}^+$ flux and $^{22}\text{Na}^+$ uptake

Active transepithelial $^{22}\text{Na}^+$ flux (from apical to basolateral aspect of the Transwell membrane supports) was performed on monolayers (grown on Costar Transwell culture filter inserts, filter pore size: 0.4 μm , Costar, Cambridge, MA) as described by Haggerty and colleagues (24). Briefly, after ouabain treatment for 1 h at the concentration indicated, both apical (upper) and basolateral (lower) compartments were rinsed with ouabain-free DMEM (0% FBS for LLC-PK1 and AAC-19 cells, and 1% FBS for HK-2 cells). 1 ml DMEM containing $^{22}\text{Na}^+$ (1 $\mu\text{Ci}/\text{ml}$) was added to the apical compartment of a filter insert, and the basolateral compartment was filled with 1 ml of DMEM. After 1 h, aliquots were removed from the basolateral compartments for scintillation counting. H^+ -driven $^{22}\text{Na}^+$ uptake were determined as described by Soleimani and colleagues (25). Briefly, the cells grown on 12-well plate were treated with ouabain at the concentration indicated and then washed three times with the Na^+ -free buffer (in mM, 140 N-methyl-D-glucammonium (NMDG^+) Cl, 4 KCl, 2 MgCl_2 , 1 CaCl_2 , 10 HEPES, pH 7.4). The cells were then incubated for 10 min in the same Na^+ -free buffer in which 20 mM NMDG^+ was replaced with 20 mM NH_4^+ . The uptake was initiated by replacing the NH_4^+ -containing buffer with Na^+ -free buffer containing 1 $\mu\text{Ci}/\text{ml}$ $^{22}\text{NaCl}$. $^{22}\text{Na}^+$ uptake was stopped after 30 min by washing four times with ice-cold saline. Cell-associated radioactivity was extracted with 1 ml of 1 N sodium hydroxide, quantified by scintillation counting, and calibrated with protein content. In both experimental settings, cells were pretreated with 50 μM amiloride to inhibit amiloride-sensitive NHE1 activity.

Assessment of endocytic recycling of NHE3 and Na/K-ATPase $\alpha 1$ subunit in LLC-PK1 cells

Endocytic recycling of $\alpha 1$ and NHE3 were assessed by the method described by the Moe Laboratory (26). Briefly, two sets of LLC-PK1 cells were biotinylated and quenched at 4°C, and then treated with ouabain (100nM) or vehicle (as control) for 1h at 37°C to induce redistribution of the Na/K-ATPase $\alpha 1$ and NHE3. After rinsing with ice-cold PBS-Ca-Mg (1x PBS with 0.1mM CaCl₂ and 1mM MgCl₂), un-internalized surface biotinylated proteins were cleaved with 50 mM glutathione-SH (GSH-SH, a reducing agent) at 4°C, and un-reacted free GSH-SH was oxidized by incubation with 30mM iodoacetamide for 10min. At this point, one set of cells was lysed with RIPA buffer to retrieve total internalized intracellular biotinylated $\alpha 1$ and NHE3 with streptavidin-agarose beads. Other set of cells was changed to serum-free DMEM medium and cultured at 37°C to permit further trafficking for 2h with or without 100nM ouabain. Biotinylated proteins that recycled back to cell surface (reinsertion) were cleaved by GSH-SH method again. The remaining intracellular biotinylated proteins after reinsertion were retrieved with streptavidin-agarose beads, which represent the internalized biotinylated $\alpha 1$ and NHE3 that were not reinserted. The difference of the total intracellular $\alpha 1$ and NHE3, before and after reinsertion, represents the $\alpha 1$ and NHE3 that was internalized and then reinserted (endocytic recycling).

Western blot

Equal amounts of total protein were resolved by 10% SDS-PAGE and immunoblotted with indicated antibodies (with dilution of 1:2000 for the Na/K-ATPase $\alpha 1$ subunit and 1:1000 dilution for NHE3, in 4% non-fat dry milk in 1x Tris-buffered saline with 0.1% Tween-20). The same membrane was also immunoblotted with antibodies against integrin- $\beta 1$ (for surface biotinylation), EEA-1 (for EE fraction) and Rab7 (for LE fraction) to serve as loading controls (data not shown), respectively, as we previously described (17–19). Signal detection was performed with an enhanced chemiluminescence super signal kit (Pierce, Rockford, IL). Multiple exposures were analyzed to assure that the signals were within the linear range of the film. The signal density was determined using Molecular Analyst software (Bio-Rad, Hercules, CA).

Statistical analysis

Data were tested for normality (all data passed) and then subjected to parametric analysis. When more than two groups were compared, one-way ANOVA was performed prior to the comparison of individual groups with an unpaired t-test. Statistical significance was reported at the $P < 0.05$ and $P < 0.01$ levels. SPSS software was used for all analysis (SPSS, Chicago, IL). Values are given as mean $\pm$ S.E.

Results

Ouabain-mediated inhibition of the Na/K-ATPase

Ouabain-induced inhibition of the Na/K-ATPase “ion-pumping” activity (ouabain-sensitive $^{86}\text{Rb}^+$ uptake) in these RPT cell lines is summarized in Fig 1. The IC₅₀ values are consistent with the established differences of $\alpha 1$ ouabain sensitivity amongst these species (see Discussion). In LLC-PK1 cells with IC₅₀ at 1 μM , 100nM ouabain is sufficient to activate the Na/K-ATPase signaling and consequent regulation of the Na/K-ATPase and NHE3 (20). According to the Na/K-ATPase $\alpha 1$ sensitivity to ouabain, we chose ouabain concentrations that are able to activate the Na/K-ATPase signaling for these three cell lines (10nM for HK-2, 100nM for LLC-PK1, and 10 μM for AAC-19 cells) without significant inhibition of Na/K-ATPase activity. No significant effect on cell viability was observed when these cells were treated for 1h with ouabain concentrations used that was evaluated by Trypan blue exclusion.

Ouabain-mediated inhibition of transepithelial $^{22}\text{Na}^+$ flux and $^{22}\text{Na}^+$ uptake

We have shown that ouabain inhibits transepithelial $^{22}\text{Na}^+$ flux by activating Na/K-ATPase signaling in LLC-PK1 cells (18, 19). To assess if this effect is species-specific, we measured H^+ -driven $^{22}\text{Na}^+$ uptake and transepithelial $^{22}\text{Na}^+$ flux in these three RPT cell lines. As shown in Fig 2 and 3, when ouabain was added in the basolateral aspect, ouabain inhibited $^{22}\text{Na}^+$ uptake (Fig 2) and active transepithelial $^{22}\text{Na}^+$ flux (Fig 3) in both HK-2 and AAC-19 cells in the same manner as in LLC-PK1 cells. The effect of ouabain on $^{22}\text{Na}^+$ flux and NHE3 activity was largely blunted when these cells were pretreated with the Src kinase inhibitor PP2 (1 μM for 30 min, at 37 °C). PP2 alone did not show significant effect. No significant inhibition of NHE3 activity was observed in all three cell lines when ouabain was added in the apical aspect (data not shown), suggesting that ouabain-induced regulation of $^{22}\text{Na}^+$ flux and NHE3 activity requires ouabain-activated Na/K-ATPase signaling.

Ouabain-induced protein trafficking of Na/K-ATPase and NHE3

In LLC-PK1 cells, ouabain-induced inhibition of $^{22}\text{Na}^+$ flux is largely due to an ouabain-mediated redistribution of the Na/K-ATPase and NHE3 via Na/K-ATPase signaling. To further test this regulatory mechanism, these three cell lines were treated with or without ouabain to assess the ouabain-induced redistribution. As shown in Fig 4, the ouabain induced redistribution of the $\alpha 1$ subunit and NHE3 was caused by a decrease in cell surface $\alpha 1$ subunit and NHE3 in both HK-2 and AAC-19 cells (as we previously reported in LLC-PK1 cells (18, 19)). Pretreatment with PP2 abolished ouabain-induced redistribution of the $\alpha 1$ subunit and NHE3 (data not shown). As shown in Table 1, ouabain (1h) caused a dose-dependent reduction of cell surface $\alpha 1$ subunit and NHE3. Furthermore, the ouabain-induced reduction of cell surface $\alpha 1$ subunit and NHE3 was closely correlated to ouabain-induced inhibition of transcellular $^{22}\text{Na}^+$ flux and NHE3 activity (Fig. 2 and 3).

Ouabain-mediated regulation of endocytic recycling of the Na/K-ATPase $\alpha 1$ subunit and NHE3 in LLC-PK1 cells

Endocytic recycling is essential for maintaining the distinction between apical and basolateral membranes in polarized cells, even though the recycling pathways may be redundant (27). It has been shown that ouabain redistributes the Na/K-ATPase into both late endosomes and lysosomes (28), presumably for degradation. To explore the underlying mechanism, we used LLC-PK1 cells to assess endocytic recycling of the $\alpha 1$ subunit and NHE3. As shown in Fig 5A, GSH-SH (glutathione-SH, a reducing agent) was able to cleave over 90% of protein-bound biotin. Ouabain (100nM, 1h) accumulated the $\alpha 1$ subunit (control $100 \pm 9.6\%$ vs. ouabain 198.3 ± 18.2 , $n=3$, $p<0.01$) and NHE3 (control $100 \pm 8.9\%$ vs. ouabain 187.4 ± 16.5 , $n=3$, $p<0.01$) in intracellular compartments (Fig 5B) after cleavage of surface protein-bound biotin with GSH-SH method. After 2h-period of recycling of internalized biotinylated proteins, recycled biotinylated proteins were cleaved again with GSH-SH. The total intracellular biotinylated $\alpha 1$ and NHE3 after recycling, which represent un-recycled biotinylated $\alpha 1$ and NHE3, are shown in Fig 5C. The reinsertion (endocytic recycling) was presented as the difference of the total intracellular $\alpha 1$ and NHE3 before and after reinsertion (Fig 5D). The present data indicate that ouabain treatment not only induced NHE3 redistribution, but it also inhibited the endocytic recycling of NHE3. Interestingly, the endocytic recycling of the $\alpha 1$ subunit was not significantly affected by ouabain. These observations suggested that, while most of the internalized $\alpha 1$ was destined for degradation, at least part of internalized NHE3 was recycled back to cell membrane surface. Despite the lack of microvilli in cultured renal proximal tubular cells (29), our observation is reminiscent of the model of NHE3 moving along the microvilli structure (30).

To further explore the destination of the $\alpha 1$ subunit and NHE3, we determined the protein content of these two transporters in EE and LE fractions in response to ouabain. As shown in

Fig. 6, ouabain treatment (100nM, 1h) stimulated accumulation of both $\alpha 1$ and NHE3 in EE fractions. However, ouabain significantly accumulated $\alpha 1$, but not NHE3 in LE fractions. The NHE3 protein content in LE fractions was not significantly increased after ouabain treatment, even in the presence of the lysosomotropic weak base agent chloroquine (0.2 mM, pretreated for 2h) which inhibits degradation by the lysosomal pathway. On the other hand, pretreatment with chloroquine caused a further accumulation of the $\alpha 1$ subunit in LE fractions in response to ouabain, suggesting that the endocytosed $\alpha 1$ subunit, but not NHE3, is degraded through the LE-lysosome pathway.

Discussion

The renal tubular Na/K-ATPase comprises a final site for the regulation of renal sodium transport by many factors (31). Our recent work indicates that ouabain is one of these factors that act via a coordinated regulation of the Na/K-ATPase and NHE3 through Na/K-ATPase signaling (17–20). In the present study, we have investigated whether this ouabain mediated regulatory model is species-specific. Our present study indicated that ouabain-induced regulation of the Na/K-ATPase and NHE3 is not species-specific. First, ouabain was able to inhibit the active transepithelial $^{22}\text{Na}^+$ flux in these three cell lines. This effect was largely prevented by blocking c-Src activation with PP2 pretreatment. Secondly, ouabain-induced redistribution and reduction of the cell surface Na/K-ATPase and NHE3 contributed to the inhibition of active $^{22}\text{Na}^+$ flux. Third, the effect of ouabain on active $^{22}\text{Na}^+$ flux was only observed when ouabain was applied in the basolateral, but not in the apical aspect. Fourth, the ouabain-induced reduction of the cell surface $\alpha 1$ subunit contributed to the ouabain-induced inhibition of $^{22}\text{Na}^+$ flux and $^{22}\text{Na}^+$ uptake. Taken together, these observations suggest that the ouabain-induced regulation of the Na/K-ATPase and NHE3 is not species-specific, and may be explained if the Na/K-ATPase is the functional receptor for ouabain-induced regulation.

Activation of c-Src is critical in the ouabain-activated Na/K-ATPase signaling and redistribution of the Na/K-ATPase and NHE3 (18–21, 32). When compared with HK-2 and LLC-PK1 cells, a higher concentration of ouabain was needed to regulate activity and redistribution of the Na/K-ATPase and NHE3 in AAC-19 cells expressing native pig NHE3 and rat $\alpha 1$. This is consistent with the established differences in ouabain sensitivity of the $\alpha 1$ subunit as well as ouabain-stimulated c-Src activation between these two cell lines (22). It is well known that there are large differences in sensitivity of the Na/K-ATPase to ouabain based on α isoforms and species (33–35). Specifically, the rodent $\alpha 1$ is far less sensitive than pig, dog, or human $\alpha 1$. Higher concentrations of ouabain were required to activate Na/K-ATPase signaling in rodents, compared to other species (22, 36–39). We have also shown that a higher concentration of ouabain (10 μM) is needed to activate c-Src in isolated renal proximal tubules of Dahl salt-resistant rats (17). Most interestingly, different natriuretic responses were observed between transgenic mice expressing ouabain-sensitive $\alpha 1$ and wild-type mice expressing ouabain-resistant $\alpha 1$ (16) in which the ouabain binding site of the $\alpha 1$ subunit plays a critical role (40). As shown in Table 1, the ouabain sensitivity of the $\alpha 1$ subunit influenced the ouabain-induced inhibition of $^{22}\text{Na}^+$ flux as well as surface reduction of the $\alpha 1$ subunit and NHE3. The present study further suggests that species-specific $\alpha 1$ sensitivity to ouabain might explain the species differences in ouabain-induced natriuresis *in vivo* (7, 10, 16, 41). Considering the high ouabain sensitivity of human $\alpha 1$ subunit, this could be a explanation how pathophysiological circulating CTS might affect renal sodium handling, especially in the view that endogenous ouabain is a natriuretic hormone and has a physiological role in controlling sodium homeostasis in normal rats (7).

After the redistribution of membrane proteins, subsequent intracellular trafficking differs among different endocytosed proteins. Receptor-mediated redistribution is believed to be an

effective pathway to reduce cell surface signaling receptors. In our experimental settings, ouabain caused accumulation of the $\alpha 1$ subunit in LE fractions, but failed to affect its endocytic recycling (Fig 5 and 6). This is a reminiscence of the early observation that ouabain caused the redistribution of the Na/K-ATPase into late endosomes and lysosomes (28), suggesting that the endocytosed Na/K-ATPase is most likely degraded in LE/lysosome pathway. On the other hand, the cell surface expression of NHE3 is likely reduced by inhibition of the recycling of internalized NHE3 (Fig 5 and 6). However, the mechanism is not clear. In both cases, we cannot exclude the possibility that both recycling and degradation were affected by ouabain, but the overall observed effect was a balance between these two processes.

Renal Na^+ reabsorption through NHE3 plays an important role in salt-sensitivity, as well as in the development and control of sodium homeostasis and blood pressure (42–45). Recently, we have demonstrated that impaired Na/K-ATPase-Src signaling contributes to salt sensitivity in Dahl rats (17). This is consistent with the observation that a high salt diet stimulates redistribution of RPT Na/K-ATPase and NHE3 (46). Although the mechanisms are still being elucidated, accumulating evidence supports the notion of coordinated regulation of the Na/K-ATPase and NHE3 (47). It appears that the pathways regulating the Na/K-ATPase and NHE3 are numerous and redundant, and CTS-induced coordinated regulation is one of the pathways that occur in response to conditions that cause an increase in endogenous CTS.

In summary, our data indicate that the Na/K-ATPase is the functional receptor for ouabain-induced regulation of the Na/K-ATPase and NHE3 (and thus transcellular Na^+ transport), as we previously proposed (20). This regulation is not species-specific, but the species-specific $\alpha 1$ ouabain sensitivity may partially account for the species differences observed in ouabain-induced natriuresis (7, 10, 16, 41). In ouabain-induced trafficking regulation, endocytic recycling of internalized NHE3, but not the $\alpha 1$ subunit, was inhibited by ouabain.

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Figure 1.

Dose-dependent effects of ouabain (Oua) on Na/K-ATPase activity. The HK-2, LLC-PK1 and AAC-19 cells were grown in 12-well plates with Transwell membrane support to form monolayer. The Na/K-ATPase activity (ouabain-sensitive $^{86}\text{Rb}^+$ uptake) was assayed as described in Experimental Methods. Data were shown as percentage of control, and each point is presented as mean $\pm$ S.E. of four sets of independent experiments. Curve fit analysis was performed by GraphPad software.

Figure 2.

Ouabain (Oua) inhibits H^+ -driven $^{22}Na^+$ uptakes. The HK-2, LLC-PK1 and AAC-19 cells were grown in 12-well plates to form a monolayer. After treatment with ouabain (1h) and/or PP2 ($1\mu M$ for 30min), $^{22}Na^+$ was added and assayed for H^+ -driven $^{22}Na^+$ uptake. To determine H^+ -driven Na^+ uptake, cells were first acid loaded in Na^+ -free buffer with 20 mM NH_4Cl and then assayed for $^{22}Na^+$ uptake. 50 μM amiloride was used to inhibit amiloride-sensitive NHE1 activity. Data are shown as mean $\pm$ S.E., percentage of control. $n=4$. ** $p<0.01$ compare to Control.

Figure 3.

Ouabain (Oua) inhibits transcellular $^{22}\text{Na}^+$ flux. The HK-2, LLC-PK1 and AAC-19 cells were grown in 12-well plates with Transwell membrane support to form a monolayer. The cells were treated with ouabain (1h) and/or PP2 (1μM for 30min) in the basolateral or apical aspect. Active transepithelial $^{22}\text{Na}^+$ flux (apical to basolateral) was determined by counting radioactivity in the basolateral aspect at 1 h after $^{22}\text{Na}^+$ addition. 50 μM amiloride was added in the basolateral aspect to inhibit amiloride-sensitive NHE1 activity. Data are shown as mean $\pm$ S.E., percentage of control. n=4. ** p<0.01 compare to Control.

Figure 4.

Ouabain (Oua) reduces cell surface expression of the $\alpha 1$ and NHE3. The HK-2, LLC-PK1 and AAC-19 cells were treated with indicated concentrations of ouabain (1h). Biotinylation of cell surface proteins was performed to assess cell surface protein contents. Data are shown as mean $\pm$ S.E., percentage of control (Con). n=4. ** p<0.01 compare to control. Insert shows a representative western blot of four separate experiments. Immunoblotting with antibody against integrin- $\beta 1$ served as loading controls (data not shown).

Figure 5.

Ouabain (Oua) inhibits endocytic recycling of NHE3 in LLC-PK1 cells. The experiments were performed as described in Experimental Methods. (A) Determination of GSH-cleavage efficiency. Cell surface proteins were biotinylated and applied with or without GSH cleavage procedure. (B) Retrieval of total internalized biotinylated $\alpha 1$ and NHE3 after treatment with or without ouabain (100nM, 1h) and GSH cleavage. (C) Assessment of the effect of ouabain on reinsertion. (D) The graph bars represented reinsertion of endocytosed $\alpha 1$ and NHE3, the difference of total endocytosed $\alpha 1$ and NHE3 before and after reinsertion procedure. $n=3$. **, $p<0.01$.

Figure 6.

Ouabain (Oua) accumulates the Na/K-ATPase $\alpha 1$ subunit, but not NHE3 in late endosome in LLC-PK1 cells. LLC-PK1 cells were treated with or without ouabain (100nM for 1h), with or without pretreatment of chloroquine (Chlor, 0.2 mM, pretreated for 2h). Early endosome (EE) and late endosome (LE) fractions were isolated at the end of ouabain treatment. Equal amount of proteins (25 μ g) was used to determine protein contents of the $\alpha 1$ and NHE3 by western blot analysis. n=4. ** p<0.01 compared to controls from EE and LE, respectively. # p<0.01, comparison of $\alpha 1$ subunit in LE fraction with or without chloroquine pretreatment. Insert shows a representative western blot of four separate experiments. Immunoblotting with antibodies against EEA-1 (for EE fractionation) and Rab7 (for LE fractionation) served as loading controls, respectively (data not shown).

Ouabain causes a dose-dependent inhibition of transcellular $^{22}\text{Na}^+$ flux, $^{22}\text{Na}^+$ uptake, and surface $\alpha 1$ and NHE3. $^{22}\text{Na}^+$ flux and $^{22}\text{Na}^+$ uptake were measured as described in Experimental Methods. Surface $\alpha 1$ and NHE3 were determined by cell surface biotinylation.

Table 1

	HK-2 cells					
	Ouabain (nM) for 1h					
	0	1	5	10	50	
$^{22}\text{Na}^+$ flux	100±4.5	102.1±3.8	81.2±5.2 ^a	68.5±4.8 ^b	61.8±5.9 ^b	
$^{22}\text{Na}^+$ uptake	100±5.1	98.4±4.2	83.6±5.6 ^a	76.6±6.2 ^b	67.8±5.3 ^b	
Surface $\alpha 1$	100±6.1	96.5±6.7	84.2±6.2 ^a	63.5±7.1 ^b	59.7±7.4 ^b	
Surface NHE3	100±4.5	99.2±6.1	86.2±6.8 ^a	67.3±7.1 ^b	69.1±7.9 ^b	

	LLC-PK1 cells				
	Ouabain (nM) for 1h				
	0	10	25	100	1,000
$^{22}\text{Na}^+$ flux	100±5.2	99.1±4.8	82.4±7.4 ^a	65.5±6.8 ^b	60.2±8.6 ^b
$^{22}\text{Na}^+$ uptake	100±5.8	95.7±6.7	88.3±8.1 ^a	64.8±7.2 ^b	58.5±7.8 ^b
Surface $\alpha 1$	100±7.9	97.3±8.1	85.6±7.2 ^a	65.2±7.7 ^b	67.6±8.4 ^b
Surface NHE3	100±6.5	98.6±8.8	83.2±7.4 ^a	72.7±6.1 ^b	69.3±7.6 ^b

	AAC-19 cells					
	Ouabain (nM) for 1h					
	0	100	1,000	10,000	25,000	
$^{22}\text{Na}^+$ flux	100±6.2	96.3±3.8	86.4±7.3 ^a	75.5±6.9 ^b	68.8±7.6 ^b	
$^{22}\text{Na}^+$ uptake	100±7.2	97.8±7.1	85.1±8.5 ^a	71.6±8.1 ^b	65.2±9.6 ^b	
Surface $\alpha 1$	100±8.2	96.5±7.6	87.4±8.1 ^a	75.2±7.9 ^b	67.4±9.1 ^b	
Surface NHE3	100±9.1	94.7±8.7	84.8±9.3 ^a	69.5±8.8 ^b	61.8±9.9 ^b	

N=3 for each treatment.

^a p<0.05 compared to controls;

$p < 0.01$ compared to controls.

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