# Ouabain induces endocytosis of plasmalemmal Na/K-ATPase in LLC-PK1 cells by a clathrin-dependent mechanism

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**Ouabain induces endocytosis of plasmalemmal Na/K-ATPase in LLC-PK1 cells by a clathrin-dependent mechanism.**

*Background.* We have demonstrated that ouabain causes dose- and time-dependent decreases in $^{86}$Rb uptake in porcine proximal tubular (LLC-PK1) cells. The present study addresses the molecular mechanisms involved in this process.

*Methods.* Studies were performed with cultured LLC-PK1 and Src family kinase deficient (SYF) cells.

*Results.* We found that 50 nmol/L ouabain applied to the basal, but not apical, aspect for 12 hours caused decreases in the plasmalemmal Na/K-ATPase. This loss of plasmalemmal Na/K-ATPase reverses completely within 12 to 24 hours after removal of ouabain. Ouabain also increased the Na/K-ATPase content in both early and late endosomes, activated phosphatidylinositol 3-kinase (PI(3)K), and also caused a translocation of some Na/K-ATPase to the nucleus. Immunofluorescence demonstrated that the Na/K-ATPase colocalized with clathrin both before and after exposure to ouabain, and immunoprecipitation experiments confirmed that ouabain stimulated interactions among the Na/K-ATPase, adaptor protein-2 (AP-2), and clathrin. Potassium (K) depletion, chlorpromazine, or PI(3)K inhibition all significantly attenuated this ouabain-induced endocytosis. Inhibition of the ouabain-activated signaling process through Src by 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) significantly attenuated ouabain-induced endocytosis. Moreover, experiments performed in SYF cells demonstrated that ouabain induced increases in the endocytosis of the Na/K-ATPase when Src was reconstituted (SYF+), but not in the Src-deficient (SYF−) cells.

*Conclusion.* These data demonstrate that ouabain stimulates a clathrin-dependent endocytosis pathway that translocates the Na/K-ATPase to intracellular compartments, thus suggesting a potential role of endocytosis in ouabain-induced signal transduction as well as proximal tubule sodium handling.

Renal sodium handling involves a complex interplay of different hormonal systems and physical factors; this topic has fascinated scientists for a number of years [1]. In the proximal tubule cell, the Na/K-ATPase resides at the basolateral surface, where it provides the force for the vectorial transport of sodium from the tubular lumen to the blood compartment [2]. The cellular distribution of the Na/K-ATPase is thought to be crucial for this function. Digoxin and ouabain, substances of plant origin, inhibit Na/K-ATPase activity by binding to an extracellular portion of the Na/K-ATPase α-subunit [3]. The fact that substances of plant origin specifically affect the activity of an important animal cell enzyme naturally led to the idea that animals may have endogenous digitalis-like substances (DLS) that can specifically regulate the activity of Na/K-ATPase. During the 1960s, it was postulated that one or more circulating inhibitors of the Na/K-ATPase, also called "3rd factor," were important in the regulation of renal sodium handling [4]. Recent studies have demonstrated that substances that are structurally similar to ouabain-like compound (OLC) as well as marinobufagenin (MBG) are increased in the serum and urine of experimental animals and patients who are volume expanded [5–8]. Moreover, in some cases, the increases in the concentrations of OLC and MBG correlate with increases in renal sodium excretion [9]. DLS are also found in the hypothalamus and adrenal glands of animals [10].

We have previously reported that in LLC-PK1 but not Madin-Darby canine kidney (MDCK) cells, ouabain administration for 12 hours decreases $^{86}$Rb uptake by 50% at concentrations less than 1/20th of that necessary to acutely (30 minutes) inhibit $^{86}$Rb uptake by 50% (IC$_{50}$). We have proposed that this mechanism might explain how DLS circulating at concentrations of $10^{-9}$ mol/L could substantially alter proximal tubule sodium handling in vivo [11]. Interestingly, regulation of Na/K-ATPase activity by dopamine also appears to require endocytosis of the enzyme [12, 13].

The clathrin-dependent pathway of endocytosis is clearly the best-characterized endocytosis pathway for membrane proteins in mammalian cells, although our understanding of caveolin-dependent processes is certainly expanding [14]. This clathrin-dependent endocytosis

pathway involves the delivery of cell surface components and solutes to early endosomes. After this, endocytosed components are either recycled back to the plasma membrane for reuse or transported to the late endosomes and lysosomes for degradation [15]. Our recent work indicates that the Na/K-ATPase can function as a signal transducer, leading to the activation of a signal transduction cascade involving Src and endothelial growth factor receptor (EGFR) [16–18]. Because many other membrane receptor ligands activate clathrin-mediated endocytosis of the receptor coincident with stimulation [19], we suspected that ouabain might also produce such an effect. To test this hypothesis, the following experiments were performed.

## METHODS

### Materials

Chemicals of the highest purity available were obtained from Sigma (St. Louis, MO, USA). Radioactive rubidium ($^{86}$Rb) was obtained from Dupont NEN Life Science Products (Boston, MA, USA). EZ-Kink sulfo-NHS-ss-Biotin and ImmunoPure immobilized streptavidin-agarose beads were obtained from Pierce Biotechnology (Rockford, IL, USA). Wortmannin and LY294002 were obtained from CalBiochem (La Jolla, CA, USA). Optitran nitrocellulose and polyvinylidine difluoride (PVDF) membranes (Hybound-P) were obtained from Schleicher&Schuell (Keene, NH, USA) and Amersham Biosciences (Piscataway, NJ, USA), respectively.

Polyclonal and monoclonal antibodies against Na/K-ATPase α1 subunit (clone C464.6), EEA-1, anti PI-3K p85α antibody coupled to protein A-agarose, and AP-2 α subunit (clone 8G8/5) were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Monoclonal antibodies against clathrin heavy chain (CHC, clone X22) were obtained from Affinity BioReagents (Golden, CO, USA), and were used in the immunoprecipitation experiments. Polyclonal antibodies against CHC, Rab5, Rab7, as well as horseradish peroxidase–conjugated goat antimouse and goat antirabbit IgG obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) were used for Western blot. Monoclonal antibody against Na/K-ATPase α1 subunit (clone α6F) obtained from Hybridoma Bank (University of Iowa, Iowa City, IA, USA) and polyclonal anti-clathrin antibodies obtained from BD Transduction Laboratories (San Diego, CA, USA) were used for immunofluorescence studies. Alexa Fluor® 488- or Alexa Fluor® 546-conjugated antimouse or antirabbit secondary antibodies were obtained from Molecular Probes (Eugene, OR, USA). Normal mouse IgG and rabbit IgG were purchased from Sigma.

### Cell culture

The pig renal proximal tubule cell line, LLC-PK1, was obtained from the American Tissue Type Culture Collection (Manassas, VA, USA), and cultured to confluent condition as previously described [11]. Cell viability was evaluated by Trypan blue exclusion. In some experiments, LLC-PK1 cells were grown to confluence (6 to 7 days) on the 12- or 24-mm polycarbonate Transwell culture filter inserts (filter pore size 0.4 μm; Costar Co., Cambridge, MA, USA). Medium was replaced daily until 12 hours before experiments, at which time the monolayer was serum starved as reported previously [11]. For some select experiments, we used SYF– cells lacking functional Src, as well as SYF+ cells in which Src was reconstituted as we have previously described [17]. The SYF– cells are derived from mouse embryos harboring functional null mutations in both alleles of the Src family kinases Src, Yes, and Fyn. The SYF+ cells are SYF– cells that have been stably transfected to express c-Src [20]. These cells were also obtained from ATCC. SYF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Medium was changed daily until the cells reached 80% to 90% confluence, at which time the medium was changed to DMEM with 1% FBS for at least 12 hours before experiments. SYF cells are not viable if they are completely serum starved [20].

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

Ouabain-sensitive uptake $^{86}$Rb uptake (of LLC-PK1 cells grown to monolayer) was performed as previously described [11]. The $^{86}$Rb uptake was calibrated with protein content. Data were expressed as the percentage of control ouabain-sensitive $^{86}$Rb uptake.

### Western blot

Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or NuPAGE 4% to 12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA), and transferred to Optitran or PVDF membrane. After transfer, the gel was stained with Coomassie Brilliant Blue to verify uniform loading and transfer. Immunoblotting was performed as described previously. Detection was performed with the enhanced chemiluminescence (ECL) super signal kit (Pierce). Multiple exposures were analyzed to assure that the signals were within the linear range of the film. Autoradiograms were scanned with a Bio-Rad GS-670 imaging densitometer (Bio-Rad, Hercules, CA, USA) to quantify the signals [11].
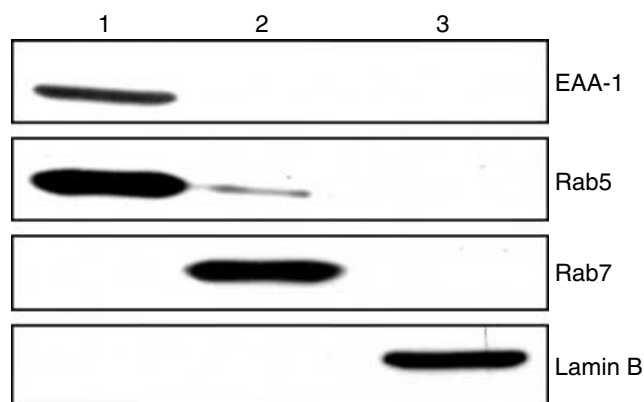
**Fig. 1. Representative Western blots of EAA-1, Rab5, Rab7, and Lamin B.** Lane 1 was loaded with protein from 16%/10% sucrose interface (early endosomes), lane 2 with protein from 10%/buffer interface (late endosomes), and lane 3 with protein from nuclear fraction. All lanes were loaded with 10 µg of protein. In $N = 3$ studies, EAA-1 and Rab5 contents in the early endosomes were more than 10× that seen in late endosomes, and conversely, Rab7 content in late endosomes was more than 10× that seen in early endosomes. Lamin B appeared to be essentially confined to the nuclear fraction, and the nuclear fraction did not contain any detectable Rab5, Rab7, or EAA-1 in each of these three samples.

## Subcellular fractionation

Nuclear fraction was isolated as previously described [11]. We stress that this methodology uses both a 2 mol/L sucrose cushion, as well as multiple washings, to minimize contamination of the nuclear compartment with either plasmalemma or endosomes. We were unable to detect either Rab 5 or Rab 7 (markers of early and late endosomes, see below) in the nuclear fraction of either normal or ouabain-stimulated cells; conversely, Lamin B was easily detected in the nuclear fraction (Fig. 1).

## Preparation of endosomes

Endosomes were fractionated on a flotation gradient using the technique of Gorvel et al [21]. Briefly, control and treated cells were washed twice with ice-cold phosphate-buffered saline (PBS)-Ca-Mg (PBS containing 100 µmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$), and once with ice-cold PBS. The cells were collected in PBS and centrifuged at 4°C at 3000$g$ for 5 minutes. The pellet was resuspended in 3 mL of the homogenization buffer (250 mmol/L sucrose in 3 mmol/L imidazole, pH 7.4) and recentrifuged at 4°C at 3000$g$ for 10 minutes. The pellet was resuspended in 1.0 mL of homogenization buffer (with 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mmol/L PMSF, and 0.5 mmol/L EDTA), and gently homogenized on ice using a Dounce homogenizer (15 to 20 strokes), followed by a centrifugation (4°C at 3000$g$ for 10 minutes). The supernatant was adjusted to 46% sucrose using a stock solution of 62% sucrose in 3 mmol/L imidazole (pH 7.4) and loaded at the bottom of a centrifuge tube, to which was sequen-

tially added 16% sucrose (4 mL) in 3 mmol/L imidazole and 0.5 mmol/L EDTA in $^2H_2O$, 10% sucrose in the same buffer (3 mL), and finally homogenization buffer (1 mL). The gradient was centrifuged at 4°C at 130,000$g$ in a Beckmann SW 40Ti rotor for 60 minutes. Early endosomes were collected at the 16%/10% sucrose interface, while the late endosomes were collected at the homogenization buffer and 10% sucrose interface. The identity of early and late endosomes was determined with polyclonal antibodies raised against Rab5, Rab7, and EEA-1 (Fig. 1).

## Preparation of clathrin-coated vesicles (CCV)

Isolation of CCV was performed as described by Hammond and Verroust [22]. Briefly, LLC-PK1 cells were collected and homogenized using a Potter homogenizer in isolation buffer (EGTA, 1 mmol/L; $MgCl_2$, 0.5 mmol/L; $NaN_3$, 0.02%; 2-(N-morpholino) ethanesulfonic acid, 100 mmol/L; pH 6.5). The homogenate was centrifuged at 15,000$g$ for 15 minutes, and the supernatant was further centrifuged at 85,000$g$ for 60 minutes (with the addition of 1 mmol/L PMSF, 1 mmol/L sodium orthovanadate, 10 µg/mL leupeptin, and 10 µg/mL aprotinin). The pellet was resuspended in isolation buffer, applied to a discontinuous sucrose gradient (wt/vol in the same buffer, 60, 50, 40, 10, and 5%), and centrifuged at 80,000$g$ for 75 minutes in a SW41Ti rotor. Samples were collected from the 10%/40% interface, washed, and resuspended in isolation buffer, and pelleted at 85,000$g$ for 60 minutes. Wheat germ agglutinin (1 mg to 10 mg protein) was added and incubated overnight at 4°C, then centrifuged at 20,000$g$ for 15 minutes. The supernatant was centrifuged at 165,000$g$ for 60 minutes, and the resulting pellet was resuspended in the isolation buffer [22].

## Labeling of cell surface Na/K-ATPase by biotinylation

Cell surface biotinylation was performed as previously described [11, 23, 24]. Proteins bound to the ImmunoPure immobilized streptavidin-agarose beads were eluted by incubation at 55°C water bath for 30 minutes with an equal volume of 2× Laemmli sample buffer, and then resolved on SDS-PAGE followed by immunoblotting.

For the biotinylation pulse-chase studies, LLC-PK1 cells were first biotinylated under the same conditions described above (except that the biotinylation buffer pH was adjusted down to 8.0, which we found did not affect cell viability), quenched with PBS-Ca-Mg-glycine, and brought back to normal incubation conditions in DMEM in the presence or absence of ouabain for different times.

## Immunoprecipitation of α1-subunit of Na/K-ATPase, clathrin, and clathrin-associated protein AP-2

Immunoprecipitation of α1-subunit, AP-2 α-subunit, and CHC proceeded mainly as described [17, 25, 26].

Briefly, after washing (2×) with PBS-Ca-Mg and with PBS, cells were solubilized in TGH buffer (1% Triton X-100, 0.25% sodium deoxycholate, 10% glycerol, 50 mmol/L NaCl, 50 mmol/L HEPES, pH 7.3, 1 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 1 mmol/L PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin). After brief centrifugation (13,000*g* for 15 minutes), supernatants containing equal amounts of protein were immunoprecipitated with a saturating amount of antibodies against α1 subunit, CHC, or AP-2 α subunit at 4°C overnight with end-to-end rotation, and then 2 hours with Protein A or G-agarose beads (Upstate). Immunoprecipitates were washed (3×) with TGH and with PBS. Proteins were eluted as described above, and resolved on SDS-PAGE followed by immunoblotting. Normal rabbit or mouse IgG was used as a control.

## Confocal microscopy

Cells grown to confluence on the 12-mm Transwell filters were fixed and permeabilized as described by Muth et al [27]. Briefly, cells were fixed with cold absolute methanol or 4% paraformaldehyde in PBS, permeabilized in permeabilization buffer [PBS-Ca-Mg with 0.3% Triton X-100 and 0.1% bovine serum albumin (BSA), freshly prepared] for 15 minutes, and blocked with GSDB buffer [20 mmol/L sodium phosphate, pH 7.4, with 150 mmol/L NaCl, 0.3% Triton X-100, and 16% (v/v) filtered normal goat or horse serum] for 30 minutes at room temperature. The cells were then probed with primary antibody for 90 minutes at room temperature or overnight at 4°C (monoclonal anti α1 antibody, Upstate; polyclonal anti-clathrin antibody, BD Transduction Laboratories; 1:100 dilution in GSDB). After 3 washes with permeabilization buffer, the cells were incubated with Alexa Fluor® 488- or Alexa Fluor® 546-conjugated antimouse or antirabbit secondary antibody for 1 hour at room temperature. After another 3 washes, specimens were mounted using Prolong Anti-fade medium (Molecular Probes) and stored at −20°C. In some cases, the cells were counterstained with propidium iodide (Molecular Probes) to localize nuclei. All images were generated with a Bio-Rad Radiance2000® laser scanning confocal microscope (Bio-Rad). Contrast and brightness were set to ensure that all pixels were within the linear range. The software LaserSharp 2000 (Bio-Rad) was used for image acquisition, storage, and visualization. Negative controls were also performed to verify the specificity of primary and secondary antibodies.

## Determination of phosphatidylinositol 3-kinase (PI(3)K) activity

Determination of PI(3)K was performed as described by Chibalin et al [28] with minor modifications. Briefly, after preincubation with ouabain under different con-ditions, the cells were homogenized in 400 μL of ice-cold lysis buffer [140 mmol/L NaCl, 10 mmol/L HEPES, 10 mmol/L sodium pyrophosphate, 1 mmol/L NaF, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1 mmol/L $Na_3VO_4$, 10% glycerol, 1% Nonidet P-40, 10 μg/mL aprotinin and leupeptin, and 1 mmol/L PMSF, (pH 8.1)] and solubilized by continuous stirring for 1 hour at 4°C. After centrifugation, 1 mg of supernatant protein was immunoprecipitated with an anti-PI-3K p85α antibody coupled to protein A-agarose beads (Upstate). The immunoprecipitates were washed 4 times with washing buffer [100 mmol/L NaCl, 1 mmol/L $Na_3VO_4$, and 20 mmol/L HEPES (pH 7.5)] and resuspended in 40 μL of resuspension buffer [180 mmol/L NaCl and 20 mmol/L HEPES (pH 7.5)]. The PI(3)K activity in the immunoprecipitate was assessed directly on the protein A-agarose beads. The reaction was initiated by addition of 20 μL of reaction buffer [50 mmol/L NaCl, 0.015% Nonidet P-40, 12.5 mmol/L $MgCl_2$, 250 μmol/L [γ-$^{32}$P]ATP (50 μCi), 1.0 mg/mL phosphatidylinositol (Avanti Biochemicals, Birmingham, AL, USA), and 20 mmol/L HEPES (pH 7.5)]. The samples were incubated for 15 minutes at room temperature, and the reaction was terminated by sequential addition of 80 μL of 1 mol/L HCl and 160 μL of chloroform/methanol (1:1, vol/vol). After vigorous vortexing and a brief centrifugation, 40 μL of the lower phase was spotted on aluminum-backed Silica Gel 60 thin layer chromatography plates (Sigma). The lipids were resolved by 1-propanol/2 mol/L acetic acid (65:35, vol/vol). The bands corresponding to phosphatidylinositol 3-phosphate were analyzed by autoradiography and quantified using phosphoimaging (Typhoon 8600, Molecular Dynamics, Sunnyvale, CA, USA).

## Chlorpromazine treatment

LLC-PK1 cells were pretreated with 50 μmol/L of chlorpromazine for 30 minutes at 37°C, and then treated with ouabain for indicated time as previously reported [29, 30]. The chlorpromazine concentration and times of incubation were chosen so that the cell viability was not affected.

## Intracellular potassium depletion treatment

Intracellular potassium depletion was carried out as previously described by Larkin et al [31]. Briefly, LLC-PK1 cells were washed twice with PBS-Ca-Mg and incubated for 5 minutes at 37°C in hypotonic medium (DMEM/water, 1:1, vol/vol). The cells were then washed with isotonic $K^+$-free buffer (100 mmol/L NaCl, 0.1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and 50 mmol/L HEPES, pH 7.4), and incubated at 37°C in the same medium to produce potassium depletion. This potassium-free media was then used for ouabain treatment experiments. More than 90% of the cells were viable at

the end of each experiment as assessed by Trypan blue exclusion.

## Statistical analysis

Data were first tested for normality (all data passed) and then subjected to parametric analysis. When more than two groups were compared, one-way analysis of variance (ANOVA) was performed before comparison of individual groups with the unpaired Student $t$ test with Bonferroni's correction for multiple comparisons. If only two groups of normal data were compared, the Student $t$ test was used without correction. SPSS software (Chicago, IL, USA) was used for all analyses.

## RESULTS

### Nontoxic concentration of ouabain inhibits the activity of Na/K-ATPase via the endocytosis of the Na/K-ATPase

In cells grown to confluence on the Transwell culture inserts, we have previously observed that addition of nontoxic concentrations of ouabain to the basolateral aspect resulted in a dose- and time-dependent decrease in ouabain-sensitive $^{86}$Rb uptake [11]. In this series of studies, we observed that while administered to the basolateral aspect at 50 nmol/L [approximately $0.05 \times$ of the acute $IC_{50}$ (determined at 30 minutes of incubation)] caused a $58 \pm 4\%$ reduction in ouabain-sensitive $^{86}$Rb uptake after 12 hours ($N = 7$, $P < 0.01$), whereas addition of this concentration of ouabain to the apical aspect resulted in essentially no alteration in the $^{86}$Rb uptake at 12 hours ($102 \pm 4\%$ of control values, $P = NS$). To understand how ouabain decreased $^{86}$Rb in these cells, we examined whether ouabain caused a reduction in surface enzyme density. We determined the level of the surface α1 and β1 subunits of Na/K-ATPase by measuring biotinylated protein densities. In response to 50 nmol/L ouabain (12 hours) applied to the basolateral aspect, biotinylated protein content of both the Na/K-ATPase α1 and β1 subunits decreased by about 60%. When ouabain was applied to the apical aspect in the same conditions, it did not alter the content of these biotinylated subunits (Fig. 2).

To determine the reversibility of these changes, confluent monolayers of LLC-PK1 cells were subjected to 12 hours of ouabain (50 nmol/L) on the basolateral aspect. At this point, the ouabain was either maintained or washed away. In monolayers in which the ouabain was maintained, the $^{86}$Rb uptake remained low, whereas once the ouabain was removed, the $^{86}$Rb uptake returned to normal over the next 24 hours (Fig. 3A). Using the same experimental design, plasmalemmal pump density was measured using the biotinylated α1 subunit density measurement described above. We observed that plasmalemmal pump recovery paralleled the recovery of $^{86}$Rb uptake (Fig. 3B).
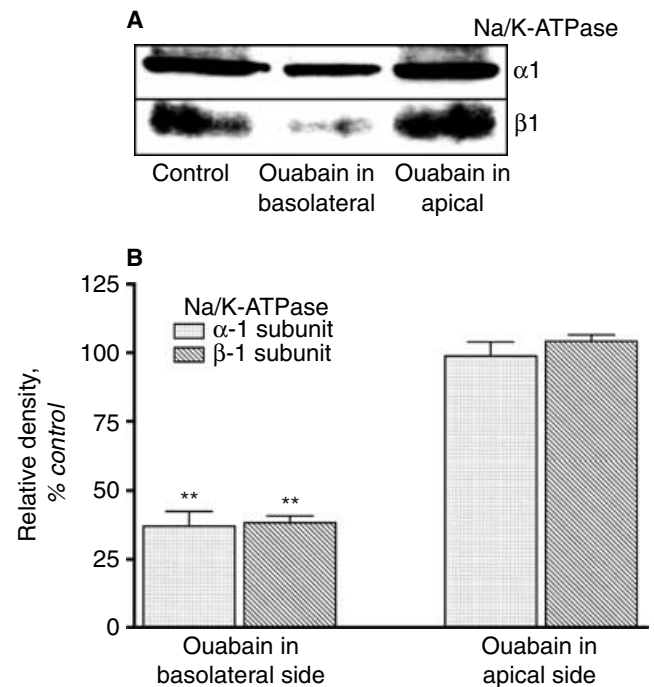


**Fig. 2. Effect of ouabain (50 nmol/L for 12 hours) on Na/K-ATPase α1 and β1 protein density when applied to the apical or basolateral aspects of an LLC-PK1 monolayer.** (*A*) Representative Western blots. (*B*) Quantification of $N = 5$ ouabain-treated experiments (5 apical and 5 basolateral) compared with $N = 5$ controls (values averaged and expressed as 100%). Data shown as mean $\pm$ SEM. ** $P < 0.01$ vs. control.

To further confirm that ouabain stimulates the internalization of the enzyme, confocal microscopy was utilized to qualitatively assess the distribution of the Na/K-ATPase in the LLC-PK1 monolayers. Cells were double-stained with anti-α1 antibody to localize α1 subunit (green signal) and by propidium iodide (PI) to localize the nuclei (red signal). In control, nonstimulated cells, we observed that the α1 subunit of Na/K-ATPase was largely confined to the basolateral aspects of the LLC-PK1 monolayer (Fig. 4A). In these control cells, no yellow signal (indicative of overlap of the green and red signals) was detectable when the two images were merged. However, in the cells treated with ouabain for 12 hours, we found a considerable portion of the Na/K-ATPase α1 subunit localized in internal portions of the cells (Fig. 4B). Interestingly, we also found that ouabain stimulated the translocation of Na/K-ATPase into nucleus. This is consistent with our earlier observation of the time-dependent accumulation of [$^3$H] ouabain in the nucleus of LLC-PK1 cells [11].

Because there is evidence that several receptor tyrosine kinases move to the nucleus where they can regulate the transcriptional activity of their targeted genes [32–34], we further examined whether ouabain stimulates the translocation of Na/K-ATPase to the nucleus. To do this, we first biotinylated the surface proteins, quenched the
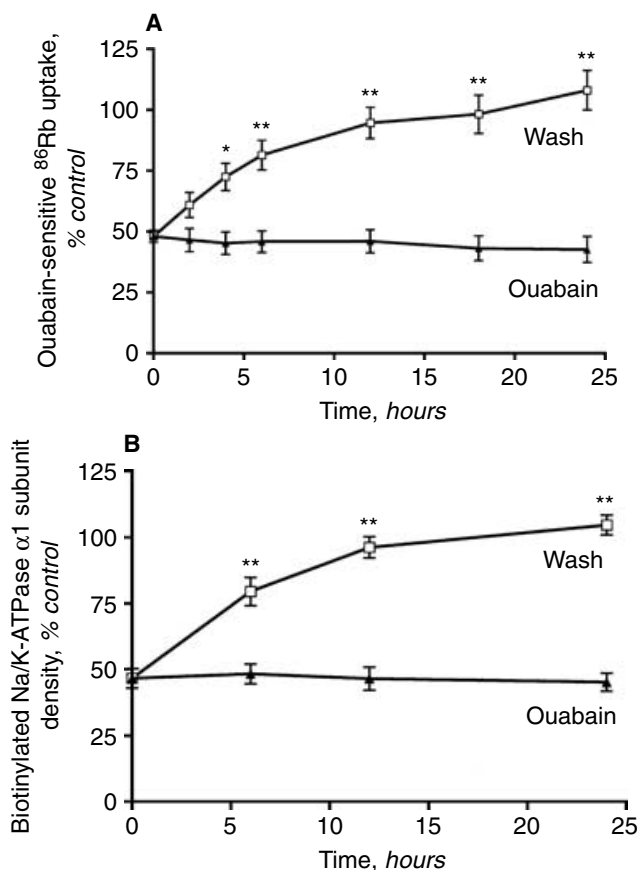
**Fig. 3. Recovery of plasmalemmal Na/K-ATPase in LLC-PK1 cell monolayers after treatment with ouabain (50 nmol/L for 12 hours) on the basolateral aspect.** (*A*) Demonstrated is the recovery of ouabain-sensitive $^{86}$Rb uptake (expressed as % control). (*B*) Recovery of Na/K-ATPase $\alpha$1 subunit by surface biotinylation method. Open squares depict those from which ouabain was removed (Wash), and solid triangles depict those where the ouabain concentration was maintained (Ouabain). $N = 4$ experiments are represented at each data point in each panel. *$P < 0.05$; **$P < 0.01$ vs. ouabain.

nonreacted biotin reagent, and then chased the biotinylated proteins for 12 hours with restoration of normal medium, with or without ouabain. To assess the possibility of the contamination in the isolation process of nuclear fraction, we also measured the biotinylated $\alpha$1-subunit without any chase to serve as a negative control. As shown in Figure 5A, we observed that the isolation of the nuclear compartment probably has a very small degree of what we would characterize as contamination during the isolation procedure, because we would not anticipate measurable biotinylated $\alpha$1-subunit to reach the nucleus after the minimal time (5 minutes) necessary for the isolation procedure itself. In pulse chase experiments performed at 12 hours of incubation with ouabain (50 nmol/L), we observed that ouabain induced marked increases of $\alpha$1-subunit in the nuclear fraction that was labeled with biotin (Fig. 5A and B).

## Ouabain induces Na/K-ATPase accumulation in CCVs and endosomes and increases the association with clathrin and AP-2

To define the mechanisms underlying this Na/K-ATPase translocation, cells were treated with 50 nmol/L ouabain for 1 hour, and CCV were isolated. Without ouabain, CCVs contained relatively little Na/K-ATPase $\alpha$1 subunit, but after ouabain treatment, a considerable amount of the sodium pump could be demonstrated (Fig. 6). At this time point, ouabain treatment induced a $174 \pm 41\%$ increase in CCV Na/K-ATPase $\alpha$1 subunit content ($N = 4$, $P < 0.01$). To further examine these mechanisms, early and late endosomes were also isolated. As shown in Figure 7, ouabain (50 nmol/L $\times$ 2 hours) induced significant increases in both early and late endosomal Na/K-ATPase $\alpha$1-subunit protein content. We next labeled the $\alpha$1 subunit with a monoclonal antibody, and clathrin heavy chain with a polyclonal antibody, and then processed these samples with an Alexa546®-conjugated secondary antibody for the Na/K-ATPase $\alpha$1 subunit (Fig. 8, left panels), and an Alexa488®-conjugated secondary antibody for clathrin heavy chain (Fig. 8, middle panels). Ouabain treatment was associated with internalization of the Na/K-ATPase (Fig. 8, bottom vs. top panels). Moreover, marked colocalization with clathrin was observed both before and after ouabain exposure (Fig. 8, right panels). It should be noted that the colocalization of $\alpha$1 subunit with clathrin mainly occurred within the basolateral membrane in the control cells, while the colocalization could be identified in both basolateral membrane and inside the cells after ouabain exposure.

We next used a coimmunoprecipitation assay to examine the protein-protein interaction between the $\alpha$1-subunit and two of the major components of the clathrin-coated pit, the AP-2 $\alpha$ subunit (AP-2), and the clathrin heavy chain. As anticipated, incubation with ouabain enhanced the interaction of the $\alpha$1-subunit with AP-2 (Fig. 9). From the same lysate obtained after ouabain treatment, ouabain increased the amount of AP-2 in the immunoprecipitated material obtained with an antibody against the Na/K-ATPase $\alpha$1-subunit and the amount of Na/K-ATPase $\alpha$1-subunit in the immunoprecipitated material obtained with an antibody against AP-2 (Fig. 9). In similar experiments addressing the protein-protein interaction between the Na/K-ATPase $\alpha$1-subunit and clathrin, we also found that ouabain increased the amount of Na/K-ATPase $\alpha$1-subunit and AP-2 in the immunoprecipitated material obtained with an antibody against CHC (Fig. 9). These coimmunoprecipitation data suggest that ouabain enhances the protein-protein interactions of the Na/K-ATPase $\alpha$1-subunit with AP-2 $\alpha$ subunit and with clathrin. Ouabain also appears to enhance the interaction of clathrin with AP-2.
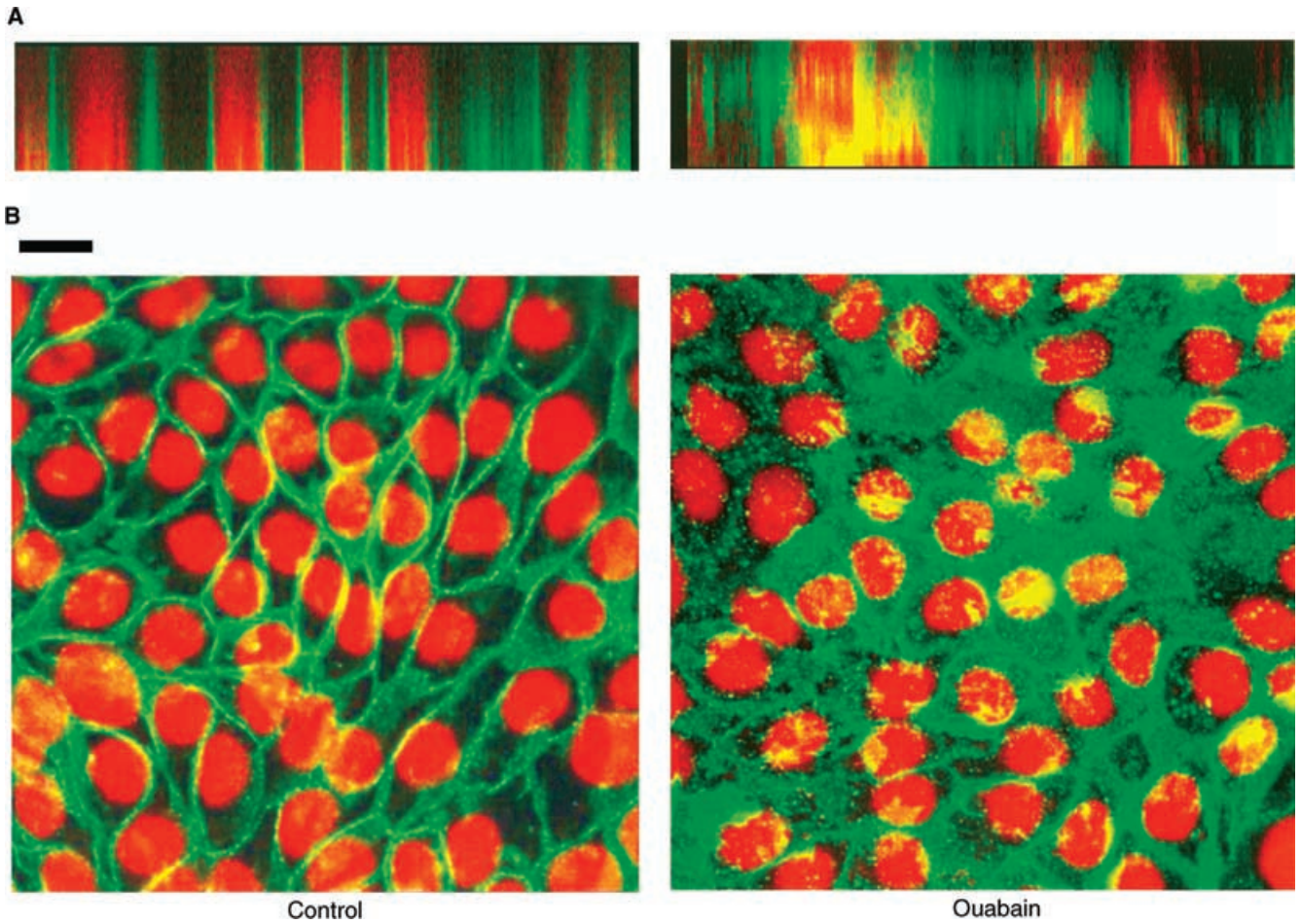
**Fig. 4. Confocal immunofluorescence images of (A) control and (B) ouabain- (50 nmol/L for 12 hours on basolateral aspect) treated LLC-PK1 cells grown to monolayer.** In these images, α1 subunit of Na/K-ATPase is labeled with Alexa Fluor® 488 labeled secondary antibody, giving green fluorescence signal. Nuclei counterstained with propidium iodide (PI) (red color) for contrast. (A) Shows Z axis reconstruction, whereas (B) shows representative XY plane. Note that the green stain is largely confined to the basolateral aspects of the control cells, whereas there is substantial internalization of this stain in the cells treated with ouabain. Size bar = 10 μm.
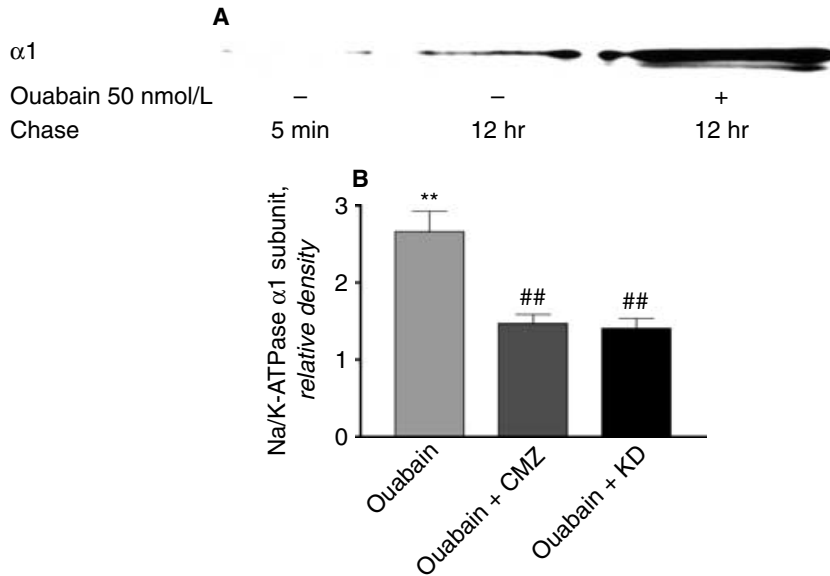


**Fig. 5. Effect of chlorpromazine (CMZ) and potassium depletion (KD) on ouabain-induced increases in nuclear accumulation of biotinylated α1 subunit of Na/K-ATPase.** (*A*) Representative Western blot after streptavidin isolation of biotinylated pump from LLC-PK1 cell nuclei from cells exposed to ouabain for 12 hours and control cells. Note that there is very little biotinylated pump appearing after 5-minute chase, confirming low levels of nuclear contamination with our isolation procedure. (*B*) Quantification of the nuclear biotinylated pump (data shown as mean ± SEM of 4 individual experiments normalized to the mean control value) in response to ouabain alone vs. ouabain + CMZ or KD. **$P < 0.01$ vs. ouabain alone; ##$P < 0.01$ in comparison with ouabain-treated cells.

**Fig. 6. Representative Western blot illustrating effect of ouabain (1 hour at 50 nmol/L) on CCV α1 subunit of Na/K-ATPase protein content.** Both lanes loaded with 5 μg of protein. Quantification of 4 experiments show the relative CCV Na/K-ATPase α1 subunit content in ouabain-treated cells was 2.74 ± 0.41 (*P* < 0.01 vs. control) compared with CCVs isolated from untreated cells.
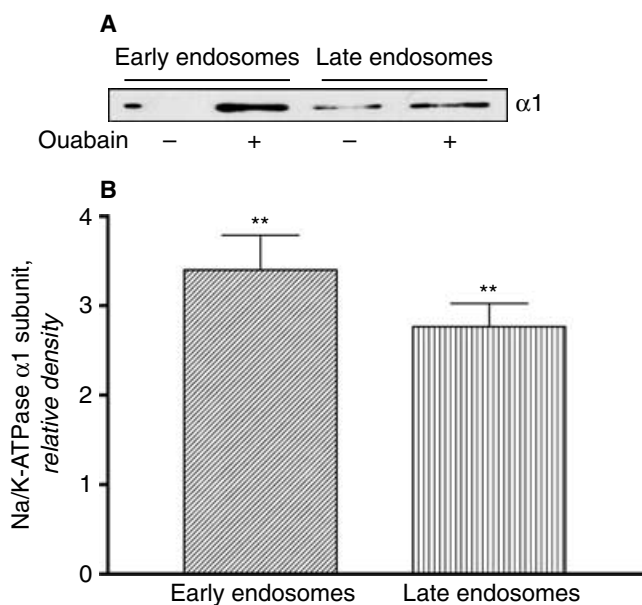


**Fig. 7. Effect of ouabain (2 hours at 50 nmol/L) on early and late endosome α1 subunit of Na/K-ATPase protein content.** (*A*) Representative Western blot. (*B*) Quantitative data expressed as fraction of control (control values = 1). *N* = 5 controls and *N* = 5 ouabain-treated cells studied. \*\**P* < 0.01 vs. control.

## Ouabain activates PI(3)K in LLC-PK1 cells

In a separate set of experiments, LLC-PK1 cells were exposed to ouabain 50 nmol/L, and PI(3)K activity was assayed. We observed that ouabain caused substantial activation of PI(3)K by as early as 15 minutes after exposure. These data are summarized in Table 2.

## Inhibition of clathrin-mediated endocytosis attenuates ouabain-stimulated Na/K-ATPase endocytosis

To confirm that ouabain-induced endocytosis of the α1-subunit of Na/K-ATPase involves a clathrin-dependent pathway, we first disrupted the clathrin-mediated endocytosis pathway by potassium depletion (KD) in conjunction with hypotonic shock [31], or treatment with a cationic amphiphilic drug, chlorpromazine [30]. In experiments similar to that shown in Figure 2, the

surface proteins of LLC-PK1 cells were first biotinylated, and the nonreacted biotin reagent was quenched. Then, the LLC-PK1 cells were exposed to ouabain (chase) for 12 hours in the presence of chlorpromazine (50 μmol/L) or after potassium depletion. Biotinylated surface proteins and nuclear fraction were isolated, and the amount of the biotinylated Na/K-ATPase α1 subunit was determined as described above. In ouabain-stimulated cells, both chlorpromazine and potassium depletion markedly attenuated ouabain-induced nuclear accumulation of the biotinylated Na/K-ATPase α1 subunit (Fig. 5B). Ouabain-induced decreases in the plasmalemmal Na/K-ATPase α1 subunit were also attenuated by both chlorpromazine and potassium depletion at 12 hours. Plasmalemmal Na/K-ATPase α1 subunit density was decreased only 12 ± 5% and 9 ± 7% by ouabain in the presence of chlorpromazine and potassium depletion, respectively, compared with control.

Because activation of PI(3)K is involved in vesicular trafficking and targeting of proteins to specific cell compartments (especially from clathrin-coated pits to early endosomes) and assembly of clathrin-coated pits [28, 35], we determined if inhibition of this enzyme attenuates ouabain-induced endocytosis of the Na/K-ATPase. LLC-PK1 cells were pretreated with 100 nmol/L wortmannin for 30 minutes, then exposed to 50 nmol/L ouabain for 2 hours. Early endosomes were isolated and assayed for the content of the Na/K-ATPase α1 subunit as described earlier. Although wortmannin alone did not have a significant effect on this measurement, wortmannin substantially attenuated the accumulation of Na/K-ATPase α1 subunit in response to ouabain as compared to ouabain treatment alone (1.28 ± 0.07 vs. 3.4 ± 0.35 relative units, normalized to control early endosomes, *N* = 12 in both groups, *P* < 0.01).

In the experiments depicted in Figure 10, LLC-PK1 cells were treated with 50 nmol/L ouabain for 12 hours in the presence or absence of 100 nmol/L wortmannin, then biotinylated. Wortmannin alone did not affect biotinylated pump density but substantially attenuated the ouabain-induced decreases in surface Na/K-ATPase (87 ± 9% vs. 48 ± 5% control values at 12 hours, both *N* = 4, *P* < 0.01). To further confirm the role of PI(3)K in ouabain-induced endocytosis of Na/K-ATPase, we performed ^{86}Rb uptake studies. These studies were complicated by the marked decreases in ouabain-sensitive ^{86}Rb uptake observed after 12-hour exposure to either wortmannin or LY294002. This phenomenon was observed over a wide range of concentrations, and was not accompanied by evidence of cellular toxicity (e.g., altered Trypan blue exclusion or altered microscopic appearance). When these inhibitors were used at the concentrations shown in Table 1 (100 nmol/L for wortmannin and 25 μmol/L for LY294002), the effect of ouabain (50 nmol/L) to induce decreases in ^{86}Rb uptake was
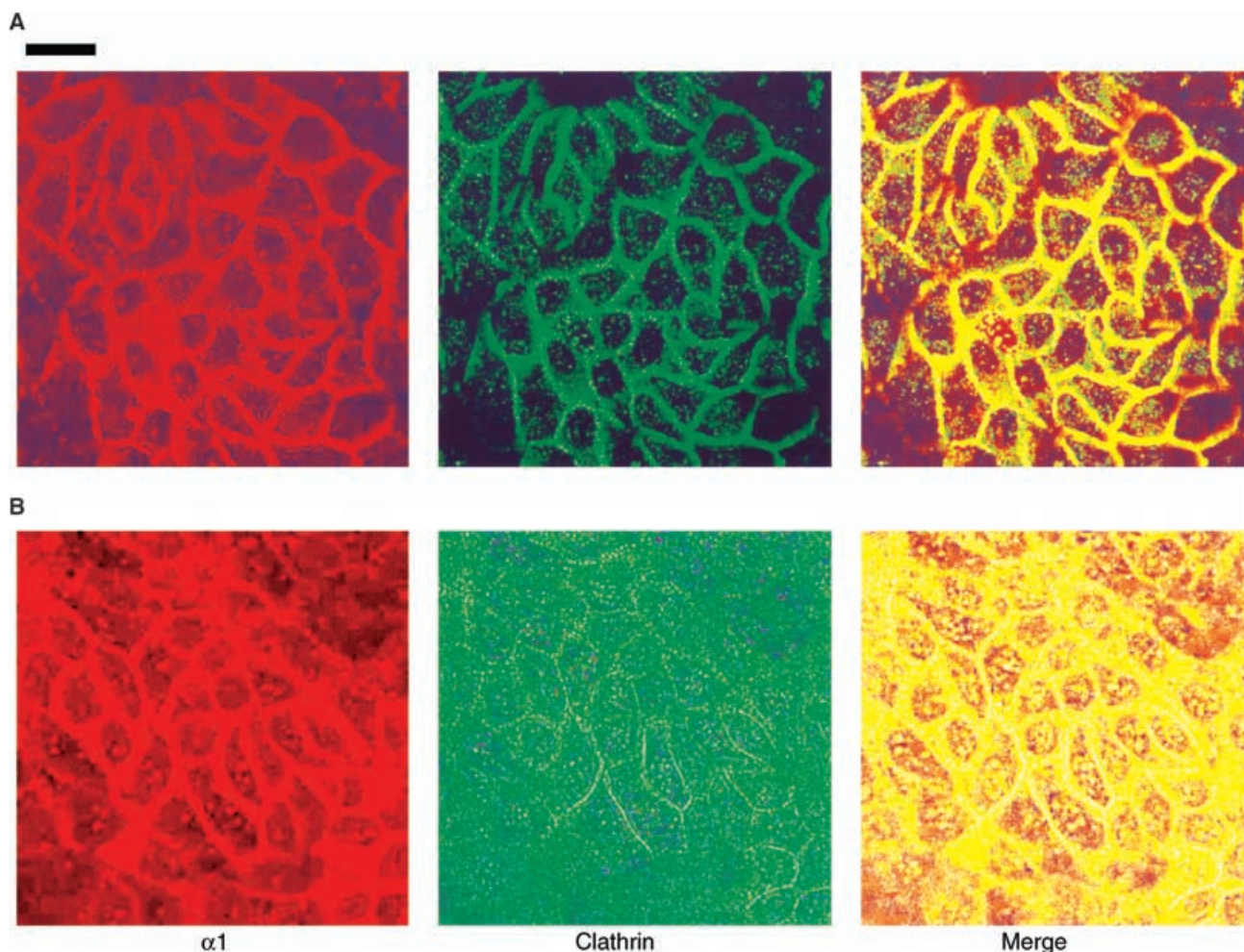
**Fig. 8. Colocalization of α1 subunit with clathrin in response to ouabain.** In these images, LLC-PK1 cells were grown to monolayer, α1 subunit of Na/K-ATPase is labeled with a Alexa Fluor® 546-conjugated secondary antibody (red, *right panels*), and clathrin is labeled with Alexa Fluor® 488-conjugated secondary antibody (*middle panels*). Right panels show the resultant images after merging without further processing. Top (*A*) panels show cells obtained without ouabain, whereas bottom (*B*) panels show cells obtained after 12 hours of exposure to 50 nmol/L ouabain. Note the compelling colocalization of the α1 subunit of Na/K-ATPase with clathrin under both control and ouabain treated conditions. Size bar = 10 μm.

markedly attenuated if we compare these values with those observed with wortmannin and LY294002 alone.

### Ouabain-induced endocytosis is not secondary to changes in membrane potential

To address whether the ouabain-induced endocytosis might be caused by changes in ion concentrations or membrane potential, we performed the following experiments. LLC-PK1 cells were exposed to different potassium concentrations for 12 hours. At this time, the cells were washed and ouabain sensitive ^{86}Rb uptake was measured under standard experimental conditions (Table 3). These data demonstrate that alterations in media potassium concentration, which would be expected to induce hypo- or hyperpolarization, did not significantly

alter ^{86}Rb uptake and, by extension, stimulate substantial Na/K-ATPase endocytosis.

### SRC kinase is involved in ouabain-induced α1-subunit endocytosis

Because we have previously observed that the activation of Src initiates the ouabain-activated signal transduction pathway through the Na/K-ATPase in LLC-PK1 cells [17], we examined the role of Src kinase in ouabain-induced internalization of the Na/K-ATPase. In experiments depicted in Figure 11, we used the Src kinase inhibitor, PP2, to block the activation of Src in response to ouabain, and then examined the accumulation of the Na/K-ATPase α1-subunit in endosomes at 2 hours in response to ouabain. LLC-PK1 cells were first pretreated with 1 μmol/L PP2 for 15 minutes at 37°C, and
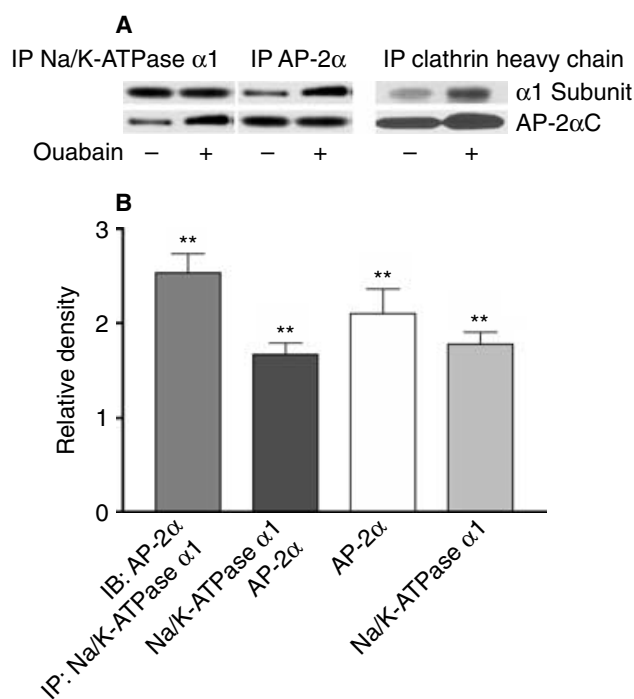
**A**

IP Na/K-ATPase α1     IP AP-2α     IP clathrin heavy chain

α1 Subunit

AP-2αC

Ouabain    −    +        −    +        −    +

**B**



**Fig. 9. Effect of ouabain (50 nmol/L for 1 hour) on the interaction between the α1-subunit of Na/K-ATPase with AP-2 and clathrin heavy chain.** (*A*) Representative Western blots. (*B*) Quantitative data. After ouabain treatment, LLC-PK1 cell lysate from control and treated cells was immunoprecipitated with either antibody against (IP) the Na/K-ATPase α1−subunit, AP−2α, or clathrin heavy chain (CHC). The immunoprecipitate was separated by SDS-PAGE and immunoblotted (IB) with antibodies against the Na/K-ATPase α1 subunit or AP-2α. Data shown as mean ± SEM of 4 individual experiments after ouabain treatment compared with control. **$P < 0.01$ vs. control.

then treated with 50 nmol/L of ouabain (2 hours, in the presence of PP2). As shown in Figure 11, PP2 significantly attenuated ouabain-induced accumulation of the Na/K-ATPase α1-subunit in early endosomes.

In additional experiments performed with SYF cells, we isolated early and late endosomes under control circumstances, as well as 30, 60, and 120 minutes after exposure to ouabain. These data are summarized in Figure 12. We observed that in the SYF− cells, ouabain did not affect the Na/K-ATPase α1 subunit density in either early or late endosomes at any of these time points, whereas in the reconstituted SYF+ cells, ouabain caused significant increases in Na/K-ATPase α1 subunit density in early endosomes at all time points studied, and in late endosomes at the 120-minute time point.

## DISCUSSION

The role of circulating inhibitors of Na/K-ATPase in renal sodium homeostasis is still unclear, despite decades of investigation [1]. Recently, it has been established that increases in the renal excretion and plasma concentration of DLS such as MBG and OLC accompany acute
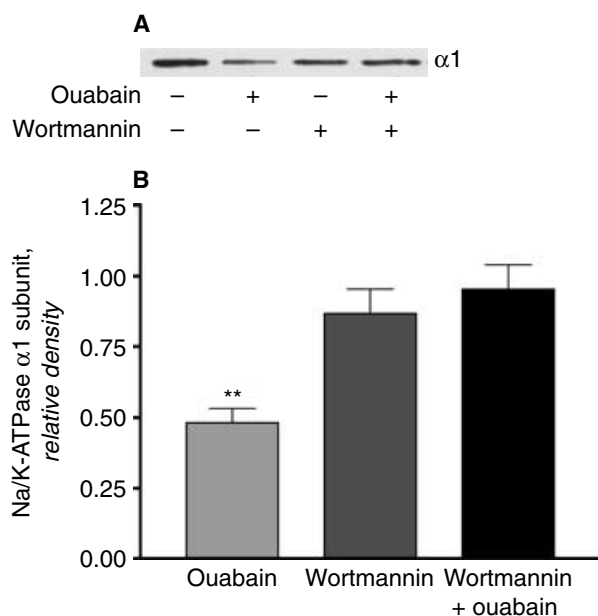
**A**

α1

Ouabain        −    +    −    +
Wortmannin     −    −    +    +

**B**



**Fig. 10. Effect of PI(3)K inhibitor on ouabain-induced decrease of surface Na/K-ATPase α1-subunit.** (*A*) Representative Western blots. (*B*) Quantitative data expressed as mean ± SEM of 4 individual experiments normalized to the mean control value. LLC-PK1 cells (grown on Transwell culture inserts) were preincubated with 100 nmol/L wortmannin for 30 minutes, and then exposed to 50 nmol/L ouabain for 12 hours (on basolateral aspect). Biotinylated α1-subunit was determined as in Figure 1. **$P < 0.01$ vs. control.

**Table 1.** Effect of PI(3)K inhibitors on ouabain-induced decreases in $^{86}$Rb uptake

| Experimental group (N) | $^{86}$Rb uptake (fraction control) |
|---|---|
| Ouabain (8) | 45.6 ± 4.1[b] |
| Wortmannin (5) | 82.5 ± 6.4[a] |
| Ly294002 (5) | 80.4 ± 5.8[a] |
| Wortmannin + ouabain (5) | 61.6 ± 3.8[b,c] |
| Ly294002 + ouabain (5) | 64.6 ± 5.4[b,c] |

Doses of pharmacologic agents: ouabain, 50 nmol/L; wortmannin, 100 nmol/L; LY294002, 25 μmol/L. All agents were administered for 12 hours. Fresh media (with agents) were changed every 4 hours.
[a]$P < 0.05$; [b]$P < 0.01$ vs control; [c]$P < 0.05$ vs. ouabain alone.

**Table 2.** Effect of ouabain (50 nmol/L) on PI(3)K activity

| Time of ouabain exposure *minutes* | PI(3)K activity *% control* |
|---|---|
| 5 | 98.7 ± 2.1[a] |
| 15 | 165.3 ± 8.5[b] |
| 30 | 144.0 ± 7.4[b] |

Data expressed as % control value ± SEM from $N = 6$ determinations.
[a]$P < 0.05$; [b]$P < 0.01$ vs. control.

volume expansion in the Dahl salt-sensitive rat, and that administration of antibody to cardiac glycoside blocks both the hypertension and the increases in urinary sodium excretion seen in this setting [5]. However, the mechanism(s) by which picomolar circulating concentrations of DLS can markedly attenuate renal sodium reabsorption is (are) still unclear.

**Table 3.** Effect of altered potassium concentration for 12 hours on $^{86}$Rb uptake [performed under standard experimental conditions (i.e., K = 5.3 mmol/L)]

| Potassium concentration (all N = 6) | $^{86}$Rb uptake (fraction control) |
|---|---|
| K = 1 mmol/L | 95.7 ± 4.3 |
| K = 3 mmol/L | 96.8 ± 6.2 |
| K = 7 mmol/L | 97.2 ± 2.8 |
| K = 10 mmol/L | 96.9 ± 2.7 |

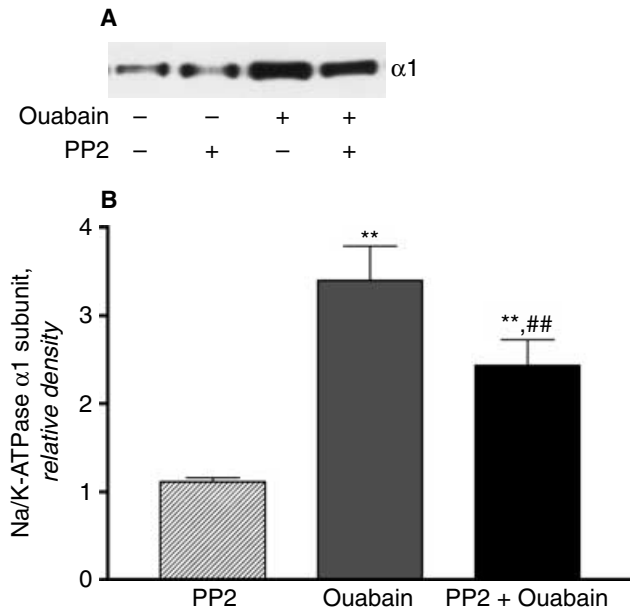Data expressed as % control value ± SEM. No value was significantly different from control (K = 5.3 mmol/L).



**Fig. 11. Effect of Src kinase inhibitor on ouabain-induced accumulation of α1-subunit in endosomes.** LLC-PK1 cells were preincubated with 1 μmol/L PP2 for 15 minutes, and then exposed to 50 nmol/L of ouabain for 2 hours in the continued presence of PP2. (*A*) Representative Western blot. (*B*) Quantitative data expressed as fraction of control (control values = 1). Quantitative data derived from 3 individual experiments. **$P < 0.01$ in comparison with control and PP2 alone; ##$P < 0.01$ in comparison with ouabain-treated cells.



**Fig. 12. Effect of ouabain (10 μmol/L) on accumulation of Na/K-ATPase α1-subunit in early (EE) and late (LE) endosomes of SYF+ and SYF− cells.** Data expressed as fraction of control (i.e., compared with no ouabain in the respective cell type), and shown as mean ± SEM of 5 experiments. *$P < 0.05$ and **$P < 0.01$ compared with control.

In this report, we make several important observations. First, a very low concentration of ouabain (50 nmol/L) rapidly induces endocytosis of the Na/K-ATPase. After this process continues for several hours, substantial depletion of the sodium pump density and function on the membrane surface in LLC-PK1 cells can be measured. In fact, the decrease in the surface α1 subunit was in concert with the decrease in the β1 subunit of the Na/K-ATPase, suggesting that ouabain binds to and removes the whole α1β1 complex from the plasmalemma. We also noted that essentially complete recovery from 12-hour exposure to this dose occurs within 12 to 24 hours. This observation provides an explanation by which circulating concentrations of DLS might decrease proximal tubular sodium reabsorption in vivo. The second observation is that the
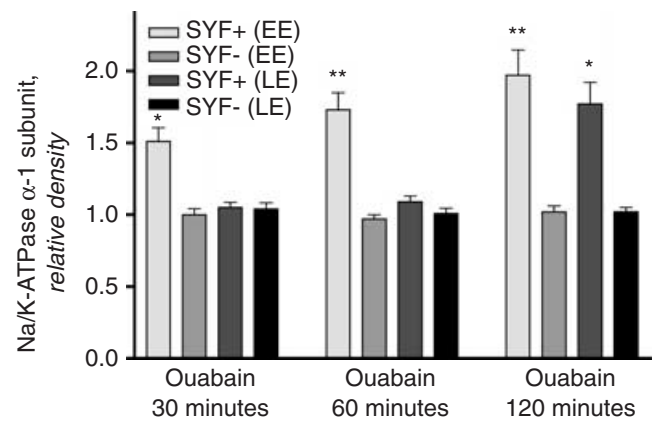
ouabain-stimulated endocytosis of the Na/K-ATPase appears to involve clathrin-dependent mechanisms. Third, and perhaps most interesting, we found that inhibition of the signal transduction cascade initiated by ouabain through the Na/K-ATPase at the level of Src also attenuated the endocytosis pathway. This latter point, to our minds, is so important because it completes the analogy of signal transduction through the Na/K-ATPase with more conventional receptor ligand systems [36].

Endocytosis of the Na/K-ATPase, especially in response to dopamine, has been clearly demonstrated [28, 37]. In addition, early studies from the laboratories of Cook and Lamb demonstrated that ouabain-bound Na/K-ATPase translocated from the plasmalemmal to intracellular compartments [38, 39]. In our previous report we observed that ouabain and marinobufagenin could induce substantial decreases in Na/K-ATPase activity (assayed as ouabain-sensitive $^{86}$Rb uptake as well as enzymatic activity) and plasmalemmal Na/K-ATPase protein content (as assayed by both ouabain binding and biotinylation) in LLC-PK1 cells, which we used as a model for proximal renal tubule epithelial cells [11].

Endocytosis of many different receptors, including G-protein–coupled receptors and receptor tyrosine kinases, is mediated through clathrin-mediated pathways. This process is initiated by tyrosine phosphorylation followed by recruitment and activation of PI(3)K, which lead to assembly of clathrin-coated pits [28, 35]. Recently, there has been ample evidence that Na/K-ATPase can function as a signal transducer as well as an ion pump. Binding of ouabain to the Na/K-ATPase activates Src, resulting in transactivation of EGFR and PI(3)K in LLC-PK1 cells [16, 17, 40]. Based on the findings presented in this report, we suggest that ouabain may actually induce the formation of an Na/K-ATPase/Src/EGFR/PI(3)K
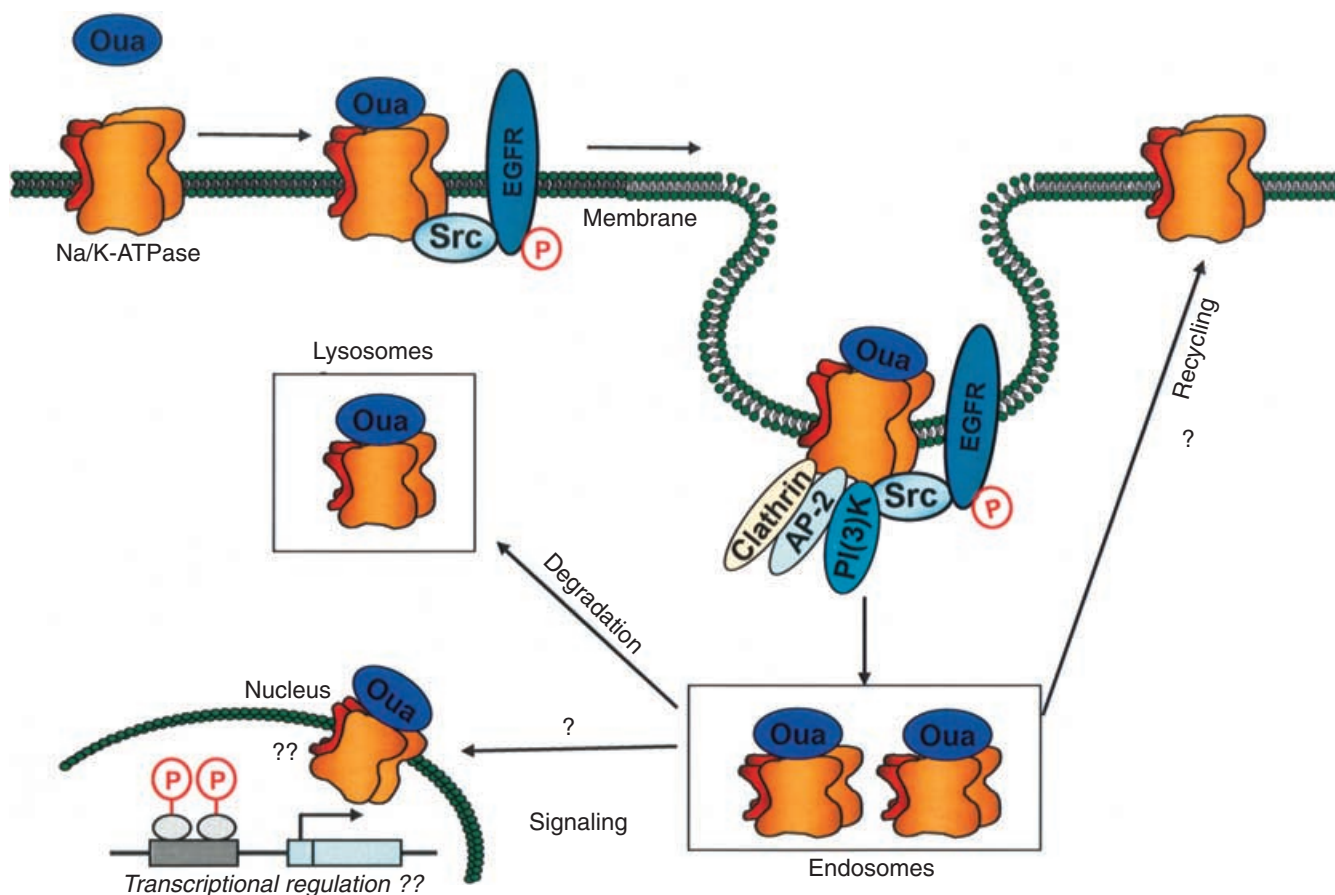
**Fig. 13. Schematic representation of the ouabain-induced endocytosis of Na/K-ATPase.** Na/K-ATPase depicted show binding to ouabain (Oua, *blue oval*), Src (*purple oval*), EGFR (*dark green oval*), and later PI(3)K (*light green oval*) and AP2 (*light blue oval*). Possible fates, including degradation in lysosomes, recycling to the plasmalemma, and translocation to the nucleus for signaling are illustrated.

complex, and this complex recruits AP-2 and clathrin to form the clathrin-coated pits, resulting in the endocytosis of the enzyme (Fig. 13). This proposal is further supported by the following evidence. First, it is well established that potassium depletion inhibits this pathway by blocking clathrin-coated pit formation [41], and that chlorpromazine causes a decrease in the assembly of coated pits on the cell surface and an increase of clathrin lattice assembly on endosomal membranes [30]. In our experiments, both chlorpromazine and potassium depletion treatments did not affect cell viability, but resulted in a significant attenuation of the ouabain-induced endocytosis of the α1-subunit of Na/K-ATPase. Moreover, inhibition of Src or PI(3)K blocked ouabain-induced accumulation of the Na/K-ATPase α1 subunit in early endosomes. Additional experiments performed with the SYF cell lines further confirmed that Src family kinases are required for ouabain-induced endocytosis of the sodium pump to proceed. Also, on a molecular level, ouabain stimulated the interactions among the Na/K-ATPase, AP-2, and clathrin. These data provide substantial evidence that ouabain induces endocytosis of the Na/K-ATPase

through a clathrin-dependent mechanism. Because the doses of ouabain employed would not be expected to substantially alter cytosolic sodium [18, 42], and because hypopolarization (or hyperpolarization) of the membrane did not cause major changes in ^{86}Rb uptake, we further suggest that ouabain-induced endocytosis of the Na/K-ATPase is part of, or a direct consequence of, signal transduction through the Na/K-ATPase.

Although dopamine-mediated endocytosis also proceeds through a clathrin-dependent pathway [13, 43], we do not yet know how much overlap there is between dopaminergic and ouabain-induced Na/K-ATPase endocytosis. Endocytosis of Na/K-ATPase in response to dopamine is triggered by the phosphorylation of Ser[18] and activation of PI(3)K [44]. The binding and activation of PI(3)K facilitates the binding of the α1-subunit with adaptor AP-2 protein, providing the inclusion of Na/K-ATPase into clathrin vesicles. However, Ser[18] is found only in rat α1-subunit and is not present in pig and dog α1-subunit [45]. This indicates that ouabain- and dopamine-induced Na/K-ATPase endocytosis may follow different initial steps and common postendocytic

steps. Interestingly, Chibalin et al found that ouabain at 1 mmol/L did not induce detectable increases in endosomal $\alpha1$ content at 2.5 minutes in the rat proximal tubule preparation [13]. However, this dose of ouabain is approximately $10\times$ the $IC_{50}$ for the $\alpha1$ isoform in the rat, and it is likely that this dose of ouabain is quite toxic to these cells.

In cardiac myocytes and several other cell types (including LLC-PK1 cells), Na/K-ATPase, in response to nontoxic concentrations of ouabain, interacts with other membrane proteins to relay signals to intracellular signaling complexes, mitochondria, and the nucleus. This led to the activation of multiple signal transduction cascades, including protein kinase C (PKC), Ras/Raf/MEK/MAPK [46, 47], and increased production of reactive oxygen species, which results in the activation of transcription factor AP-1 and nuclear factor $\kappa$B (NF-$\kappa$B), and alteration of the transcriptional regulation of several growth-related genes [48].

From our observations, it is very clear that there is considerable opportunity for "cross-talk" between ouabain-induced signaling pathways and ouabain-induced endocytosis of Na/K-ATPase. While receptor-mediated endocytosis has been traditionally considered an effective mechanism to attenuate ligand-activated responses, more recent observations have led to the view that signaling continues on the endocytic pathway, including from endosomes [49, 50]. Endocytic vesicles can function as a signaling compartment distinct from the plasma membrane, and endocytosis plays an important role in the activation and propagation of signaling pathways. It appears that endocytosis of activated EGFR, which recruits signaling proteins directly onto endosomes, is necessary for full-scale mitogen-activated protein kinase (MAPK) activation. MEK1-ERK complex could be formed intracellularly, and activated Ras, Raf1, and MAP kinase/ERK kinase (MEK) can be found on endosomes [51]. In some circumstances, endocytosis is necessary to allow the signaling protein complex, like MEK1, access to extracellular signal-regulated protein kinase (ERKs) in an intracellular compartment [52], and H-Ras signaling through Raf/MEK/MAPK requires endocytosis and endocytic recycling [53]. On the other hand, signal transduction can also regulate endocytosis. For example, EGF stimulation causes rapid phosphorylation of clathrin heavy chain in an Src-dependent process, which leads to increased recruitment of clathrin onto the plasma membrane [54], and over-expression of c-Src increases the rate of endocytosis of EGF/EGFR complexes [55]. As discussed above, ouabain inhibits Na/K-ATPase activity and induces endocytosis of the enzyme; ouabain also activates Src and PI(3)K (both involved in ouabain-induced endocytosis of Na/K-ATPase), and transactivates EGFR, leading to the activation of MAPK. It is therefore

quite reasonable to propose that there is cross-talk between ouabain-induced Na/K-ATPase endocytosis and ouabain-induced signaling transduction. In other studies examining signal transduction in myocytes, our group has observed that caveolae may also be involved in signal transduction resulting in changes in cytosolic calcium and inotropy [56, 57]. This, of course, raises the possibilities that interactions between the Na/K-ATPase and caveolins, as well as caveolins and proteins important in clathrin-dependent endocytosis, may also be involved in the endocytosis pathway in renal epithelium examined in this paper. Certainly, additional studies are necessary to examine these extremely important issues in detail.

In this report, we observed that some of the Na/K-ATPase appears to translocate to the cell nucleus after ouabain stimulation. While we must concede that contamination of the nuclear preparation with endosomes or plasmalemma after ouabain could potentially obfuscate our findings, we stress that our isolation procedure should minimize such contamination. Although it is not yet clear what role endocytosis may play in the Na/K-ATPase signal cascade, this translocation of Na/K-ATPase into the nucleus immediately suggests two potential mechanisms. First, it is possible that translocation of the pump to the nucleus allows for an active sodium "pump" to be inserted into the nuclear membrane, where changes in ion concentrations across this membrane might result as recently suggested [58]. Alternately, the Na/K-ATPase $\alpha1$ subunit (or a fragment of this subunit) might actually be transported into the nucleus, where it might have direct genomic effects. Specifically, we would speculate that the translocation of the Na/K-ATPase to the nucleus would induce down-regulation of other ion pumps important in sodium handling by the proximal tubule, such as the NaH(3) exchanger. Down-regulation of these ion pumps is known to occur in proximal tubules in vivo during salt loading [59]. The presence of the full-length Na/K-ATPase $\alpha1$-subunit and the accumulation of [$^3$H]ouabain in the nucleus is, however, also consistent with previous reports [60]. Moreover, our Western blot studies would argue against degradation or modification of the $\alpha1$-subunit along the endocytic pathway because the $\alpha1$-subunit found in the nucleus is identical to that of the plasma membrane on SDS-PAGE analysis; this suggests that the catalytic $\alpha1$-subunit of Na/K-ATPase may be still functional as a ion transporter or as a transcriptional regulator on the nuclear membrane or inside the nucleus. It was shown that an intramolecular domain (Ser$^{692}$-Ser$^{740}$) of chicken Na/K-ATPase $\alpha1$-subunit fused with the GAL4 DNA binding domain can regulate reporter gene expression in yeast and mammalian cells [60]. To further support this concept, many transmembrane receptors have also been detected in the nucleus [61], such as the receptors for EGF [32] and insulin [62]. Nuclear localization might therefore be a general feature of many

transmembrane receptors. Interestingly, after translocation to the nucleus, the EGFR appears to directly affect gene expression [32]. However, further studies are necessary to examine whether endosomal and nuclear translocated α1-subunits do communicate with ouabain-induced signaling pathways and contribute to ouabain-induced gene regulation.

## CONCLUSION

We demonstrated that low concentrations of ouabain induce substantial endocytosis of the Na/K-ATPase in a clathrin-dependent manner. Further studies will be necessary to define the degree of overlap and cross-talk with the dopaminergic pathway, the role that the internalized Na/K-ATPase plays in ouabain- or DLS-induced signal transduction, and the in vivo physiologic importance of this pathway with respect to renal sodium handling.

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