

Identification of a Pool of Non-Pumping Na/K-ATPase*

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Summary

Recent studies have ascribed many non-pumping functions to the Na/K-ATPase. Here, we present experimental evidence demonstrating that over half of the plasma membrane Na/K-ATPase in LLC-PK1 cells is performing cellular functions other than ion pumping. This “non-pumping” pool of Na/K-ATPases, like the pumping pump, binds ouabain. Depletion of either cholesterol or caveolin-1 moves some of the “non-pumping” Na/K-ATPase into the pumping pool. Graded knock-down of the $\alpha 1$ subunit of the Na/K-ATPase eventually results in loss of this “non-pumping” pool while preserving the pumping pool. Our prior studies indicate that a loss of the non-pumping pool is associated with a loss of receptor function as evidenced by the failure of ouabain administration to induce the activation of Src and/or ERK. Therefore, our new findings suggest that a substantial amount of surface-expressed Na/K-ATPase, at least in some types of cells, may function as non-canonical ouabain-binding receptors.

Introduction

Na/K-ATPase is an integral membrane protein found in most mammalian cells. It was

initially discovered as an energy transducing ion pump that transports 3 Na⁺ out and 2 K⁺ into the cell by hydrolysis of one molecule of ATP (1,2). This pumping process generates the transmembrane chemical and electrical gradients that are essential to the excitable activity of muscle and nerve tissue, as well as the regulation of cell volume and a number of Na⁺-coupled transporters. Interestingly, evidence accumulated during the last ten years indicates that the same Na/K-ATPase also serves as a signaling receptor. This non-canonical function depends on interactions of the Na/K-ATPase with various proteins including protein and lipid kinases, membrane transporters, channels and cellular receptors (3-6). Binding of either endogenous or exogenous cardiotonic steroids, such as ouabain, to this receptor/protein complex evokes protein and lipid kinase cascades, thus generating a large number of secondary messengers (4,7-10). Specifically, we have shown that the Na/K-ATPase is capable of interacting with Src via multiple domains *in vitro*. Furthermore, the interaction between the Na/K-ATPase and Src keeps Src in an inactive state. Binding of ouabain to the Na/K-ATPase/Src complex changes the interaction, resulting in activation of the associated Src (4). In cultured

cells, the receptor Na/K-ATPase resides with Src in caveolae (11). Once the Na/K-ATPase/Src complex is activated by ouabain, it stimulates tyrosine phosphorylation of down-stream effectors, which in turn recruits additional kinases and adaptor proteins to the activated receptors, resulting in the activation of protein kinase cascades and the generation of second messengers (4).

To further prove the above Na/K-ATPase-specific signaling mechanism, we have generated several $\alpha 1$ knock-down cell lines using a siRNA-based approach. These cell lines (e.g., A4-11 and PY-17) were cloned from LLC-PK1 cells that were transfected with an $\alpha 1$ -specific siRNA expressing vector (12). While A4-11 cells express about 44% of $\alpha 1$ subunit in comparison to the control P-11 cells, PY-17 cells contain only 8% of the $\alpha 1$ subunit. Functional studies indicated that graded knock-down of $\alpha 1$ decreased the interaction between the Na/K-ATPase and Src, and resulted in an increase in basal Src activity. Concomitantly, it also abolished ouabain-induced activation of Src and the down-stream protein kinase cascade (12). These findings clearly demonstrate that there is a pool of Src-interacting Na/K-ATPase, thus providing further support to the above newly discovered cellular signaling mechanism. They also suggest that there may be two functionally separable pools of Na/K-ATPase in the plasma membrane, one performing the canonical (pumping) function, the other carrying out non-canonical functions such as signal transduction and scaffolding. To test this hypothesis, we have further characterized the ion-pumping activity of Na/K-ATPase in the established $\alpha 1$ knock-down cells. The data presented here clearly demonstrate the existence of a pool of non-pumping Na/K-ATPase in the plasma membrane.

Experimental Procedures

Materials: Image-iT FX signal enhancer, Antifade kit, Alexa Fluor 488-conjugated anti-mouse/rabbit IgG and Alexa Fluor 546-conjugated anti-rabbit IgG antibodies were obtained from Molecular Probes (Eugene, OR). Anti-Na/K-ATPase $\beta 1$ (clone C464.8) antibody was from Upstate (Lake Placid, NY); Anti-Giantin antibody was from Covance (Berkeley, CA). The monoclonal anti- $\alpha 1$

antibody ($\alpha 6F$) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. All the secondary horseradish peroxidase-conjugated antibodies were from Santa Cruz (Santa Cruz, CA). Radioactive $^{86}\text{Rb}^+$ and ^3H -ouabain were from NEN Life Science Products (Boston, MA).

Cell culture: The $\alpha 1$ knock-down stable cell lines were generated from LLC-PK1 cells and cultured in Dulbecco's Modification of Eagle's Medium (DMEM) as described (12). Most of the experiments were performed after the cells reached 90% confluence. To test whether polarity affects the conclusion of our studies, cells were also cultured on Transwell inserts and used when the monolayer was formed (13). Because serum-starved cells were used for our prior studies of non-pumping function of the Na/K-ATPase (12), in order to be consistent, cells were serum-starved for 12 h and then used for studies of pumping functions.

Cell surface biotinylation, streptavidin precipitation, and immunoblot: Labeling with biotin of plasmalemma proteins was performed with modifications according to the protocol described by Gottardi *et al.* (14). Specifically, cells grown on 60 mm Petri dishes were washed once with a nominally Ca^{2+} -free DMEM (15), and placed on ice. Afterwards, the cells were rinsed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.4) and incubated with 2 ml of NHS-ss-biotin freshly diluted into biotinylation buffer (10 mM triethanolamine pH 9.0, 150 mM NaCl) for 25 min at 4°C with very gentle horizontal motion to ensure mixing. Cells were rinsed twice with PBS containing 100 mM glycine, and washed in this buffer for 20 min at 4°C to make certain that all of the un-reacted biotin was quenched. Cells were then rinsed twice with PBS to wash away the quenched biotin, and solubilized in 900 μl lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5) for 60 min on ice. Cell lysates were clarified by centrifugation at $14,000 \times g$ for 10 min at 4°C . Control experiments showed that nearly all of the cellular Na/K-ATPase was solubilized, which is consistent with prior observations (16). To label the cells grown on Transwell inserts, the same protocol was followed after the cells were first incubated in PBS solution containing 1 mM EDTA for 5 min (14).

Biotinylation buffer was then added to the apical side of the Transwell. According to Gottardi *et al.* (14), pre-treatment of polarized cell cultures with PBS-EDTA solution completely opens the tight junction for the apically added biotinylation buffer to access the basolateral Na/K-ATPase (14). The cleared cell lysate (750 μ l) was collected and incubated overnight with 100 μ l packed streptavidin-agarose beads at 4°C with end-over-end rotation. The supernatant was then collected, and 1/10 of the total volume was loaded on the polyacrylamide gel as unbound fraction (U), together with 1/10 of the cleared lysate as total (T). Protein signal was detected using an ECL kit and quantified by Bio-Rad GS-670 imaging densitometer.

Confocal fluorescence microscopy: The subcellular localization of Na/K-ATPase β 1 subunit and Giantin was analyzed by immunofluorescence staining. Cells cultured on cover slips were washed twice with PBS and fixed with methanol pre-chilled at -20°C. The fixed cells were then rinsed with PBS for 3 times and blocked with 200 μ l Image-iT FX signal enhancer for 30 min at room temperature. Cells were then incubated with primary antibodies (anti-Na/K-ATPase β 1 and anti-Giantin) in PBS containing 1% BSA for 1 h, followed by incubation with secondary Alexa Fluor-conjugated antibodies. Image visualization was performed by Leica TCS-SP2 laser scanning microscope (Leica, Mannheim, Germany).

Ouabain-sensitive $^{86}\text{Rb}^+$ uptake: Ouabain-sensitive $^{86}\text{Rb}^+$ uptake was employed to measure the transport function of Na/K-ATPase (17). Cells were cultured in 12 well plates to 90% confluence, washed, and serum-starved overnight. After being washed once with the fresh medium, the cells were incubated in the same medium at 37°C for 10 min in the presence of different concentrations of ouabain. Monensin (20 μ M) was added to clamp intracellular Na^+ to ensure maximal capacity of active uptake (18). Then, $^{86}\text{Rb}^+$ as the tracer for K^+ (1 μ Ci/well) was added to start the uptake experiment. After 10 min, 3 ml of ice-cold 100 mM MgCl_2 was added to stop the $^{86}\text{Rb}^+$ uptake. Cells were then washed three times with the same solution. Trichloroacetic acid (TCA)-soluble $^{86}\text{Rb}^+$ was measured by a liquid scintillation counter. TCA-precipitated protein was

dissolved in 0.1 N NaOH/0.2% SDS solution for protein assay. Preliminary experiments showed that the uptake was a linear function of time for up to 20 min. Ouabain-sensitive $^{86}\text{Rb}^+$ uptake was about 85% of the total in control P-11 and LLC-PK1 cells, and about 70% in PY-17 cells. To test whether tight junction affects the measurement of $^{86}\text{Rb}^+$ uptake in different cell lines, cells were incubated in nominally Ca^{2+} -free DMEM medium containing 0.1 mM EGTA (15). Afterwards, the $^{86}\text{Rb}^+$ uptake was conducted in this Ca^{2+} -free medium as described in the above paragraphs. Prior studies have demonstrated that removal of extracellular Ca^{2+} was sufficient to open up tight junction (14,19,20). For experiments using cells cultured on Transwell inserts, $^{86}\text{Rb}^+$ solution was added to both apical and basolateral sides to measure the total pumping activity. Control experiments showed that the basolateral activity accounted for about 93% of total ouabain-sensitive $^{86}\text{Rb}^+$ uptake. To measure Na/K/2Cl cotransporter activity, bumetanide-sensitive $^{86}\text{Rb}^+$ uptake was conducted as previously described (21). This activity in P-11 and PY-17 cells accounted for less than 10% and 16% of the total uptake activity, respectively.

^3H -ouabain binding: The number of ouabain binding sites in cultured cells was estimated by the protocol that had been previously used (with modifications) and discussed (22-24). Briefly, cells were seeded into 12 well plates, cultured to 90% confluence, and then serum-starved overnight. Afterwards, cells were rinsed, incubated in K^+ -free Krebs's solution (NaCl 142.4 mM; CaCl_2 2.8 mM; NaH_2PO_4 0.6 mM; MgSO_4 1.2 mM; dextrose 10 mM; Tris 15 mM; pH 7.4) in the presence of 20 μ M monensin for 10 min, and then exposed to 2 μ M ^3H -ouabain for 15 min at 37°C. Monensin was added to clamp intracellular Na^+ and to prevent recycling of the Na/K-ATPase from the intracellular pool to the plasma membrane (25,26). Control experiments showed that maximal binding was reached at 2 μ M ouabain after 15 min exposure in both P-11 and PY-17 cells. At the end of incubation, cells were washed four times with ice-cold K^+ -free Krebs's solution, solubilized in 0.1 N NaOH/0.2% SDS, and measured by a scintillation counter. Non-specific binding, measured in the presence of 5 mM unlabeled ouabain, was less than 3% of total binding, and was subtracted from the total binding. Control

experiments showed that PY-17 cells contained 0.21 ± 0.03 ng protein/cell whereas P-11 had 0.19 ± 0.02 ng protein/cell. The sizes of these cells are also similar: 15.0 ± 2.6 μm in diameter for P-11 cells and 16.0 ± 2.9 μm for PY-17 cells. Thus, the binding data were normalized by the cell numbers counted in paralleled dishes.

To test whether tight junction affects the measurement of ouabain binding, the ouabain binding assay was performed in a nominally Ca^{2+} - and K^+ -free Krebs solution in the presence of 0.1 mM EGTA as described above. The ouabain binding sites in P-11 cells under this condition were $8 \times 10^5/\text{cell}$, which is comparable to that ($8.5 \times 10^5/\text{cell}$) in Ca^{2+} -containing solution. For binding experiments in cells cultured on Transwell inserts, ^3H -ouabain solution was added to both apical and basolateral sides to measure the total binding. The binding activity was normalized per mg total cellular proteins ($3.6 \times 10^{12}/\text{mg}$ protein in P-11 cells).

$[\text{Na}^+]_i$ measurement: The relative intracellular Na^+ content was measured according to Kim *et al.* (27) with minor modifications (28). Briefly, cells cultured in 12 well plates were incubated at 37°C in DMEM containing trace amounts of $^{22}\text{Na}^+$ (0.5 $\mu\text{Ci}/\text{ml}$) for 60 min. Control experiments showed that 60 min incubation was sufficient to fully equilibrate the exchangeable intracellular Na^+ with $^{22}\text{Na}^+$ in both control and $\alpha 1$ knock-down cells. Cells were then washed three times with ice-cold 100 mM MgCl_2 , and precipitated by TCA. The TCA-extractable $^{22}\text{Na}^+$ was measured by a scintillation counter and the data were normalized by the cell number.

$[\text{Na}^+]_i$ measurement with fluoroprobe sodium-binding benzofuran isophthalate (SBFI): Intracellular Na^+ concentration was measured as previously described (15). Briefly, P-11 cells and PY-17 cells were loaded with 5 μM SBFI-AM in the presence of 0.075% pluronic for 30 min at 37°C and then washed to remove the excess fluorescent dye. The cells were alternately illuminated by 340 nm and 380 nm UV light and the emission light at 520 nm was recorded. The emission ratio of 340/380 was used to calculate intracellular Na^+ concentration based on the calibration curve constructed by varying extracellular Na^+ in the presence of 10 μM

gramicidin. The basal $[\text{Na}^+]_i$ concentration in P-11 cells was 20 ± 2 mM ($n=15$).

Data analysis: Data presented are mean \pm SEM of at least three independent experiments and statistical analysis was performed using Student's *t*-test.

Results and Discussion

Pumping and non-pumping pools of Na/K-ATPase: We have recently demonstrated that knock-down of the cellular Na/K-ATPase reduces the size of a pool of Src-interacting Na/K-ATPase, resulting in an increase in basal Src activity and an inhibition of ouabain-induced Src activation (12). These new findings led us to propose that there may be two functionally separable pools of Na/K-ATPase in cultured LLC-PK1 cells, one being the canonical ion pumping pool and the other being the non-pumping pool. Because cells need the pumping Na/K-ATPase to survive, we reasoned that knock-down of the cellular $\alpha 1$ subunit might preferentially deplete the non-pumping pool of Na/K-ATPase to preserve the pumping pool if these two different pools of Na/K-ATPase co-exist in the plasma membrane. Indeed, when total Na/K-ATPase activity was measured in the control and the $\alpha 1$ knock-down cells, we observed that while PY-17 cells expressed only 8% of the $\alpha 1$ protein, they exhibited 21% of ouabain-sensitive ATPase activity in comparison to the control P-11 cells (12). These data support the notion that there is a pool of non-pumping Na/K-ATPase in the control P-11 cells. They also indicate that depletion of the $\alpha 1$ preferentially reduces the size of this non-pumping pool, resulting in a mismatch between the ouabain-sensitive ATPase activity and the $\alpha 1$ protein amount in the knock-down PY-17 cells.

Because the ATPase activity was measured in the presence of alamethicin, the above assay determined the total Na/K-ATPase activity including the enzyme in the plasma membrane and other intracellular compartments (29), which could over-estimate the actual pumping pool of Na/K-ATPase in the plasma membrane. Thus, to further assess how $\alpha 1$ knock-down affects the size of the pumping pool of Na/K-ATPase in the plasma membrane, we measured the ouabain-sensitive $^{86}\text{Rb}^+$ uptake. Control experiments showed that

ouabain-sensitive $^{86}\text{Rb}^+$ uptake accounted for more than 85% and 70% of total uptake in the control and PY-17 cells, respectively. As shown in Table I, the reduction of the $\alpha 1$ subunit content caused a roughly proportional decrease of the maximum pumping activity in A4-11 cells when compared to that of control P-11 cells ($46 \pm 2\%$ of control). However, PY-17 cells that express 8% of the $\alpha 1$ subunit still exhibited about 42% of pumping activity. These findings provide further support to the notion that the control P-11 cells contain a large pool of non-pumping Na/K-ATPase.

It is important to note that the above ouabain-sensitive $^{86}\text{Rb}^+$ uptakes were done in the presence of 20 μM monensin. Monensin increases the intracellular Na^+ to a degree sufficient to maximally stimulate Na/K-ATPase activity (18). In the absence of monensin, PY-17 cells exhibited about 66% of pump activity compared to P-11 cells (Table II). When intracellular Na^+ was measured by $^{22}\text{Na}^+$ equilibrium assay, we observed that the $\alpha 1$ knock-down significantly increased intracellular Na^+ per cell. For example, intracellular Na^+ in PY-17 cells was elevated to about 194% of that in P-11 cells (Table II). To confirm these observations, we also measured intracellular Na^+ concentration using a fluorescence probe SBFI in both P-11 and PY-17 cells, and observed a similar relative change as measured by $^{22}\text{Na}^+$ equilibrium assay (Table II). Importantly, while addition of monensin caused an 86% increase in ouabain-sensitive $^{86}\text{Rb}^+$ uptake due to the increased turnover rate (or reserved pump capacity) in P-11 cells (18), it failed to do so in PY-17 cells (Table II). These data indicate that the pumps in PY-17 cells operated at the maximal pumping capacity, which is consistent with the fact that the Na^+ content was almost doubled in these cells.

Because LLC-PK1 cells can form tight junction when they are cultured at confluent density on Transwell porous inserts, it is prudent to rule out the possibility that the potential difference in tight junction formation among different cell lines may cause the observed mismatch between the pumping activity and the amount of Na/K-ATPase. Since the formation of tight junction requires the presence of extracellular Ca^{2+} (14,19), we repeated the above functional assays after the cultures were incubated in a

nominally Ca^{2+} -free DMEM medium. As shown in Table III, the pumping activity in PY-17 cells was 46% in comparison to that in control P-11 cells, which is essentially the same as that measured in the presence of extracellular Ca^{2+} .

Since the above studies were done on cells cultured in Petri dishes, to further test whether fully polarized LLC-PK1 cells also contain a large pool of non-pumping Na/K-ATPase, we measured the pumping activity in cells cultured at confluent density on Transwell inserts. As shown in Table III, PY-17 cells had about 32% of pumping activity in comparison to the control P-11 cells. Taken together, our findings clearly show that there is a large pool of non-pumping Na/K-ATPase in LLC-PK1 cells, cultured either in Petri dishes or on Transwell inserts.

To test whether the $\alpha 1$ knock-down changes other K^+ uptake mechanism, we measured Na/K/2Cl cotransporter mediated- $^{86}\text{Rb}^+$ uptake, and observed that bumetanide-sensitive uptake activity in PY-17 cells was comparable to that in P-11 cells ($91 \pm 3\%$ of PY-17 cells, $n=5$, $p>0.05$). However, since intracellular Na^+ was much higher in PY-17 cells, these data suggest that $\alpha 1$ knock-down probably up-regulated Na/K/2Cl cotransporter to maintain the similar transporter activity as in control P-11 cells. Because PY-17 cells exhibited a similar cell size as control P-11 cells, the above findings also suggest that $\alpha 1$ knock-down cells are likely adapted to the decreased Na/K-ATPase activity.

Surface distribution of Na/K-ATPase: The above data indicate that there may be a large pool of non-pumping Na/K-ATPase in the control P-11 cells. The formation of a functional pumping Na/K-ATPase requires the assembly of the $\alpha\beta$ complex and the subsequent delivery of this complex to the plasma membrane. Although it is less likely, if most Na/K-ATPase in P-11 cells resided in cellular compartments other than the plasma membrane, this could account for the observed non-pumping pool. Thus, to rule this out, we performed the following two sets of experiments.

Because the β subunit is essential for targeting Na/K-ATPase to the plasma membrane (30), in the first set of experiments we determined the effects of $\alpha 1$ knock-down on the expression

and cellular distribution of the $\beta 1$ subunit. As depicted in Fig. 1A, control P-11 cells presented at least three glycosylated $\beta 1$ bands. The expression of these glycosylated $\beta 1$ was significantly reduced in both A4-11 and PY-17 cells, and the reduction was correlated to the degrees of the $\alpha 1$ knock-down (Fig. 1A). When the density of the $\beta 1$ band was normalized against all three glycosylated $\beta 1$ bands in P-11 cells, we found that the $\beta 1$ in PY-17 cells was about 25% of that in P-11 cells. This was further confirmed by Western blot analysis of deglycosylated $\beta 1$ prepared from these cell lysates (data not shown). Interestingly, knock-down of the $\alpha 1$ caused almost complete depletion of the two $\beta 1$ bands that are highly glycosylated. As depicted in Fig. 1A, under normal exposure conditions, these two species were barely detectable in PY-17 cells. However, when the film was over-exposed, we were able to detect the expression of these two species. The quantitative data indicated that PY-17 cells expressed less than 5% of these two species in comparison to P-11 cells. These findings were consistent with the immunostaining results depicted in Fig. 1B. While the majority of the Na/K-ATPase $\beta 1$ resided in the plasma membrane in control P-11 cells, it was largely retained in the intracellular compartments in TCN23-19 cells, another $\alpha 1$ knock-down cell line that behaves like PY-17 (12). In addition, co-immunostaining indicated that a portion of the retained Na/K-ATPase $\beta 1$ in TCN23-19 cells was clearly co-localized with the Golgi marker protein, giantin. Taken together, the data showed that knock-down of the $\alpha 1$ caused a significant reduction of the plasma membrane $\beta 1$.

To determine surface distribution of the Na/K-ATPase and to calculate how much surface Na/K-ATPase is required for cellular pumping activity, we performed surface biotinylation assay. As depicted in Fig. 2A, about 30% of $\alpha 1$ in the control P-11 cells remained in the unbound fraction, indicating that the other 70% was accessible to biotinylation, thus distributed in the plasma membrane. Using the same protocol, we detected about 19% $\alpha 1$ in the unbound fraction in A4-11 cells. However, we failed to detect any $\alpha 1$ subunit in the unbound fraction in PY-17 cells even when the amount of cell lysate used for the biotinylation assay was doubled (Fig. 2A). These data suggest that most of the $\alpha 1$ in PY-17 cells

must be targeted to the plasma membrane. Thus, if we assume that all of the $\alpha 1$ (8% of control) in PY-17 cells resided in the plasma membrane and functioned as ion pumps, about 27% [$Y=(8 \times 100/70)/42=0.27$] of the surface $\alpha 1$ in control P-11 cells would be required for maintaining the maximal pumping activity. Apparently, knock-down of the $\alpha 1$ initially reduces both pumping and non-pumping pools as demonstrated in A4-11 cells. Once the maximal pumping activity is reduced to half, further reduction in $\alpha 1$ causes a preferential depletion of the non-pumping pool of Na/K-ATPase, which is consistent with our prior observations (12).

To further confirm this, we repeated the same measurements in cells cultured on Transwell inserts. Interestingly, we observed that only 13% of $\alpha 1$ in control P-11 cells was in the unbound fraction (Fig. 2B), indicating that the other 87% was distributed in the plasma membrane. As in cells cultured in Petri dishes, we failed to detect any $\alpha 1$ in the unbound fraction in PY-17 cells (data not shown). Because high density cell cultures were used, we re-measured the relative amount of $\alpha 1$ in both control and PY-17 cells. As shown in Fig. 2C, the $\alpha 1$ in PY-17 cells was about $11 \pm 3\%$ of that in P-11 cells, which is similar to that measured in cells cultured in Petri dishes (Table I). Thus, if we assume that all of the $\alpha 1$ (11% of control) in PY-17 cells resided in the plasma membrane, about 40% of surface Na/K-ATPase in control P-11 cells would be involved in pumping. This calculation suggests that a higher percentage of surface Na/K-ATPase may pump in the fully polarized cells than that in cells cultured in Petri dishes. Although it is unlikely, we can not exclude the possibility that the activity assay conducted in cells cultured in Petri dishes might underestimate the pumping activity due to impaired substrate accessibility.

Ouabain binding and turnover rates: The above biotinylation data indicate that there is a large pool of non-pumping $\alpha 1$ in the plasma membrane of P-11 cells. Although the $\alpha 1$ subunit in the plasma membrane of control P-11 cells are likely to be paired with the $\beta 1$ subunit based on the prior studies (31,32), it is prudent to conduct additional experiments to ensure that the non-pumping pool we have detected is constituted by the Na/K-

ATPase (i.e., the $\alpha\beta$ complex). To do so we measured ^3H -ouabain binding in both control and the knock-down cells because prior studies have shown that only the $\alpha\beta$ complex binds ouabain (33).

As depicted in Table I, P-11 cells contained about 850,000 ouabain binding sites per cell, which is similar to the reported value in cultured Hela cells (34). However, this number is lower than the value previously reported for LLC-PK1 cells (35). Since serum can increase the expression of Na/K-ATPase in cultured cells (36,37), we believe that 12 h serum starvation used in our experimental condition might significantly reduce the number of ouabain binding sites. When ouabain binding was measured in PY-17 cells, we found that these cells contained about 115,000 sites per cell, thus 14% of the control P-11 cells. A4-11, on the other hand, expressed about 381,000 sites per cell, 45% of that in control P-11 cells.

Quantitatively, with 8% of the $\alpha 1$ content, PY-17 cells manifest 14% of the ouabain binding sites and carry out 42% of the ion transport activity. We can analyze this if we make certain assumptions. First, we assume (as supported in ref. (12)) that there is close to zero signaling (non-pumping) pool in PY-17 cells. Second, we assume that the reason that they manifest 14% of the ouabain binding sites with 8% of the $\alpha 1$ subunit means that a proportion of the $\alpha 1$ is inaccessible to ouabain (e.g., the Na/K-ATPases in recycling vesicles and the unpaired $\alpha 1$ in the ER and later endosomes) and thus not contributing to either $^{86}\text{Rb}^+$ uptake or ^3H -ouabain binding in P-11 cells, but they are all accessible in PY-17. Thus, 42% of the transport activity can be carried out by 14% of the ouabain binding sites, giving a ratio of 3 arbitrary units of pump activity per site in PY-17 cells. For P-11 cells, the corresponding ratio is obviously 1.0. This indicates that in P-11 cells, the maximum activity per site, even in the presence of monensin where Na^+ is not rate-limiting, is $1/3 = 33\%$. This implies that 67% of the ouabain-binding sites in P-11 cells are non-pumping pumps, but may perform cellular functions other than ion pumping. The only alternative interpretation is that all of the sites are pumping, but the turnover number (pumping per site) is increased 3-fold as a result of $\alpha 1$ knockdown, which seems very unlikely. Moreover, this

estimation is essentially similar to that (73%) calculated based on surface $\alpha 1$ biotinylation and the activity data. The similar ratio (46% activity versus 14% ouabain binding site) was also observed in cells cultured in nominal calcium-free DMEM as shown in Table III. In cells cultured on Transwell inserts, PY-17 cells contained about 14% of ouabain binding sites and had 32% of pumping activity in comparison to the control P-11 cells. Thus, about 44% of pumps in fully polarized P-11 cells are involved in pumping. This is similar to that estimated with biotinylation data. Taken together, both estimations indicate that a large number of surface Na/K-ATPase is likely engaged in cellular activities other than ion pumping.

It is known that purified renal Na/K-ATPase has a turnover rate of 8,000 to 10,000 cycles per min for ATP hydrolysis. However, when the same turnover rate was measured in cultured cells or isolated renal tubules, the number was much lower, and ranged from 1500 to 5000 cycles per min depending on cell type (22,34,38,39). Using the above ouabain-binding and $^{86}\text{Rb}^+$ uptake data, we calculated turnover rates of the Na/K-ATPase-mediated ATP hydrolysis (two K^+ per pump cycle per ATP hydrolysis) in the different cell lines. These calculations revealed that the Na/K-ATPase in P-11 cells operated at about 1,600 cycles per min while the pump in PY-17 cells ran at 5,100 cycles per min. These findings are consistent with the reported values in the literature, and with the notion that these control cells contain a large pool of non-pumping Na/K-ATPase. They also suggest that interaction of the Na/K-ATPase with other cellular proteins is a key to the generation of a pool of non-pumping Na/K-ATPase. Removal of the interacting proteins from the Na/K-ATPase during the purification may disinherit the enzyme, converting the non-pumping pump into pumping pump.

In short, the above data indicate that a large pool of non-pumping Na/K-ATPase exists in cultured cells. Importantly, this pool of Na/K-ATPase, like the pumping pump, is capable of binding ouabain. If this non-pumping pool is involved in transmitting the extracellular ouabain signal, it is estimated that ouabain at physiological concentration (e.g., 0.1 nM) can activate about 2000 receptor sites per cell in LLC-PK1 cells.

This activation should generate enough second messengers to change cellular function, especially when the initial signal can be amplified via the protein kinase cascades.

A role for caveolae: We have shown that caveolae contain a pool of Na/K-ATPase that interact with caveolin-1, Src and other signaling proteins (11,40). Based on the structural information from SERCA and the Na/K-ATPase (41), both A (actuator) and N (nucleotide binding) domains of the $\alpha 1$ may undergo a large and rapid conformational change during each pump cycle. Because these domains are involved in interaction with the signaling and structural proteins (41,42), these interactions, in principle, might either slow down or inhibit the movement of these functional domains; thus, the pumping activity of the Na/K-ATPase. Conceptually, this could generate a pool of non-pumping Na/K-ATPase. Of course, not all interacting proteins will inhibit the pumping activity of the Na/K-ATPase. For example, when adducin was added to the purified Na/K-ATPase, it stimulated the ATPase activity by accelerating the E2 to E1 conformational change (43). Nevertheless, by concentrating Na/K-ATPase and its signaling partners, caveolae could facilitate the interactions between the Na/K-ATPase and inhibitory proteins, thus playing a role in the generation of a pool of non-pumping Na/K-ATPase.

We previously demonstrated that the disruption of caveolae structure by either cholesterol depletion or siRNA-mediated knock-down of caveolin-1 could redistribute the Na/K-ATPase from caveolar fraction, and inhibit ouabain-activated signal transduction in LLC-PK1 cells (11). If caveolae are involved in assembly of the non-pumping pool of Na/K-ATPase, we would expect that depletion of cholesterol by methyl- β -cyclodextrin (M β CD) should increase Na/K-ATPase-mediated $^{86}\text{Rb}^+$ uptake. Indeed, as illustrated in Fig. 3A, pre-treatment of P-11 cells with 10 mM M β CD resulted in a significant increase (40% over control) in the Na/K-ATPase-mediated $^{86}\text{Rb}^+$ uptake. The same effect was observed when the parent LLC-PK1 cells were exposed to 10 mM M β CD (data not shown). It is important to note that the M β CD treatment did not change the maximal ouabain binding sites in these

cells (data not shown). Significantly, when the same treatment was applied to the knock-down cells, we found no change in the pumping activity in PY-17 cells. On the other hand, it caused a significant, but much smaller increase in A4-11 cells. These data are consistent with the notion that most of the Na/K-ATPase in PY-17 cells operate as a pump whereas A4-11 cells contain reduced pools of both pumping and non-pumping Na/K-ATPase. To further confirm the above findings, we also measured $^{86}\text{Rb}^+$ uptake and ^3H -ouabain binding in both control P-11 cells and caveolin-1 knock-down C2-9 cells that were derived, like P-11, from LLC-PK1 cells (40). As depicted in Fig. 3B, depletion of caveolin-1 also resulted in a comparable increase in the pumping activity per ouabain binding site. Clearly, disruption of caveolae can convert a portion of non-pumping Na/K-ATPase into pumping pumps. It is also clear that caveolae in LLC-PK1 cells only contain less than half of cellular non-pumping Na/K-ATPase assuming that these treatments release all of caveolar Na/K-ATPase from interactions with the inhibitory proteins. Furthermore, these data indicate that the caveolar signaling Na/K-ATPase is capable of pumping ions once the interacting proteins (or factors) are removed by depletion of cholesterol or caveolin-1, which is consistent with the findings that the partially purified caveolar Na/K-ATPase has the normal ouabain-sensitive ATPase activity (31).

To test whether cells other than LLC-PK1 contain a pool of non-pumping Na/K-ATPase, we treated cultured fibroblasts derived from the rat heart with M β CD, and then measured for the Na/K-ATPase-mediated $^{86}\text{Rb}^+$ uptake. We found that the depletion of cholesterol doubled the pumping activity in cultured fibroblasts ($232 \pm 16\%$ of control, $n = 6$). These results suggest that an even greater majority of plasma membrane Na/K-ATPase belongs to the non-pumping pool in fibroblasts than that in the cells derived from renal epithelium. This is not surprising as renal epithelial cells express a large number of Na^+ and K^+ channels and transporters, thus requiring more “pumping pumps” to keep up with the inward sodium and outward potassium “leaks”. Fibroblasts, on the other hand, are far less “leaky”. Therefore, their pump population would contain relatively less “pumping pumps”. Interestingly, reduction of cellular cholesterol has also been

reported to increase the Na/K-ATPase activity in several other types of cells (44,45). For example, depletion of cholesterol by 5-25% could increase the pumping function by 15% in human red blood cells (44).

To assess whether the presence of a large pool of non-pumping Na/K-ATPases affect ouabain-induced inhibition of the pumping activity, we measured the dose-dependent effects of ouabain on $^{86}\text{Rb}^+$ uptake in both P-11 and PY-17 cells in the presence of 20 μM monensin. As shown in Fig. 4, we found that depletion of the cellular Na/K-ATPase increased the potency of ouabain on cellular pumping activity. Specifically, while 357 nM ouabain was sufficient to cause 50% inhibition of ouabain-sensitive $^{86}\text{Rb}^+$ uptake in PY-17 cells, the same inhibition in control P-11 cells required 2131 nM ouabain (Fig. 3). Consistently, the apparent $K_{1/2}$ for A4-11 cells was between P-11 and PY-17 cells. Although we do not know how depletion of the cellular Na/K-ATPase sensitizes ouabain-induced inhibition of the pumping activity, several possibilities are worthy of discussion. First, the existence of a large pool of non-pumping Na/K-ATPases may make the access of ouabain to the pumping pumps more restricted in P-11 cells. Because the expression of $\beta 1$ was significantly reduced in PY-17 cells, it could alter the formation of tight junction (46), thus making PY-17 cells much leakier than P-11 cells. However, this appears to be an unlikely explanation because if this is true, we would detect a large difference in ouabain binding and total pump activity among these cells when the formation of tight junction was disrupted by removal of extracellular Ca^{2+} (Table III). Alternatively, the on-rate of ouabain binding might be faster in PY-17 cells due to increased intracellular Na^+ . However, since the assays were conducted in the presence of monensin, it is less likely that differences in intracellular Na^+ among these cells can fully account for the observed ouabain sensitivity. Finally, an increase in ouabain sensitivity could be due to decreased endocytosis of the Na/K-ATPase in PY-17 cells (24,47). It is known that activation of the Na/K-ATPase/Src complex by ouabain can stimulate the endocytosis of the Na/K-ATPase in P-11 cells (40). Since depletion of the non-pumping pool of Na/K-ATPases blocks ouabain-induced activation of Src (12), it is conceivable that ouabain would be

less effective in stimulation of the endocytosis of Na/K-ATPase. Clearly, these issues remain to be resolved experimentally.

In short, the above data indicate that caveolae play an important role in the organization of non-pumping pool of Na/K-ATPase. Moreover, the size of the non-pumping pool may vary depending on the function of different cells as well as culture conditions (Table III). This is important because the size could determine the mode of ouabain action (Fig. 4). For example, if the size of the non-pumping pool is large enough, the physiological effects of ouabain are most likely mediated by the activation of protein kinase cascades as we demonstrated (4,12) or by stimulation/inhibition of other unknown protein complexes. On the other hand, if the non-pumping pool is small or depleted, the ouabain effect is most likely due to inhibition of the pumping activity as depicted in Fig. 4. Since Na/K-ATPase is very important in the economy of cells, the existence of a pool of non-pumping Na/K-ATPase could also serve as a reservoir for cells to rapidly and appropriately adjust to different conditions.

In summary, the evidence provided in the present study strongly suggests the existence of a large pool of non-pumping Na/K-ATPase in cultured cells. However, it is important to point out an alternative possibility that this so-called non-pumping pool is constituted by a number of slow pumping pumps that are in complex with signaling, structural and other unknown proteins. Apparently, this non-pumping pool of Na/K-ATPase resides in the plasma membrane. Like the pumping pumps, they are capable of binding ouabain. These findings are significant. First, they are consistent with recent studies from many laboratories, showing that the cellular Na/K-ATPase can transmit an extracellular ouabain signal independent of its pumping function. Second, they suggest that over half of plasma membrane Na/K-ATPase, at least in some cell types, is engaged in activities other than ion-pumping. It is important to note that these activities include, but are not limited to, the well-characterized signal transducing functions of Na/K-ATPase. The Na/K-ATPase is known to interact with many protein partners. For example, Na/K-ATPase binds phospholemman and agrin, and these interactions can either slow the pump or keep the pump in an inactive state (48-51). Recent

work has also revealed the interaction between the Na/K-ATPase and cofilin. Interestingly, this interaction appears to play a role in the regulation of cellular metabolic activity (52). Third, the new findings bring about many new and important issues regarding the Na/K-ATPase. For example, how are these two pools of Na/K-ATPase

assembled, delivered, and disassembled? Furthermore, are the sizes of these pools dynamically regulated? Finally, they call for the engagement of more investigators to unravel many unknown non-canonical functions of the Na/K-ATPase, and delineate the roles of these functions in cell biology and animal physiology.

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Figure legend

Figure 1. Na/K-ATPase β 1 expression in knock-down cells. (A): Total cell lysates were analyzed by Western blot using anti-Na/K-ATPase β 1 antibody. P-11 is the control cell line. A4-11, PY-17 and TCN23-19 are different α 1 knock-down cell lines. A representative Western blot is shown and quantitative data are from four independent experiments. Values are mean \pm SEM. (B): Immunostaining of β 1 subunit in P-11 and TCN23-19 cells was performed as described in "Experimental Procedures". Giantin was used as the Golgi marker. The same experiments were repeated three times.

Figure 2. Cell surface biotinylation. (A): Two 60 mm dishes of PY-17 cells, one dish of P-11 cells and one dish of A4-11 cells were biotinylated as described in "Experimental Procedures". Afterwards, the cells were solubilized in 900 μ l lysis buffer, and then analyzed. The same volume of the unbound fraction (U) and total cell lysate (T) was subjected to SDS-PAGE and probed with α 6F antibody. (B): Biotinylation assay was performed in P11 cells cultured in Transwell inserts as described in "Experimental Procedures". (C): Total α 1 was measured by Western blot from cell lysates collected from P-11 and PY-17 cells cultured on Tranwell inserts. Representative Western blots are shown in each panel and quantitative data are calculated based on at least three independent experiments. Values are mean \pm SEM.

Figure 3. (A): Effects of cholesterol depletion on Na/K-ATPase pumping activity. The control cells (P-11) and α 1 knock-down cells (A4-11 and PY-17) were pre-treated with M β CD (10 mM) at 37 $^{\circ}$ C for 1 h, washed, and then assayed for the ouabain-sensitive 86 Rb $^{+}$ uptake activity as described. Data are shown as % of control value of each cell line and are presented as the mean \pm SEM (n = 3). * P<0.05 ** P<0.01. (B): **Effects of knock-down of caveolin-1 on Na/K-ATPase pumping activity.** The ouabain-sensitive 86 Rb $^{+}$ uptake was assayed in both control P-11 cells and caveolin-1 knock-down C2-9 cells as described in "Experimental Procedures". Values are normalized per ouabain binding site, and then calculated as % of P-11 cells. Data are presented as the mean \pm SEM (n = 4). ** P<0.01.

Figure 4. Ouabain dose-response curve. Cells were pre-treated with different concentrations of ouabain as indicated for 10 min and assayed for 86 Rb $^{+}$ uptake as described in "Experimental Procedures". Data are shown as % of respective control value and are presented as the mean \pm SEM of 3 independent determinations. Curve fit analysis was performed by Graphpad software.

Table I. Comparison of Na/K-ATPase Activities in Different Cells

Cell lines	P-11	A4-11	PY-17
α 1 content (%), n=4	100	44 \pm 2	8 \pm 3
Pump activity (%), n=4 (20 μ M monensin)	100	46 \pm 2	42 \pm 3
Bmax (%), n=3 (ouabain binding sites, $\times 10^4$ /cell)	100 (85 \pm 8)	45 \pm 4 (38 \pm 1)	14 \pm 4 (12 \pm 1)

Table II. Intracellular Na⁺ Content and Monensin-Induced Changes in the Pump Activity in Different Cells

Cell lines	P-11	A4-11	PY-17
Pump activity in the absence of monensin (%), n=4	100	78 ± 3	66 ± 9
Pump activity in the presence of monensin (% of P-11 activity in the absence of monensin)	186 ± 9*	81 ± 2	74 ± 8
²² Na ⁺ _i (% of control), n =4	100	161 ± 11	194 ± 7
[Na ⁺] _i (mM), n = 8	20 ± 2	N/A	36 ± 5

* p<0.05 compared to P-11 in the absence of monensin.

Table III. Pumping activity and ouabain binding sites in cells cultured either in Ca²⁺-free medium or on Transwell inserts (monolayer).

Cell lines	Ca ²⁺ -Free		Monolayer	
	P-11	PY-17	P-11	PY-17
Pump activity (%), n=4 (20 μM monensin)	100	46 ± 3	100	32 ± 5
Ouabain binding site (%), n =4	100	14 ± 4	100	14 ± 2

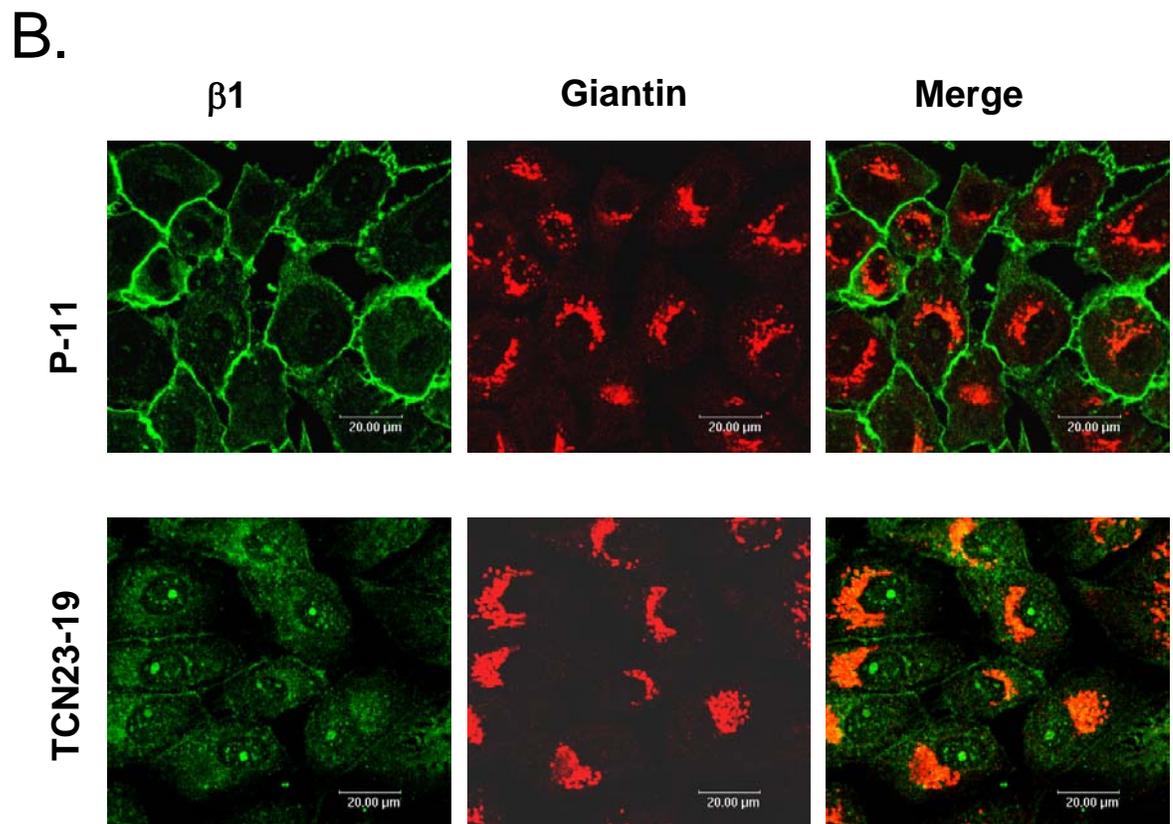
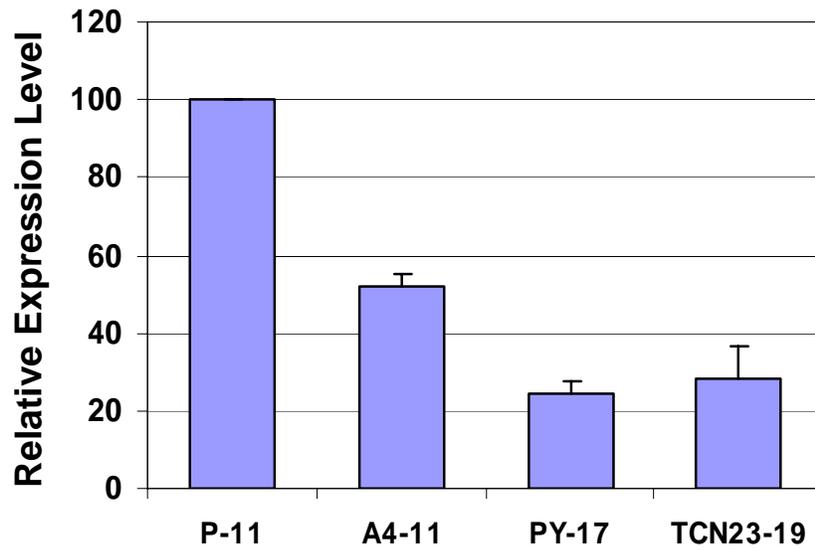
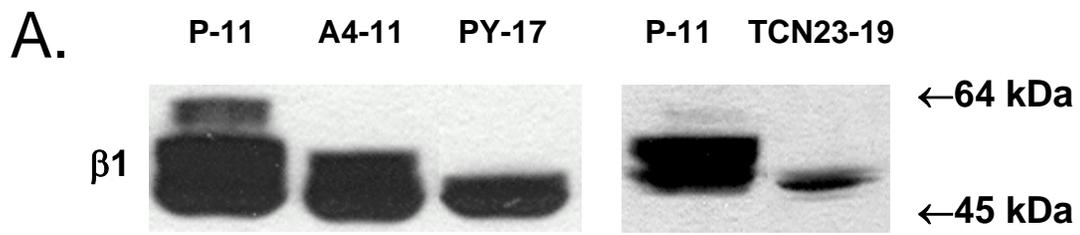
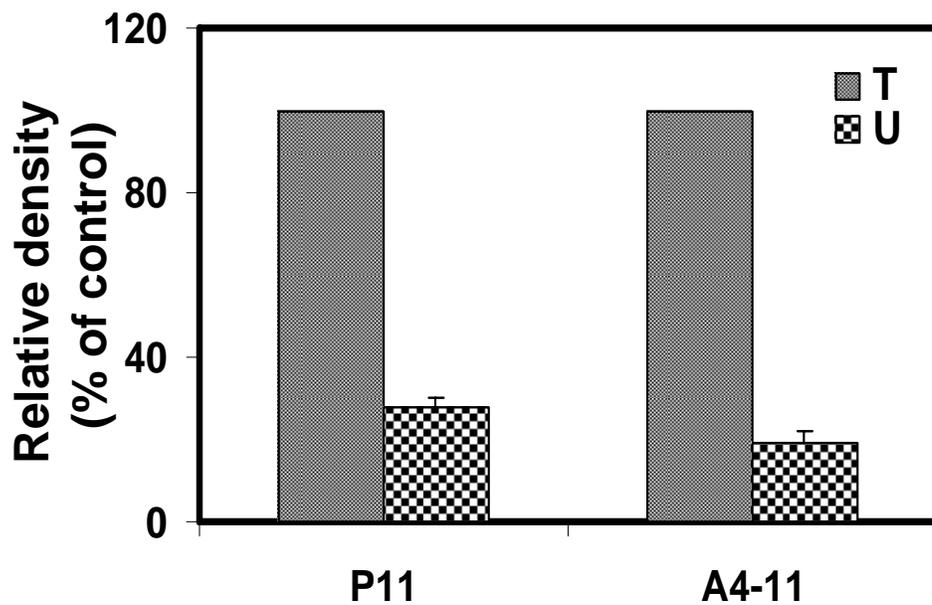
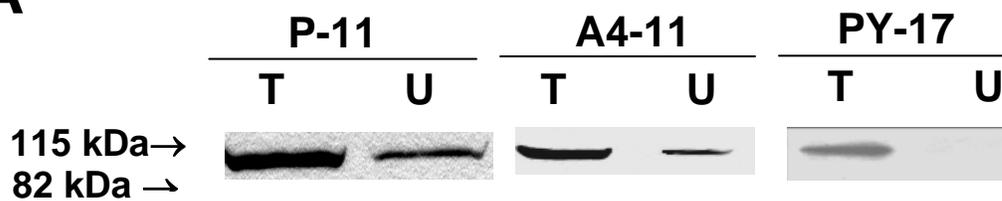
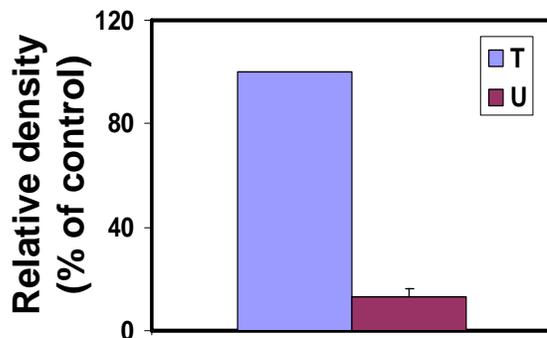


Figure 1

A



B



C

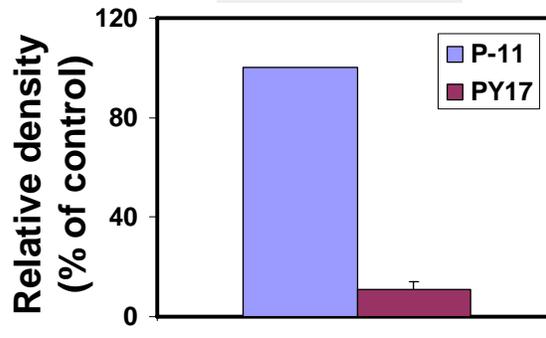
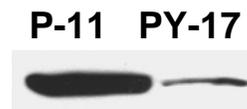


Figure 2

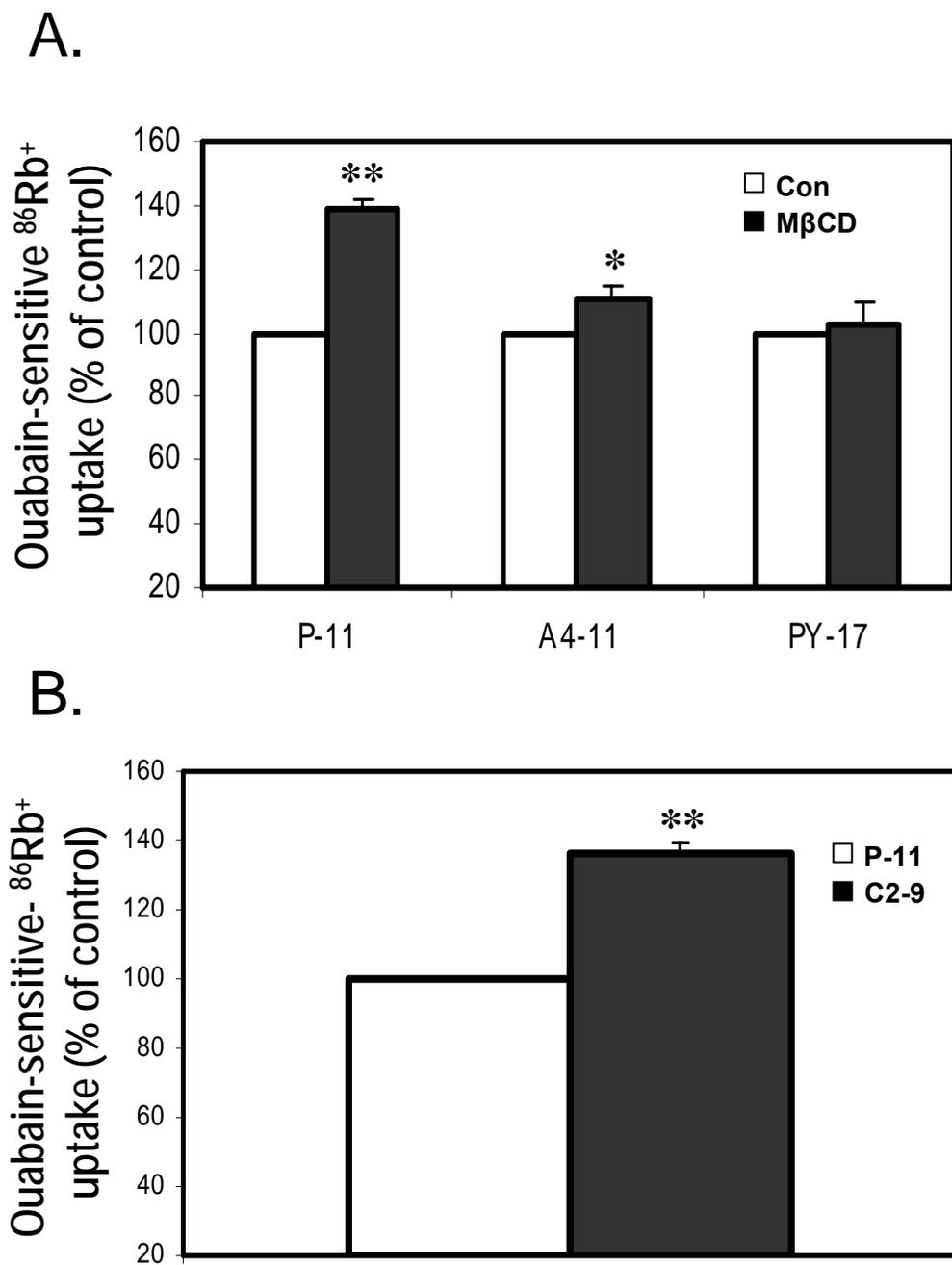
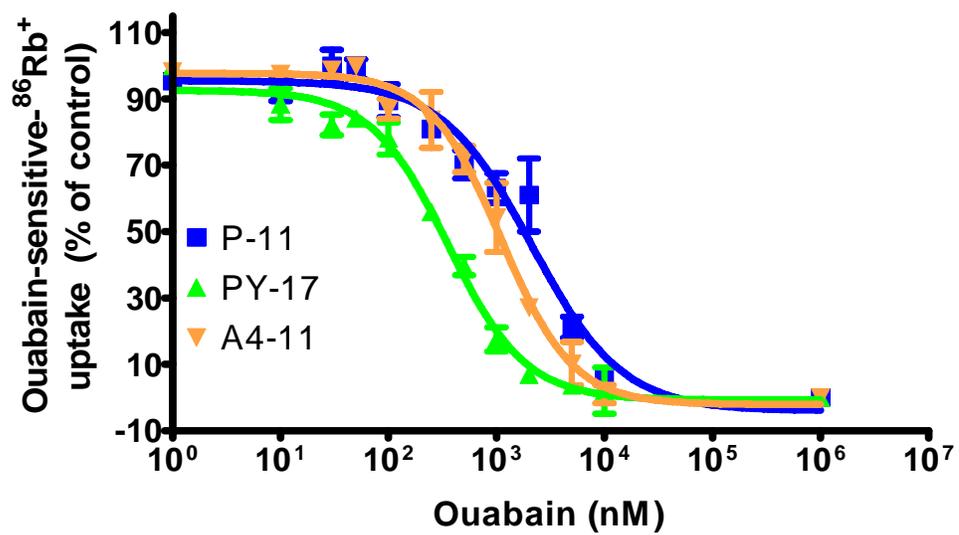


Figure 3



	P-11	PY-17	A4-11
Ouabain EC ₅₀ (nM)	2131	356.9	1101

Figure 4