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Effects of cardiac glycosides on sodium pump expression and function in LLC-PK1 and MDCK cells

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Effects of cardiac glycosides on sodium pump expression and function in LLC-PK1 and MDCK cells.

Background. The decreases in proximal tubule sodium reabsorption seen with chronic renal failure and volume expansion have been ascribed to circulating digitalis-like substances (DLS). However, the circulating concentrations of DLS do not acutely inhibit the sodium pump to a degree consistent with the observed changes in proximal tubule sodium reabsorption.

Methods. We examined how cell lines that simulated proximal (LLC-PK1) and distal tubule (MDCK) cells responded to acute (30 min) and long-term (up to 12 hours) Na^+,K^+-ATPase inhibition with DLS.

Results. In LLC-PK1, but not MDCK cells, low concentrations of ouabain decreased ^{86}Rb uptake profoundly in a time and dose dependent manner. In LLC-PK1 cells grown to confluence, transcellular ^{22}Na flux was markedly reduced in concert with the decreases in ^{86}Rb uptake. Similar findings were observed with marinobufagenin (MBG) and deproteinated extract of serum derived from patients with chronic renal failure. However, inhibition of the Na^+,K^+-ATPase with low extracellular potassium concentrations did not produce any of these effects. Western and Northern blots detected no change in alpha1 Na^+,K^+-ATPase protein and message RNA, respectively, in LLC-PK1 cells treated with ouabain for 12 hours. However, the decrease in enzymatic activity of Na^+,K^+-ATPase of these cells was comparable to observed decreases in ^{86}Rb uptake. Differential centrifugation as well as biotinylation experiments demonstrated a shift of the Na^+,K^+-ATPase from the plasmalemma with prolonged ouabain treatment.

Conclusions. The results show that binding of cardiac glycosides by proximal (but not distal) tubular cells results in internalization of Na^+,K^+-ATPase with the net effect to amplify inhibition of the Na^+,K^+-ATPase. As the circulating concentrations of DLS increase with chronic renal failure and volume expansion, we suggest that this phenomenon explains some of the decreased sodium reabsorption by the proximal tubule seen in these conditions.

Key words: proximal tubule sodium reabsorption, Na,K-ATPase, kidney, trafficking, inhibition, digitalis-like substance, chronic renal failure, volume expansion.

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There has been considerable interest in the topic of digitalis-like substances (DLS) and the potential effects on renal sodium handling for some time [1]. A number of laboratories have measured several DLS in the serum and urine of experimental animals and clinical subjects [2–5]. Specifically, substances that are identified on immunoassays such as ouabain and marinobufagenin (MBG) have been measured in the concentration range of several hundred picomolar to several nanomolar [2, 6–9]. Under conditions of volume expansion, increases in the plasma and urine concentrations of ouabain and MBG have been observed along with alterations in renal sodium handling. Members of our group found that in some experimental animals, blockade of DLS with specific antibodies has blunted the alterations in renal sodium handling associated with volume expansion [6]. However, the measured concentrations of MBG and ouabain may not be sufficient to explain all of the observed changes in proximal tubule sodium handling, at least based on Michelis-Menton kinetics.

Somewhat surprisingly, the possibility that binding of DLS to the sodium pump results in its internalization has received relatively little attention. Griffiths and colleagues reported that prolonged exposure to ouabain altered the apparent K_d for ouabain binding in HeLa cells [10]. This provocative study demonstrated that extremely low doses of ouabain over 48 hours caused a marked decrease in the number of unoccupied sites for ouabain binding. Interestingly, differences between the cardiac glycosides studies, in terms of causing this decrease in receptor number, were documented. The authors speculated that trafficking of the sodium pump was likely to be involved in this phenomenon. Regarding renal tubular cells, altered trafficking of the sodium pump related to receptor-mediated endocytosis has received tremendous attention with respect to dopamine in the recent past. A number of investigators have demonstrated that this receptor-mediated endocytosis does occur in renal epithelium, and that it is mediated through

the phosphatidylinositol-3 (PI3) kinase, clathirin-coated pit endocytosis pathway [11–14]. Depending on the cell type or line, protein kinase C (PKC) or protein kinase A (PKA) modulation of this pathway have been demonstrated [14–16].

Based on this background, we speculated that the binding of DLS to the Na^+,K^+-ATPase might induce internalization and/or inactivation of the sodium pump in a time dependent fashion in some renal tubular epithelial cells. In this way, the effects of the relatively low circulating concentrations of the DLS might be amplified. To test this hypothesis, the following studies were performed.

METHODS

Materials

Chemicals of the highest purity available were obtained from Sigma (St. Louis, MO, USA), Gibco BRL Life Technologies (Rockville, MD, USA), and Molecular Probes (Eugene, OR, USA). All radionucleotides (^{32}P-labeled, about 3000 Ci/mmol) and Rubidium were obtained from Dupont NEN Life Science Products (Boston, MA, USA).

Cell culture

The pig renal proximal tubule cell line, LLC-PK1, and canine renal distal tubule cell line, Madin-Darby canine kidney (MDCK), were obtained from the American Tissue Type Culture Collection (ATCC, Manassas, VA, USA), and maintained at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Sigma) with 10% (vol/vol) fetal bovine serum (FBS; Sigma), 2 mmol/L L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL) to subconfluent conditions. Cells were passaged twice a week with 0.05% trypsin and 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA; Sigma). The numbers of cells were quantified by microcytometry, and cell viability was evaluated by Trypan blue exclusion. After 24 to 48 hours, the cells were serum-starved for 12 hours, at which time all experiments were performed.

In select experiments, LLC-PK1 cells were grown to confluence on the 12-mm polycarbonate Transwell culture filter inserts (filter pore size 0.4 μmol/L; Costar Co., Cambridge, MA, USA) and grown for seven days. Medium was replaced daily. These cells were studied for both ^{86}Rb uptake and transcellular ^{22}Na transport as described by Haggerty and colleagues [17]. Briefly, for transcellular ^{22}Na transport, the upper compartment of a filter insert was filled with 700 μL DMEM containing 15 mmol/L 22Na (1 μCi/mL), and the lower compartment was filled with 1 mL of DMEM. Aliquots were removed at intervals as indicated from the lower compartment for scintillation counting. We studied seven-day-old monolayers. Each assay was done with a 15 minute preincubation in the presence and absence of 1 mmol/L ouabain to assess the ouabain-sensitive transport.

Patient serum samples

Serum samples were collected from 12 patients with ESRD treated with hemodialysis for more than one year. The serum samples were pooled and deproteinated using the method of Stokes [5], which we have recently employed [18]. Informed consent was obtained prior to drawing blood samples in accordance with the Medical College of Ohio IRB.

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

For the assay of ^{86}Rb uptake, cells were cultured in 12-well plates to subconfluent condition and treated with different conditions. Monensin (20 μmol/L) was added to the medium prior to the initiation of the ^{86}Rb uptake assay to assure that the maximal capacity of active uptake was measured. ^{86}Rb uptake was initiated by the addition of 1 μCi of ^{86}Rb, and the reaction was stopped after 15 minutes by washing four times with 3 mL of ice-cold 0.1 mol/L $MgCl_2$. Trichloroacetic acid (TCA)-soluble ^{86}Rb was extracted with 10% trichloroacetic acid (TCA) and counted with a β-counter (Beckman LS 6000SC; Beckman, Fullerton, CA, USA). TCA-precipitated cellular protein was dissolved in 0.1 N NaOH with 0.2% sodium dodecyl sulfate (SDS), and protein content was determined by Lowry's assay, using bovine serum albumin as standard. The ^{86}Rb uptake was calibrated with protein content of each treatment [19]. Each assay was done with a 15 minute pre-incubation in the presence and absence of 1 mmol/L ouabain to assess the ouabain-sensitive uptake.

Western blot

After being grown and treated with different conditions, cells were washed with ice-cold phosphate-buffered saline (PBS) and solubilized in ice-cold modified RIPA buffer [10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L NaF, 1 mmol/L Na_3PO_4, 1 mmol/L egtazic acid (EGTA), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 50 mmol/L tetrasodium pyrophosphate, 10 nmol/L okadaic acid, 1% Triton X-100, 0.25% sodium deoxycholate, 10 μg/mL aprotinin, and 10 μg/mL leupeptin] on ice. The cell lysates were cleared by microcentrifugation, and protein concentrations were determined. For Western blot analysis, cell lysates (60 to 80 μg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Optitran membrane (Schleicher & Schuell, Keene, NH, USA). After transfer, the gel was stained with Coomassie Brilliant Blue to verify uniform loading and transfer. Membranes were blocked with 4% milk in TBS-T (Tris-HCl 10 mmol/L, NaCl 150 mmol/L, Tween 20, 0.05%; pH 8.0) for one

hour at room temperature and subsequently probed with the mouse monoclonal antibody recognizing α-1 subunit of Na^+,K^+-ATPase (1:1000, clone α6F; Hybridoma Bank, University of Iowa, Iowa City, IA, USA) overnight at 4°C. The membrane was washed with TBS-T three times and incubated for one hour at room temperature with goat anti-mouse antibody (1:1000) conjugated to horse-radish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Detection was performed using the enhanced chemiluminescence (ECL) super signal kit (Pierce, Rockford, IL, USA). 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 signals [20].

Northern blot

Northern blot was done as described previously. Routinely, about 20 μg of total RNA was subjected to gel electrophoresis, transferred to a Nytran membrane (Schleicher & Schuell), UV-immobilized, and hybridized to ^{32}P-labeled probes. Autoradiograms obtained at −70°C were scanned with a Bio-Rad GS-670 imaging densitometer. Multiple exposures were analyzed to assure that the signals were within the linear range of the film. The relative amount of RNA in each sample was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA to correct for differences in sample loading and transfer [20].

Determination of Na^+,K^+-ATPase enzymatic activity

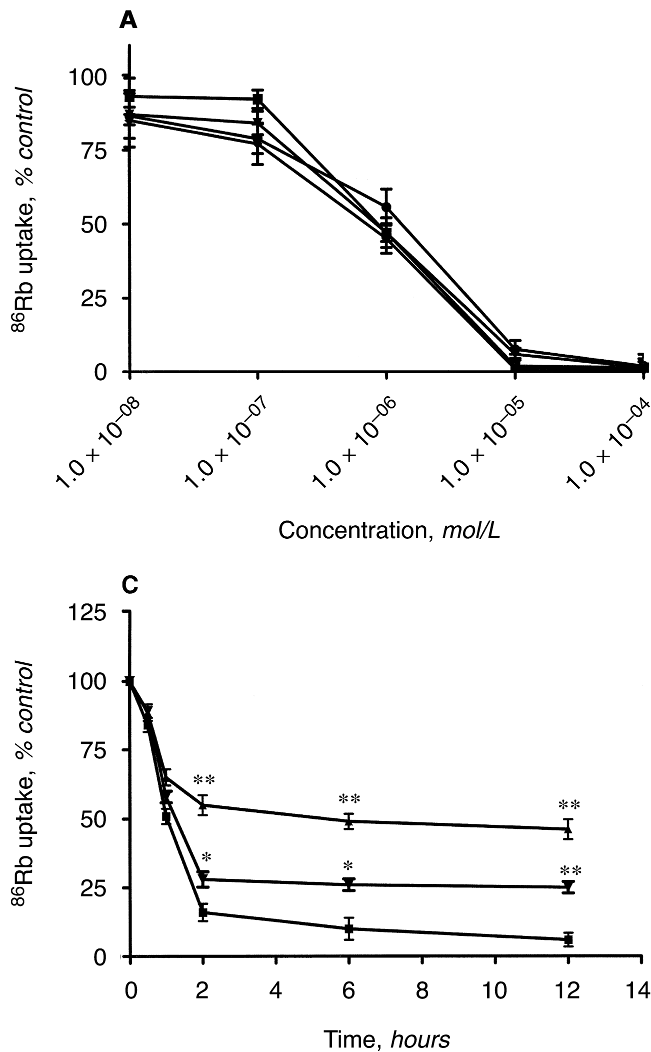
Determination of Na^+,K^+-ATPase enzymatic activity was performed by the previously described method with minor modification. Briefly, cells were collected in ice-cold Skou C (with 1 mmol/L PMSF) solution, ultrasonicated, and centrifuged at 100,000 × *g* for 45 minutes. The pellet containing plasma membrane was re-suspended in Skou C solution and kept in −80°C before use. The samples were treated with alamethicin (1:10, alamethicin: protein) for 30 minutes at room temperature. In studies examining a range of alamethicin concentrations ranging from 1:100 to 1:1, this ratio was found to yield the highest activities on these cells (data not shown). Na^+,K^+-ATPase enzymatic activity assay was performed at 37°C by measuring the initial rate of release of ^{32}P from [γ-^{32}P]ATP (Dupont NEN) in a reaction solution containing 100 mmol/L NaCl, 25 mmol/L KCl, 3 mmol/L $MgCl_2$, 1 mmol/L EGTA, 2 mmol/L adenosine 5′-triphosphate (ATP), 5 mmol/L NaN_3, and 50 mmol/L Tris-HCl (pH 7.4). Each ATPase assay was done with a 15 minute pre-incubation in the presence and absence of 1 mmol/L ouabain to assess the ouabain-sensitive component of the activity [21].

Separation of Na^+,K^+-ATPase from different cellular compartments (subcellular fractionation)

LLC-PK1 cells were collected with hypotonic buffer (10 mmol/L Tris-HCl, pH 7.2, 1 mmol/L PMSF, 10 μg/mL of aprotonin, and 10 μg/mL of leupeptin) on ice and allowed to sit on ice for another 15 minutes. The cell suspension was then passed through a syringe needle (26½ gauge) ten times and then homogenized 15 times with a ball bearing cell homogenizer (H&Y Enterprise, Redwood City, CA, USA). An equal volume of 0.5 mol/L sucrose buffer (in 10 mmol/L Tris-HCl, pH 7.2) was added to make the solution isotonic. The cell suspension was centrifuged at 700 to 800 × *g* at 4°C for ten minutes to get post-nuclear supernatant and nuclear pellet (which may include unbroken cell and cell debris). Post-nuclear supernatant was centrifuged at 17,000 × *g* at 4°C for 15 minutes to get the heavy membrane pellet, and then the supernatant from 17,000 × *g* centrifugation was further centrifuged at 100,000 × *g* at 4°C for 60 minutes to get the light membrane pellet. The nuclear pellet was re-suspended and passed through a 26½-gauge needle several times, and then centrifuged at 430,000 × *g* for two hours to pass through a 2 mol/L sucrose cushion. All pellets were re-suspended in ice-cold PBS, stored at −80°C before use.

Measurement of plasmalemmal Na^+,K^+-ATPase (cell surface biotinylation)

Cell surface biotinylation experiments were performed as described by Caplan and coworkers [22, 23]. Briefly, LLC-PK1 cells were grown in 35 mm petri dishes. After three washes with ice-cold PBS-Ca-Mg (PBS containing 100 μmol/L $CaCl_2$, and 1 mmol/L $MgCl_2$), the surface proteins were biotinylated with EZ-Kink sulfo-NHS-ss-Biotin (1.5 mg/mL in 10 mmol/L triethanolamine pH 9.0, containing 2 mmol/L $CaCl_2$ and 150 mmol/L NaCl) for 25 minutes on ice two times. Non-reacted sulfo-NHS-ss-biotin was washed twice with ice-cold PBS-Ca-Mg-glycine (PBS-Ca-Mg containing 100 mmol/L glycine) and quenched with the same solution for 20 minutes on ice to make sure the entire biotinylation reagent was quenched. After two washes with PBS-Ca-Mg, cells were lysed with 1.0 mL cell lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 7.5) for 60 minutes on ice. The cell lysates were clarified by sedimentation at 14,000 × *g* for 10 minutes at 4°C. Biotinylated proteins were separated with a 50% slurry of ImmunoPure immobilized streptavidin-agarose beads (Pierce) overnight at 4°C, with end-to-end rotation. The beads were washed three times with lysis buffer, two times with high-salt washing buffer (0.1% Triton X-100, 500 mmol/L NaCl, 5.0 mmol/L EDTA, 50 mmol/L Tris-Cl, pH 7.5), and two times with no-salt washing buffer (10 mmol/L Tris-Cl, pH 7.5). Proteins bound to the beads were eluted by incubated at 55°C water bath for 30 minutes in an equal volume of 2× Laemmli sample buffer. Proteins were then applied to SDS-PAGE and Western blot as described above.



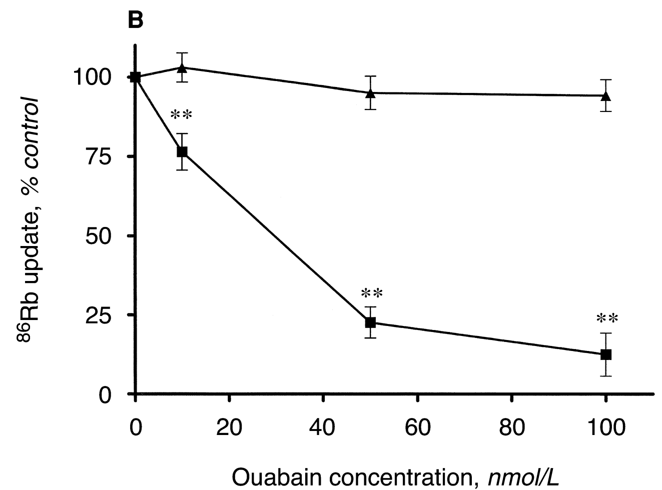


Fig. 1. (*A*) Effect of ouabain and marinobufagenin (MBG) on ^{86}Rb uptake in LLC-PK1 and MDCK cells. Cells were pre-treated with ouabain (O) or MBG (M) for 30 minutes, and assayed for ^{86}Rb uptake as described in the **Methods** section. Data are shown as % control value and are presented as the mean ± SEM of 3 determinations. Symbols are: (●) O-LLC-PK1; (▼) M-LLC-PK1; (■) O-MDCK; (◆) M-MDCK. (*B*) Effect of 12 hours of incubation of ouabain on ^{86}Rb uptake in LLC-PK1 (■) and MDCK (▲) cells. Cells were treated with different concentrations of ouabain as indicated for 12 hours, then washed once with the medium and assayed for ouabain-sensitive ^{86}Rb uptake. Data are shown as % control value and presented as mean ± SEM of 3 determinations. $*P < 0.05$, $**P < 0.01$ vs. MDCK. (*C*) Time course showing the effects of ouabain (■; 100 nmol/L), MBG (▲; 100 nmol/L) and digoxin (▼; 100 nmol/L) on ^{86}Rb uptake in LLC-PK1 cells. Data are shown as % control values and presented as mean ± SEM of 4 determinations at each time point. $*P < 0.05$, $**P < 0.01$ vs. ouabain.

Measurement of ^{3}H labeled ouabain binding

The ouabain-binding assay was performed as previously described with minor modification [20, 24]. After treatments, LLC-PK1 cells, grown in 12-well plates (Corning-Costar, Cambridge, MA, USA) in triplicate, were washed once with DMEM, then DMEM containing 100 nmol/L [^{3}H]ouabain (2.3 μCi/mL, for total binding) or containing 100 nmol/L [^{3}H]ouabain and 100 μmol/L cold ouabain (for nonspecific binding) was added and incubated at 37°C for 30 minutes. The reaction was stopped by adding ice-cold 100 mmol/L $MgCl_2$, and washed three times with ice-cold 100 mmol/L $MgCl_2$. Cells were lysed in 0.2% SDS containing 0.1 N NaOH at room temperature for 45 minutes with gently shaking. Cell lysate was counted with a β-counter (Beckman LS 6000SC; Beckman Instruments, Fullerton, CA, USA). The count was calibrated with protein concentration. The specific ouabain binding was calculated as the difference between the counts of the total and the nonspecific binding.

Statistical analysis

Data were compared using the unpaired Student *t* test employing Bonferroni's correction for multiple comparisons [25]. Statistical analysis was performed using SIGMA-STAT™ Software (SPSS, Inc. Chicago, IL, USA).

RESULTS

Acute effects of cardiac glycosides on ^{86}Rb uptake in LLCPK1 and MDCK cells

The effects of ouabain and MBG on ^{86}Rb uptake in the MDCK and LLC-PK1 cell lines are shown in Figure 1A. The concentration that inhibited enzymatic activity by 50% under these conditions (IC_{50}) for both ouabain and MBG was quite similar in the LLC-PK1 and MDCK cell lines.

Effects of prolonged incubations of cardiac glycosides on ^{86}Rb uptake in LLCPK1 and MDCK cells

Prolonged incubation with ouabain leads to marked decreases in ^{86}Rb uptake in LLC-PK1 but not MDCK

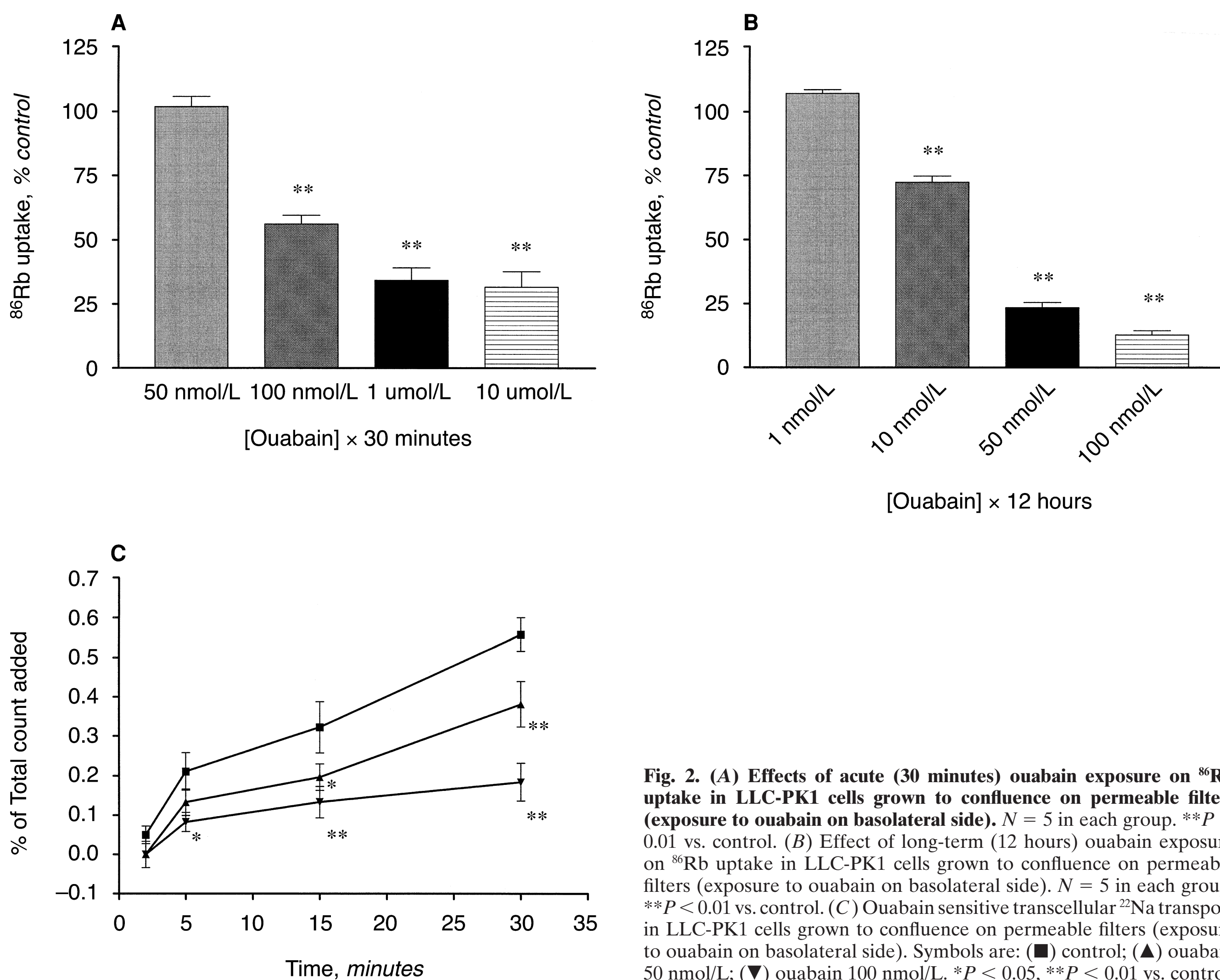


Fig. 2. (*A*) Effects of acute (30 minutes) ouabain exposure on ^{86}Rb uptake in LLC-PK1 cells grown to confluence on permeable filters (exposure to ouabain on basolateral side). $N = 5$ in each group. $^{**}P < 0.01$ vs. control. (*B*) Effect of long-term (12 hours) ouabain exposure on ^{86}Rb uptake in LLC-PK1 cells grown to confluence on permeable filters (exposure to ouabain on basolateral side). $N = 5$ in each group. $^{**}P < 0.01$ vs. control. (*C*) Ouabain sensitive transcellular ^{22}Na transport in LLC-PK1 cells grown to confluence on permeable filters (exposure to ouabain on basolateral side). Symbols are: (■) control; (▲) ouabain 50 nmol/L; (▼) ouabain 100 nmol/L. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

Table 1. Effects of 12 hours of ouabain (50 nmol/L) on Na,K-ATPase mRNA, protein content (alpha1 subunit) and enzymatic activity (plasma membrane) in LLC-PK1 cells

Measurement (*N*)	Control	Ouabain
MRNA *arbitrary units* ($N = 3$)	100 ± 2.2	96.8 ± 2.9
Protein *arbitrary units* ($N = 4$)	100 ± 4.3	94.3 ± 4.6
Enzymatic activity of purified pig kidney enzyme *µmol/h/mg protein* ($N = 4$)	510.2 ± 4.7	448.6 ± 5.8[a]
Enzymatic activity of plasma membrane *µmol/h/mg protein* ($N = 7$)	3.59 ± 0.42	0.90 ± 0.22[a]

Data expressed as mean ± SEM.
[a] $P < 0.01$ vs. Control

cells. After 12 hours of incubation, sub-nanomolar doses of ouabain cause significant decreases in ^{86}Rb uptake of LLC-PK1 cells (Fig. 1B). Despite having similar IC_{50} values in LLC-PK1 cells, ouabain is more effective at causing decreases in ^{86}Rb uptake than MBG and digoxin (Fig. 1C). Interestingly, in LLC-PK1 cells, reductions in extracellular potassium concentration that cause acute decreases in ^{86}Rb uptake in a dose-dependent fashion do not cause any long-term changes in ^{86}Rb uptake (data not shown).

Effects of prolonged incubations of LLCPK1 cells grown to confluence with ouabain on ^{86}Rb uptake and transcellular ^{22}Na transport

LLC-PK1 cells were grown to confluence on polycarbonate membranes and exposed to different doses of ouabain for 30 minutes (Fig. 2A) or 12 hours (Fig. 2B) on the basolateral aspect. Exposure of the LLC-PK1 cells to 1 mmol/L ouabain (30 min) on the apical side did not significantly affect ^{86}Rb uptake (data not shown). This confirmed that the LLC-PK1 cells been polarized with the Na^+,K^+-ATPase largely on the basolateral aspect of the cells when grown in this manner. Moreover, they had a similar IC_{50} for acute exposure to ouabain,

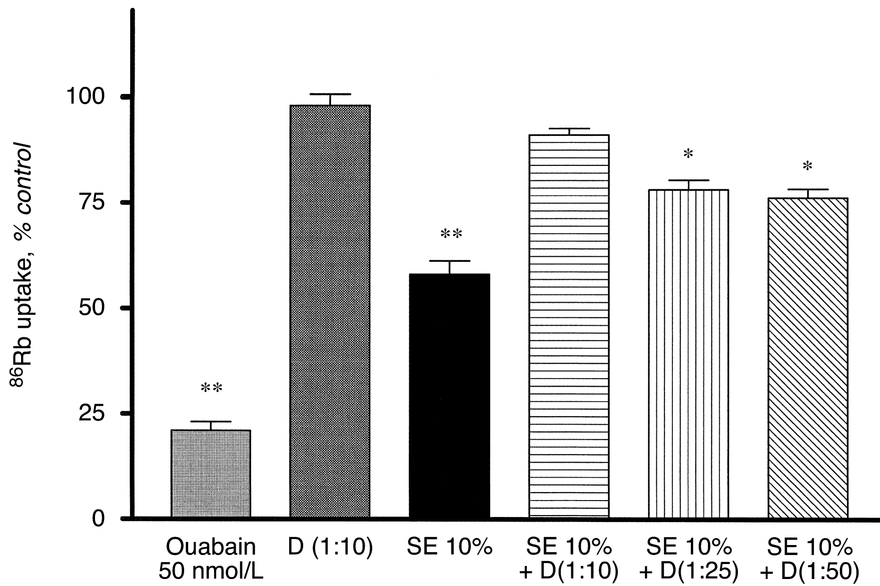


Fig. 3. Digibind (D) blocks the effects of 12 hours of incubation with uremic serum extract (SE) on ^{86}Rb uptake. Data are shown as % control values and presented as mean ± SEM of 3 determinations. Concentration of D before dilution is 1.3 mg/mL. *$P < 0.05$, **$P < 0.01$ vs. Control.

A
Relative density values, %
125
100
75
50
25
0
Ouabain 4 hours
Ouabain 6 hours
Ouabain 12 hours

B
Ouabain binding capacity *arbitrary unit*
40
30
20
10
0
0 1 2 3 4 5 6 7.5 10.0 12.5
Time, *hours*

C
CPM/mg protein
2500
2000
1500
1000
500
0
Control
[^{3}H]oua 100 nmol/L 15 minutes
[^{3}H]oua 100 nmol/L 12 hours

Fig. 4. (*A*) Effects of exposure to ouabain for different time points on plasma membrane Na^+,K^+-ATPase (α1 subunit) density in LLC-PK1 cells. Data are shown as a fraction of control. Each data point represents the mean ± SEM of 5 determinations. **$P < 0.01$ vs. Control. (*B*) Effects of control (■) and ouabain exposure (●; 50 nmol/L for 12 h) on [^{3}H] ouabain binding. Data are shown as mean ± SEM of 5 determinations. **$P < 0.01$ vs. Control. (*C*) Effects of ouabain exposure (50 nmol/L) on [^{3}H] ouabain binding in the nuclear fraction. Data are shown as the mean ± SEM of 3 determinations.

so lower doses of ouabain induced a time-dependent decrease in ^{86}Rb uptake, as also observed in the cells studied under subconfluent conditions. As well, ouabain-sensitive transcellular ^{22}Na transport was markedly decreased with 12 hours of exposure to 50 or 100 nmol/L ouabain on the basolateral aspect (Fig. 2C).

Effects of prolonged incubations of LLCPK1 cells with ouabain on Na^+,K^+-ATPase mRNA, protein expression and enzymatic activity

Exposure of LLC-PK1 cells to 50 nmol/L ouabain did not have significant effects on Na^+,K^+-ATPase mRNA (Northern blot) or protein content (Western blot); however, enzymatic activity of the Na^+,K^+-ATPase was decreased to a comparable degree as the decrease in ^{86}Rb uptake (Table 1).

Effects of human uremic serum extract on ^{86}Rb uptake, Na^+,K^+-ATPase mRNA, protein expression and enzymatic activity in LLC-PK1 cells

Uremic serum extract (SE, 10%) had similar effects on ^{86}Rb uptake as ouabain (100 nmol/L) concentration. However, whereas the SE inhibited the pump acutely by about 25%, the 12 hour incubation resulted in a 45% inhibition in the LLC-PK1 cells (Fig. 3). Anti-digitalis antibody (Digibind) was effective at preventing the inhibition of ^{86}Rb uptake, although very high amounts of the antibody was necessary to block this effect of the SE.

Effect of ouabain on Na^+,K^+-ATPase trafficking in LLC-PK1 cells

Ouabain exposure (50 nmol/L) caused marked reductions in biotinylation of the sodium pump in LLC-PK1 cells in a time-dependent manner (Fig. 4A). In contrast, 12 hours of exposure to 50 nmol/L ouabain did not significantly affect the biotinylation of the sodium pump in MDCK cells (data not shown). [^{3}H]Ouabain binding also was reduced markedly in the LLC-PK1 cells after 12 hours of exposure (Fig. 4B), but binding of [^{3}H]ouabain to the nucleus increased dramatically over time (Fig. 4C). When Western blots of whole cells were examined, heavy membrane and nuclear fractions, the ouabain exposure did not significantly alter whole cell Na^+,K^+-ATPase α-1 subunit density, whereas significant reductions in heavy membrane fraction (containing plasmalemma) and significant increases in nuclear fraction were observed (Table 2). Interestingly, the enzymatic activity of the pump studied in these fractions was decreased substantially by 12 hours of ouabain treatment (Table 2).

Table 2. Effects of 12 hours of ouabain (50 nmol/L) on Na,K-ATPase protein content (alpha1 subunit) and enzymatic activity in different subcellular fractions of LLC-PK1 cells

Compartment	Protein density	Enzymatic activity
Heavy membrane	71.2 ± 4.8^b	32.3 ± 3.9^b
Light membrane	119 ± 3.6^a	Not determined
Nuclear fraction	168 ± 5.2^b	29.6 ± 4.1^b

Results are expressed as percent of control. $N = 5$ measurements in the ouabain treated and control treated cells for each determination. Data are expressed as mean ± SEM. Enzymatic activity was not determined in the light membrane compartment.
$^a P < 0.05$, $^b P < 0.01$

DISCUSSION

The fascination with DLS has continued since the mid 1960s when they were first postulated to play a role in altered sodium handling by Bricker and colleagues [26–28]. However, those DLS that have been best characterized, including ouabain and marinobufagenin, circulate in concentrations that may not acutely inhibit the sodium pump to the degree necessary to produce the physiological effects attributed to them [8, 29]. The interested reader is referred to several recent symposia and texts on this topic [30–33].

Our studies demonstrate that a proximal tubule cell line, the LLC-PK1 cell have exquisite sensitivity to low doses of ouabain and other DLS because of the translocation/inactivation of the sodium pump in this setting. We also observed that this occurred when the cells were grown to confluence and became polarized, and that this down-regulation of the sodium pump had dramatic effects on transcellular sodium transport. In contrast, MDCK cells, which are derived from more distal tubular cells, demonstrated relatively little translocation/inactivation related to long-term (hours) exposure to ouabain. We suggest that this discrepancy between the proximal and distal cell lines is quite consistent with the alterations in renal sodium handling that are known to accompany chronic reductions in renal mass. In this setting, distal mechanisms for sodium handling are largely intact and sensitive to the renin-angiotensin system and atrial natriuretic peptide, whereas the proximal tubule handling of sodium is substantially altered. Other authors have speculated that this allows the "fine tuning" of sodium balance, mediated largely by the distal nephron, to proceed under this clinical condition [34, 35].

We are still unclear as to which mechanisms are involved in the inactivation and internalization of the sodium pump in the LLC-PK1 cells. Our data demonstrate some discordance between the internalization of the pump (as assessed by the biotinylation studies and cell compartmentalization studies) and the enzymatic activity of the pump. Specifically, the decreases in ^{86}Rb uptake appear to be greater than the decreases in plasmalemmal pump density. Moreover, the actual enzymatic activity of the pump is decreased even in the plasmalemmal fraction of the cell. These results suggest that a two-step process may be involved; the pump may be inactivated

prior to translocating from the surface of the cell. Clearly, further studies are necessary to elucidate this important issue. Along these lines, it is clear from our data that some of the sodium pump localizes to the nucleus, and that this nuclear fraction increases following 12 hours of exposure to DLS. Although the movement of the sodium pump to the nucleus immediately suggests the model of a steroid hormone–receptor complex, the role of the sodium pump in the nucleus as well as the mechanisms by which it gains access to this compartment are still undefined [36]. Again, further studies addressing this important topic are certainly indicated.

In summary, we observed that small amounts of cardiac glycosides caused marked decreases in sodium pump activity in a time-dependent fashion in LLC-PK1 but not MDCK cells. We suggest that this effect of cardiac glycosides may be relevant to their effects on renal sodium handling in the intact organism.

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