

# Ouabain-induced endocytosis of the plasmalemmal Na/K-ATPase in LLC-PK1 cells requires caveolin-1

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## Ouabain-induced endocytosis of the plasmalemmal Na/K-ATPase in LLC-PK1 cells requires caveolin-1.

**Background.** We have demonstrated that ouabain causes dose- and time-dependent decreases in  $^{86}\text{Rb}$  uptake in pig renal proximal tubule cell line (LLC-PK1) cells; and ouabain induces endocytosis of plasmalemmal Na/K-ATPase in LLC-PK1 cells in a clathrin-dependent pathway. Our data also suggest a role of endocytosis in both ouabain-induced signal transduction and proximal tubule sodium handling. The present study addresses the molecular mechanisms involved in this process.

**Methods.** Studies were performed with cultured LLC-PK1 and a stable-expressed caveolin-1 knockdown LLC-PK1 cell line by SiRNA method.

**Results.** In wild-type LLC-PK1 cells, depletion of cholesterol by methyl  $\beta$ -cyclodextrin reduced ouabain-induced accumulation of Na/K-ATPase  $\alpha$ -1 subunit, EGFR, Src, and MAPKs in clathrin-coated vesicles, as well as in endosomes. Depletion of cholesterol also significantly reduced the protein-protein interaction among  $\alpha$ -1 subunit, AP2, PI-3K, and clathrin heavy chain. In LLC-PK1 cells expressing mock-vehicle and caveolin-1 siRNA, depletion of caveolin-1 abolished ouabain-induced decrease in Rb uptake and decrease in the plasmalemmal Na/K-ATPase content. Depletion of caveolin-1 also significantly reduced the ouabain-induced accumulation of Na/K-ATPase  $\alpha$ -1 subunit, EGFR, Src, and MAPKs in clathrin-coat vesicles, as well as early and late endosomes. In addition, depletion of caveolin-1 also significantly reduced the protein-protein interaction among  $\alpha$ -1 subunit, AP2, PI-3K, and clathrin heavy chain. These data suggest that caveolae are involved in ouabain-induced endocytosis and signal transduction by initiating assembly of signaling cascades through the caveolar Na/K-ATPase and/or the interaction with clathrin-mediated endocytosis of the Na/K-ATPase.

In the proximal tubule cell, the Na/K-ATPase resides at the basolateral surface and provides the driving force for the transport of sodium from the tubular lumen into the extracellular space [1]. The cellular distribution of the Na/K-ATPase is thought to be crucial for this function.

**Key words:** sodium, potassium, ATPase, endocytosis, caveolin, clathrin.

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Endogenous cardiac steroids (also referred to as endogenous digitalis like substances, or DLS), including ouabain, are now accepted as a class of hormones involved in blood pressure regulation and renal sodium handling [2]. Endogenous DLS are also found in hypothalamus and the adrenal glands of animals [2, 3]. These DLS are known to inhibit the enzymatic activity of the Na/K-ATPase activity by binding to an extracellular portion of the Na/K-ATPase  $\alpha$ -subunit [4].

Our group has previously reported that in pig renal proximal tubule cell line (LLC-PK1) cells but not MDCK cells, low concentrations of ouabain induced significantly depletion of the basolateral Na/K-ATPase through a clathrin-dependent endocytic pathway [5, 6]. A body of work from our laboratory indicates that the Na/K-ATPase can function as a signal transducer, leading to the activation of a signal transduction cascade involving c-Src and EGFR [7–9]. It has been proposed that the ouabain-bound (activated) Na/K-ATPase is capable of recruiting and activating protein tyrosine kinases through specific protein-protein interactions [10]. Our data also suggest a strong link between ouabain-induced signal transduction and endocytosis. We observed that ouabain-induced activation of c-Src was necessary in order to induce endocytosis [6]. In a separate paper, workers from our laboratory reported that signal transduction through the Na/K-ATPase could be localized to caveolar structures, and that depletion of these caveolae prevented ouabain-induced signaling through the Na/K-ATPase [11, 12].

Caveolae and lipid rafts are now believed to play important roles in endocytosis [13–17]. Caveolae were first identified as flask-shaped, noncoated membrane vesicular invagination, and are enriched in cholesterol, glycosphingolipids, and sphingomyelin [14, 17–19]. Caveolins are 21 to 24 kD membrane-associated scaffold proteins (a substrate of v-Src [17]) and the major structural components of caveolae [13, 14, 17]. Many signaling molecules and membrane receptors are dynamically associated with caveolae mainly through their interactions with caveolins [16, 20, 21]. Caveolins stabilized caveolae and modulated signal transduction by attracting signaling molecules to caveolae and regulating their activity [21].

There is now strong evidence that caveolins may modulate endocytosis through their interactions with clathrin [22–25].

We propose that there is a crosstalk between ouabain-induced endocytosis and signaling transduction of the Na/K-ATPase. 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 ( $^{86}\text{Rb}^+$ ) 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). Polyvinylidene (PVDF) membranes (Hybound-P) were obtained from Amersham Biosciences (Piscataway, NJ, USA).

Polyclonal and monoclonal antibodies against Na/K-ATPase  $\alpha$ -1 subunit (clone C464.6), EGFR, EEA-1, anti PI-3K p85 $\alpha$  antibody coupled to protein A-agarose, and AP-2  $\alpha$  subunit (clone 8G8/5) were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Antibody against caveolin-1 (clone C060) was obtained from BD Transduction Laboratories (Lexington, KY, USA). Monoclonal antibodies against clathrin heavy chain (CHC, clone  $\times$ 22) were obtained from Affinity BioReagents (Golden, CO, USA). Polyclonal antibodies against caveolin-1, c-Src, CHC, Rab5, Rab7, total ERK, as well as horseradish peroxidase-conjugated goat antimouse and goat antirabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and were used for Western blots. Monoclonal antibody against Na/K-ATPase  $\alpha$ -1 subunit (clone  $\alpha$ 6F) was obtained from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA). Alexa Fluor<sup>®</sup> 488- and Alexa Fluor<sup>®</sup> 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 described before [5]. Cell viability was evaluated by Trypan blue exclusion. In case of experiments of immunostaining and cell surface protein biotinylation, LLC-PK1 cells were grown to confluence (6–7 days) on the 12- or 24-mm polycarbonate Transwell culture filter inserts (filter pore size 0.4  $\mu\text{m}$ , Costar Co.; Cambridge, MA, USA). Medium was replaced daily until 12 hours

before experiments, at which time the cells were serum starved as reported previously [5]. LLC-PK1 cells expressing mock-vehicle (P-11, as control) and caveolin-1 siRNA (C2-9, as caveolin-1 depleted cell) were cultured in the same manner as the parent LLC-PK1 cells.

### Generation of caveolin-1 knockdown cells

Caveolin-1-specific siRNA was constructed using the GeneSuppressor Construction Kit (BioCarta, San Diego, CA, USA) following the manufacturer's protocol, as described before [11]. For the plasmid construct of Cav-1 siRNAs, the insert was prepared by annealing two oligonucleotides: sense TCG AGC CAG AAG GGA CAC ACA GTT TTC AAG AGA AAC TGT GTG TCC CTT CTG GTT TTT; antisense CTA GAA AAA CCA GAA GGG ACA CAC AGT TTC TCT TGA AAA CTG TGT GTC CCT TCT GGC. The annealed insert was cloned into pSuppressor<sup>TM</sup>-U6 vector (BioCarta) digested with Sal I and Xba I. The structure of the positive clone was confirmed by nucleotide sequencing.

Transfection and selection of stable cell lines were performed as previously described [11]. Briefly,  $1 \times 10^6$  LLC-PK1 cells grown on 60-mm Petri dishes were cotransfected with either 7  $\mu\text{g}$  pSuppressor-Cav-1 or pSuppressor (empty vector), together with 1  $\mu\text{g}$  pBabe-Puro (provided by Dr. Hanfei Ding, Medical College of Ohio, Toledo, OH, USA) using 20  $\mu\text{L}$  Lipofectamine<sup>TM</sup> 2000. pBabe-Puro allows for the selection of transfected cells by puromycin resistance (1  $\mu\text{g}/\text{mL}$ ). Expression of caveolin-1 was detected by Western blot using anti-Cav-1 rabbit polyclonal antibody (Santa Cruz).

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

Ouabain-sensitive uptake  $^{86}\text{Rb}^+$  uptake was performed as previously described [5]. The  $^{86}\text{Rb}^+$  uptake was calibrated with protein content. Data were expressed as the percentage of ouabain-sensitive  $^{86}\text{Rb}^+$  uptake in control cells.

### Western blot

Immunoblotting was performed as described previously [6]. Detection was performed using the enhanced chemiluminescence (ECL) super signal kit (Pierce).

### Subcellular fractionation

The isolation of the nuclear fraction and preparation of clathrin-coated vesicles, early and late endosomes was performed as described previously [5, 6].

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

Cell surface protein biotinylation was performed as described before [5, 6, 26, 27]. Proteins bound to the

ImmunoPure™ immobilized streptavidin-agarose beads were eluted and then resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting.

### Coimmunoprecipitation

Immunoprecipitation of  $\alpha$ 1-subunit, AP-2  $\alpha$ -subunit, and clathrin heavy chain (CHC) proceeded as we have previously described [6, 8, 25, 28]. Proteins were resolved on SDS-PAGE followed by immunoblotting.

### Confocal microscopy

Cell growth, immunostaining, and confocal microscopy were performed as previously described [6].

### Cholesterol depletion and repletion

Cholesterol depletion and repletion was performed as described previously using methyl-beta-cyclodextrin (M $\beta$ -CD) [11, 29, 30]. Cholesterol depletion was carried out by incubating the cells in the presence of M $\beta$ -CD [5%, w/v, in Dulbecco's modified Eagle's medium (DMEM)] for 30 minutes at 37°C. The cells were washed twice with serum-free medium before the experiments. Cholesterol repletion was done as previously reported [29, 30]. Briefly, 400  $\mu$ L of a cholesterol/M $\beta$ -CD stock solution was added to 10 mL of DMEM, and cholesterol-depleted cells were incubated in this medium for 1 hour at 37°C. A stock solution of cholesterol/M $\beta$ -CD mixture was prepared by adding 100  $\mu$ L of cholesterol (20 mg/mL in ethanol) to 10 mL of 5% M $\beta$ -CD solution and mixing at 40°C.

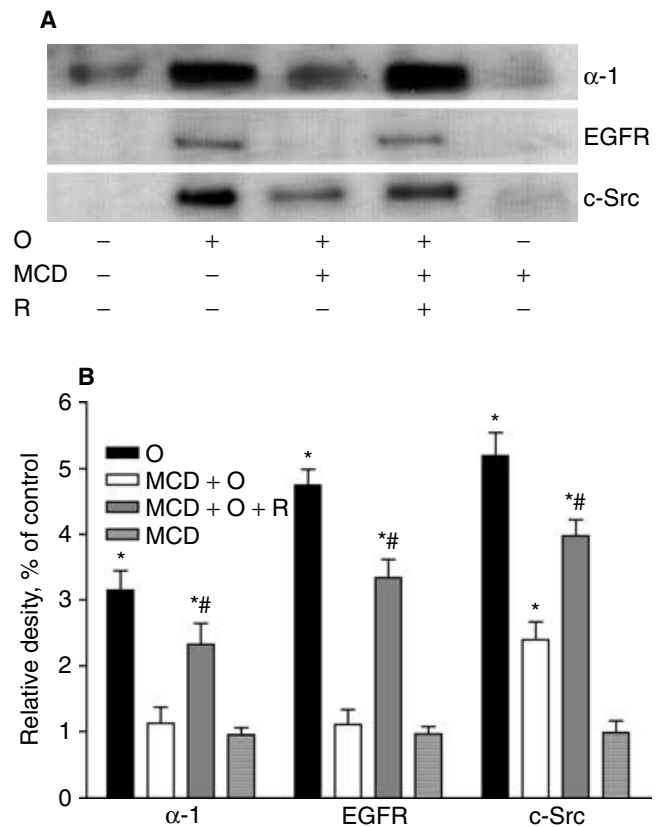
### Statistical analysis

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 prior to 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 [31]. SPSS software (Chicago, IL, USA) was used for all analysis.

## RESULTS

### Depletion of cholesterol inhibits ouabain-induced endocytosis of the Na/K-ATPase $\alpha$ -1 subunit in LLC-PK1 cells

To test whether ouabain-activated signals originate from caveolae and lead to the endocytosis of the Na/K-ATPase, we first disrupted the caveolar structure by acute cholesterol depletion by preincubating LLC-PK1 cells with M $\beta$ -CD for 30 minutes. Following this cholesterol depletion, the cells were washed twice with serum-free

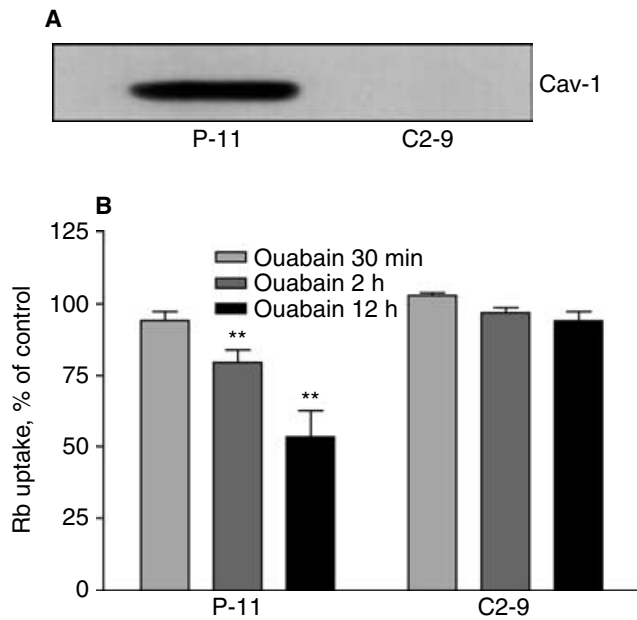


**Fig. 1. Depletion of cholesterol prevents ouabain-induced early endosomal accumulation of the Na/K-ATPase, EGFR, and c-Src in response to ouabain, and cholesterol repletion restores the control pattern.** (A) Shown are representative Western blots. Ten  $\mu$ g of total protein was applied to each lane. (B) Shown is quantification of *N* = 5 ouabain treated early endosome fractions compared with *N* = 5 control early endosome fractions (values averaged and expressed as fraction of control). O, ouabain; MCD, methyl  $\beta$ -cyclodextrin; R, cholesterol repletion. Data shown as mean  $\pm$  SEM. \**P* < 0.01 vs. control, #*P* < 0.01 vs. MCD + O.

medium and then treated with ouabain. We then isolated early endosomes from control and ouabain-treated (50 nmol/L, 2 hours) LLC-PK1 cells, and immunoblotted for  $\alpha$ 1 subunit, EGFR, and c-Src. M $\beta$ -CD treatments clearly blocked ouabain-induced accumulation of  $\alpha$ 1 subunit, EGFR, and c-Src in the early endosomes. Moreover, cholesterol repletion restored the endosomal accumulation of these proteins following ouabain treatment (Fig. 1).

### Ouabain reduces the activity of Na/K-ATPase in LLC-PK1 cells expressing mock vector (P-11 cells), but not in LLC-PK1 cells expressing caveolin-1 siRNA (C2-9 cells)

To further explore the role of caveolae in ouabain-induced endocytosis, we used the P-11 and C2-9 cells and examined the effects of ouabain on the expression of plasmalemmal Na/K-ATPase. First, we measured the



**Fig. 2. Caveolin-1 expression in P-11 and C2-9 cells (A) and ouabain-sensitive  $^{86}\text{Rb}^+$  uptake assay in P-11 and C2-9 cells (B, expressed as % control).** In (A), 20  $\mu\text{g}$  of whole cell lysate was applied to each lane and immunoblotted for caveolin-1. In (B), both cells were treated with or without (as control) ouabain (50 nmol/L) for different amounts of time.  $N = 4$  experiments are represented at each data point in each panel. \*\* $P < 0.01$  vs. control. (C) Confocal immunofluorescence images of control (upper panel) and ouabain (50 nmol/L for 12 hours on basolateral aspect, lower panel) treated P-11 cells grown to monolayer. In these images,  $\alpha 1$  subunit of Na/K-ATPase is labeled with Alexa Fluor<sup>®</sup> 488, and caveolin-1 is labeled with Alexa Fluor<sup>®</sup> 546-conjugated secondary antibody. (D) Same conditions as (C), but in C2-9 cells. Size bar = 10  $\mu\text{m}$ .

caveolin-1 expression levels in the P-11 cells and C2-9 cells. C2-9 cells expressed only a small fraction of the caveolin-1 seen in the P-11 cells (expressing mock vector) (Fig. 2A). P-11 cells expressed the same amount of cav-1 as LLC-PK1 cells. Next, we determined if depletion of caveolin-1 affected the Na/K-ATPase enzymatic activity following different durations of exposure to 50 nmol/L ouabain. Ouabain at 50 nmol/L represents approximately 1/20th of the IC<sub>50</sub> for acute (30 minutes) inhibition of ouabain-sensitive  $^{86}\text{Rb}^+$  uptake in LLC-PK1 cells [5] and, as expected, does not cause any major changes in ouabain-sensitive  $^{86}\text{Rb}^+$  uptake in either the C-11 or P2-9 cells at 30 minutes. Moreover, as we previously observed in wild-type LLC-PK1 cells [5], addition of 50 nmol/L ouabain causes a time-dependent decrease in ouabain-sensitive  $^{86}\text{Rb}^+$  uptake in P-11 cells. However, no time-dependent enhancement of ouabain-induced inhibition of  $^{86}\text{Rb}^+$  uptake was observed in C2-9 cells (Fig. 2B). The ouabain-sensitive  $^{86}\text{Rb}^+$  uptake assay also showed that ouabain-induced inhibition of the Na/K-ATPase enzymatic activity in P-11 cells was also found to be reversible, as we have previously demonstrated in wild-type LLC-PK1 cells [6] (data not shown).

Confocal immunofluorescence microscopy also showed that, in P-11 cells, caveolin-1 was expressed on the cell surface and colocalized with Na/K-ATPase  $\alpha-1$  subunit, but in C2-9 cells, only weak signal of caveolin-1 was observed and colocalized with  $\alpha-1$  subunit (Fig. 2C and D). In P-11 cells, both  $\alpha-1$  subunit and caveolin-1 diffused into cytosolic part in response to ouabain treatment, but not in C2-9 cells.

We next determined the level of the surface  $\alpha 1$  subunit of Na/K-ATPase by measuring biotinylated protein densities. In response to 50 nmol/L ouabain (12 hours), biotinylated protein content of the Na/K-ATPase  $\alpha-1$  and  $\beta-1$  subunits of P-11 cells decreased by about 64% and 70%, respectively. However, when ouabain was applied to the C2-9 cells at the same concentration, it did not alter the content of the biotinylated  $\alpha 1$  subunit (Fig. 3).

Confocal immunofluorescence microscopy also demonstrated that ouabain induced internalization of the plasmalemmal Na/K-ATPase  $\alpha-1$  subunit in P-11 but not in C2-9 cells (data not shown).

#### Ouabain-induced endocytosis of the Na/K-ATPase $\alpha-1$ subunit is caveolin-1 dependent

We next examined whether ouabain-stimulated translocation of Na/K-ATPase to the nucleus [6] was blunted by depletion of caveolin-1. To do this, we first biotinylated the cell surface proteins, quenched the non-reacted biotin reagent, and then chased the biotinylated proteins for 12 hours with restoration of normal medium, with or without ouabain (as previously described [6]). 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 in P-11 cells, but totally abolished in C2-9 cells (Fig. 4).

To further define the role of caveolin-1 in the Na/K-ATPase translocation, P-11 and C2-9 cells were treated with or without (as control) 50 nmol/L ouabain for 1 hour, and clathrin-coated vesicles (CCVs) 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. 5A and B). At this point, ouabain treatment induced a  $317 \pm 43\%$  increase Na/K-ATPase  $\alpha 1$  subunit content in CCVs ( $N = 4$ ,  $P < 0.01$ ). To further examine these mechanisms, early endosomes were also isolated. As shown in Figure 6C, ouabain (50 nmol/L  $\times$  2 hours) induced significant increases in early endosomal Na/K-ATPase  $\alpha 1$ -subunit protein content. As expected, depletion of caveolin-1 by siRNA also totally abolished the ouabain-induced accumulation of Na/K-ATPase  $\alpha 1$ -subunit in early endosomes, like we observed in CCVs.

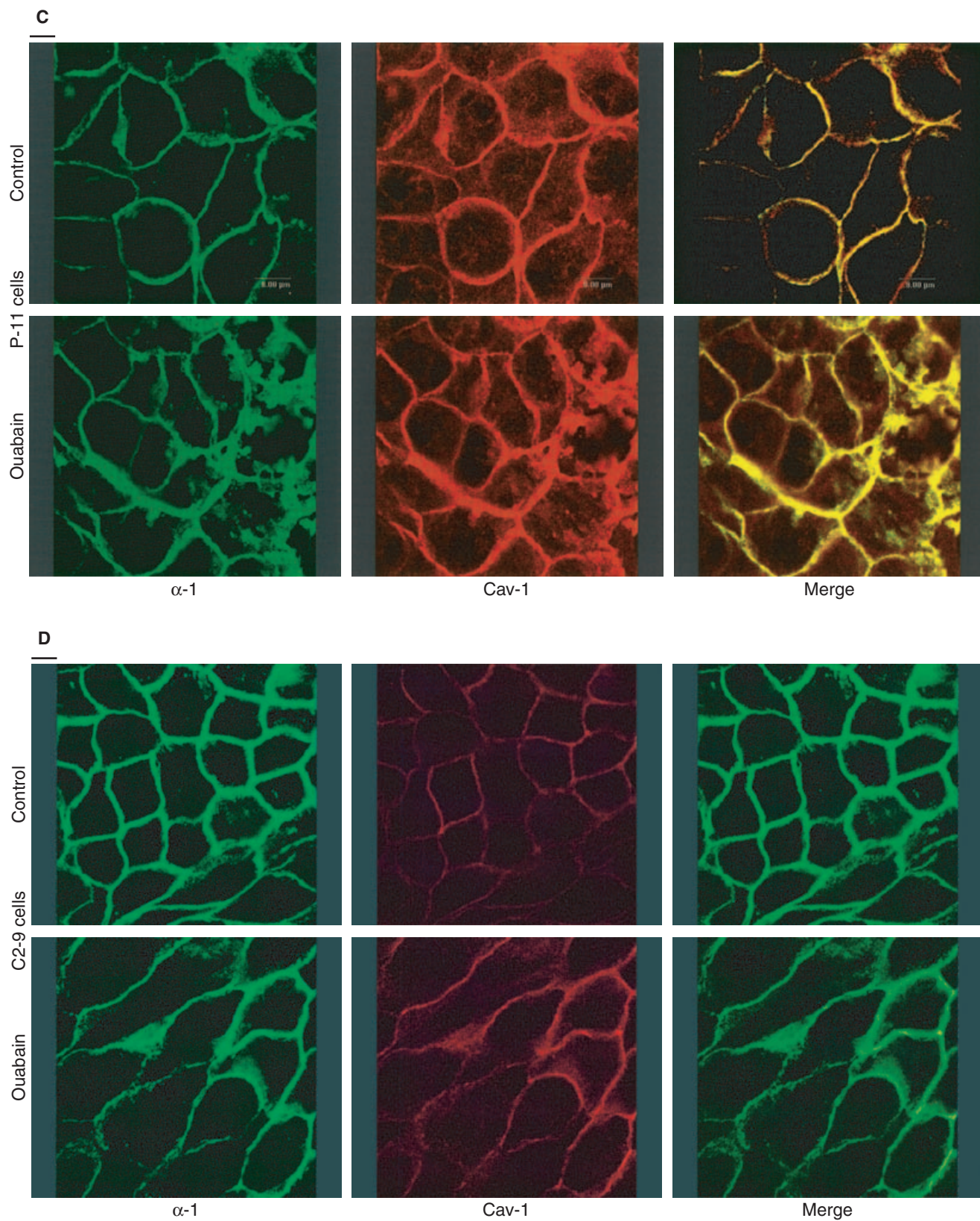
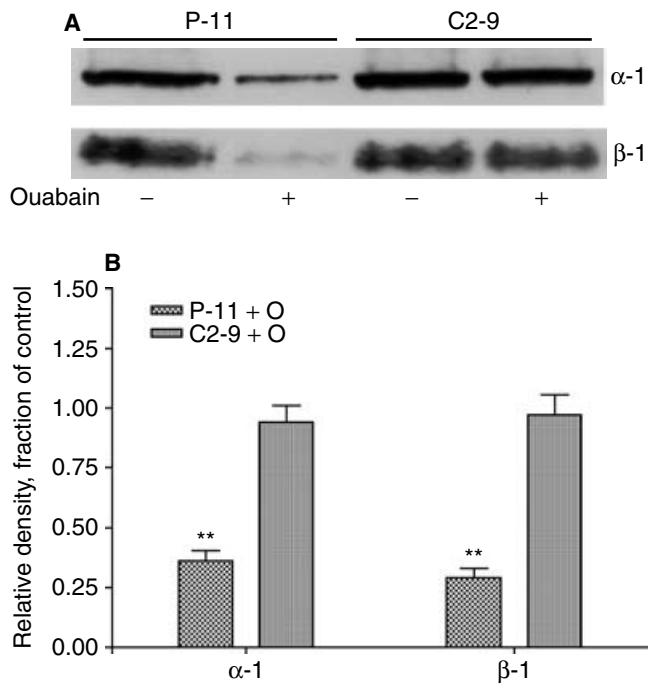


Fig. 2. (Continued)

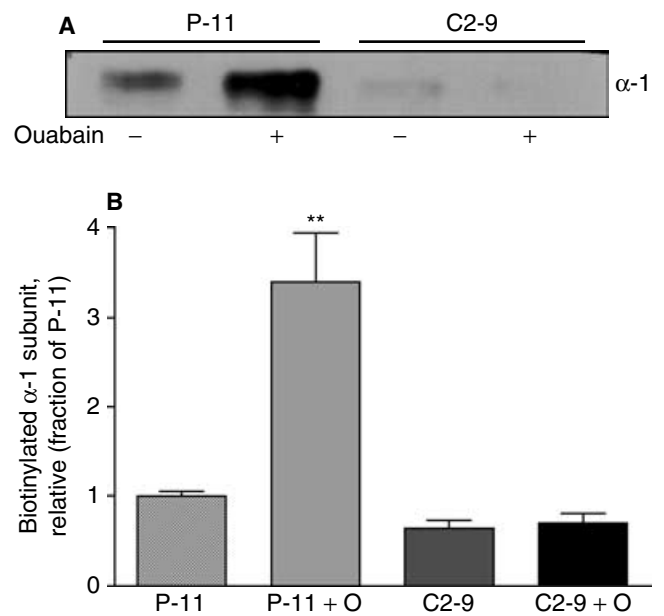


**Fig. 3. Depletion of caveolin-1 prevents ouabain-induced decreases of the plasmalemmal Na/K-ATPase  $\alpha$ -1 subunit.** (A) Shown is the biotinylated surface Na/K-ATPase  $\alpha$ -1 subunit in P-11 and C2-9 cells. After treatment with or without (as control) ouabain (50 nmol/L, 12 hours), cell surface proteins were biotinylated, pull-down with streptavidin, and immunoblotted with the Na/K-ATPase  $\alpha$ -1 subunit. O, ouabain.  $N = 4$  experiments are represented at each data point in each panel, \*\* $P < 0.01$  vs. control. (B) shows quantification data of (A).

We next performed immunoprecipitation studies to examine the physical interactions between the Na/K-ATPase  $\alpha$ 1-subunit and other proteins important in endocytosis. We found that in P-11 cells, but not in C2-9 cells, ouabain stimulates the protein-protein interaction of the Na/K-ATPase  $\alpha$ -1 subunit and AP-2 (Fig. 6), similar to that which was previously demonstrated in wild-type LLC-PK1 cells [6]. We also observed an enhancement of protein-protein interaction among the  $\alpha$ -1 subunit, clathrin heavy chain (CHC), and PI-3K (Fig. 6) in P-11 cells, but not in C2-9 cells.

#### Depletion of caveolin-1 attenuates ouabain-induced compartmentalization of signaling molecules

We next treated P-11 cells and C2-9 cells with ouabain for different amounts of time (50 nmol/L, 1 hour for CCV isolation; 50 nmol/L, 2 hours for early endosomes isolation), and then isolated CCV and early endosomes, immunoblotting for the signaling molecules EGFR, c-Src, and ERK. As shown in Figure 7, ouabain significantly stimulated the internalization of these signaling molecules in endosomes in P-11 cells, but depletion of caveolin-1 (C2-9 cells) totally abolished this compartmentalization; the same results were also observed in



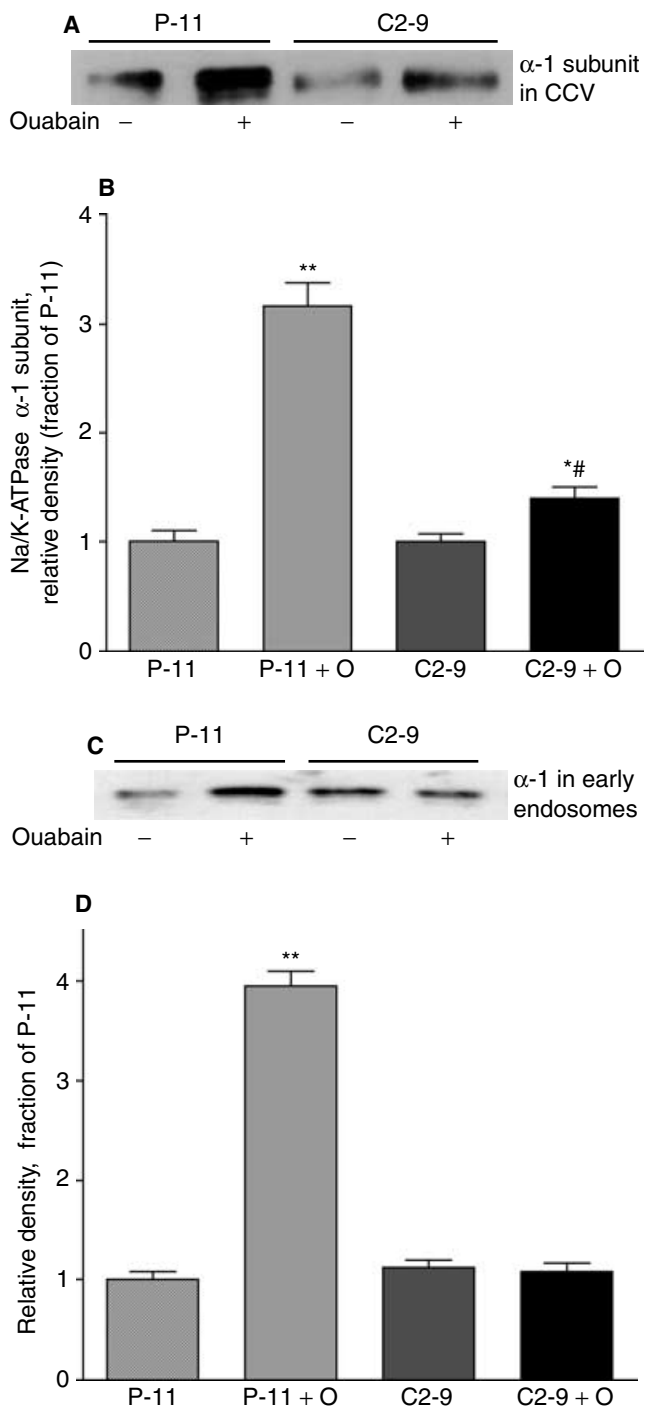
**Fig. 4. Depletion of caveolin-1 prevents ouabain-induced increases in nuclear accumulation of biotinylated  $\alpha$ -1 subunit of Na/K-ATPase.** (A) Shown is representative Western blot following streptavidin isolation of biotinylated  $\alpha$ -1 subunit from P-11 and C2-9 cell nuclei from cells exposed to ouabain for 12 hours and control cells. Twenty  $\mu$ g of total protein from nuclear fraction was applied to each lane and immunoblotted with antibody against  $\alpha$ -1. (B) Shown is quantification of the nuclear biotinylated pump (data shown as mean  $\pm$  SEM of 4 individual experiments normalized to the mean control value) in response to ouabain. \*\* $P < 0.01$  vs. control.

CCV isolation (data not shown), supporting the central role of caveolin-1 in ouabain-induced endocytosis of Na/K-ATPase and signaling transduction of Na/K-ATPase through inhibition of the enzyme.

#### Activation of EGFR alone is not sufficient to induce endocytosis of Na/K-ATPase

Ouabain transactivated the EGFR by activation of Src kinase and stimulation of Src binding to the EGFR, providing the scaffolding for the recruitment of adaptor proteins and Ras and the activation of Ras/MAPK cascade [7, 8]. But unlike the stimulation by its cognate ligand, ouabain-induced transactivation of EGFR was phosphorylated on site(s) different from the receptor's major autophosphorylation site.

To distinguish the possible different mechanisms evoked by ouabain and EGF, EGF was used to stimulate EGFR activation in P-11 and C2-9 cells. P-11 and C2-9 cells were treated with EGF (50 ng/mL) for 15 minutes, and the protein contents of EGFR,  $\alpha$ -1 subunit, c-Src, ERK were detected by Western blot in early endosomal fraction. In the experiment shown in Figure 8, EGF induced EGFR internalization in both P-11 and C2-9 cells, and compartmentalized Src kinase in P-11, but not in C2-9 cells. No EGF-induced accumulation of ERK or  $\alpha$ -1



**Fig. 5. Depletion of caveolin-1 prevents ouabain-induced endocytosis of the Na/K-ATPase  $\alpha$ -1 subunit.** (A) Representative Western blot illustrating effect of ouabain (1 hour at 50 nmol/L) on CCV  $\alpha$ -1 subunit of Na/K-ATPase protein content. Five  $\mu$ g of protein was applied to each lane. Quantification of 4 experiments showed that, in P-11 cells, the relative CCV Na/K-ATPase  $\alpha$ -1 subunit content in ouabain-treated cells was  $2.42 \pm 0.45$  ( $P < 0.01$  vs. control) compared with CCVs isolated from untreated cells; there is no significant change in C2-9 cells. (B) Quantification of data of (A). (C) Representative Western blot illustrating effect of ouabain (O) at 50 nmol/L for 2 hours on endosomal  $\alpha$ -1 subunit of Na/K-ATPase protein content. Ten  $\mu$ g of protein were applied to each lane. (D) Quantification of data of (C). For both (B) and (C),  $N = 4$ , data shown as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$  vs. control, # $P < 0.01$  vs. P-11+ouabain.

subunit was observed in early endosomes at this time point. In another set of experiments, EGF also failed to induce  $\alpha$ -1 subunit internalization after 2-hour treatment ( $\alpha$ -1 subunit content in early endosomes with EGF treatment was  $104 \pm 4\%$  relative to control,  $N = 4$ ,  $P = \text{NS}$ ).

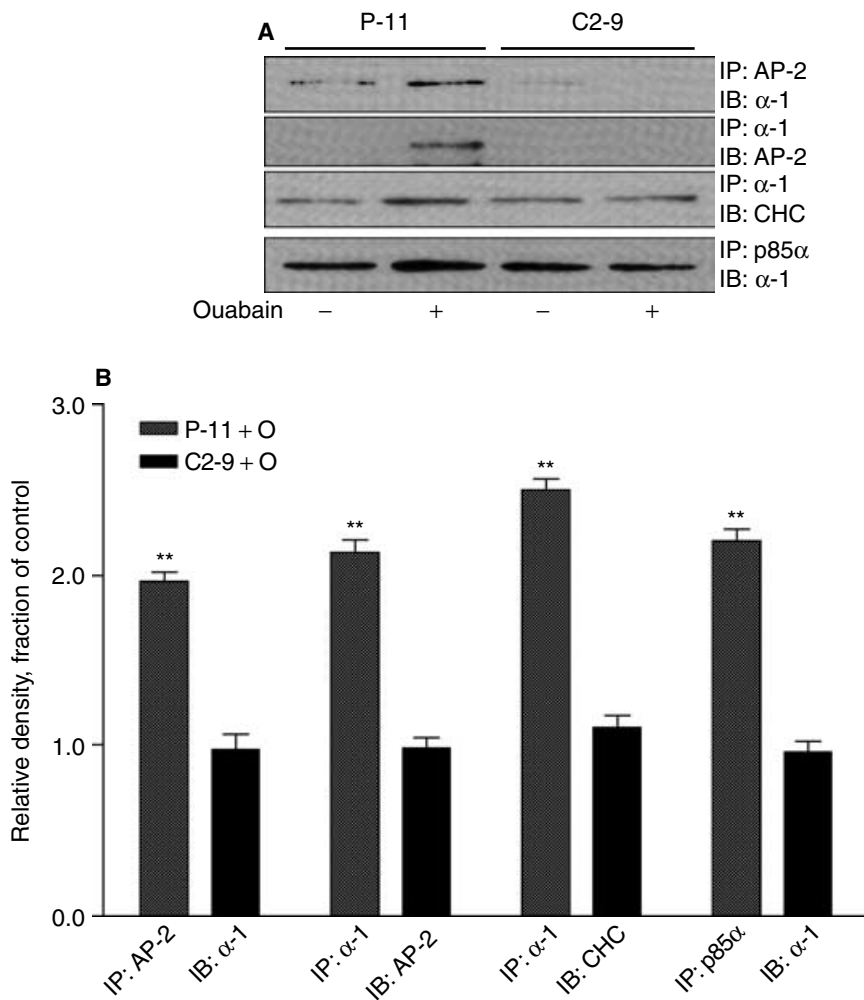
## DISCUSSION

We have demonstrated that low concentrations of ouabain induce substantial endocytosis of the Na/K-ATPase in a clathrin- and Src-dependent manner [6], supporting the analogy of signal transduction through the Na/K-ATPase with more conventional receptor ligand systems [10], and our notion that ouabain-induced endocytosis of the Na/K-ATPase is part of or a direct consequence of signal transduction through the Na/K-ATPase.

Many signaling molecules and membrane receptors are dynamically associated with caveolae, such as the Src-family kinase, Ras, PKC, ERK, insulin receptor, platelet-derived growth factor receptor (PDGFR), EGFR, and some entire signaling modules like PDGFR-Ras-ERK, mainly through their interactions with caveolins [16, 20, 21]. There is also evidence that caveolins may modulate endocytosis through their interactions with clathrin [22–25]. Free cholesterol is believed to be critical for maintaining the shape of caveolae and clathrin-coated pit, because depletion of cholesterol correlated directly with the flattening of caveolae and clathrin-coated pits, indicating that cholesterol affects the morphology and curvature of the plasma membrane [32]. In B lymphocytes, the ligand-induced endocytosis of its antigen receptor (BCR) only occurs when clathrin is associated with lipid rafts and is tyrosine phosphorylated following BCR crosslinking, suggesting that receptor uptake may be regulated not only by the interplay between components of signaling and endocytic pathway, but also by their relative spatial organization in membrane microdomain [25].

Ouabain-induced interaction of Na/K-ATPase with caveolin-1 is essential for ouabain-activated signal transduction in LLC-PK1 cells, and the caveolar Na/K-ATPase, but not the pump in other membrane fractions, most likely behaves as a signal transducer for ouabain [11]. Both caveolin and CHC are substrates of Src kinase [17, 33], and ouabain-induced endocytosis of the Na/K-ATPase is Src dependent [6]. These raise the possibility that both ouabain-induced endocytosis and signaling transduction are under control of Src kinases, but further investigations are needed to define their crosstalking and interplay crossing different membrane microdomains.

From Figures 1 and 7, it is clear that ouabain compartmentalizes and enhances the protein-protein interaction between the caveolar Na/K-ATPase and Src, leading to the compartmentalization of EGFR, c-Src, and ERK in CCVs and endosomes. This compartmentalization of signaling molecules may play an important role



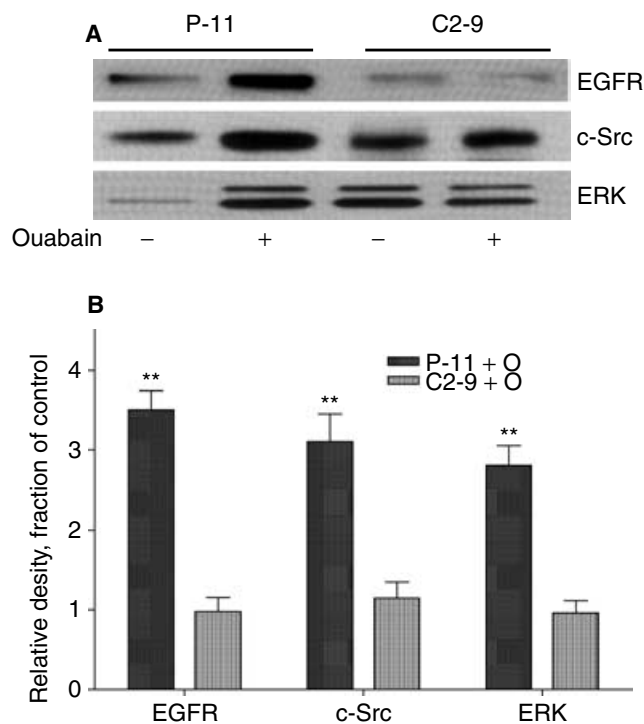
**Fig. 6. Depletion of caveolin-1 abolishes ouabain (O)-stimulated protein-protein interaction between the Na/K-ATPase  $\alpha$ -1 subunit, PI(3)K p85 $\alpha$  subunit, CHC, and AP-2.** Coimmunoprecipitate experiments were performed as described before [6]. One mg of total protein was used for each sample.  $N = 4$ , \*\* $P < 0.01$  vs. control.

in the activation and propagation of ouabain-induced signaling pathways, and on the other hand, the activation and propagation of ouabain-induced signal transduction can also regulate endocytosis. These data are consistent with previous observations that activation and propagation of Ras/Raf/MEK/MAPK signal cascade requires the endocytosis and endocytic recycling pathways [34–36], and overexpression of c-Src increases the rate of endocytosis of EGF/EGFR complexes [33, 37]. These data also support our proposed model that ouabain may actually induce the formation of an Na/K-ATPase/Src/EGFR/PI(3)K complex, and this complex recruits AP-2 and clathrin to form the clathrin-coated pits, resulting in the endocytosis of the enzyme [6]. However, it is not clear whether the Src-dependent endocytosis and signal transduction occur in parallel or sequence.

Binding of ouabain to the Na/K-ATPase activates multiple signal transduction pathways, and regulates transcription and translation of many genes in cardiac myocytes and other cell types [38, 39], including Src-mediated transactivation of EGFR and subsequent

recruitment and assembly of the Shc/Ras/Raf/ERKs in several different cell lines [7, 8]. Interaction of caveolin scaffolding domain with putative caveolin-binding motifs in a large number of signaling proteins such as Src, EGFR, and Ras concentrates these proteins in caveolae [17]. It was also demonstrated that caveolins may modulate endocytosis through their interactions with clathrin [22–25]. Our data also emphasize the importance of the integrity of the caveolae/lipid rafts in ouabain-induced endocytosis and signaling propagation, because depletion of cholesterol or caveolin-1 abolished ouabain-induced endocytosis of the Na/K-ATPase and compartmentalization of signaling molecules in endocytic compartments, and repletion of cholesterol restored these processes. There is evidence that recycling endosomes (in MDCK cells), and perhaps in other endocytic compartments, are also enriched in caveolin-1 [40], which raises the possibility that depletion of cholesterol or caveolin-1 may also disrupt the integrity of the structure of the endocytic compartments or reduce the protein-protein interaction of caveolin-1 with signaling molecules, leading to the inhibition of

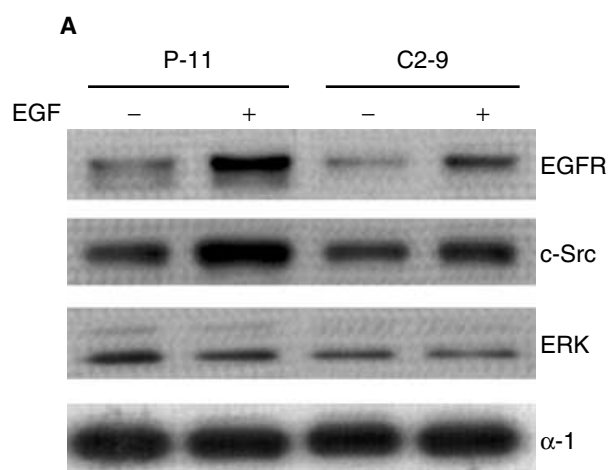




**Fig. 7. Depletion of caveolin-1 prevents ouabain-induced compartmentalization of signaling molecules in endosomes.** Both P-11 and C2-9 cells were treated with (50 nmol/L, 2 hours) or without (as control) ouabain. (A) A representative Western blot (for EGFR, c-Src, and total ERK) from early endosomal isolation. Ten  $\mu$ g of total protein was applied to each lane, and immunoblotted with antibodies against EGFR, c-Src, and total ERK. (B) Quantitative data expressed as fraction of control (control values = 1).  $N = 4$  control and  $N = 4$  ouabain-treated cells studied. \*\* $P < 0.01$  vs. control. P-11 + O and C2-9 + O were compared to P-11 and C2-9 control cells, respectively. O, ouabain.

the endocytosis pathway and the compartmentalization of the signaling molecules. This phenomenon also supports our previous proposal that ouabain-induced endocytosis of the Na/K-ATPase is a ligand-receptor process that cross-talks with ouabain-activated signaling pathway in which the (caveolar) Na/K-ATPase functions as a signaling transducer. From our observations, it is also very clear that there is a “cross-talk” between ouabain-induced signaling pathways and ouabain-induced endocytosis of Na/K-ATPase.

Transactivation of EGFR played an important role in ouabain-induced signaling transduction, ouabain-induced activation of Src kinase phosphorylated EGFR, and provided the scaffolding to recruit adaptor proteins [7, 8], but this transactivation of EGFR was phosphorylated on site(s) different from the receptor’s major autophosphorylation site by its cognate ligand [7, 8]. The transactivated EGFR has been identified as a critical element in the signaling transduction pathways using G protein-coupled receptors [41]. Our previous study indicated that Src activation is essential for ouabain-induced endocytosis of Na/K-ATPase [6]. Present data



**Fig. 8. EGF-induced activation of EGFR is not sufficient to induce Na/K-ATPase internalization.** Both P-11 and C2-9 cells were treated with (50 ng/mL, 15 minutes) or without (as control) EGF. (A) A representative Western blot from early endosomal fractions. Ten  $\mu$ g of total protein was applied to each lane and immunoblotted with antibodies against EGFR, c-Src, total ERK, and  $\alpha$ -1 subunit. (B) Quantitative data expressed as fraction of control (control values = 1).  $N = 3$  controls and  $N = 3$  ouabain-treated cells studied. \*\* $P < 0.01$  vs. control. P-11 + EGF and C2-9 + EGF were compared to P-11 and C2-9 control cells, respectively.

(Fig. 8) suggest that activation of EGFR by EGF is not sufficient to internalize Na/K-ATPase, but ouabain-induced transactivation of EGFR, phosphorylated at different site(s), may be essential to use its phosphorylation site(s) as docking site(s) to form proper signaling complex in caveolae or/and clathrin-coated pits. It has been reported that c-Src may interact with autophosphorylated EGFR through its SH2 domain, and EGF-activated EGFR may require its interaction with c-Src to be internalized; furthermore, c-Src-dependent autophosphorylation and transactivation of EGFR may enhance their interaction in cases of oxidative stress and GPCR activation (for review, see [42]). EGF-induced endosomal accumulation of EGFR, but not c-Src in C2-9 cells, suggests that caveolin may be involved in EGF-stimulated Src-EGFR interaction and internalization. Although the mechanism for this is still unclear, we would propose the following. The EGF-EGFR signaling stimulates Src kinase-mediated phosphorylation of clathrin, leading to the redistribution of clathrin to facilitate internalization [33]. Clathrin is constitutively associated with lipid rafts, wherein it is efficiently phosphorylated and redistributed [25]. Moreover, clathrin phosphorylation outside lipid rafts is not sufficient for accelerated ligand induced receptor internalization in B lymphocytes [25]. Depletion of caveolin-1 may, therefore, cause “structure damaged” caveolae (lipid rafts) and redistribution of lipids, leading to the inefficient clathrin phosphorylation, assembly of the clathrin-coated pits, and internalization.

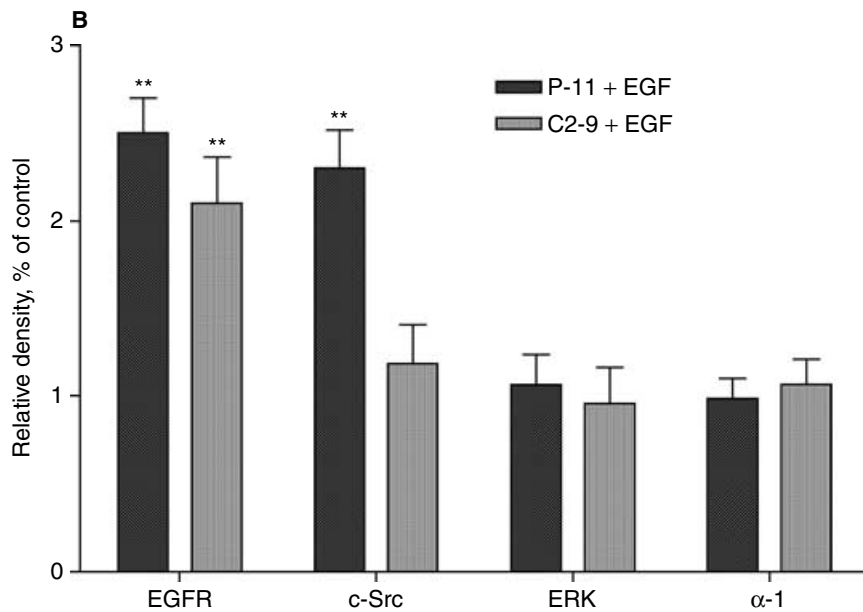


Fig. 8. (Continued)

## CONCLUSION

We demonstrated that caveolae are involved in ouabain-induced endocytosis of the Na/K-ATPase. Further studies will be necessary to define the role that the internalized Na/K-ATPase plays in ouabain or endogenous DLS-induced signal transduction, and the in vivo physiologic importance of this pathway with respect to renal sodium handling.

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## REFERENCES

- CAPLAN MJ: Ion pumps in epithelial cells: Sorting, stabilization, and polarity. *Am J Physiol* 272:G1304–G1313, 1997
- SCHONER W: Endogenous cardiac glycosides, a new class of steroid hormones. *Eur J Biochem* 269:2440–2448, 2002
- DORIS P: Regulation of Na,K-ATPase by endogenous ouabain-like materials. *Proc Soc Exp Biol Med* 205:202–212, 1994
- LINGREL JB, HUYSSSE J, O'BRIEN W, et al: Structure-function studies of the Na,K-ATPase. *Kidney Int* 44:S32–S39, 1994
- LIU J, PERIYASAMY SM, GUNNING W, et al: Effects of cardiac glycosides on sodium pump expression and function in LLC-PK1 and MDCK cells. *Kidney Int* 62:2118–2125, 2002
- LIU J, KESIRY R, PERIYASAMY S, et al: Ouabain induces endocytosis of plasmalemmal Na/K-ATPase in LLC-PK1 cells by a clathrin-dependent mechanism. *Kidney Int* 66:227–241, 2004
- HAAS M, ASKARI A, XIE Z: Involvement of Src and epidermal growth factor receptor in the signal-transducing function of Na<sup>+</sup>/K<sup>+</sup>-ATPase. *J Biol Chem* 275:27832–27837, 2000
- HAAS M, WANG H, TIAN J, XIE Z: Src-mediated inter-receptor cross-talk between the Na<sup>+</sup>/K<sup>+</sup>-ATPase and the epidermal growth factor receptor relays the signal from ouabain to mitogen-activated protein kinases. *J Biol Chem* 277:18694–18702, 2002
- LIU J, TIAN J, HAAS M, et al: Ouabain interaction with cardiac Na<sup>+</sup>/K<sup>+</sup>-ATPase initiates signal cascades independent of changes in intracellular Na<sup>+</sup> and Ca<sup>2+</sup> concentrations. *J Biol Chem* 275:27838–27844, 2000
- XIE Z, ASKARI A: Na<sup>+</sup>/K<sup>+</sup>-ATPase as a signal transducer. *Eur J Biochem* 269:2434–2439, 2002
- WANG H, HAAS M, LIANG M, et al: Ouabain assembles signaling cascades through the caveolar Na<sup>+</sup>/K<sup>+</sup>-ATPase. *J Biol Chem* 279:17250–17259, 2004
- LIU L, MOHAMMADI K, AYNAFSHAR B, et al: Role of caveolae in signal-transducing function of cardiac Na<sup>+</sup>/K<sup>+</sup>-ATPase. *Am J Physiol Cell Physiol* 284:C1550–1560, 2003
- ANDERSON RG: Caveolae: Where incoming and outgoing messengers meet. *Proc Natl Acad Sci U S A* 90:10909–10913, 1993
- ANDERSON RG: The caveolae membrane system. *Annu Rev Biochem* 67:199–225, 1998
- BICKEL PE: Lipid rafts and insulin signaling. *Am J Physiol Endocrinol Metab* 282:E1–E10, 2002
- LIU P, RUDICK M, ANDERSON RG: Multiple functions of caveolin-1. *J Biol Chem* 277:41295–41298, 2002
- SCHLEGEL A, LISANTI MP: The caveolin triad: Caveolae biogenesis, cholesterol trafficking, and signal transduction. *Cytokine Growth Factor Rev* 12:41–51, 2001
- PELKMANS L, HELENIUS A: Endocytosis via caveolae. *Traffic* 3:311–320, 2002
- SCHNITZER JE: Caveolae: From basic trafficking mechanisms to targeting transcytosis for tissue-specific drug and gene delivery in vivo. *Adv Drug Deliv Rev* 49:265–280, 2001
- GALBIATI F, RAZANI B, LISANTI MP: Emerging themes in lipid rafts and caveolae. *Cell* 106:403–411, 2001
- NABI IR, LE PU: Caveolae/raft-dependent endocytosis. *J Cell Biol* 161:673–677, 2003
- SHIGEMATSU S, WATSON RT, KHAN AH, PESSIN JE: The adipocyte plasma membrane caveolin functional/structural organization is necessary for the efficient endocytosis of GLUT4. *J Biol Chem* 278:10683–10690, 2003
- SLEIGHT S, WILSON BA, HEIMARK DB, LARNER J: G(q/11) is involved in insulin-stimulated inositol phosphoglycan putative mediator generation in rat liver membranes: Co-localization of G(q/11) with the insulin receptor in membrane vesicles. *Biochem Biophys Res Commun* 295:561–569, 2002

24. SCHERER PE, LISANTI MP, BALDINI G, et al: Induction of caveolin during adipogenesis and association of GLUT4 with caveolin-rich vesicles. *J Cell Biol* 127:1233–1243, 1994
25. STODDART A, DYKSTRA ML, BROWN BK, et al: Lipid rafts unite signaling cascades with clathrin to regulate BCR internalization. *Immunity* 17:451–462, 2002
26. GOTTARDI CJ, CAPLAN MJ: Delivery of Na<sup>+</sup>,K<sup>+</sup>-ATPase in polarized epithelial cells. *Science* 260:552–554, 1993
27. GOTTARDI CJ, DUNBAR LA, CAPLAN MJ: Biotinylation and assessment of membrane polarity: Caveats and methodological concerns. *Am J Physiol* 268:F285–295, 1995
28. SORKIN A, MCKINSEY T, SHIH W, et al: Stoichiometric interaction of the epidermal growth factor receptor with the clathrin-associated protein complex AP-2. *J Biol Chem* 270:619–625, 1995
29. FURUCHI T, ANDERSON RGW: Cholesterol depletion of caveolae causes hyperactivation of extracellular signal-related kinase (ERK). *J Biol Chem* 273:21099–21104, 1998
30. LABRECQUE L, ROYAL I, SURPRENANT DS, et al: Regulation of vascular endothelial growth factor receptor-2 activity by caveolin-1 and plasma membrane cholesterol. *Mol Biol Cell* 14:334–347, 2003
31. WALLENSTEIN S, ZUCKER CL, FLEISS JL: Some statistical methods useful in circulation research. *Circ Res* 47:1–9, 1980
32. D'HONDT K, HEESE-PECK A, RIEZMAN H: Protein and lipid requirements for endocytosis. *Annu Rev Genet* 34:255–295, 2000
33. WILDE A, BEATTIE EC, LEM L, et al: EGF receptor signaling stimulates SRC kinase phosphorylation of clathrin, influencing clathrin redistribution and EGF uptake. *Cell* 96:677–687, 1999
34. DI GUGLIELMO GM, BAASS PC, OU WJ, et al: Compartmentalization of SHC, GRB2 and mSOS, and hyperphosphorylation of Raf-1 by EGF but not insulin in liver parenchyma. *EMBO J* 13:4269–4277, 1994
35. KRANENBURG O, VERLAAN I, MOOLENAAR WH: Dynamin is required for the activation of mitogen-activated protein (MAP) kinase by MAP kinase kinase. *J Biol Chem* 274:35301–35304, 1999
36. ROY S, WYSE B, HANCOCK JF: H-Ras signaling and K-Ras signaling are differentially dependent on endocytosis. *Mol Cell Biol* 22:5128–5140, 2002
37. WARE MF, TICE DA, PARSONS SJ, LAUFFENBURGER DA: Overexpression of cellular Src in fibroblasts enhances endocytic internalization of epidermal growth factor receptor. *J Biol Chem* 272:30185–30190, 1997
38. KOMETIANI P, LI J, GNUDI L, et al: Multiple signal transduction pathways link Na<sup>+</sup>/K<sup>+</sup>-ATPase to growth-related genes in cardiac myocytes. The roles of Ras and mitogen-activated protein kinases. *J Biol Chem* 273:15249–15256, 1998
39. XIE Z, KOMETIANI P, LIU J, et al: Intracellular reactive oxygen species mediate the linkage of Na<sup>+</sup>/K<sup>+</sup>-ATPase to hypertrophy and its marker genes in cardiac myocytes. *J Biol Chem* 274:19323–19328, 1999
40. GAGESCU R, DEMAUREX N, PARTON RG, et al: The recycling endosome of Madin-Darby canine kidney cells is a mildly acidic compartment rich in raft components. *Mol Biol Cell* 11:2775–2791, 2000
41. LUTTRELL LM, DAAKA Y, LEFKOWITZ RJ: Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr Opin Cell Biol* 11:177–183, 1999
42. LEU TH, MAA MC: Functional implication of the interaction between EGF receptor and c-Src. *Front Biosci* 8:s28–38, 2003