

ROLE OF THE Na,K-ATPase IN POLYCYSTIC KIDNEY DISEASE

BY

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Dedicated with great love and respect to my family

Tin and Canh Nguyen

Sam and Alison Sul

Anh and Johnny Vinh & family

Thuylien Duong & family

Ngoc-Lan and Jon Knoff & family

Trung Nguyen

Tung Nguyen

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## **ABSTRACT**

Autosomal dominant polycystic kidney disease (ADPKD) is the most common monogenic disease, and is characterized by multiple fluid-filled cysts that impair the organ, ultimately leading to renal failure. Formation and enlargement of the cysts require abnormal proliferation and cell death, as well as changes in the transport properties of the renal tubule epithelial cells. Because of its primary role in the vectorial movement of salt and water in the kidney, the Na,K-ATPase has been the focus of investigation to understand the pathophysiology of ADPKD. However, the precise role of the transporter has not been identified. In this dissertation, we describe studies designed to characterize the Na,K-ATPase in ADPKD, and examine the mechanisms underlying its role in the disease.

The transport properties of the Na,K-ATPase is regulated by the hormone ouabain, which inhibits its movement of the cations. In addition, ouabain binding to the Na,K-ATPase has been found to activate a cascade of phosphorylating events, leading to cell growth. Interestingly, we have found a population of the Na,K-ATPase in human cystic epithelial cells to have a higher affinity for ouabain, at concentrations consistent with circulating levels of the hormone. Thus, nanomolar concentrations of ouabain that do not normally affect the activity of the Na,K-ATPase in the kidney partially inhibit the enzyme of cystic tissue and cells. The Na,K-ATPase has been found to interact with polycystin-1, and we have found this to association to increase the sensitivity of the enzyme to ouabain.

Our hypothesis is that due to their increased sensitivity to the hormone in ADPKD, the cystic renal epithelium is more susceptible to the effects of endogenous ouabain. We found ouabain to stimulate both proliferation and apoptotic death of the ADPKD cells, causing a disbalance that favors increased cell growth. While the mitogenic effect of ouabain is mediated by activation of the epidermal growth factor receptor (EGFR), Src kinase and extracellular signal-regulated kinase (ERK) pathway, its apoptotic effect was found to be through activation of the intrinsic pathway of apoptotic cell death. We also found ouabain to exacerbate the development and growth of cysts, both in cultured human ADPKD cells and metanephric organ cultures from *Pkd1<sup>m1Bei</sup>* mice, a well characterized model of ADPKD. Collectively, these results demonstrate ouabain, acting through the Na,K-ATPase, is a novel agent that can adversely affect the progression of ADPKD.



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## CHAPTER 1

### INTRODUCTION

#### *Na,K-ATPase*

The Na,K-ATPase or Na pump is a plasma membrane enzyme that establishes and maintains the high external  $\text{Na}^+$  and high intracellular  $\text{K}^+$  concentrations typical of most animal cells [1]. By using the energy from the hydrolysis of ATP, it transports 3  $\text{Na}^+$  out in exchange for 2  $\text{K}^+$  that are taken into the cell [2, 3]. The Na,K-ATPase plays a key role in numerous cell processes that depend directly or indirectly on the transmembrane gradients of  $\text{Na}^+$  and  $\text{K}^+$ . In this manner, the enzyme is essential in maintaining cell osmotic balance, volume and pH, in maintaining the cell resting membrane potential, and in creating the inward gradient of  $\text{Na}^+$  that provides the chemical energy for the secondary  $\text{Na}^+$ -coupled transport of other ions, solutes, and water across the cell membrane [1, 4, 5]. In the kidney, the adequate function of the Na,K-ATPase at the basolateral membrane of the tubular epithelial cells is essential for reabsorption of  $\text{Na}^+$  and water [1].

Structurally, the Na,K-ATPase consists of distinct molecular forms of two main polypeptides, the  $\alpha$  and  $\beta$  subunits [6, 7]. The  $\alpha$  polypeptides constitute the catalytic subunit of the Na,K-ATPase, directly participating in the ion transport and catalytic activity of the enzyme [6]. The  $\alpha$  isoforms are ten membrane-spanning proteins that contain the binding sites for  $\text{Na}^+$ ,  $\text{K}^+$  and ATP. In addition, the  $\alpha$  isoforms bind cardenolides, such as ouabain [2]. The  $\beta$  subunits are single membrane spanning glycoproteins that are important during synthesis of the Na,K-

ATPase, controlling the proper folding and trafficking of the  $\alpha$  subunit to the plasma membrane [8-10]. An interesting characteristic of the Na,K-ATPase is its molecular heterogeneity. Four structural variants of the  $\alpha$  ( $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\alpha 4$ ) and three different  $\beta$  ( $\beta 1$ ,  $\beta 2$  and  $\beta 3$ ) isoforms that are the product of different genes have been discovered in mammalian tissues [11-13]. Association of the various  $\alpha$  and  $\beta$  polypeptides in different heterodimers gives rise to multiple Na,K-ATPase isozymes, each of which exhibit a particular tissue localization and unique functional characteristics [13-17]. The  $\alpha 1$  isoform is expressed in almost every cell, while the other  $\alpha$  polypeptides show a tissue specific pattern of expression [13]. The  $\alpha 2$  isoform predominates in adipocytes, muscle, heart and brain [18-24],  $\alpha 3$  is abundant in nervous tissues [22, 24, 25], and the  $\alpha 4$  isoform is a sperm-specific isoform [26]. Also, the  $\beta$  isoforms are distributed in a tissue-dependent manner. The  $\beta 1$  polypeptide is widely distributed [13],  $\beta 2$  is found in skeletal muscle, pineal gland and nervous tissues [27, 28], while  $\beta 3$  is expressed in testis, retina, liver and lung [29]. The pattern of Na,K-ATPase isoform expression is subjected to developmental as well as hormonal regulation and can be altered during disease [13, 30, 31]. Na,K-ATPase isozymes composed of different  $\alpha$  isoforms have unique kinetic properties reflected by their differential affinities for the transported ions,  $\text{Na}^+$  and  $\text{K}^+$ , the substrate, ATP, and ouabain.

Also, the activity of the various Na,K-ATPases exhibit a distinctive response to a series of physiological stimuli, such as hormones and autacoids. The differences in expression, activity and regulation of the Na,K-ATPase isoforms

appear to be essential in adapting cellular Na,K-ATPase activity to the specific physiological requirements of each cell. Current information suggests that the  $\alpha 1\beta 1$  isozyme functions as the housekeeping Na,K-ATPase maintaining the basic  $\text{Na}^+$  and  $\text{K}^+$  homeostasis in the cell, whereas the other isozymes mediate tissue-specific roles. Importantly, the  $\alpha 1\beta 1$  heterodimer constitutes the only Na,K-ATPase present in normal kidney and may be the isozyme primarily suited for salt and water transport in this organ [1, 13].

#### ***Na,K-ATPase as a signal transducer of ouabain effects***

Several transport molecules have been shown to associate with an array of proteins, including kinases and phosphatases, cytoskeletal tethers and adaptor proteins to form regulatory complexes in the cell. Some of these protein complexes are involved in signal transduction events that extend beyond the plasma membrane to transmit signals into the cell [32]. Interestingly, a role in signal transduction has also been reported for the Na,K-ATPase [33-37]. The Na,K-ATPase is the natural receptor for cardenolides, a family of steroidal substances that share the unique property of binding to the external surface of the  $\alpha$  subunit of the Na,K-ATPase [6, 7]. One of the most common cardenolides, extensively used as a tool to specifically define Na,K-ATPase activity in a sample, is ouabain. At high millimolar concentrations, ouabain has a toxic effect, inhibiting the cycling, transport and catalytic activity of the total Na,K-ATPase in the cell [2, 3]. In contrast, low ouabain concentrations (in the nanomolar range), exerts other effects that are

independent from the inhibition of the ion transport function of the Na,K-ATPase. In several cell types, ouabain-Na,K-ATPase binding triggers a cascade of cell events that include interaction of the enzyme at the plasma membrane with neighboring proteins and the activation of the tyrosine kinase Src [38, 39]. This subsequently causes the recruitment and phosphorylation of a series of proteins including the mitogen activated protein kinases (MAPK), the extracellular regulated kinase, ERK pathway, and serine and tyrosine protein kinases [40]. The process ultimately results in regulation of several cell processes, including cell motility and growth [34, 41, 42].

For many years, ouabain was thought to be strictly a product of plants. However, the compound was more recently discovered as an endogenous substance present in nanomolar amounts in the fluids of several mammals, including man [41, 43-46]. Ouabain is synthesized in the adrenal glands and is released to the circulation by several stimuli, such as increase  $\text{Na}^+$  levels, hypoxia and physical exercise [42, 45]. In addition, elevated levels of ouabain have been found under a number of conditions, such as chronic renal failure, hyperaldosteronism and preeclampsia [42].

### ***Na,K-ATPase regulation***

Due to the central role of the Na,K-ATPase in fluid movement across the renal epithelium, the enzyme is subjected to strict regulatory mechanisms. These mechanisms act at different levels to produce both acute and chronic changes in

Na,K-ATPase activity. Long-term regulation of Na,K-ATPase function depends on changes in the transporter expression at the cell plasma membrane through changes in the rate of synthesis/degradation or redistribution with intracellular stores [13, 47]. Short-term regulation of Na,K-ATPase is achieved through direct modification of the activity of the enzyme [13]. This is caused by interaction of the enzyme with several ligands, mainly ions, such as  $\text{Na}^+$  and  $\text{Ca}^{+2}$ . In addition, another important mechanism to regulate Na,K-ATPase transport function is interaction with other proteins [13]. Use of the yeast two-hybrid system and co-immunoprecipitation assays have identified a series of proteins that associate with the Na,K-ATPase. Interestingly, among these proteins is polycystin-1 (PC-1), one of the polypeptides known to be altered in polycystic kidney disease.

### ***ADPKD as a disease***

ADPKD is one of the most common, potentially lethal inherited disorders, with a prevalence of 1 in 500 to 1,000 births nationwide. ADPKD afflicts more individuals than cystic fibrosis, sickle cell anemia, hemophilia, muscular dystrophy, Down's syndrome and Huntington's disease combined. Although ADPKD is a systemic disorder with multiple manifestations, including hypertension, renal stones, and extrarenal cysts in the pancreas, liver and intestines, its most prominent feature is the formation of numerous fluid-filled cysts that originate from the epithelial cells within all nephron segments. Progressive expansion of the cysts causes a 4-8 fold enlargement of the kidney that grossly distorts the structure of the



renal parenchyma and reduces its function. Approximately one half of ADPKD patients progress to chronic renal failure and require dialysis or kidney replacement by the age of 60, and ADPKD is responsible for approximately 8-10% of all cases of end-stage renal failure [48-51]. End stage renal disease caused by polycystic kidney disease (PKD) alone costs the federal government more than \$2 billion per year in Medicare and Medicaid costs. The burden caused by ADPKD and its economic costs fuels the need to understand the molecular basis of the disease to find appropriate interventional therapies that can slow or stop its progression.

### ***Etiology of ADPKD***

ADPKD is caused by mutations in the *Pkd1* and *Pkd2* genes, which encode for polycystin-1 (PC-1) and 2 (PC-2) respectively [50, 52]. PC-1 is a 450,000-460,000 kDa glycoprotein composed of a large extracellular region that has adhesion and protein-protein interaction domains, 11 transmembrane spanning domains, and a short C-terminal portion involved in cell signaling[53, 54]. On the other hand, PC-2 is a 6-membrane spanning protein that appears to function as a  $\text{Ca}^{+2}$  permeable non-selective cation channel [55]. Although the precise functions of PC-1 and PC-2 are not known, current evidence suggests that PC-1 and PC-2 interact to form a macromolecular signaling system that acts as a mechanosensor capable of receiving extracellular signals and transducing them into cellular responses that regulate fundamental aspects of renal epithelial development and cell biology [55]. Mutations in PC-1 and PC-2, or an imbalance in expression of either

protein results in deregulation of signaling pathways that are important for the control of renal epithelial proliferation, migration, differentiation and nephron tubular morphogenesis [48, 56].

In addition to the genetic causes of ADPKD, non-genetic factors such as pharmacological agents and compounds circulating in blood play an important role in influencing the rates of cell proliferation and fluid secretion by the renal epithelium, contributing to the development and progression of renal cysts [57-60]. The role of non-genetic factors explains in part the high degree of variability in the age of onset and progression of the disease, even among family members that carry the same mutation.

### ***ADPKD and renal cystogenesis***

Studies in humans and a variety of experimental models of PKD implicate several major factors in the formation and maintenance of renal cysts. One is the abnormal proliferation or hyperplasia of the tubular cells; the second is the change in the salt and fluid transport properties of the epithelium, which modifies its reabsorptive capacity to favor secretion of fluid into the lumen of the vesicles [60, 61]. The first event in cystogenesis appears to be the uncontrolled cell growth, with the subsequent expansion of the tubular renal epithelium that constitutes “blister like” structures. These structures eventually pinch off and lose contact with the parent nephron from which they originated. Cysts then continue to develop, increasing their size through the combined effect of proliferation of the cells

surrounding the cyst, and the continuous secretion of fluid by the epithelium that accumulates and enlarges the cyst cavity [62, 63]. Agents such as cAMP, steroids and epidermal growth factor (EGF) have been shown to alter growth as well as ion and water transport in ADPKD [58, 59, 64-66].

In addition to abnormal cell proliferation and fluid transport, apoptotic cell death has been shown to be dysregulated in PKD. Apoptosis involves a complex and highly regulated process of programmed cell death that is important during normal embryonic development and maintenance of normal adult tissues [67]. Both proliferation and apoptosis have been reported to be up-regulated in the cystic epithelium [51], and inhibition of apoptosis has been shown to slow development of the disease [68]. This suggests that an imbalance in proliferation and apoptosis may promote cystogenesis and progression of PKD.

At present, the precise mechanisms that cause the changes in cell growth and fluid transport characteristic of ADPKD are unknown. Although the genetic factor is important, it remains unclear how it contributes to the pathophysiology of the disease. Several circulating agents have been identified to have a crucial role in progression of ADPKD. Understanding the factors and mechanism(s) involved in formation and maintenance of renal cysts in ADPKD is essential in predicting the outcome of the disease, as well as identifying targets for future development of pharmacological approaches to treat this illness.

### *Specific aims of this work*

The major objective of this study was to determine the role of the Na,K-ATPase in ADPKD. Previous results from our laboratory have shown that the Na,K-ATPase of animal models of PKD, such as the Han:SPRD cy/cy rat and cpk mouse, has an abnormally high affinity for ouabain. Therefore, it is possible that this will render the renal epithelial cells more susceptible to the effect of the hormone ouabain. The overall hypothesis motivating this work is the high ouabain-sensitive phenotype causes ADPKD cells to be more prone to the effects of circulating levels of the hormone; endogenous ouabain excessively stimulates Na,K-ATPase signaling to cause hyperplastic growth and fluid secretion by the renal epithelium. We reasoned that ouabain represents a novel factor capable of exacerbating the development of ADPKD renal cysts, thus contributing to the progression of the disease.

The following specific aims were directed to elucidate our hypothesis:

1. Determine the role of ouabain/Na,K-ATPase signaling in proliferation of human renal epithelial cells from patients with ADPKD (Chapter 2).
2. Identify the signaling pathway involved in the ouabain-induced proliferation of ADPKD cells (Chapter 3).
3. Examine the effects of ouabain on apoptosis of ADPKD cells (Chapter 4).
4. Assess the influence of ouabain on fluid secretion by the ADPKD epithelium (Chapter 5).

5. Establish the role of PC-1 in modulating the ouabain affinity and signaling function of the Na,K-ATPase (Chapter 6).

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## CHAPTER 2

### OUABAIN INDUCES PROLIFERATION IN ADPKD CELLS

#### ABSTRACT

In autosomal dominant polycystic kidney disease (ADPKD) cyst formation and enlargement requires proliferation of mural renal epithelial cells and the transepithelial secretion of fluid into the cyst cavity. Na,K-ATPase is essential for solute and water transport in ADPKD cells, and ouabain blocks fluid secretion in these cells. By binding to the Na,K-ATPase, ouabain also induces proliferation in some cell types. Surprisingly, we found that nanomolar concentrations of ouabain, similar to those circulating in blood, induced ADPKD cell proliferation, but had no statistically significant effect on normal human kidney cells (NHK). Ouabain, acting from the basolateral side of the cells, also caused an increase in the level of phosphorylated extracellular regulated kinases (ERK). MEK inhibitor U0126 blocked ouabain-induced ERK activation and cell proliferation, suggesting that the effect of ouabain is mediated through the MEK-ERK pathway. In contrast to NHK cells, the dose response curve for ouabain inhibition of Na,K-ATPase activity indicated that approximately 20% of the enzyme in ADPKD cells exhibits a higher affinity for ouabain. The increased ouabain affinity of ADPKD cells was not due to differences in Na,K-ATPase isoform expression since these cells, like NHK cells, only possess the  $\alpha 1$  and  $\beta 1$  subunits. The  $\gamma$  variants of the Na,K-ATPase are also expressed in the cells, but are elevated in ADPKD cells. Currently, the basis for the differences in ouabain sensitivity of NHK and ADPKD cells is unknown. We

conclude that ouabain stimulates proliferation in ADPKD cells by binding to the Na,K-ATPase with high affinity, and via activation of the MEK-ERK pathway.

## INTRODUCTION

The Na,K-ATPase is an enzyme of the plasma membrane of most cells that uses cellular ATP to exchange cytoplasmic  $\text{Na}^+$  for extracellular  $\text{K}^+$  [1]. The function of the Na,K-ATPase is essential for the generation and maintenance of the electrochemical gradients that drive the secondary movement of solute and fluid across renal epithelia [2]. Structurally, the Na,K-ATPase is an oligomer composed of distinct molecular forms of two major polypeptides, the  $\alpha$  and  $\beta$  subunits [3]. At present, four structural variants of the  $\alpha$  polypeptide ( $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\alpha 4$ ), and three  $\beta$  ( $\beta 1$ ,  $\beta 2$  and  $\beta 3$ ) subunits have been identified in mammals. Association of the  $\alpha$  and  $\beta$  polypeptides in different oligomers results in multiple isozymes of the Na,K-ATPase that have unique functional properties, and a tissue-specific pattern of expression [3]. The  $\alpha 1$  and  $\beta 1$  isoforms constitute the Na,K-ATPase isozyme ubiquitously expressed in tissues and the prevalent isozyme in kidney [2-4]. In addition to the  $\alpha$  and the  $\beta$  subunits, the renal Na,K-ATPase contains a third subunit, the  $\gamma$  polypeptide that exists as two different splice variants,  $\gamma a$  and  $\gamma b$  [5].

Cardiotonic steroids comprise a family of compounds that bind to the  $\alpha$  subunit of the Na,K-ATPase to inhibit the function of the enzyme. These compounds are clinically used to increase the force of heart contraction and cardiac output in patients with heart failure [6, 7]. Ouabain, a cardiotonic steroid has been shown to be an endogenous factor secreted by the adrenal glands in humans and other mammals, and is present in blood at nanomolar concentrations [8]. The mechanisms of action of



ouabain have been classically attributed to ion changes that are secondary to inhibition of the catalytic and transport activity of the Na,K-ATPase [6].

More recently, it has been shown that ouabain also induces proliferation of several cell types, including myocardial cells, smooth muscle cells, astrocytes, and renal proximal tubule cells [9-14]. The mechanisms for the mitogenic effect of ouabain are not completely understood. In myocardial cells, binding of ouabain to the Na,K-ATPase activates Src kinase and tyrosine phosphorylation of proteins including the epidermal growth factor receptor (EGFR). Transactivation of EGFR then leads to a cascade of phosphorylating events that include the mitogen activated protein kinase kinase MEK and extracellular regulated kinases (ERK) [15]. Activation of the MEK-ERK pathway results in expression of genes involved in cell growth [15].

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the formation and progressive enlargement of cysts in the kidney that disrupt the structure and severely compromises renal function in about half of the patients [16, 17]. In ADPKD, mutations of at least two genes, *Pkd1* and *Pkd2* that encode for polycystin 1 and 2 respectively, initiate cyst formation. However, the progressive enlargement of cysts appears to be regulated by a variety of factors, including growth hormones and cAMP agonists [18-20]. Arginine vasopressin (AVP) and epidermal growth factor (EGF) stimulate cell proliferation of human ADPKD cells through activation of the MEK-ERK pathway [21-24]. Cyclic AMP causes cell proliferation in human cells and animal models of polycystic kidney disease, also via the MEK-

ERK pathway, through B-Raf, a kinase that phosphorylates and activates MEK [25, 26]. In addition, cyst expansion is the result of transepithelial fluid secretion driven by the cAMP-dependent secretion of  $\text{Cl}^-$  [27]. Because of its central role in salt and water transport, the Na,K-ATPase has been the focus of investigation to understand the pathophysiology of PKD [17]. Relatively high concentrations of ouabain added to basolateral, but not apical, membranes of ADPKD cell monolayers [28] and intact cysts dissected from ADPKD kidneys [24] have been shown to inhibit fluid secretion. However, the effect of low concentrations of ouabain, similar to those circulating in blood, on renal cyst growth has not been examined.

In the present study, we investigated the effect of physiological concentrations of ouabain on the growth and MEK-ERK pathway of cells derived from normal human kidneys and from renal cysts of patients with ADPKD. This is the first report to demonstrate that human ADPKD cells present an abnormally high sensitivity to ouabain, and that the steroid stimulates proliferation of ADPKD cells at a higher rate than NHK cells via activation of the MEK-ERK pathway.

## METHODS

### *Cell culture*

Primary cell cultures derived from nephrectomy specimens of normal human kidney cortex (NHK cells) or surface cysts of ADPKD kidneys (ADPKD cells) were generated by the PKD Biomaterial Core at University of Kansas Medical Center (KUMC). A protocol for the use of discarded human kidney tissues was approved by the Institutional Review Board at KUMC. Primary cultures were prepared as described [29]. Cells were seeded and grown in DME/F12 supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin G and 0.1 mg/ml streptomycin, 5  $\mu$ g/mL insulin, 5  $\mu$ g/mL transferrin, and 5 ng/mL sodium selenite (ITS). Cells were cultured on both filter supports (Transwell Costar, Corning, NY) and on plastic culture dishes. When the cells were grown to confluency on filter supports, cell polarity of the cultures was confirmed by the achievement of a transepithelial electrical resistance by the cell monolayer, measured with an EVOM<sup>TM</sup> voltohmmeter (World Precision Instruments, Sarasota, Fl) as described [30]. In culture, both ADPKD and NHK cells are able to establish a monolayer, form tight junctional complexes, develop a transepithelial electrical potential difference and carry out an active transepithelial transport of salt and fluid [27, 29, 31]. As previously shown, these cells are epithelial in nature and the majority of the cells stain positive for specific lectin markers for the collecting duct and distal nephron, such as *Arachis hypogaea* (PNA) and *Dolichos biflorus agglutinin* (DBA) [25]. Altogether, this suggests that both cell types are enriched in cells derived from distal

nephrons and collecting tubules. In agreement with this observation, aquaporin-2, a marker for collecting tubules, was detected by immunoblot analysis in both NHK and ADPKD cells (Figure 1).

### *Biochemical Assays*

Protein assays were performed using the dye-binding assay from Bio-Rad (Hercules, CA). Na,K-ATPase activity was determined on homogenates of NHK and ADPKD cells grown to confluency on filter supports or plastic dishes. Specific ATP hydrolysis was determined by measuring the initial rate of release of  $^{32}\text{P}_i$  from  $\gamma[^{32}\text{P}]\text{-ATP}$  for 30 min at  $37^\circ\text{C}$  in a final volume of 0.25 mL of medium containing 120 mM NaCl, 30 mM KCl, 3 mM  $\text{MgCl}_2$ , 0.2 mM EGTA, 1 mM sodium azide, 30 mM Tris-HCl (pH 7.4), 3 mM ATP with 0.2  $\mu\text{Ci}$   $\gamma[^{32}\text{P}]\text{ATP}$ , in the absence and presence of different ouabain concentrations [32].

### *Reverse transcriptase-polymerase chain reaction (RT-PCR) Analysis*

Total RNA was isolated using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). Complementary DNA was generated by reverse transcription using the SuperScript<sup>TM</sup> First-Strand Synthesis System (Invitrogen Corp., Carlsbad CA) and oligo (dT) primers as described [32]. The resulting first-strand cDNA was amplified using Na,K-ATPase isoform specific primers, and under PCR conditions that assure no cross-reactivity among the Na,K-ATPase isoforms. The sequences of the primers used, their annealing properties and the size of the amplified cDNAs are described in

Table 1. The amplified DNA fragments were identified by electrophoresis in a 1% agarose gel stained with ethidium bromide.

#### *Immunoblot analysis of Na,K-ATPase isoforms*

Cell proteins (30  $\mu$ g) from homogenates of NHK and ADPKD cells grown to confluency on filter supports or plastic were analyzed by 8% SDS-PAGE for the  $\alpha$  and  $\beta$  isoforms, or 16% tricine gels for the  $\gamma$  subunit and blotted onto nitrocellulose membranes (Osmonics Inc, Minnetonka, MN) as described [32, 33]. Primary antibodies specific for each Na,K-ATPase isoform were incubated overnight at 4°C. Horseradish-peroxidase conjugated secondary antibodies and chemiluminescence was used for detection.

#### *Measurement of cell proliferation*

NHK and ADPKD cells (4,000 cells/well) were seeded onto a 96-well plate with culture medium supplemented with 1% FBS and ITS. After 24 h, ITS was removed and the serum concentration was reduced to 0.002%. After an additional 24 h, the cells were treated with control medium or with various concentrations of ouabain for 24 h. Cell proliferation was determined using the Promega CellTiter 96 MTT Assay according to the manufacturer recommendations (Promega Corp., Madison, WI). This method is based on the metabolism of a tetrazolium salt by the cells, and the optical density of the formazan product formed is proportional to the metabolic activity, and the number of cells in the culture [34]. Proliferation rates

were also determined by counting cells with a hemocytometer. In all proliferation assays, data are presented as a percent of proliferation of untreated cells.

#### *Measurement of cell mitosis*

Cells ( $1 \times 10^5$ ) were seeded onto glass coverslips in culture medium supplemented with 1% FBS and ITS for 24 h. After an additional 24 h in 0.002% FBS, subconfluent cell monolayers were treated with control medium or medium containing  $3 \times 10^{-9}$  M ouabain for 1, 3, 5, 8 and 24 h. The cells were then fixed in 100% methanol for 45 min at  $-20^\circ\text{C}$  and washed three times for 5 min each in 0.1% Triton X-100 in PBS. Samples were blocked for 1 h with 3% BSA in PBS and were incubated for 1 h with an antibody against Ser-10-phospho-Histone H3, a marker for mitosis (Upstate Biotechnology, Lake Placid, NY). Following three washes for 15 min each in 0.1% Triton X-100 in PBS, cells were incubated for 1 h with an Alexa 488 secondary goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Samples were stained with DAPI and mounted with SlowFade<sup>R</sup> mounting solution (Molecular Probes, Eugene, OR). Slides were analyzed using a Zeiss LSM510 confocal microscope. Data were expressed as number of green fluorescent cells relative to the total cells per unit area. At least 10 random fields were analyzed and a minimum of 1,000 cells per time point were counted.

#### *Immunoblot analysis of phosphorylated ERK levels*

NHK and ADPKD cells ( $1 \times 10^5$ ) were grown in 6-well plates under the same conditions as described above, and then treated with varying ouabain concentrations for 0, 15, 30, 60, 90, and 120 min. In some experiments, ouabain was added to either the apical or basolateral side of ADPKD cell monolayers grown on filter supports, to determine if the ouabain-induced ERK activation was unique to one membrane domain. In all cases, cells were washed with ice-cold PBS and lysed with 1% NP-40, 0.25% sodium deoxycholate, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF, 150 mM NaCl, 1 mM EDTA, 50 mM Tris, and protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The cleared lysates (15  $\mu\text{g}$  total protein) were analyzed by 10% SDS-PAGE and blotted onto nitrocellulose membranes. The immunoblots were first analyzed for levels of phosphorylated ERK, and then the membranes were stripped and probed for total ERK, using antibodies from Santa Cruz Biotechnology, Santa Cruz, CA. After detection by chemiluminescence, the images were scanned and quantified for band intensity using the Gel-Pro software (Media Cybernetics, Inc., Silver Spring, MD). Levels of P-ERK and total ERK were expressed as density units relative to the untreated controls, and presented as P-ERK to total ERK ratios.

#### *Immunocytochemistry of Na,K-ATPase in ADPKD cells*

Cells were grown on filter supports until they were confluent, confirmed by the development of transepithelial electrical resistance. Cells were fixed in 100% methanol for 45 min at  $-20^\circ\text{C}$  and were subjected to immunocytochemistry as described [30]. An anti- $\alpha 1$  monoclonal antibody (6F, Developmental Studies

Hybridoma Bank, University of Iowa) was used, followed by an Alexa Fluor 594 conjugated secondary antibody (Molecular Probes, Eugene, OR). Slides were analyzed using a Zeiss LSM510 confocal microscope. Images were acquired in Multitrack channel mode with LSM510 (v 3.2) software and a Plan-Apochromat 63X/1.4 Oil DIC objective with a frame size of 1024X1024 pixels and a zoom factor of 2. z line views were obtained by averaging 10 sections over a line at each z position in 1.0  $\mu\text{m}$  steps.

#### *Data analysis*

Curve fitting of the experimental points was performed using a Marquardt least-squares non linear regression computing program (Sigma Plot, Jandel Scientific, San Rafael, CA). Dose-response relations for the ouabain inhibition of Na,K-ATPase activity were fitted by equations assuming the presence of one, or two enzyme populations with different affinity for ouabain as previously described [32]. The validity of using a two versus a single component regression model for ouabain binding was statistically supported by applying the Snedecor's F test [35]. Statistical significance of the differences between ouabain treated and untreated controls was determined by ANOVA, while differences between normal and cystic cells were analyzed by Student's *t*-test. Statistical significance was defined as  $P < 0.05$ .



## RESULTS

### *Effect of ouabain on proliferation of NHK and ADPKD cells*

Ouabain binding to the Na,K-ATPase causes induction of various transcription factors and up-regulation of genes involved in cell growth [15]. To determine whether ouabain affects the proliferation of NHK and ADPKD cells, we measured the rate of cell proliferation in the presence of various concentrations of ouabain using a proliferation assay. Surprisingly, concentrations of ouabain as low as  $10^{-10}$  M and in the nanomolar range ( $1 \times 10^{-10}$  M to  $3 \times 10^{-8}$  M) significantly increased the rate of proliferation of ADPKD cells (30% to 40%) compared to ADPKD cells in control medium (Figure 2A). Concentrations below  $10^{-10}$  M had no significant effect, while concentrations at  $10^{-7}$  M and higher instead inhibited cell proliferation, corresponding with extensive inhibition of Na,K-ATPase activity [4, 19]. In contrast, nanomolar ouabain concentrations did not have a statistically significant effect on the proliferation of NHK cells. The ouabain effect on cell proliferation was also determined by direct cell count using a hemocytometer (Figure 2B). These data confirm that ouabain increased the proliferation of ADPKD cells in a dose dependent manner, but had no significant effect on the growth of NHK cells. The same results were obtained when the NHK cells were grown at a lower cell density (data not shown), suggesting that the lack of effect of ouabain on cell proliferation was not due to the extent of cell confluency, but rather depended on the particular response of the cells to the steroid.

To further examine the effect of ouabain on cell proliferation, we measured

the number of mitotic cells in cultures of NHK and ADPKD cells 1, 3, 5, 8 and 24 h following treatment with  $3 \times 10^{-9}$  M ouabain (Figure 3). Phosphorylation of histone H3 is a commonly used marker for cell division [11], which correlates with the chromosomal condensation that accompanies the onset of mitosis. Treatment with ouabain for 1 h increased the number of ADPKD cells that stained positive for phospho-histone H3 (% of total cell number) approximately two-fold compared to the untreated cells. The mitotic index (% of phospho-histone H3 positive cells) for ADPKD cells treated with ouabain increased three-fold at 3 h, and remained elevated throughout the 24 h incubation period (Figure 3B). In contrast, ouabain caused only a small increase in the mitosis of NHK cells at 3 h of incubation, with no significant difference between the ouabain treated and control groups at other timepoints (Fig. 3A). Altogether, these results demonstrate that nanomolar concentrations of ouabain promote the proliferation of human ADPKD cells grown in culture.

#### *Effect of ouabain on ERK activity in human ADPKD cells*

In several cell types, ouabain has been shown to stimulate cell growth through activation of the MEK-ERK pathway [15]. To determine if these mediators are involved in the ouabain induced proliferation of ADPKD cells, we measured the levels of phosphorylated ERK (P-ERK) in the cells after treatment with different concentrations of ouabain for various times, from 0 to 120 min. The effect of  $3 \times 10^{-9}$  M ouabain on P-ERK to total ERK ratios for NHK and ADPKD cells is

presented in Figures 4A and 4B. Consistent with the lack of an effect of ouabain on cell proliferation, ouabain did not significantly alter ERK activity in NHK cells (Figure 4A). In contrast, ouabain increased P-ERK levels in ADPKD cells within 15 min after treatment with the steroid (Figure 4B). ERK phosphorylation remained elevated during the following 60 min incubation and, although levels were slightly lower, significant amounts of P-ERK were still observed by 120 min. There was no change in P-ERK levels in ADPKD cells incubated with control medium without ouabain for each time point (data not shown). Similar time-course responses were observed at  $10^{-9}$ ,  $10^{-8}$  and  $3 \times 10^{-8}$  M ouabain (data not shown). Analysis of the relative levels of ERK activation by different concentrations of ouabain indicated a dose dependent relationship. Below concentrations of  $10^{-10}$  M, ouabain had no significant effect, while induction of ERK phosphorylation was detected with  $10^{-10}$  M ouabain, and P-ERK increased at ouabain concentrations between  $1 \times 10^{-9}$  M and  $3 \times 10^{-8}$  M. In contrast, higher ouabain amounts ( $10^{-7}$  M), known to significantly block Na,K-ATPase activity, failed to elevate P-ERK levels (Figure 4C). Altogether, these results suggest that ouabain induced ADPKD cell proliferation is mediated via ERK activation, and this response occurs within minutes of the addition of nanomolar concentrations of the steroid.

To determine whether ERK activation by ouabain occurs from the apical or basal side of the ADPKD cells, levels of P-ERK were determined in confluent cultures grown on filter supports. Apical application of ouabain had no effect on P-ERK levels. In contrast, ouabain applied to the basal compartment of the culture

chamber caused activation of ERK (Figure 5A). In addition, as was observed in the subconfluent cultures (Figures 2, 3 and 4), the NHK monolayer grown on filter support did not show ERK activation after treatment with ouabain, either when the steroid was applied from the apical or basolateral surface of the cells (Fig. 5B). In agreement with the effect of ouabain occurring through the Na,K-ATPase in ADPKD cells, immunocytochemical analyses of the  $\alpha 1$  isoform of the enzyme showed it to be restricted to the basolateral membrane domain of the ADPKD cells, a distribution that is similar to that of the normal kidney cells (Figure 5C). These results suggest that ouabain acts from the serosal side of ADPKD cells, and are in agreement with the notion that ERK activation is mediated by the Na,K-ATPase restricted to the basolateral membrane of the cells.

To confirm that the MEK-ERK pathway is central for ouabain-induced proliferation of ADPKD cells, we compared the effect of ouabain in the absence and presence of the MEK inhibitor U0126. We found that 1  $\mu$ M U0126 completely blocked the ouabain induced proliferation of ADPKD cells (Figure 6A). Accordingly, U0126 prevented the phosphorylation of ERK caused by ouabain (Figure 6B). Also, U0126 decreased ADPKD cell proliferation to levels lower than untreated controls. This suggests an important role of the MEK-ERK pathway in the growth of ADPKD cells. More importantly, the ability of U0126 to counteract the effects of ouabain strongly suggests that ouabain-induced proliferation in ADPKD cells is mediated through activation of MEK-ERK and not through another pathway.

*Ouabain sensitivity of Na,K-ATPase in NHK and ADPKD Cells.*

To determine whether the difference in the proliferative response of NHK and ADPKD cells to ouabain was due to differences in the affinity of the Na,K-ATPase to the steroid, we examined the ouabain kinetic response of the Na,K-ATPase of NHK and ADPKD cells. For this, dose-response curves for the inhibition of Na,K-ATPase activity by ouabain were determined (Figure 7). NHK cells exhibited a monophasic ouabain inhibition profile (Figure 7A), with a ouabain inhibition constant ( $K_i$ ) of  $0.48 \pm 0.05 \times 10^{-6}$  M, consistent with what has been described in normal kidney [3, 4, 36]. In contrast, ADPKD cells showed a heterogeneous response, with two Na,K-ATPase populations with different affinities for ouabain. Approximately 80% of the total Na,K-ATPase had a  $K_i$  for ouabain ( $1.3 \pm 2.0 \times 10^{-6}$  M) similar to that of normal kidney cells. Surprisingly, the remaining 20% of the enzyme showed a sensitivity for ouabain that was three orders of magnitude higher, with a  $K_i$  of  $1.7 \pm 4.7 \times 10^{-9}$  M (Figure 7B). The finding that an important fraction of Na,K-ATPase expressed in ADPKD cells has a high affinity for ouabain suggests that the enzyme would be more susceptible to binding the steroid at nanomolar concentrations such as those found in plasma.

*Na,K-ATPase isoform expression in NHK and ADPKD cells*

Generally, heterogeneous response to ouabain reflects the expression of

different isozymes of the Na,K-ATPase [3]. Normal kidney expresses predominantly one isozyme of the Na,K-ATPase, composed of  $\alpha 1$  and  $\beta 1$  isoforms [3, 4]. In the current study, the  $K_i$  for the more resistant Na,K-ATPase component observed in both the NHK and ADPKD cells is consistent with the ouabain kinetics corresponding to the  $\alpha 1\beta 1$  isozyme [36]. The high ouabain sensitive Na,K-ATPase unique to the cystic cells, suggested the presence of additional  $\alpha$  or  $\beta$  isoforms. To explore the expression pattern of the Na,K-ATPase  $\alpha$  and  $\beta$  isoforms in the kidney cells at the RNA level, we used RT-PCR employing the primer sets described in Table 1. Figures 8A and 8C show that cDNAs for only the  $\alpha 1$  and  $\beta 1$  isoforms were amplified from the normal and ADPKD cells. This agrees with previous results showing that only the  $\alpha 1\beta 1$  isozyme is present in kidneys [3]. The specificity of the Na,K-ATPase primers used was confirmed by the lack of cross reactivity with cDNAs of  $\alpha$  isoforms other than that containing the corresponding complementary sequence. In addition, PCR reactions performed in the absence of reverse transcription yielded no Na,K-ATPase isoform products, indicating the lack of genomic DNA contamination in the RNA isolation step (data not shown).

To determine the expression of the Na,K-ATPase isoforms at the protein level, immunoblot analysis of kidney cell total proteins was performed using antibodies specific for the  $\alpha$  and  $\beta$  isoforms. In agreement with the RT-PCR analysis, the protein profile of Na,K-ATPase subunits demonstrated that only the  $\alpha 1$  and  $\beta 1$  isoforms were present in NHK and ADPKD cells (Figures 8B and 8D). The  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\beta 2$  and  $\beta 3$  isoforms were absent, and only observed in human brain and

testis (used as positive controls), which express different combinations of the Na,K-ATPase isoforms (first lane in Figures 8A-D).

In addition, RT-PCR and immunoblot analysis was performed for the Na,K-ATPase  $\gamma$  subunit (Figures 8E and 8F). This showed that both  $\gamma_a$  and  $\gamma_b$  variants of the enzyme are expressed in the cells. Interestingly, relative to the ubiquitously expressed glyceraldehyde phosphate dehydrogenase (GAPDH), the levels of  $\gamma$  expression were higher in ADPKD than NHK cells, both at the RNA and protein levels. Based on the RT-PCR and immunoblot analysis, we conclude that NHK and ADPKD cells present similar Na,K-ATPase isozyme expression profiles and that the  $\gamma$  variants of the enzyme are up-regulated in ADPKD cells.

## DISCUSSION

The present results demonstrate that epithelial cells derived from renal cysts of patients with ADPKD have an abnormal mitogenic response to nanomolar concentrations of ouabain. This was reflected by an increase in cell proliferation as detected by the MTT assay, by an enhanced mitotic index, and by an increase in cell number. These findings provide new evidence that ouabain binding to the Na,K-ATPase not only modulates the ion transport of the enzyme, but also induces growth signals in cells [15]. The proliferative action of ouabain is apparent in ADPKD cells, and NHK cells are not significantly affected by the steroid. The NHK and ADPKD cells used in this study appear to be largely derived from distal convoluted and collecting tubules, based on lectin markers and by the existence of transport mechanisms expressed in those nephron segments ([25, 29, 31] and Figure 1). The similar origin of the cells suggests that the changes in ouabain response observed are a consequence of the cellular phenotype of normal renal epithelial cells versus mural cystic cells, and not due to differences in the progenitor cell type. The proliferative effects of ouabain have been described in various cell types, including normal proximal tubule cells of the rat kidney [11, 37]. Although we could not detect statistically significant differences in the NHK cells, we observed a trend for an increase in ouabain-dependent proliferation in these cells. The different magnitude in ouabain response reported for normal rat cells, compared to human cells may just reflect differences between species.



Sensitivity of the Na,K-ATPase to ouabain is of critical importance in the response to the cardiotonic steroid [3, 4]. In contrast to NHK cells, ADPKD cells express a population of Na,K-ATPase that has an abnormally high affinity for ouabain. The cause of the unique ouabain sensitivity phenotype of ADPKD cells was not due to the aberrant expression of Na,K-ATPase isoforms, since only the  $\alpha 1\beta 1$  isozyme was present in the cells. The  $\alpha 1$  isoform expression we observe agrees well with previous reports for normal and cystic human kidney [38]. Our results also confirm the expression of  $\beta 1$  in ADPKD. Although the potential for expression of the  $\beta 2$  subunit has been reported in human ADPKD kidneys [38], we were unable to detect it in the cultured cyst epithelial cells. It is well established that the binding sites for ouabain are located on the  $\alpha$  subunit, and the  $\beta$  polypeptide does not influence the ouabain binding kinetics of the Na,K-ATPase [3, 35]. Therefore, it is clear that the change in proliferative response to ouabain in the ADPKD cells was not due to expression of  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$  or the  $\beta$  isoforms. Interestingly, we found that while  $\gamma a$  and  $\gamma b$  are both present in NHK and ADPKD cells, the  $\gamma$  polypeptides are up-regulated in ADPKD cells. The  $\gamma$  subunit has been shown to be a regulator of Na,K-ATPase function and it influences the kinetic properties of the enzyme [33]. It is possible then, that the increased  $\gamma$  polypeptide content of ADPKD cells may favor its interaction with the enzyme and thus, the response to ouabain. Additional explanations for the particular ouabain affinity of ADPKD cells include the expression of a yet to be identified isoform of the Na,K-ATPase, or mutations in the  $\alpha 1$  polypeptide that render the enzyme more sensitive to ouabain. Alternatively,

ouabain affinity may depend on interaction between the Na,K-ATPase and other proteins. The large intracellular region of the Na,K-ATPase between transmembrane domains 4 and 5 has been shown to interact with the C-terminus of polycystin 1 [39, 40]. Interestingly, we found that, in insect cells exogenous expression of a construct containing the transmembrane and C-terminal domains of polycystin 1 increases the sensitivity of the Na,K-ATPase to ouabain, with the enzyme exhibiting an inhibition constant for the steroid that is similar to that found in ADPKD cells [41]. Therefore, Na,K-ATPase-protein interaction represents another potential mechanism involved in the response of the cells to ouabain. Despite the mechanisms involved, ADPKD cells express a pool of the Na,K-ATPase that is more susceptible to bind and transmit the mitogenic signal of ouabain.

Activation of the mitogen-activated protein kinase (MAPK) pathway plays a primary role in proliferation of ADPKD cells. For example, the epidermal and transforming growth factors (EGF and TGF $\alpha$  respectively) elicit mitogenic effect in ADPKD cells by the MEK-ERK pathway through binding to receptor tyrosine kinase [21-24], and cAMP promotes growth and secretion of ADPKD cells via B-Raf/MEK/ERK [21, 42]. Our results show that ouabain caused phosphorylation of ERK in ADPKD cells within 15 min of addition of the steroid, ERK phosphorylation was maximal at nanomolar ouabain concentrations, and inhibition of MEK blocked the effect. These results confirm the role of the MEK-ERK pathway in the mitogenic effect of ouabain, and identify MEK and ERK as mediators of ouabain proliferation in ADPKD cells [15]. The kinetics of ERK phosphorylation and cell proliferation

induced by ouabain are in good agreement. The relatively higher response in cell proliferation compared to P-ERK levels at  $10^{-10}$  M ouabain may reflect differences in the sensitivity of the experimental methods used, or it may indicate that submaximal ERK activation may be sufficient to cause a prominent effect on ADPKD cell proliferation.

Importantly, the ERK phosphorylation mediated by ouabain was observed not only in ADPKD cells that had not reached confluency (see Figures 2, 3, 4 and 6), but also in confluent cell cultures (see Figure 5), suggesting that the steroid is also capable of inducing growth of the cyst epithelium. A relatively lower ouabain dependent ERK phosphorylation level was found in confluent compared to subconfluent cultures. We reason that the culture filter support limits ouabain accessibility to the basolateral side of the cells, where the Na,K-ATPase is localized. Alternatively, different from the subconfluent cultures, the tight monolayer established by ADPKD cells, and the extensive formation of junctional complexes in the cultures may restrict the access of ouabain to the basolateral surface of the cells. In any case, both under subconfluent or confluent conditions, ADPKD cells exhibit a ouabain response that is not observed in NHK cells.

Importantly, ERK activation by ouabain in ADPKD cells is only elicited if the cardiotoxic steroid is applied to the basal side of the culture. Our observation that Na,K-ATPase expression is limited to the basolateral membrane domain of the cells agrees with previous observations [24, 28, 29], and with the notion that ouabain signal is transmitted through the Na,K-ATPase.

The effect of ouabain through its receptor, the Na,K-ATPase resembles the non-genomic actions of other steroids. Strong evidence now supports the idea that steroids can bind to cell surface proteins to trigger downstream signaling cascades in the cell [43]. Rapid effects of estrogen and aldosterone via plasma membrane transport mechanisms, such as  $\text{Ca}^{2+}$ - and voltage-activated  $\text{K}^+$  channels and the  $\text{Na}^+/\text{H}^+$  exchanger have been reported [44, 45]. The effect of ouabain in ADPKD cells is consistent with that observed in several other cell types, where ouabain binding to the Na,K-ATPase triggers ERK phosphorylation [15]. The presence of the lactone ring and sugar moieties in the steroidal structure of ouabain are important for specific binding to the Na,K-ATPase, the only known receptor to date for the compound [15, 46]. This, together with the presence of Na,K-ATPase high affinity ouabain sites, and the restricted basolateral effect of ouabain that coincides with the localization of the enzyme in ADPKD cells, strongly supports that ouabain dependent intracellular signaling in the cells occurs through the Na,K-ATPase.

The effect of ouabain on proliferation of ADPKD cells takes place at nanomolar concentrations. These ouabain amounts correspond to the levels of ouabain reported in blood [8]. Therefore, under physiological conditions, endogenous ouabain can readily activate the MEK-ERK pathway in the cyst cells of ADPKD kidneys, representing a factor that may directly promote the growth of the cysts. A variety of circulating factors, hormones and autacoids have been shown to stimulate the proliferation of renal cyst epithelial cells [21-23, 26, 27, 47, 48]. This study is the first to demonstrate that ouabain, an endogenous steroid hormone has a positive effect

on proliferation of ADPKD cells. Compounds that antagonize the action of ouabain are under development [49] and will represent an important tool for further understanding ouabain effect in ADPKD and for an eventual clinical intervention of the disease.

In conclusion, we found that ADPKD cells express a population of Na,K-ATPase that has a high affinity for ouabain, and respond to the steroid with an increase in proliferation mediated by the MEK-ERK pathway. The potential for endogenous ouabain as well as exogenous cardiotonic steroids to influence cyst mural cell growth is of clinical relevance for the progression of the disease.

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Figure 1

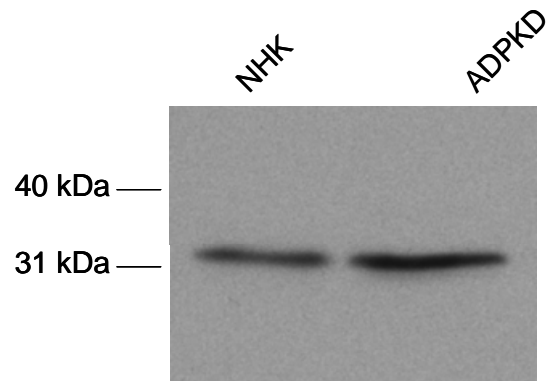


Figure 1. Expression of Aquaporin-2 in NHK and ADPKD cells. Proteins from normal and cystic cell homogenates were separated by SDS/PAGE, transferred to nitrocellulose and immunoblotted using an anti-Aquaporin-2 antibody (Sigma Chemical Co., St. Louis, MO). A horseradish peroxidase conjugated secondary antibody and chemiluminescence was used for detection.

Figure 2

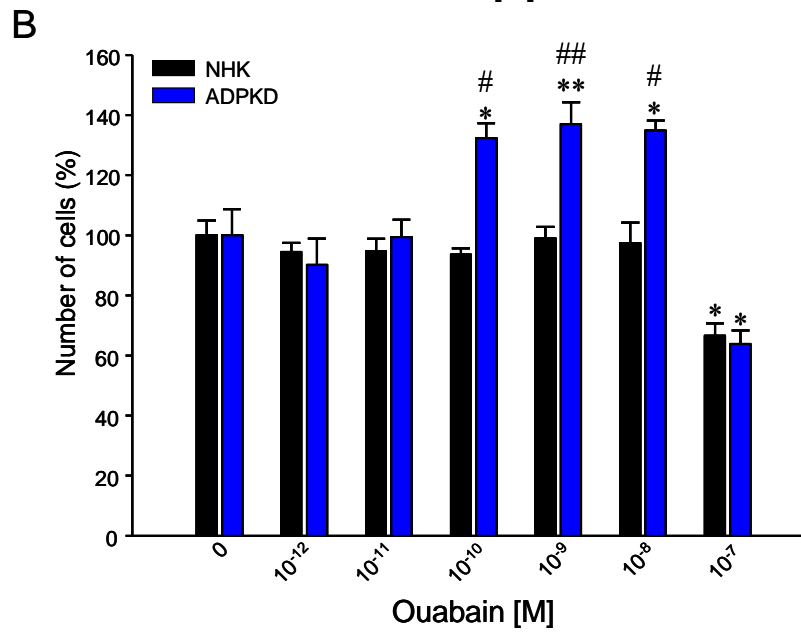
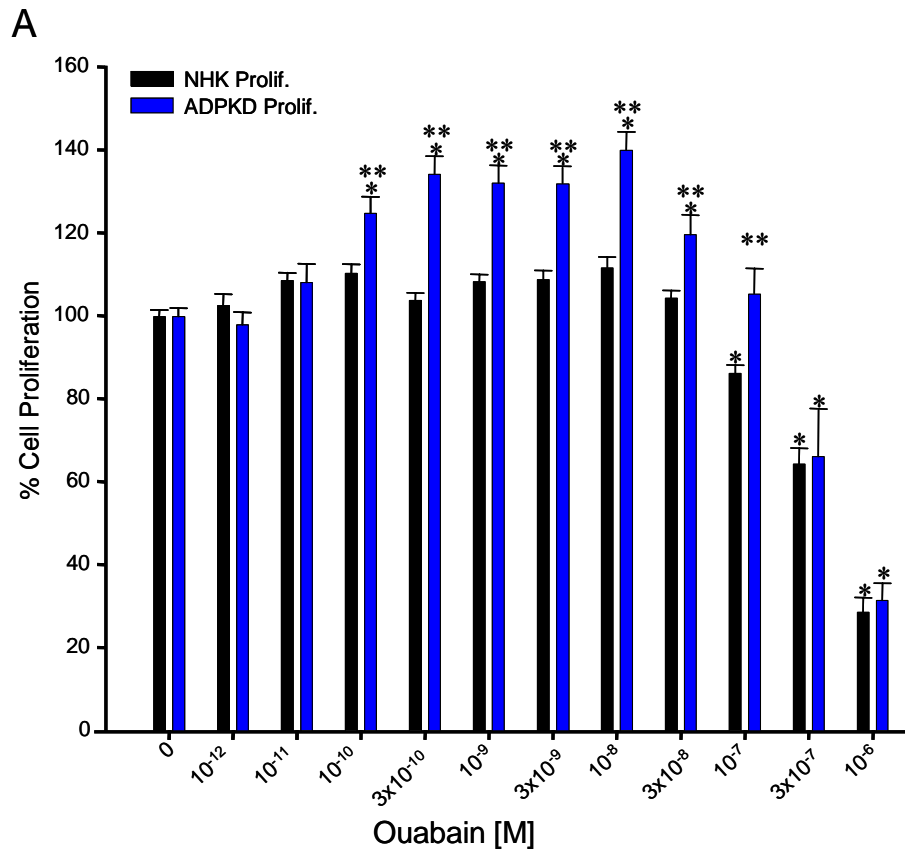


Figure 2. Effect of ouabain on NHK and ADPKD cell proliferation. (A) Cells were grown in 96 well plates, serum starved for 24 h and treated with control medium or media containing the indicated ouabain concentrations for an additional 24 h. Cell number was determined using the CellTiter 96 MTT assay. For ADPKD cells, data represent 18 experiments performed in sextuplicate on cells obtained from 8 different ADPKD kidneys. For NHK cells, data represent 15 experiments performed in sextuplicate on 7 different normal kidneys. Bars represent the mean  $\pm$  SE normalized to the respective untreated controls. Absolute optical density (OD) values at zero ouabain were  $0.23 \pm 0.04$  and  $0.15 \pm 0.01$  for NHK and ADPKD respectively. (\*)  $P < 0.001$  compared to untreated controls; (\*\*)  $P < 0.001$  compared to the corresponding values in NHK cells. (B) Cells were grown in 24 well plates, and treated without and with the indicated ouabain concentrations as mentioned in A. Cell number was determined with a hemocytometer. Data represent the mean  $\pm$  SE of 16-32 determinations performed in cells obtained from two kidneys. Average cell number at zero ouabain was  $68 \pm 5 \times 10^4$  and  $40 \pm 4 \times 10^4$  for NHK and ADPKD cells respectively. (\*)  $P < 0.05$ , and (\*\*),  $P < 0.001$  compared to untreated controls; and (#)  $P < 0.05$  and (##)  $P < 0.001$  compared to the corresponding values in NHK cells. For NHK cells, similar results were obtained at lower cell densities, such as those of the ADPKD cells. This was observed both with the CellTiter 96 MTT proliferation and cell count assays, in which the average OD and cell number values at zero ouabain were  $0.15 \pm 0.02$  and  $39 \pm 2 \times 10^4$  cells respectively (data not shown).

Figure 3

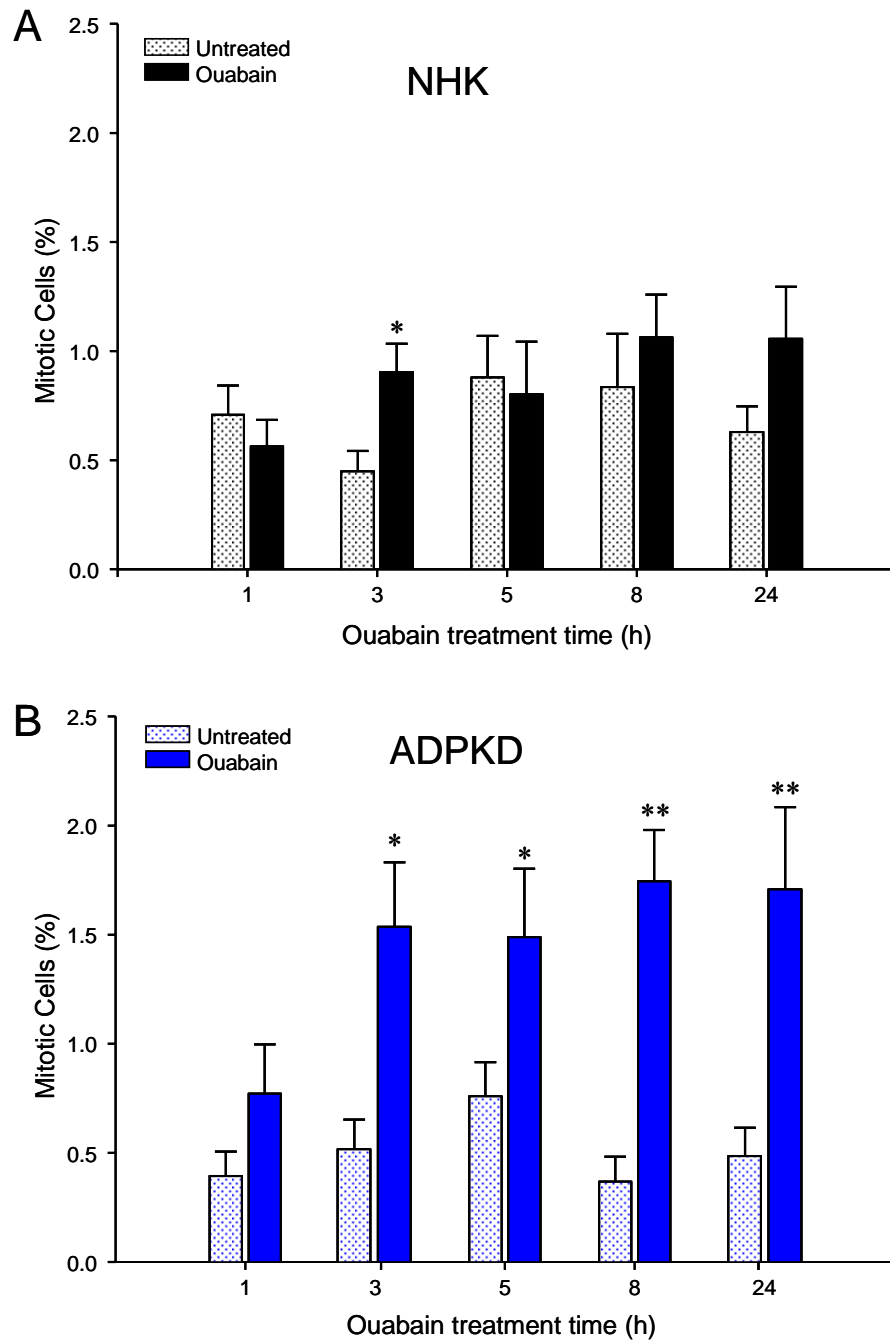




Figure 3. Ouabain effect on cell mitosis in (A) normal and (B) cystic kidney cells. NHK and ADPKD cultures grown at the same cell density were treated for the indicated times with  $3 \times 10^{-9}$  M ouabain and mitosis was determined by detection of phospho-Histone H3 using immunocytochemistry. The number of mitotic cells are presented as percent of phospho-histone H3 positive cell nuclei relative to total cell nuclei, identified by DAPI staining. Bars represent the mean  $\pm$  SE of 3 separate experiments (with at least 1,000 cells/time point analyzed per experiment) from 3 different kidneys. Asterisks indicates significant differences from the corresponding untreated controls, with \*,  $P < 0.05$ ; and \*\*  $P < 0.001$ .

Figure 4

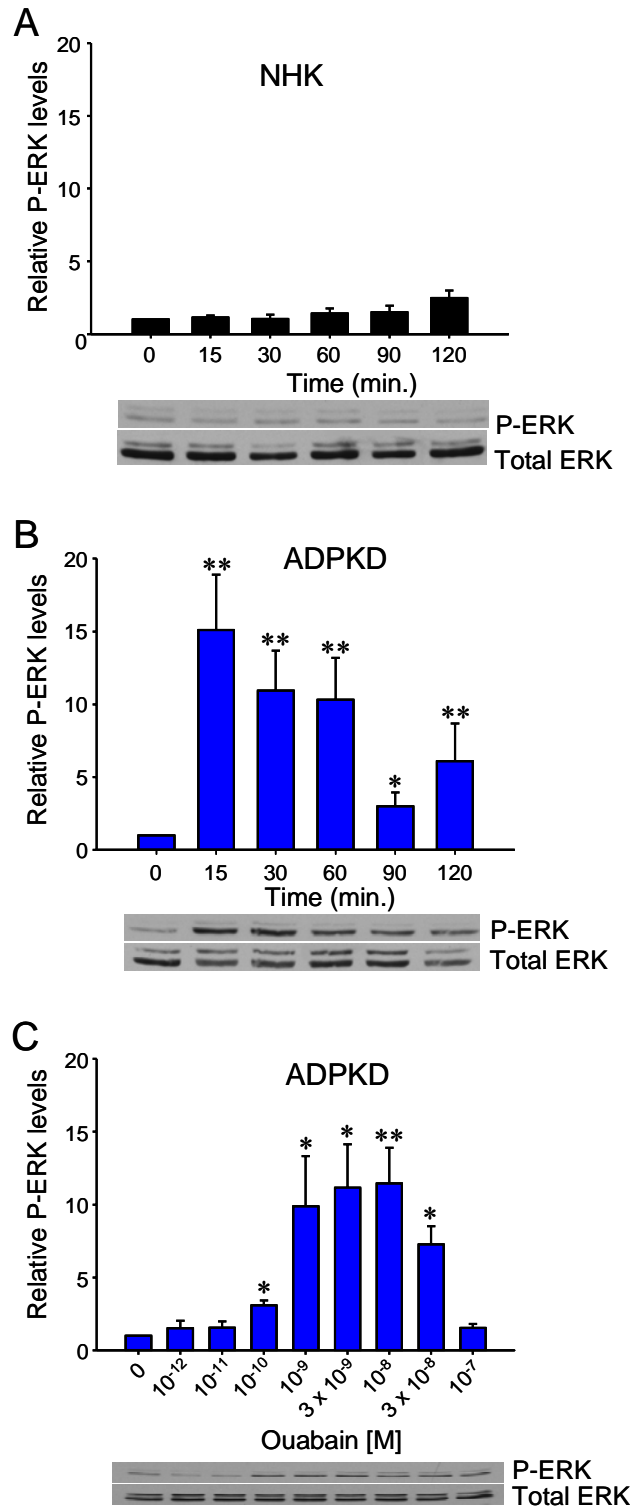


Figure 4. Ouabain activation of ERK in NHK and ADPKD cells. (A,B) time-course effect in NHK and ADPKD cells respectively. Same number of NHK and ADPKD cells were treated with  $3 \times 10^{-9}$  M ouabain for the indicated times. Vehicle controls without ouabain performed at each time point showed similar ERK phosphorylation levels as zero time. (C) Dose-response effect of ouabain in ERK phosphorylation in ADPKD cells. Cells were treated with the indicated ouabain concentrations for 15 min. Cells were lysed and cell proteins were subjected to immunoblot for determination of the total and phosphorylated forms of ERK. Representative immunoblots are shown. Bar graphs represent the densitometric analysis of the blots. Values are relative P-ERK/total ERK, presented as a ratio of the untreated controls. Bars indicate the mean  $\pm$  SE of six determinations from three different kidneys for NHK (A), ten determinations from three kidneys for ADPKD (B), or 3 to 12 determinations depending on ouabain concentration from two kidneys. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ . compared to zero time in B, or to untreated control in C.

Figure 5

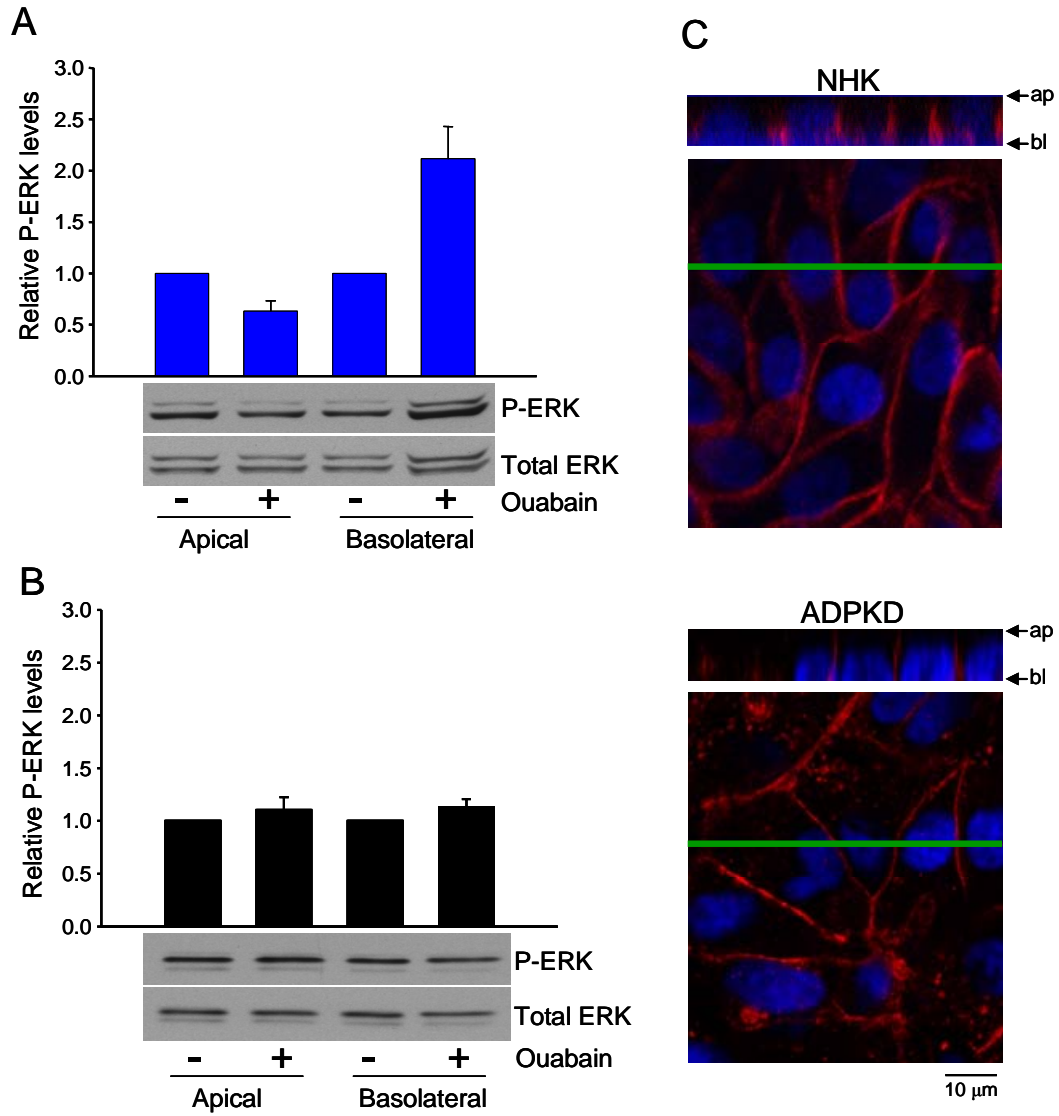


Figure 5. Effect of apical and basolateral ouabain on ERK activation in polarized ADPKD and NHK monolayers. Activation of ERK was determined in confluent polarized ADPKD (A) and NHK (B) cells after treatment with  $3 \times 10^{-9}$  M ouabain for 30 min. Ouabain was applied either to the apical or basolateral side of the culture. In the controls, the media from the apical or basolateral side of the culture chambers was also replaced with media lacking ouabain. Cells were then lysed and cellular proteins were subjected to immunoblot for determination of the total and phosphorylated forms of ERK. A representative immunoblot is shown. Values are P-ERK/total ERK, relative to the untreated controls. Bars indicate the mean  $\pm$  SE of six determinations performed on three different kidneys for ADPKD cells, and four determinations performed on two different kidneys for NHK cells. Asterisks indicates  $P < 0.001$  compared to the respective control without ouabain. (C) Immunocytochemical analysis of Na,K-ATPase expression in NHK and ADPKD cells. Cells were fixed and the Na,K-ATPase  $\alpha 1$  isoform was detected by immunofluorescence using a monoclonal 6F antibody. Alexa Fluor 594 conjugated goat anti-mouse antiserum was used as the secondary antibody. DAPI was included to stain the nuclei. Top panels show z-line views positioned at the lines indicated in the x-y views shown in the bottom panels. ap, apical; bl, basolateral side of the cells.

Figure 6

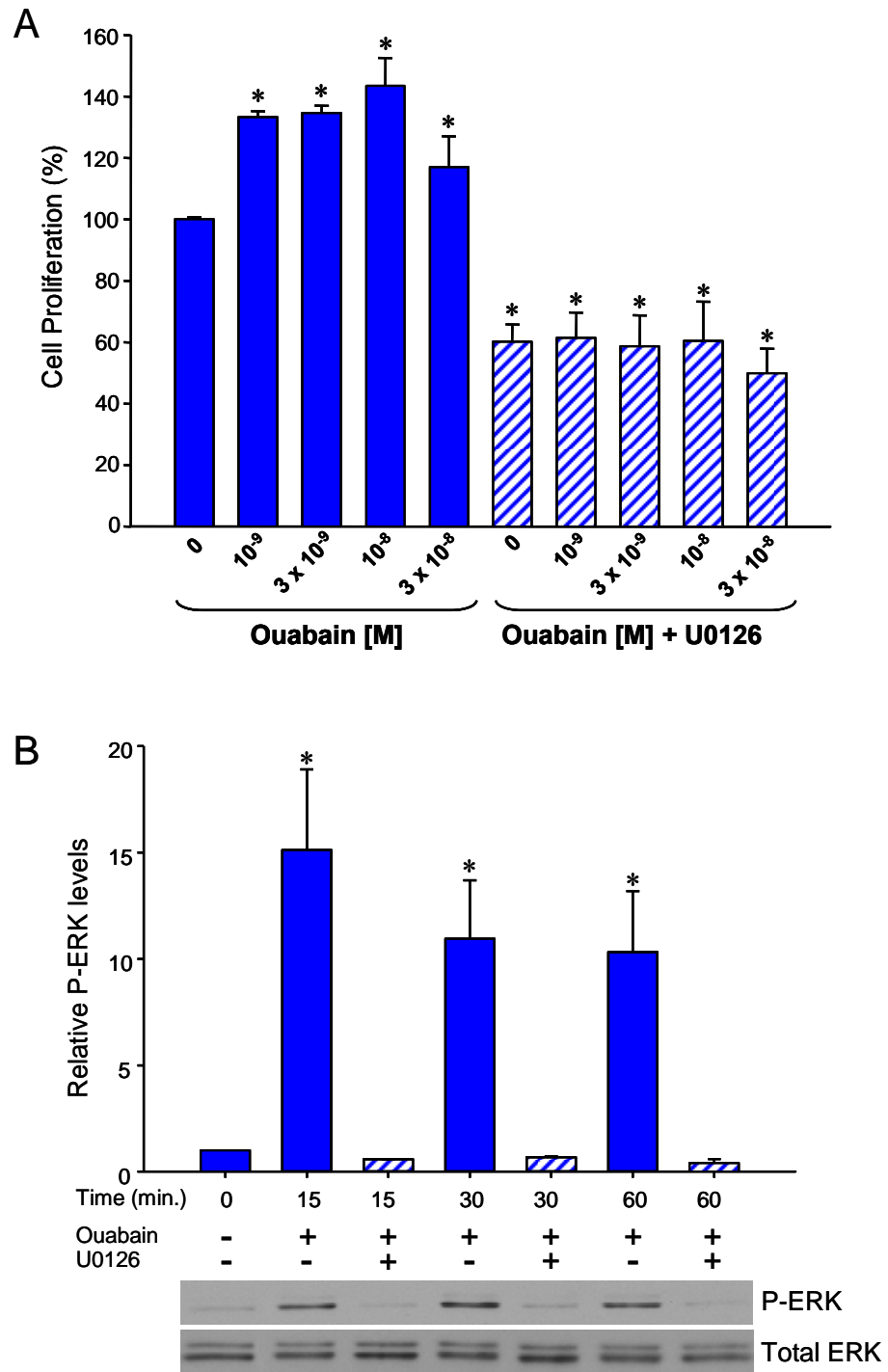


Figure 6. Effect of MEK inhibition on ouabain-induced cell proliferation and ERK activation in ADPKD cells. ADPKD cells were treated with  $3 \times 10^{-9}$  M ouabain in the absence and presence of 1  $\mu$ M of the MEK inhibitor U0126. Then, the proliferation of ADPKD cells (A) and ERK phosphorylation levels in the cells (B) were evaluated. In A, bars indicate the mean  $\pm$  SE of three experiments performed in sextuplicates in cells obtained from 3 kidneys. In B, bars represent the mean  $\pm$  SE of six determinations performed on cells obtained from 2 kidneys. A representative immunoblot is shown. Asterisks indicate significant differences compared to untreated controls, with  $P < 0.001$ .

Figure 7

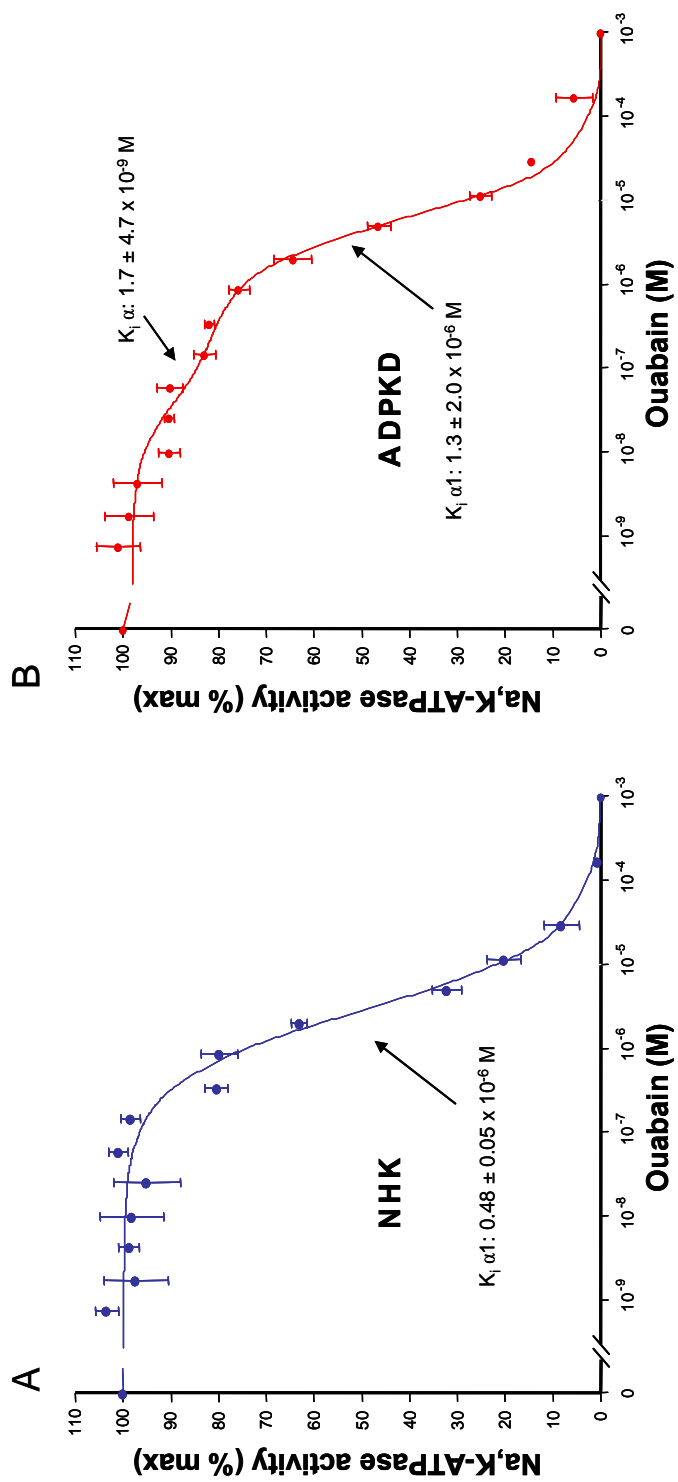




Figure 7. Dose-response curves for the ouabain inhibition of Na,K-ATPase from human renal normal, NHK (A) and cystic, ADPKD (B) epithelial cells. Na,K-ATPase was determined in medium containing 120 mM NaCl, 30 mM KCl, 3 mM MgCl<sub>2</sub>, 0.2 mM EGTA, 1 mM sodium azide, 30 mM Tris-HCl (pH 7.4), and 3 mM ATP, in the absence and presence of the indicated concentrations of ouabain. Na,K-ATPase activity was determined from the difference between the Na<sup>+</sup> and K<sup>+</sup>-dependent hydrolysis of ATP in the absence or presence of the different ouabain concentrations. Data are expressed as percentage of the Na,K-ATPase activity in the absence of the inhibitor. Dose-response curves represent the best fit of the data assuming the presence of one (NHK) or two (ADPKD) Na,K-ATPase populations with different affinities for ouabain. The validity of the use of a two component fit model for the ADPKD cells was corroborated using a F test, with  $P < 0.01$ . The inhibition constants ( $K_i$ ) for each ouabain sensitive enzyme population are shown. Each data point is the mean  $\pm$  SE of the mean of quintuplicate determinations from 3 experiments performed in cells from two different kidneys.

Figure 8

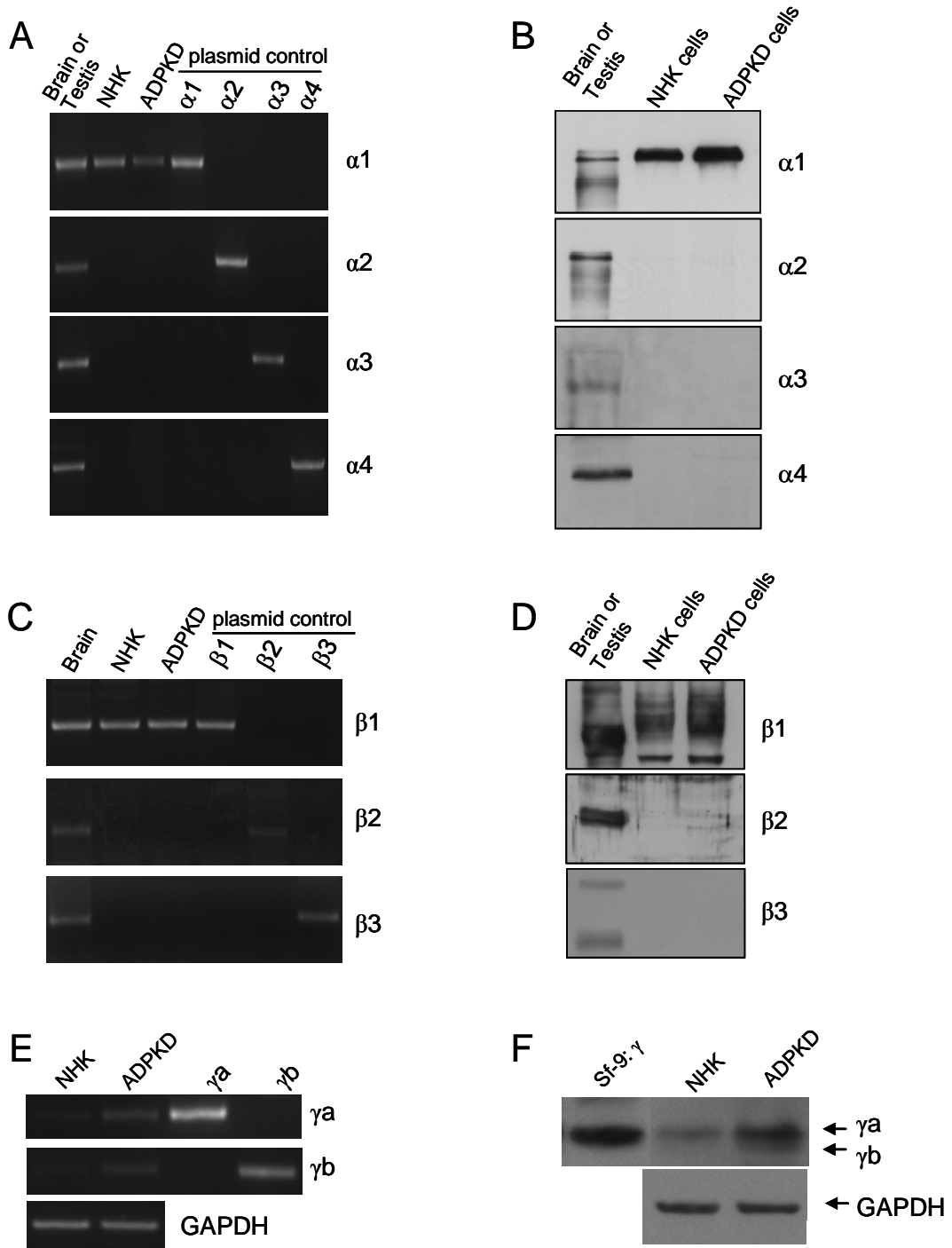


Figure 8. Na,K-ATPase isoform expression in normal and cystic human kidney cells. (A,C,E) Total RNA isolated from NHK and ADPKD cells was subjected to reverse transcription and the cDNA was amplified by PCR using oligonucleotides specific for each  $\alpha$  and  $\beta$  isoforms, and for the  $\gamma$  subunit of the Na,K-ATPase (see Table 1). Full-length cDNA for each subunit served as a control for specificity of the primers used. (B,D,F) Immunoblot analysis of  $\alpha$  and  $\beta$  isoforms and  $\gamma$  variants in NHK and ADPKD cells. Proteins from the different cell types were separated by SDS/PAGE, transferred to nitrocellulose and subjected to immunoblot analysis. For the  $\alpha 1$  isoform, the monoclonal 6F antibody was used. For  $\alpha 2$ , the MCB2 antibody, kindly provided by K Sweadner (Massachusetts General Hospital) was used. The  $\alpha 3$  and  $\beta 1$  isoforms were detected with monoclonal antibodies MA3-915 and M17-P5-F11 respectively (Affinity Bioreagents, Golden, CO). An anti- $\alpha 4$  antiserum raised against the N-terminus of the protein was used to identify  $\alpha 4$ . For  $\beta 2$ , an antiserum generously provided by P. Martin-Vasallo was used (Universidad de La Laguna, Tenerife, Spain). For  $\beta 3$ , a monoclonal antibody from BD Biosciences (San Jose, CA) was used. For the  $\gamma$  subunits, a polyclonal antibody  $\gamma 969$  provided by R. Mercer (Washington University in St. Louis) was used. Expression of GAPDH was determined, using a monoclonal antibody from Abcam Inc, Cambridge, MA. As a positive control for  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\beta 1$ ,  $\beta 2$  and  $\beta 3$ , human brain was used, for  $\alpha 4$ , human testis was used and the  $\gamma$  control was produced in Sf-9 cells using baculoviruses.

Table 1. Characteristics of the primers that were used for RT-PCR analysis of Na,K-ATPase isoforms in normal and cystic human kidney epithelial cells<sup>a</sup>

Gene Targeted (Accession No.)	Primer Sequence	Primer Position	Product Size (bp)	Annealing Conditions
$\alpha$ 1 (NM_000701)	5'-CTT AGC CTT GAT GAA CTT CA-3' (S) 5'-ACT TCC TCC GCA TTT ATG CTC ATT-3' (AS)	136 to 535	399	55°C, 30s
$\alpha$ 2 (NM_000702)	5'-ATG ACC ACA AGC TGT CCT TG-3' (S) 5'-GTT GAT CTG CAT CTT CTC TC-3' (AS)	120 to 538	418	65°C, 5s
$\alpha$ 3 (NM_152296)	5'-AAT CGA CGA GAT CCT GCA GAA T-3' (S) 5'-GCT TTC GGC AGC CTC GTA C-3' (AS)	2001 to 2456	455	50°C, 30s
$\alpha$ 4 (NM_086379)	5'-CCA TAG CCA CCA AAG GGC AA-3' (S) 5'-CAG CCA ACC ATA GCC CAA GA-3' (AS)	211 to 959	748	55°C, 30s
$\beta$ 1 (NM_001677)	5'-TTG AAT GGC TGG GAA ATT GCT CTG GA-3' (S) 5'-TTC TCA CCG TAC GCC TTA CAC TCT ATG-3' (AS)	578 to 965	387	55°C, 30s
$\beta$ 2 (NM_001678)	5'-CAG GTG GTT GAG GAG TGG AAG G-3' (S) 5'-CTT GCA TAG AGT CGT TGT AAG GCT C-3' (AS)	34 to 350	316	50°C, 30s
$\beta$ 3 (NM_001679)	5'-GAC CAG ATT CCT AGC CCA GGA C-3' (S) 5'-TTA AGT CTA TCA TTC CAT TAT GAG GAT AAA CTG CT-3' (AS)	220 to 637	417	50°C, 30s
$\gamma$ a (NM_001680)	5'-ACT GGG TTG TCG ATG GAC GGT-3' (S) 5'-CGG CTC ATC TTC ATT GAT TTG-3' (AS)	4 to 192	188	44°C, 30s
$\gamma$ b (NM_021603)	5'-GAC AGG TGG TAC CTG-3' (S) 5'-CGG CTC ATC TTC ATT GAT TTG-3' (AS)	4 to 192	188	44°C, 30s

<sup>a</sup>AS, antisense primer; RT-PCR, reverse transcriptase-PCR; S, sense primer

### CHAPTER 3

#### Na,K-ATPase-OUABAIN SIGNALING PATHWAY IN ADPKD CELLS

##### ABSTRACT

The Na,K-ATPase acts as a signal transducer that mediates the effects of the hormone ouabain in several cell types. We have shown that concentrations of ouabain, such as those circulating in blood, stimulated growth of epithelial cells derived from renal cysts of patients with autosomal dominant polycystic kidney disease (ADPKD cells). In this study, we investigated the signaling pathway triggered by ouabain in the cells. Ouabain stimulated phosphorylation of the epidermal growth factor receptor (EGFR) and the tyrosine kinase Src in ADPKD cells. Ouabain also enhanced interaction between the Na,K-ATPase and EGFR. Tyrphostin AG1478 and PP2, inhibitors of EGFR and Src respectively, blocked the proliferative effects of ouabain in the cells. Ouabain-Na,K-ATPase signaling in ADPKD cells required the integrity of plasma membrane caveolae, and disruption of caveolae by cholesterol depletion with methyl- $\beta$ -cyclodextrin prevented ouabain-induced effects in the cells. Moreover, ouabain promoted phosphorylation of caveolin-1, a major protein of cell caveolae. Downstream effects of ouabain included phosphorylation of the kinase B-Raf, and the extracellular regulated kinase ERK. Activation of ERK by ouabain resulted in translocation of this kinase into the cell nuclei. Finally, ouabain produced internalization of the Na,K-ATPase to the cell cytoplasm. Altogether, these results suggest that ouabain-stimulated proliferation of ADPKD cells is mediated by transduction pathways that require the Na,K-ATPase

signaling complex within cell caveolae, the EGFR, Src, and the downstream B-Raf and MEK-ERK pathway.

## INTRODUCTION

The enzyme Na,K-ATPase functions not only as a transporter that generates the Na<sup>+</sup> and K<sup>+</sup> gradients that typically exist across the cell plasma membrane, but is also a signaling molecule capable of mediating the effects of ouabain in the cell [1, 2]. The Na,K-ATPase is an oligomer composed of two major polypeptides, a catalytic  $\alpha$  and a glycosylated  $\beta$  subunit [3]. The  $\alpha$  polypeptide represents the primary receptor and transducer of the effects of cardenolides, such as ouabain in the cell [4, 5]. Ouabain is a well characterized steroidal hormone that is synthesized in the adrenal glands and is released into the circulation upon several stimuli [6]. Once in the bloodstream, ouabain reaches the target cells, and through the Na,K-ATPase, activates signaling events in the cells that induce changes in metabolism, motility and growth [7-12].

The signaling apparatus of the Na,K-ATPase, as well as the intracellular transduction pathways involved in ouabain-stimulated effects, have been characterized in several cell types [7-12]. For example, in myocardial and epithelial cells, a subpopulation of Na,K-ATPase molecules has been identified as part of a signaling protein complex that resides within plasma membrane caveolae [13]. Upon ouabain binding, the Na,K-ATPase initiates a series of reactions that include interaction of the enzyme at the plasma membrane with neighboring proteins, and activation of the kinase Src. This subsequently causes the recruitment and phosphorylation of the epidermal growth factor receptor (EGFR), and of downstream members of the mitogen activated protein kinase (MAPK) pathway, such as the

extracellular regulated kinase ERK. This finally results in expression of a series of genes in the cells [1, 2, 9, 10, 13-16].

Studies in our laboratory have shown that signaling through the Na,K-ATPase is functional and has important effects in epithelial cells from kidney cysts of patients with autosomal dominant polycystic kidney disease (ADPKD cells) [15]. ADPKD is the most common inherited disease of the kidney, characterized by the formation and progressive expansion of multiple fluid-filled cysts that distort the structure and severely compromises the function of the organ. Interestingly, we have found that ADPKD cells exhibit an abnormally high affinity for ouabain, and in concentrations similar to those circulating in blood, ouabain stimulates ADPKD cell growth. In contrast, ouabain does not significantly affect proliferation of normal human kidney epithelial cells (NHK cells) [15].

The aberrant proliferation of the renal epithelial cells is one of the hallmarks of ADPKD, and represents an essential event in the development of renal cystogenesis [17-19]. Uncontrolled cell growth of the renal epithelium initially causes a focal expansion of the tubular renal epithelium, which eventually separate from the originating nephron and progress into fluid-filled vesicles [20-22]. Cysts then continue increasing their size through the combined effect of proliferation of the cells surrounding the cyst, and the constant secretion of fluid by the epithelium [21-23]. Although initiation of cyst formation is controlled by genetic events, identified as alterations in the *Pkd1* and *Pkd2* genes that encode for polycystin-1 and -2 (PC-1 and PC-2) respectively, the progressive enlargement of cysts appears to be regulated by a



variety of non-genetic factors [17-19, 24]. Various pharmacological and physiological agents have been shown to stimulate ADPKD cystogenesis. For example, arginine vasopressin (AVP) and epidermal growth factor (EGF) stimulate cell proliferation of human ADPKD cells through activation of the MEK-ERK pathway; and cyclic AMP causes cell proliferation in human cells and animal models of polycystic kidney disease, also via the MEK-ERK pathway, through B-Raf, a kinase that phosphorylates and activates MEK [25-27]. Because of its mitogenic action and its stimulation of cell proliferation, ouabain emerges as a factor capable to affect renal cystic epithelial growth. At present, the pathways involved in ouabain-induced and Na,K-ATPase-mediated effects in ADPKD cells are unknown. In the present study, we have investigated the signaling events triggered by physiological concentrations of ouabain in ADPKD cells. We show that ouabain effects on proliferation of the cystic cells require interaction of the Na,K-ATPase with the caveolar protein caveolin, Src kinase, and the epidermal growth factor receptor (EGFR). In addition, ouabain-Na,K-ATPase interaction results in activation of members of the MAPK pathway, and translocation of ERK to the cell nucleus.

## METHODS

### *Cell culture*

Primary cell cultures derived from nephrectomy specimens of normal human kidney cortex (NHK cells) or surface cysts of ADPKD kidneys (ADPKD cells) were generated by the PKD Biomaterial Core at University of Kansas Medical Center (KUMC). The use of discarded human kidney tissues was approved by the Institutional Review Board at KUMC. Primary cultures were prepared as described [23]. Cells were seeded and grown in DME/F12 supplemented with 1% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin G and 0.1 mg/ml streptomycin, 5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL sodium selenite (ITS). 24 hours before cells were subject to experimental manipulation, the serum was reduced to 0.002% and ITS was removed. Cells were cultured on both filter supports (Transwell Costar, Corning, NY) and on plastic culture dishes. When the cells were grown to confluency on filter supports, cell polarity of the cultures was confirmed by the achievement of a transepithelial electrical resistance by the cell monolayer, measured with an EVOM™ voltohmmeter (World Precision Instruments, Sarasota, Fl) as described [28]. ADPKD cells are able to establish a monolayer, form tight junctional complexes, develop a transepithelial electrical potential difference and they carry out an active transepithelial transport of salt and fluid [22, 23, 29]. As previously shown, these cells are epithelial in nature and the majority of the cells stain positive for specific lectin markers for the collecting duct and distal nephron, such as *Arachis hypogaea* (PNA) and *Dolichos biflorus agglutinin* (DBA), in addition

to specific transport proteins of those nephron segments [26]. This suggests that the cell are primarily derived from distal nephrons and collecting tubules.

#### *Measurement of cell proliferation*

Cell proliferation was determined using the Promega CellTiter 96 MTT Assay according to the manufacturer recommendations (Promega Corp., Madison, WI). This method is based on the metabolism of a tetrazolium salt by the cells, and the optical density of the formazan product formed is proportional to the metabolic activity, and the number of cells in the culture [30]. In all proliferation assays, data are presented as a percent of proliferation of untreated cells.

#### *Immunoblot analysis*

Cells were treated with and without  $3 \times 10^{-9}$  M ouabain for 30 min and in the absence and presence of the indicated inhibitors. Cells were washed with ice-cold PBS and lysed with RIPA buffer containing, 1% NP-40, 0.25% Sodium deoxycholate, 1 mM Sodium orthovanadate, 1 mM NaF, 150 mM NaCl, 1 mM EDTA, 50 mM Tris, and 1x Protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Total protein content in the cleared lysates was determined using the dye-binding assay from Bio-Rad (Hercules, CA). Equal amounts of total protein (15-30  $\mu$ g) were analyzed by 10% SDS-PAGE and blotted onto nitrocellulose membranes (NitroBind, GE Water and Process Technologies, Trevose, PA). Immunoblots were first analyzed for levels of the phosphorylated forms of the proteins of interest, and then the

membranes were stripped and probed for the non-phosphorylated, or total forms of the polypeptides. The antibodies used to detect to the total and phosphorylated forms of the proteins of interest included the anti-Src from Biosource-Invitrogen, Faraday, CA; anti-EGFR from Upstate Cell Signaling, Lake Placid, NY; anti-caveolin from BD Biosciences, San Jose, CA; anti-B-Raf and anti-ERK from Santa Cruz Biotechnology, CA. Horseradish-peroxidase conjugated secondary antibodies and chemiluminescence was used for detection. The images were scanned and quantified for band intensity using the Gel-Pro software (Media Cybernetics, Inc., Silver Spring, MD). Levels of phospho- and total protein were expressed as density units relative to the untreated controls, and presented as phospho-to-total ratios.

#### *Immunoprecipitation assays*

Immunoprecipitations were used to determine the ouabain-dependent association of the Na,K-ATPase with EGFR. ADPKD cells were treated with  $3 \times 10^{-9}$  M ouabain for 30 min. Cells were then lysed in RIPA buffer and subjected to immunoprecipitation. The anti-EGFR antibody and magnetic beads coated with secondary anti-rabbit antibody were used to pull down the polypeptide. After overnight incubation on a rocking surface at 4°C, the beads were isolated with a magnet and washed three times in RIPA buffer. The precipitated proteins were eluted in sample buffer (100 mM Tris HCl pH 6.8, 2% SDS, 33% glycerol, 100 mM DTT) for 15 min at 65°C, and were separated by SDS-PAGE (7.5% gel). Proteins were

transferred to nitrocellulose and were immunoblotted for detection of the Na,K-ATPase  $\alpha$  subunit.

#### *Cholesterol Depletion and Repletion*

Cholesterol depletion was obtained by treating the cells with methyl- $\beta$ -cyclodextrin (M $\beta$ CD), 10 mM for 30 min at 37 °C as previously described [16]. Cells were washed twice in culture medium and incubated with or without 3 nM ouabain in the presence of 0.5 mM M $\beta$ CD. Cells were then analyzed for proliferation, or for determination of activated ERK. In the cholesterol repletion experiments, cells that had been depleted from cholesterol, were treated in the culture medium with 40  $\mu$ l/ml of cholesterol/ M $\beta$ CD stock solution. The cholesterol/ M $\beta$ CD solution was prepared by mixing 200  $\mu$ l of a 20 mg/ml solution of cholesterol in ethanol with 10 ml of 10% M $\beta$ CD.

#### *Immunocytochemistry in ADPKD cells*

Cells were grown on glass coverslips or filter supports to confluency, confirmed by the development of transepithelial electrical resistance. Cells were fixed in 100% methanol for 45 min at -20°C and were subjected to immunocytochemistry as described [28]. To detect the Na,K-ATPase, an anti- $\alpha$ 1 monoclonal antibody was used (6F, Developmental Studies Hybridoma Bank, University of Iowa), while P-ERK and P-B-Raf were detected using antibodies mentioned above, followed by an Alexa Fluor 488 (green) or 594 (red) conjugated secondary antibody (Molecular

Probes, Eugene, OR). Slides were analyzed using a Zeiss LSM510 confocal microscope. Images were acquired in Multitrack channel mode with LSM510 (v 3.2) software and a Plan-Apochromat 63x/1.4 Oil DIC objective with a frame size of 1024x1024 pixels and a zoom factor of 2. z line views were obtained by averaging 10 sections over a line at each z position in 1.0  $\mu\text{m}$  steps.

#### *Biotinylation assays*

ADPKD cells grown in 6-well plates and were treated with  $3 \times 10^{-9}$  M ouabain for 24h. Cells were treated with 0.75 mg/ml Biotin in 1 ml of PBS, pH 8.0 and incubated for 30 minutes at 4°C. After washing with PBS and 100 mM glycine, cells were lysed in RIPA buffer. The soluble supernatants were isolated after 3 min centrifugation at 2,000 x g. Then, streptavidin coated magnetic beads were added and samples were incubated overnight at 4 °C. Samples were finally washed and subjected to SDS-PAGE and immunoblot for detection of the Na,K-ATPase  $\alpha$  and  $\beta$  subunit.

#### *Data analysis*

Statistical significance of the differences between ouabain treated and untreated controls was determined by one way analysis of variance (ANOVA), followed by Student's T test. Statistical significance was defined as  $P < 0.05$ .

## RESULTS

### *Ouabain-stimulated proliferation in ADPKD cells requires activation of Src kinase and EGFR*

In a previous report, we have shown that physiological concentrations of ouabain, acting through the Na,K-ATPase stimulates the proliferation of ADPKD cells as determined by an increase in the mitotic index and number of cells [15]. In other cell types, ouabain binding to the Na,K-ATPase has been shown to trigger a cascade of intracellular phosphorylating events [2, 10, 14, 16, 31]. Although the Na,K-ATPase has no intrinsic kinase activity, its coupling with Src family of kinases converts it into a functional receptor tyrosine kinase that operates in a manner similar to the classical receptor tyrosine activators. Ouabain-induced stimulation of Src induces secondary tyrosine phosphorylation of a number of proteins, including EGFR. To investigate if ouabain enhancement of growth in ADPKD involves the EGFR and Src, we determined whether the proliferation of ADPKD cells induced by ouabain is sensitive to inhibitors of the kinase and EGFR. For this, cells were treated in the absence and presence of  $3 \times 10^{-9}$  M ouabain for 30 min, and with the Src kinase inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine), or the EGFR blocker tyrphostin AG1478. The mentioned amounts and incubation time with ouabain were previously described to result in maximal effect in the cells [15]. As shown in Figure 1, the ouabain-induced growth of the cells, measured using the CellTiter 96 MTT Assay, was blocked by inhibition of Src or EGFR.

*Ouabain stimulates phosphorylation of Src kinase and EGFR in ADPKD cells*

Because activation of Src and EGFR requires the incorporation of phosphate to the proteins [32], we determined the effect of ouabain in inducing phosphorylation of Src and EGFR in ADPKD cells. For this, the levels of phosphorylated Src and EGFR were measured in ADPKD cells treated in the absence and presence of  $3 \times 10^{-9}$  M ouabain, and without or with PP2 and tyrphostin AG1478. Western blot analyses of cell lysates were performed using antibodies specific to each form of the polypeptides. Figures 2A and 2B present the phosphorylated Src (P-Src) and EGFR (P-EGFR) to total Src and EGFR ratios respectively for ADPKD cells. Consistent with an involvement of Src and EGFR in Na,K-ATPase signaling in ADPKD cells, phosphorylated levels of both polypeptides was significantly increased in the presence of ouabain (Figures 2A and 2B, respectively). Moreover, as shown in Figures 2A and 2B, treatment of the cells with PP2 and tyrphostin AG1478 abrogated the ouabain-dependent phosphorylation of Src and EGFR. These results demonstrate that the kinase Src and EGFR are intermediaries in the Na,K-ATPase signaling cascade that transduces the proliferative effects of ouabain in ADPKD cells.

Ouabain has also been shown to regulate the interaction between the Na,K-ATPase and EGFR in epithelial cells [1, 2, 13, 14, 16]. We have explored whether activation of EGFR by ouabain requires association of the Na,K-ATPase with the polypeptide in ADPKD cells. This was assessed by immunoprecipitation assays.



Thus, cells treated without and with  $3 \times 10^{-9}$  M ouabain alone, or in the presence of PP2 and tyrphostin AG1478. The cleared lysates were immunoprecipitated with a polyclonal antibody against EGFR. The immunocomplex was subjected to analysis by SDS-PAGE and immunoblot to determine the presence of the  $\alpha$  subunit of the Na,K-ATPase. As shown in Figure 3, the Na,K-ATPase catalytic polypeptide co-immunoprecipitates with the EGFR. Interestingly, the association between both polypeptides was enhanced by ouabain, and was reduced by inhibition of Src and EGFR. These results further suggest the involvement of EGFR in Na,K-ATPase signaling in ADPKD cells.

*Ouabain-Na,K-ATPase signaling in ADPKD cells requires intact cell caveolae*

Recent studies indicate that two pools of Na,K-ATPase exist in cells. One is involved in ion transport and is diffusely distributed across the plasma membrane. The other enzyme population is involved in signaling and is confined to cell surface caveolae [16]. Within caveolae, the Na,K-ATPase colocalizes with several proteins constituting a signaling complex. This complex is embedded in a cholesterol-containing microdomain or lipid rafts of the plasma membrane [13]. The role of caveolae in ouabain effects in ADPKD cells is unknown. To explore this, we investigated the dependence of Na,K-ATPase signaling on caveolae by disrupting these membrane structures in the cells through cholesterol depletion with (M $\beta$ CD). Cholesterol sequestration with M $\beta$ CD represents a common tool that has been extensively used for the study of the function of cell caveolae [33]. As a first

approach, we studied the effect of M $\beta$ CD on the ouabain-stimulated proliferation of ADPKD cells. Treatment of the ADPKD cells in the culture medium with M $\beta$ CD prevented the increase in cell number caused by ouabain (Figure 4A). The requirement of structurally intact caveolar membrane domains for Na,K-ATPase signaling was confirmed by restoring cholesterol to the cells. Thus, delivery of cholesterol reverted the effect of M $\beta$ CD in ADPKD cell growth (Figure 4A). In addition, and as expected by its effect in cell proliferation, M $\beta$ CD also interfered with the ability of ouabain to induce phosphorylation of ERK in the cells. Moreover, the response of M $\beta$ CD in ERK activation was counteracted by replenishing cholesterol in the culture medium, which resulted in a significant increase in ouabain-induced P-ERK levels in the cells (Figure 4B). Another indication that Na,K-ATPase signaling requires normal function of cell caveolae was obtained by studying the effect of M $\beta$ CD on the association of the Na,K-ATPase with the EGFR. For this, after treatment with ouabain and M $\beta$ CD, cells were subjected to immunoprecipitation assays. As shown in Figure 4C, cholesterol depletion blocked the ouabain-dependent interaction of the Na,K-ATPase  $\alpha$  subunit with EGFR. Accordingly, addition of cholesterol to the cell culture media was able to offset the effect of M $\beta$ CD and recovered the Na,K-ATPase-EGFR association that ouabain promotes in the cells (Fig. 4C). Altogether, these results suggests that the Na,K-ATPase signaling complex of ADPKD cells depends on the presence of normal caveolar domains at the plasma membrane of the cells.

*Ouabain promotes phosphorylation of caveolin-1 in ADPKD cells*

A major component of caveolae are the caveolins, a family of membrane associated scaffolding proteins intimately involved in a variety of cellular processes including signal transduction, endocytosis and tumorigenesis. Caveolin-1 in particular is important in Na,K-ATPase mediated signaling. Interestingly, ouabain regulates phosphorylation of caveolin-1, which favors Na,K-ATPase-caveolin-1 interaction and the formation of a Na,K-ATPase-Src-caveolin complex, in what has been named the Na,K-ATPase signalosome [2, 16, 34]. To determine whether Na,K-ATPase signaling complex in ADPKD cells involves caveolin-1, we explored the ability of ouabain to stimulate phosphorylation of caveolin-1. For this, after treatment with or without  $3 \times 10^{-9}$  M ouabain, cells were lysed and subjected to immunoblot analysis for phosphorylated caveolin-1 (P-Caveolin). As presented in Figure 5, the levels of P-Caveolin, relative to the total caveolin amounts in the cells significantly increases after treatment with ouabain. Interestingly, the ouabain-induced incorporation of phosphate into caveolin requires activation of Src and the EGFR. Thus, addition of the inhibitors tyrphostin AG1478 and PP2 interfered with P-Caveolin formation in the cells. This set of experiments highlight the role of caveolae in the effects induced by ouabain in ADPKD cells, and suggests a direct involvement of caveolin-1 in the process.

*Ouabain-Na,K-ATPase signaling of ADPKD cells involve activation of components of the MAPK pathway*

The downstream effects of ouabain include phosphorylation and subsequent activation of multiple kinases. Notably, physiological levels of the hormone have been shown to stimulate ERK1/2, the final in a series of serine/threonine kinases in the MAPK cascade, leading to cell growth. In ADPKD, the effect of factors known to increase cell proliferation, such as EGF and adenylyl cyclase agonists, have been shown to converge on activation of the MEK/ERK pathway [26, 27, 35]. Several protein kinases are capable of activating the MAPK kinase MEK, including the Raf proteins, A-Raf, B-Raf, and c-Raf-1. While c-Raf-1 is ubiquitously expressed in most cells, A-Raf and B-Raf are more restricted [36]. Interestingly, B-Raf is highly restricted to the cerebrum and testes, and has been found to be abnormally expressed in many types of cancer cells as well as ADPKD epithelial cells [26]. Furthermore, cAMP-dependent-PKA activation of B-Raf has been demonstrated as an important mediator that functions upstream of the MEK/ERK pathway in ADPKD to promote proliferation of the cells [26].

To investigate the role of B-Raf in ouabain-induced signaling, ADPKD cells were treated with or without ouabain in the presence and absence of tyrphostin AG1478 and PP2. Phosphorylation of B-Raf was determined by Western blot and immunofluorescence analysis. As shown in Figure 6A, ouabain stimulated the incorporation of phosphate into B-Raf at Thr<sup>598</sup>/Ser<sup>601</sup>, detected equally as bands at 60 kDa and 90 kDa. As expected, blockage of Src and EGFR, which are essential for Na,K-ATPase signaling, precluded B-Raf activation in the cells. Likewise, labeling of ADPKD cells for phosphorylated B-Raf following experimental treatment revealed

an increase in fluorescence when ADPKD cells were exposed to ouabain; the signal was noticeably diminished when EGFR and Src activity were inhibited (Figure 6B). Altogether, these results suggest the importance of B-Raf as a mediator of ouabain induced Na,K-ATPase signaling in ADPKD cells.

A key kinase in the ouabain-activated cell pathway is ERK. Our previous observations (see Chapter 2) have shown that the kinase ERK is a mediator of ouabain-Na,K-ATPase signaling, and ouabain stimulates phospho-ERK (P-ERK) formation in ADPKD cells [15]. Here, we have confirmed this, and as shown in Figure 7, addition of the MEK inhibitor U0126 prevents the ouabain-dependent phosphorylation of ERK in the cells. Interestingly, when cells were treated with PP2 and tyrphostin AG1478, phosphorylation of ERK was also inhibited (Figure 7). These results are consistent with the effect of these inhibitors on proliferation of ADPKD cells and further support the role of ERK in ouabain signaling, suggesting that activation of Src and EGFR is an upstream event required for ERK phosphorylation in ADPKD cells.

Once activated, ERK is capable of translocating to the cell nucleus, where it regulates gene expression by stimulating the function of a series of transcription factors [37]. To further explore the role of ERK in ouabain signaling, we treated ADPKD cells in the absence and presence of ouabain and determined the distribution of P-ERK by immunocytochemistry. Figure 8 shows that ouabain increased the levels of P-ERK in the cells. In addition, P-ERK was found to be present not only at the cell cytoplasm, but also in the cell nucleus. This suggests that ERK is a mediator

in the ouabain signal transduction pathway and may be involved in nuclear activation of genes responsible for the proliferation of the cells.

*Ouabain down-regulates expression of the Na,K-ATPase at the plasma membrane*

In different receptor-mediated mechanisms, ligand binding results in endocytosis and retrieval of the activated receptors from the cell plasma membrane [32]. This event is important for controlling receptor signaling levels, availability and activation. To determine whether ouabain binding to ADPKD cells results in changes of the Na,K-ATPase at the plasma membrane, we performed biotinylation assays. Thus, cells treated with or without  $3 \times 10^{-9}$  M ouabain for 24 hours were incubated with biotin. Then, total cell surface proteins were immunoprecipitated with streptavidin coated magnetic beads, and the samples were subjected to immunoblot for identification of the  $\alpha$  and  $\beta$  subunits of the Na,K-ATPase. In this manner, changes in Na,K-ATPase polypeptide levels at the plasma membrane of the cells could be followed. As shown in Figure 9A, ouabain caused a decrease of the  $\alpha$  and  $\beta$  polypeptides of the Na,K-ATPase at the surface of ADPKD cells.

In agreement with these results, immunocytochemical analysis showed that in the absence of ouabain, ADPKD cells shows a typical plasma membrane distribution. In contrast, when the cells are exposed to ouabain, label for the Na,K-ATPase is not limited strictly to the cell surface, but becomes more diffusely distributed into the cell cytoplasm (Figure 9B). These results suggest that, as occurs with tyrosine kinase

receptors, the Na,K-ATPase of ADPKD cells undergoes endocytosis after binding to its natural ligand, ouabain [32].

## DISCUSSION

Abnormal proliferation of the renal epithelium is a key component in cyst formation and enlargement in ADPKD [19-21, 38, 39]. As the disease progresses, the cysts expand due to both fluid secretion into the cyst cavity, and proliferation of the cells lining the cyst walls. In ADPKD, the MEK-ERK pathway has been shown to be activated by several mitogenic agents, including growth factors and cAMP agonists, resulting in increased cell numbers [24, 40-42]. We have found that ouabain, at low concentrations such as those found in the circulation, is an additional factor with a positive effect on proliferation of ADPKD cells.

In this work, we have characterized the events involved in ouabain-Na,K-ATPase signaling in ADPKD cells. We show that integrity of cell caveolae and phosphorylation of caveolin-1 is required for stimulation by ouabain in the cells. This agrees with the notion that there is a specific population of the Na,K-ATPase in these cells that constitutes the caveolar signalosome. We also show that upon ouabain treatment of ADPKD cells, both EGFR and Src are activated, and association of the Na,K-ATPase with the EGFR is promoted. This agrees with previous observations in other cell types. Activation of the EGFR induces autophosphorylation and dimerization of the receptor, and triggers a multitude of intracellular responses, including the MAP kinase cascade [32]. Activation of the EGFR in ADPKD is particularly relevant in progression of the disease in a mouse model of ADPKD, where inhibition of the EGFR slowed progression of cystic disease [43].



Our results showing ouabain-dependent phosphorylation of Src kinase presents the hormone as a factor that stimulates signaling pathways that are common to those activated by other ligands known to induce cell proliferation in ADPKD. Increases in Src activity have been found to correlate with increasing severity of cystic disease [44, 45], whereas inhibition of Src kinase has been shown to delay progression of cystic kidney disease [44]. Our results show that in ADPKD cells, ouabain-induced activation of the EGFR and Src is followed by downstream stimulation of the MEK-ERK pathway, an important signaling event in cystic disease [37].

Epidermal growth factor (EGF) and cAMP have been shown to increase proliferation of ADPKD cells via distinct mechanisms that converge on activation of the MEK-ERK pathway. The intermediaries between the growth factor receptor and the MEK-ERK pathway include a family of GTPase activating proteins, such as Ras and Raf. EGF-stimulation of ADPKD cells has been shown to activate Ras, which directly phosphorylates Raf-1, with subsequent activation of MEK and ERK. On the other hand, cAMP-activation of PKA leads to phosphorylation of B-Raf, directly upstream of the MEK-ERK cascade. The involvement of B-Raf in ADPKD is unique in two respects. First, B-Raf is aberrantly expressed in the cystic renal epithelial cells [37]. Secondly, unlike normal cells, a PKA-dependent activation of B-Raf has been reported in ADPKD cells [26]. B-Raf can also be activated independently of PKA via Src-mediated activation of Rap-1 [46]. Our results show that in ADPKD cells, ouabain stimulated EGFR and Src, in the absence of EGF. In addition, exposure of

ADPKD cells to ouabain increased the levels of phosphorylated B-Raf. Because ouabain induced activation of Src, it is possible that it may lead to activation of B-Raf via Rap-1. Importantly, Rap-1 expression and activity has been described in ADPKD cells [26]. The expression and heightened sensitivity of B-Raf to activation in ADPKD has been shown as an important mechanism leading to increased cell growth, and was previously shown to be phosphorylated by cAMP-mediated activation of PKA [26, 47]. Thus, ouabain may be an additional effector that activates PKA in order to increase P-B-Raf levels in ADPKD cells. The intracellular signals involved in the ouabain stimulation of ADPKD cells are represented in Figure 10. At present, we are continuing experiments to more precisely define the intracellular events involved in ouabain signaling in ADPKD cells.

Our findings showed ouabain-mediated activation of the EGFR in the absence of EGF; this presents an alternative mechanism by which this receptor is involved in cell proliferation in ADPKD. In addition, ouabain stimulated interaction of the Na,K-ATPase with Src and EGFR, in conjunction with activation of B-Raf and the MAPK pathway. This reveals ouabain as a novel factor that activates intracellular signals converging on a key pathway to promote cell proliferation in ADPKD. Furthermore, the central role the MEK-ERK pathway plays in proliferation in ADPKD has led to suggestion that it be targeted for therapy [37]. Thus, inhibiting the signaling events triggered by ouabain may be able to halt or delay progression of the disease.

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Figure 1

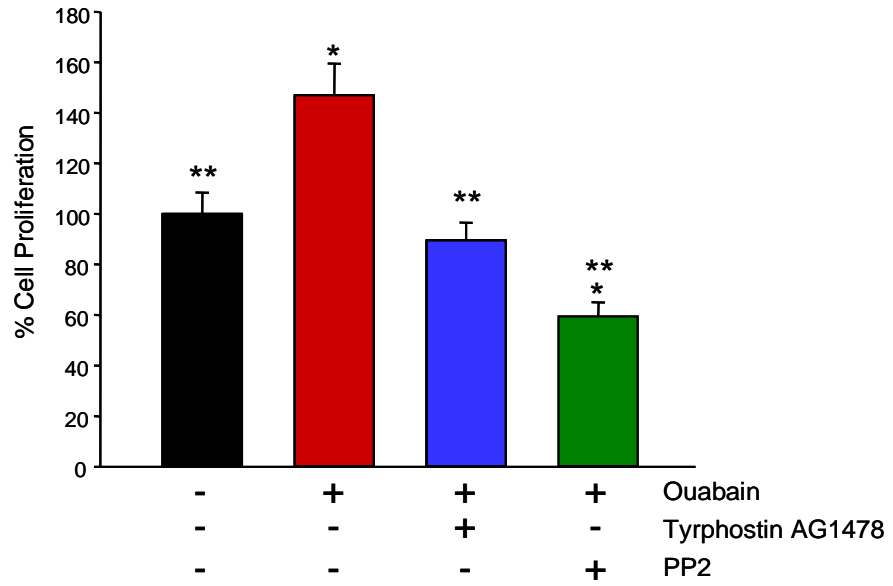


Figure 1. Mitogenic effect of ouabain on ADPKD cells requires Src and the EGF Receptor. Cells grown in 96-well plates were treated for 24 h with  $3 \times 10^{-9}$  M ouabain in the absence and presence of 2  $\mu$ M Tyrphostin AG1478, an inhibitor of the epidermal growth factor receptor protein, or 10  $\mu$ M PP2, an inhibitor of the Src family of protein tyrosine kinases. ADPKD cells were analyzed for (A) cell proliferation after 24 h using the CellTiter 96 MTT Assay. Bars represent the means  $\pm$  SE normalized to the untreated control of 8 experiments in 8 different ADPKD kidneys. (B) Levels of phosphorylated ERK after 30 minutes. Bars represent the relative phosphorylated ERK (P-ERK)/total ERK, presented as a ratio of the untreated controls of 12 determinations on 8 ADPKD kidneys. \* $P < 0.001$  vs respective untreated control, \*\* $P < 0.001$  vs. ouabain treated cells.

Figure 2

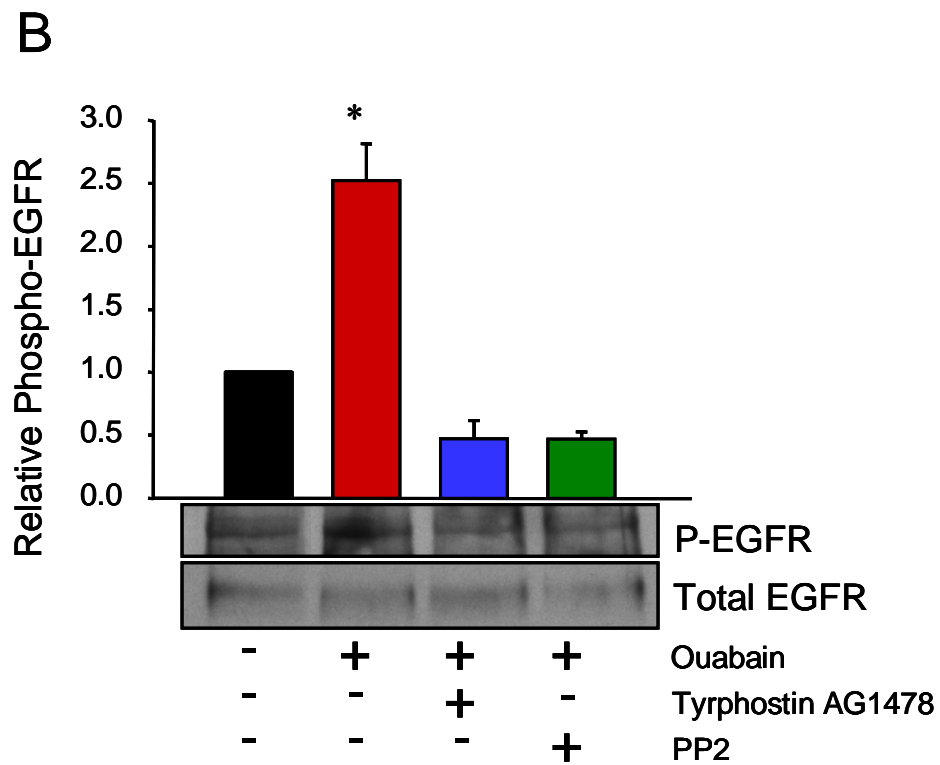
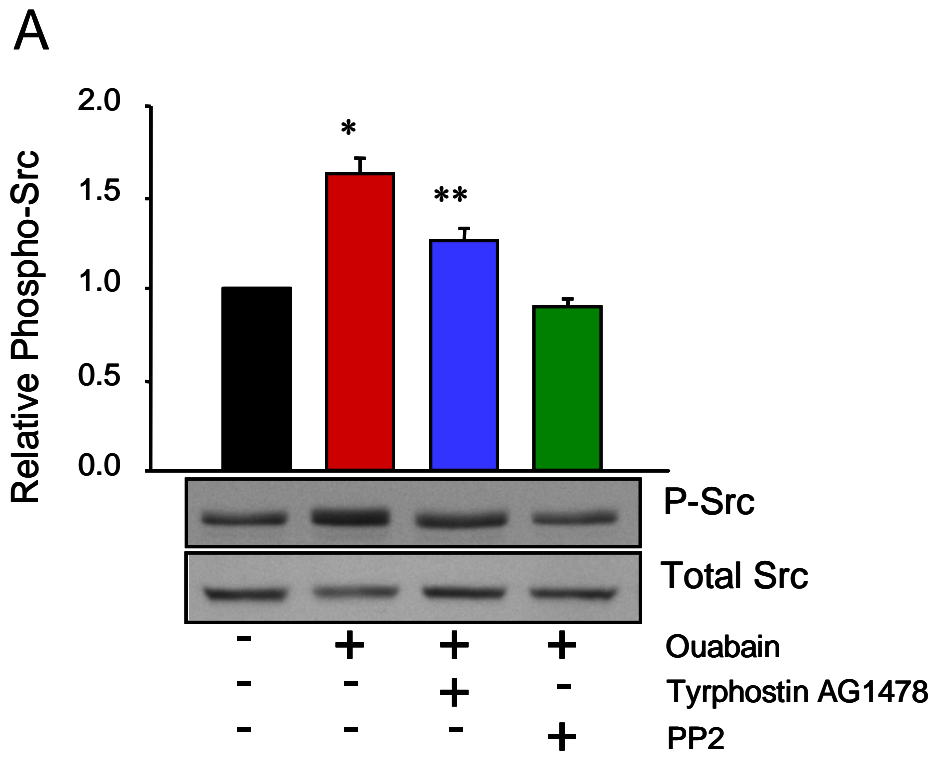


Figure 2. Effect of ouabain on tyrosine phosphorylation of Src and the EGFR. Cells were exposed to  $3 \times 10^{-9}$  M ouabain alone or in the presence of AG1478 or PP2 for 30 minutes. Lysates from untreated control and treated cells were analyzed by 10% SDS-PAGE and immunoblotted with (A) anti-Src-Y<sup>418</sup> antibody. Blots were then stripped and immunoblotted with anti-Src antibody to insure equal loading. (B) Ouabain-induced phosphorylation of EGFR was analyzed by immunoprecipitation of lysates with a polyclonal anti-EGFR antibody. Immunoprecipitates were analyzed by 8% SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. Blots were then stripped and immunoblotted with anti-EGFR antibody to insure equal loading. Bars represent the relative phosphorylated Src (P-Src)/Total Src or P-EGFR/Total EGFR, presented as a ratio of the untreated controls of 4 determinations on 4 ADPKD kidneys. \* $P < 0.001$ , \*\* $P < 0.01$  vs untreated control.

Figure 3

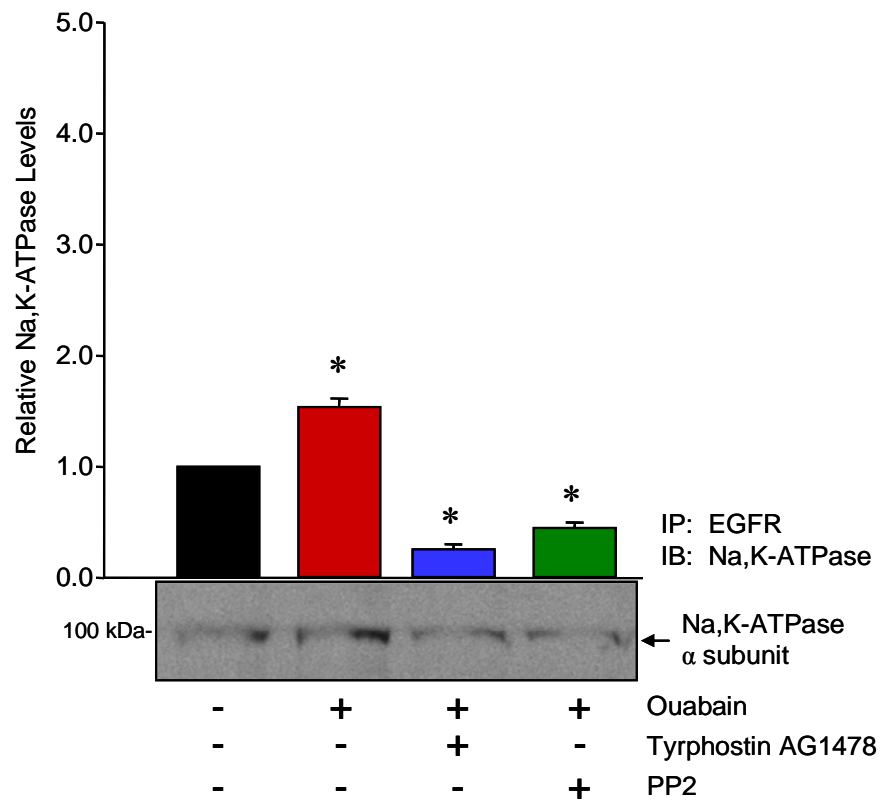


Figure 3. Interaction of the Na,K-ATPase with EGFR is enhanced by ouabain. Cells were exposed to  $3 \times 10^{-9}$  M ouabain alone or in the presence of AG1478 or PP2 for 30 minutes. Lysates from untreated control and treated cells were immunoprecipitated with a polyclonal anti-EGFR antibody. Immunoprecipitates were analyzed by 10% SDS-PAGE and immunoblotted with the monoclonal 6F antibody to detect  $\alpha 1$  of the Na,K-ATPase. Values are presented as means  $\pm$  SE of the ratio of the untreated controls from 3 determinations on 3 ADPKD kidneys. \* $P < 0.01$  vs untreated control.

Figure 4

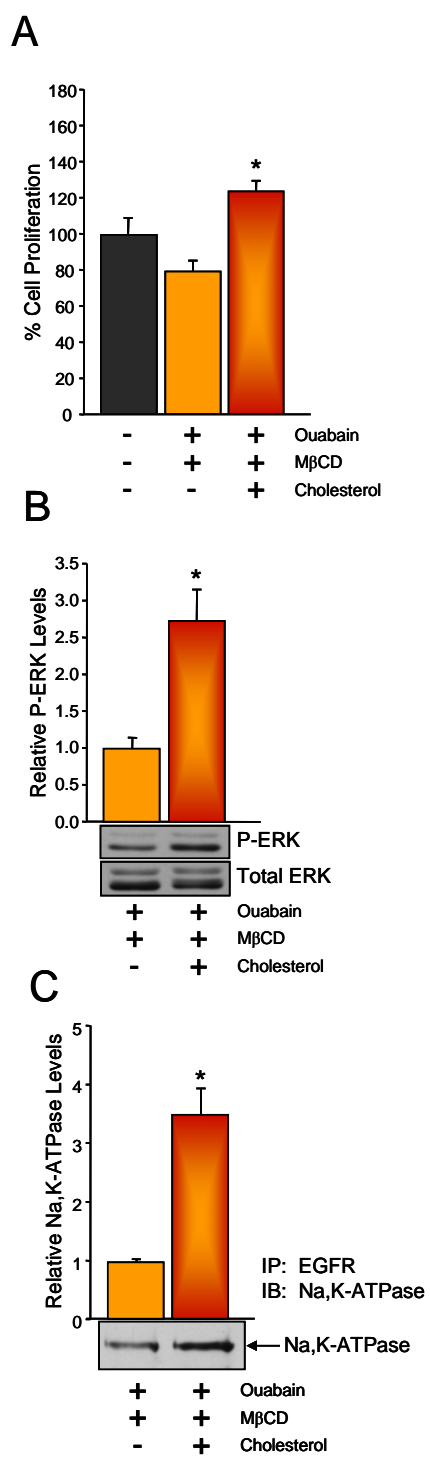


Figure 4. The signalosome is essential for ouabain signaling in ADPKD. Cholesterol was depleted from the cultures for 30 min. with 10 mM methyl- $\beta$ -cyclodextrin at 37°C to disrupt caveolae, a critical component to assemble the signalosome. Cells were then washed twice in serum-free media and exposed to  $3 \times 10^{-9}$  M ouabain and 0.5 mM methyl- $\beta$ -cyclodextrin to maintain a cholesterol-free environment. Alternatively, cholesterol was repleted in the culture media for 1 h at 37°C and subsequently treated with  $3 \times 10^{-9}$  M ouabain for (A) 24 h to analyze ouabain-induced proliferation in the absence or presence of caveolae; (B) 30 min. to measure p42/p44 MAPK activation or (C) 30 min. to analyze association of the Na,K-ATPase with EGFR. Bars represent the means  $\pm$  SE of 3 determinations from 3 ADPKD kidneys. \*  $P < 0.01$  compared to untreated controls and/or cultures where cholesterol is depleted.



Figure 5

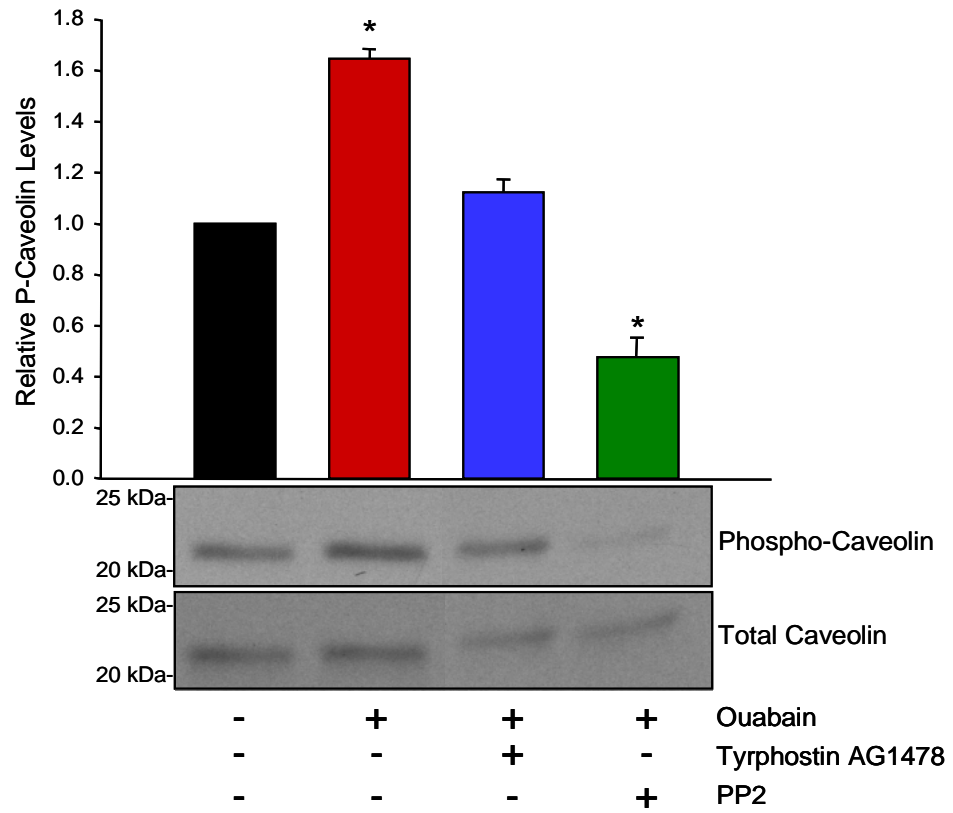


Figure 5. Ouabain stimulates tyrosine phosphorylation at Tyr<sup>14</sup> of caveolin-1. Cells were treated with  $3 \times 10^{-9}$  M ouabain for 30 minutes in the absence or presence of the EGFR inhibitor, AG1478, or the Src inhibitor, PP2. Cleared lysates were analyzed by 15% SDS-PAGE and immunoblotted with a monoclonal anti-caveolin-Y<sup>14</sup> antibody. The blots were then stripped and re-probed with a polyclonal anti-caveolin antibody. Bars represent the relative phosphorylated caveolin-1 (P-Caveolin)/Total Caveolin, presented as the mean  $\pm$  SE of the ratio of the untreated controls from 3 determinations on 3 ADPKD kidneys. \* $P < 0.01$  compared to untreated control.

Figure 6

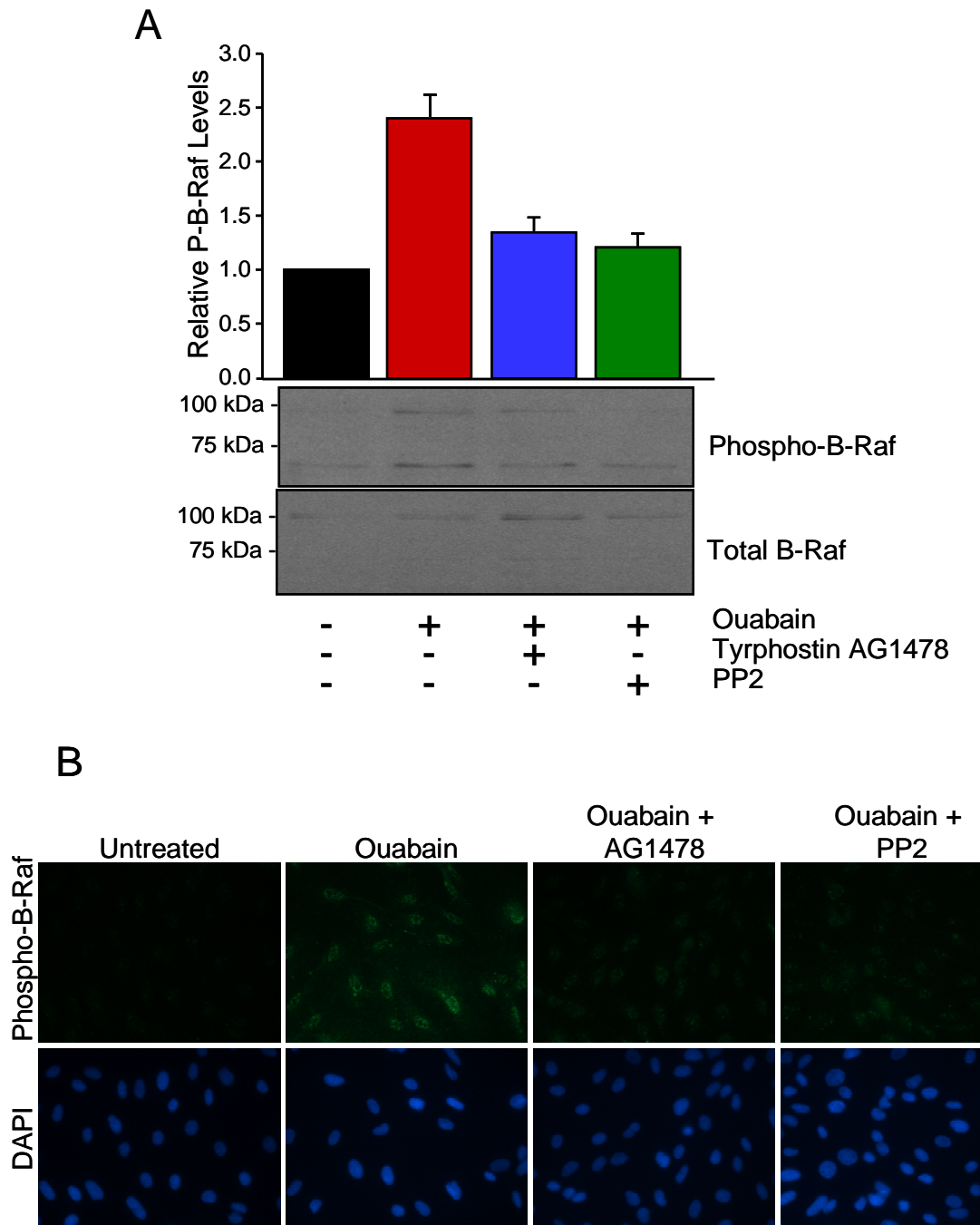


Figure 6. Ouabain-mediated activation of B-Raf requires EGFR and Src kinase activity. Cells were treated without and with  $3 \times 10^{-9}$  M ouabain for 30 minutes in the absence and presence of AG1478 and PP2. Phosphorylation of B-Raf in ADPKD was determined by (A) immunoblot analysis and (B) immunofluorescence microscopy. Inhibition of EGFR or Src activation blocked recruitment of B-Raf in the ouabain signaling cascade in both methods of detection. Bars represent the relative phosphorylated B-Raf (P-B-Raf)/Total B-Raf, presented as the mean  $\pm$  SE of the ratio of the untreated controls from 3 determinations on 3 ADPKD kidneys.

\* $P < 0.01$  compared to untreated control.

Figure 7

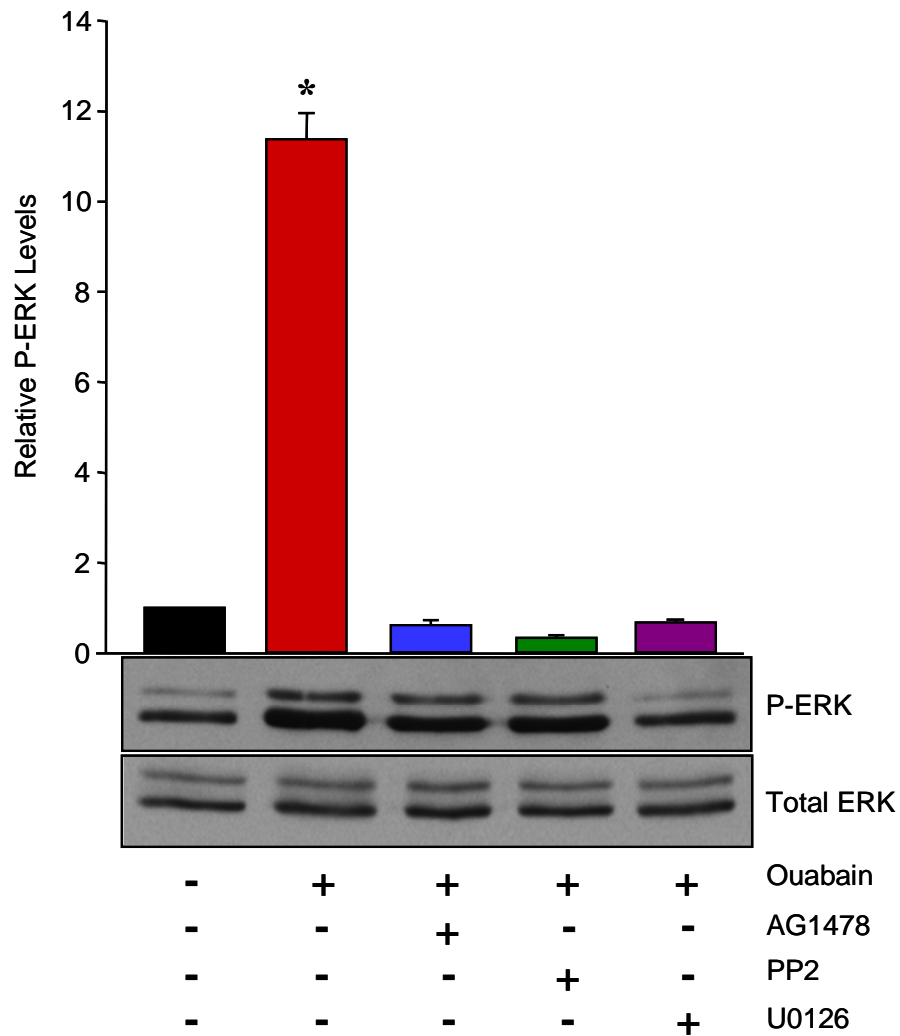


Figure 7. Ouabain activates ERK via the EGFR-Src-MEK pathway in ADPKD cells. Ouabain-dependent ERK phosphorylation requires activation of the EGFR, Src and MEK. Cells were treated for 30 min. without or with  $3 \times 10^{-9}$  M ouabain alone, or following inhibition of the EGFR (AG1478), Src (PP2) or MEK (U0126). The phosphorylated and total forms of ERK were determined. Ouabain stimulated ERK activation, and this effect was blocked by inhibition of EGFR, Src or MEK. Bars represent the ratio of phosphorylated ERK (P-ERK)/Total ERK, relative to the untreated controls. Bars represent the mean  $\pm$  SEM of 12 determinations performed on cells obtained from 8 ADPKD kidneys. \* $P < 0.001$  vs untreated control.

Figure 8

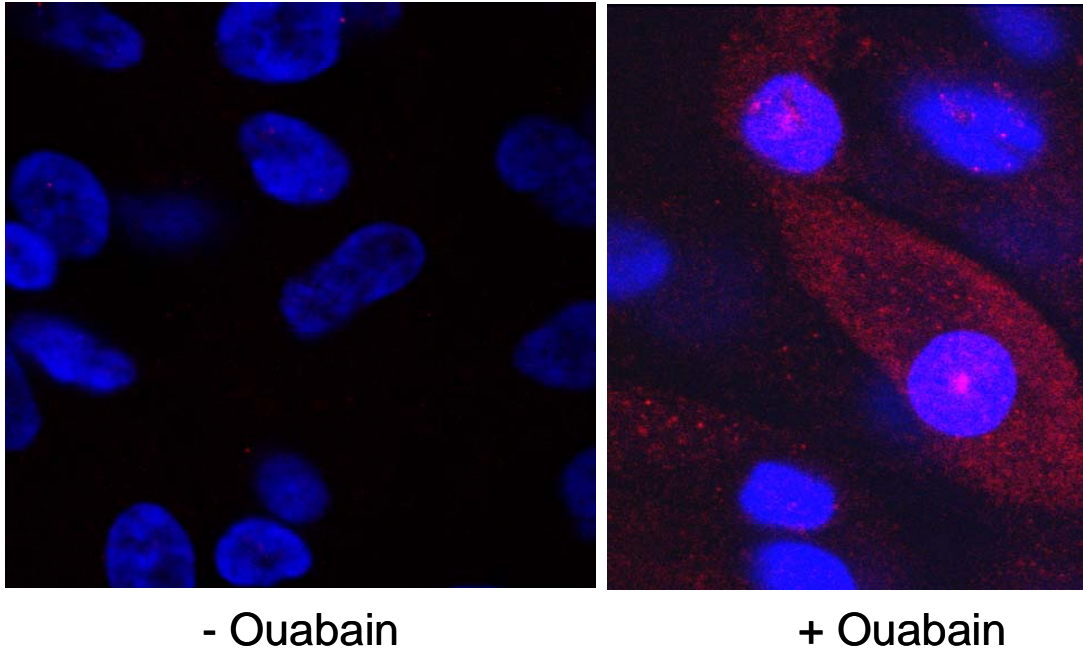
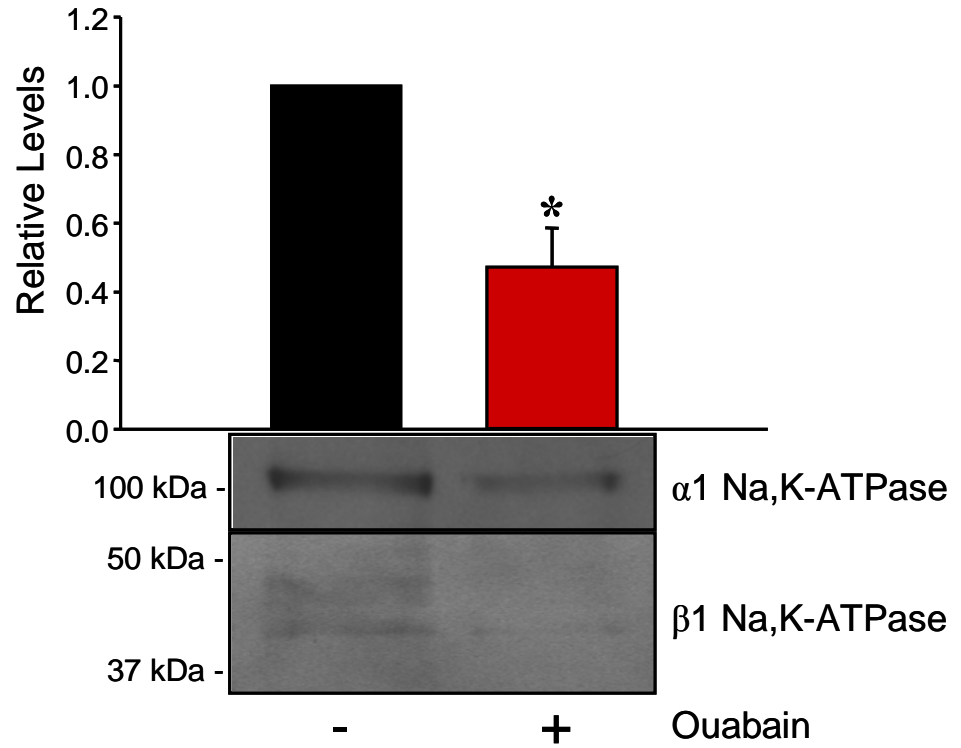


Figure 8. Ouabain induces ERK activation and translocation to the nucleus. ADPKD cells grown on glass coverslips were treated in the absence and presence of  $3 \times 10^{-9}$  M ouabain for 30 min. and labeled with an anti-phospho-ERK antibody, followed by an Alexa Fluor 594 secondary antibody. Cell nuclei were stained with DAPI.



Figure 9

A



B

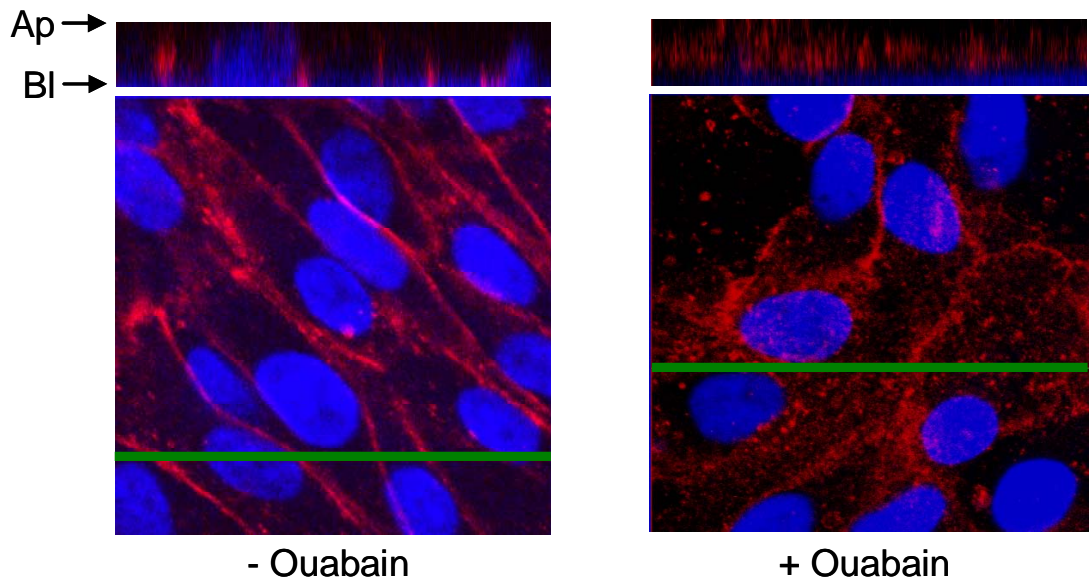


Figure 9. Na,K-ATPase expression at the plasma membrane is down-regulated by ouabain. ADPKD cells were treated in the absence and presence of  $3 \times 10^{-9}$  M ouabain for 24 h and examined for expression of the Na,K-ATPase by (A) Western blot analysis following biotinylation of surface proteins. Expression of both the  $\alpha 1$  and  $\beta 1$  subunits of the Na,K-ATPase were found to be reduced by  $\sim 50\%$  after treatment with ouabain for 24 h. Bars represent the mean  $\pm$  SEM of 3 determinations performed on cells obtained from 3 ADPKD kidneys.  $*P < 0.01$  vs untreated control. (B) Immunofluorescence microscopy of confluent cultures grown on filter supports treated with ouabain for 24 h and labeled for the  $\alpha 1$  subunit of the Na,K-ATPase. Top panels show z-line views positioned at the lines indicated in the x-y views shown in the bottom panels. Ap, apical; Bl, basolateral side of the cells.

Figure 10

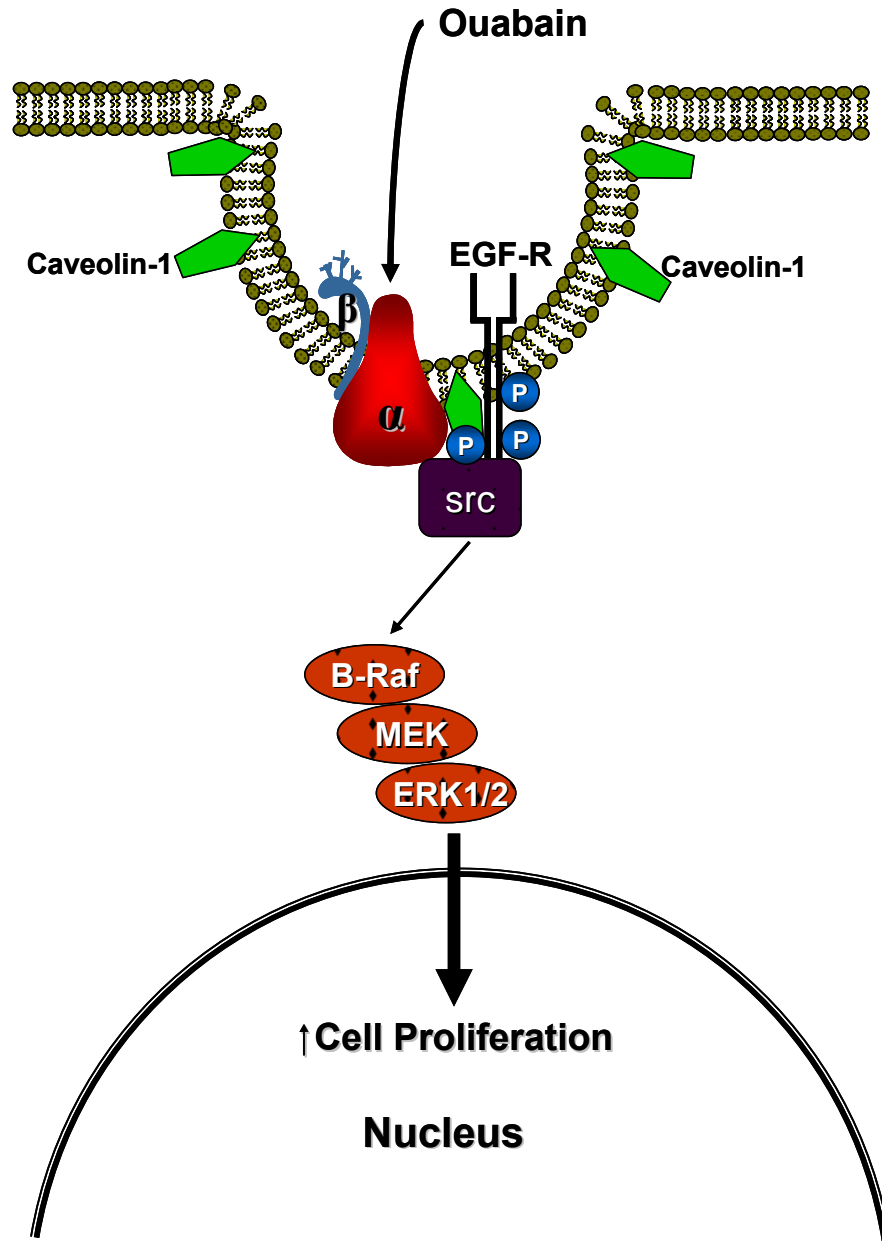


Figure 10. Signaling pathway activated by ouabain in ADPKD. Ouabain binding to the Na,K-ATPase induces phosphorylation and activation of the EGFR and Src kinase. Src, through intermediary steps to be defined, leads to activation of B-Raf, MEK and ERK. Phosphorylated ERK translocates to the nucleus to regulate gene expression, leading to increased cell proliferation.

## **CHAPTER 4**

### **OUABAIN ENHANCES PROGRAMMED CELL DEATH IN ADPKD CELLS**

#### **ABSTRACT**

The progression of autosomal dominant polycystic kidney disease (ADPKD) is characterized by increased cell proliferation and apoptosis, or programmed cell death. Inhibition of either process reduces cystogenesis and slows progression of the disease. Previous work in our laboratory has shown that physiological levels of ouabain stimulate proliferation of ADPKD cells via interaction with the Na,K-ATPase and subsequent activation of the epidermal growth factor receptor (EGFR), Src kinase, and the MAP kinase pathway. In this work, we found that the same levels of ouabain also promote apoptotic death of ADPKD cells, as determined by TUNEL staining, a biochemical marker of DNA fragmentation. In addition, ouabain induced activation of caspase-3, a key executioner of the cell apoptotic pathway. Activation of caspase-3 was not mediated by ouabain-stimulation of EGFR or Src, suggesting that the pathway triggered by ouabain to promote cell proliferation is distinct from the pathway that activates apoptosis. Apoptosis induced by ouabain resulted in release of cytochrome c from mitochondria, indicating that the hormone is able to activate the intrinsic pathway that leads to programmed cell death. The dual effect of ouabain in cell proliferation and apoptosis of ADPKD cells suggests an important role of the hormone in regulating epithelial cell growth, an event that is intimately related to progression of the disease.

## INTRODUCTION

Apoptosis is a process of programmed cell death that is tightly regulated during normal development and aging, and is critical in maintaining the homeostasis of many cell populations [1]. Changes in the rate of apoptosis have been found in several pathological situations [1-5]. Autosomal dominant polycystic kidney disease (ADPKD) is one of many diseases in which the precise regulation of apoptosis is altered [6-8]. One of the characteristics of ADPKD is that the disease progresses relatively slowly throughout the lifetime of the affected individual [9, 10]. This characteristic is difficult to explain if increased cell proliferation is the only defect. Interestingly, increased rates of apoptosis have been reported in animal models of PKD, and in kidneys from humans with ADPKD, and is now recognized as a histopathologic feature of the disease [8, 11, 12]. Furthermore, cystogenesis has been found to be attenuated when apoptosis is inhibited [13]. It has been proposed that in the epithelium of ADPKD cysts, the balance between cell proliferation and death is perturbed, and that loss of some tubular cystic cells by apoptosis may stimulate proliferation of surrounding cells [8]. Thus, the aberrant increase in programmed cell death of the cystic epithelium is a mechanism that influences progression of ADPKD [11, 14]. Although apoptosis has been described in ADPKD, further studies are required to elucidate the mechanisms by which the process is regulated in cystic cells. Understanding the factors influencing ADPKD apoptosis is important since this event affects cyst progression.

The mechanisms of apoptosis are complex, involving an intricate cascade of molecular events. A family of cysteine proteases, the caspases, are major mediators of the apoptotic process [1]. These include the “initiator” caspases-8, -9 and -10, and the “executioner” caspases-3 and -7. There are two main pathways of caspase-mediated apoptosis. The extrinsic pathway involves transmembrane receptor mediated interaction, and is characterized by the activation of caspase-8 and -10. The intrinsic pathway is distinguished by the disruption of mitochondrial function, allowing the release of pro-apoptotic proteins, such as cytochrome c, from the mitochondrial intermembrane to the cytosol. Here, cytochrome c forms part of a complex that includes pro-caspase-9, known as the "apoptosome". Subsequently, pro-caspase-9 is cleaved to its active form. Both the intrinsic and extrinsic pathways converge at this point to stimulate the activities of caspases-3 and -7. These last caspases are responsible for the typical events of apoptosis, including DNA fragmentation, protein cross-linking and degradation, and disintegration of the cell into apoptotic bodies [4].

The Na,K-ATPase is a plasma membrane enzyme essential in maintaining the normal gradients of  $\text{Na}^+$  and  $\text{K}^+$  in the cell. While maintenance of cellular ion homeostasis by the Na,K-ATPase is critical to cell survival and function, the Na,K-ATPase also serves as a signal transducer for the effects of the hormone ouabain in cells. Upon ouabain binding to the Na,K-ATPase, multiple intracellular signaling events are activated that affect cell growth, survival, differentiation and cell death [15]. By acting through the Na,K-ATPase, cardiac glycosides, such as ouabain, have

been shown to regulate apoptosis through the Na,K-ATPase in a cell type specific fashion [15-19]. In T-cell leukemia, inhibition of the Na,K-ATPase renders the cells more susceptible to ligand-induced apoptotic cell death [20]. On the other hand, ouabain has been shown to protect human umbilical vein endothelial cells against apoptosis [21]. Our previous work has shown that ouabain has a mitogenic effect in ADPKD cells. In this work, we describe that the hormone also enhances apoptosis in ADPKD cells. Because the overall effect of ouabain results in ADPKD cell growth, stimulation of cell proliferation must predominate over cell death, suggesting that ouabain creates a disbalance in the ADPKD cell growth in favor of proliferation [14].



## METHODS

### *Cell culture*

Primary cell cultures derived from surface cysts of ADPKD kidneys (ADPKD cells) were generated by the PKD Biomaterial Core at University of Kansas Medical Center (KUMC). A protocol for the use of discarded human kidney tissues was approved by the Institutional Review Board at KUMC. Primary cultures were prepared as described [22]. Cells were seeded and grown in DME/F12 supplemented with 1% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin G and 0.1 mg/ml streptomycin, 5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL sodium selenite (ITS). Twenty-four hours before cells were subjected to experimental manipulation, the serum was reduced to 0.002% and ITS was removed. As previously shown, these cells are epithelial in nature and the majority of the cells stain positive for specific lectin markers for the collecting duct and distal nephron, such as *Arachis hypogaea* (PNA) and *Dolichos biflorus agglutinin* (DBA) [23]. This suggests that the cultures are enriched in cells derived from distal nephrons and collecting tubules.

### *Measurement of fragmented DNA by TUNEL assay*

Cells cultured on glass coverslips were treated with ouabain and analyzed for apoptosis using the DeadEnd fluorometric TUNEL system (Promega, Madison, WI). This method determines nuclear fragmentation, an important biochemical marker for cell apoptosis, by using terminal deoxynucleotidyl transferase to transfer fluorescein

(FITC)-12-dUTP to the free 3'-OH of cleaved DNA. The cells are then counter-stained with DAPI. The percentage of cells undergoing apoptosis is determined by direct visualization with fluorescence microscopy.

#### *Immunoblot analysis for activated caspase-3*

Cells treated without and with ouabain for 24 h were washed once with ice-cold PBS and lysed with buffer containing 10 mM Tris-Cl (pH 7.4), 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.1% NP-40. 50 µg of the cleared lysates were analyzed by 15% SDS-PAGE and blotted onto nitrocellulose membranes. The immunoblots were probed with an antibody that simultaneously recognizes the inactive pro-form and the active cleaved forms of caspase-3 (Cell Signaling Technology, Boston, MA).

#### *Analysis of cytochrome c translocation*

Cells treated without and with ouabain for 24 h were washed with ice-cold PBS and fractionated into mitochondrial and cytosolic proteins using the Active Motif Mitochondrial Fractionation Kit according to manufacturer's recommendations (Active Motif, Carlsbad, CA). Briefly, cells were collected and disrupted by glass-homogenization (40 strokes). The supernatant containing the cytosolic and mitochondrial proteins were separated by centrifugation at 800xg for 20 min at 4°C. The cytosolic fraction contained in the supernatant was separated by centrifugation at 10,000xg for 20 min at 4°C, and further cleared by centrifugation at 16,000xg for 20 min at 4°C. The mitochondrial proteins were lysed and resuspended in Complete

Mitochondrial Buffer. The fractions were analyzed by 15% SDS-PAGE and blotted onto nitrocellulose membranes. The immunoblots were probed with a monoclonal antibody against cytochrome c (BD Pharmingen, San Diego, CA).

#### *Measurement of caspase-3/7 activity*

Caspase-3/7 activity was determined using the Caspase-Glo 3/7 Assay according to manufacturer's instructions (Promega, Madison, WI). Briefly, cells were seeded into black-walled, clear-bottomed 96-well plates (Corning Inc., Corning, NY) at a density of 4000 cells per well and treated without or with ouabain in the presence and absence of tyrphostin AG1478 and PP2, inhibitors of the EGFR and Src, respectively. The luminogenic caspase-3/7 substrate was added; any active caspase-3 or -7 would cleave this substrate and release the luminescent signal that is proportional to the amount of caspase activity present. The data are expressed as a percentage of untreated controls.

#### *Data analysis*

Statistical significance of the differences between ouabain treated and untreated controls was determined by one way analysis of variance (ANOVA), followed by Student's T test. Statistical significance was defined as  $P < 0.05$ .

## RESULTS

### *Ouabain stimulates apoptotic death of ADPKD cells.*

Ouabain has been shown to stimulate apoptosis in several cell types [16, 17, 24, 25]. To establish whether ouabain influences apoptosis in ADPKD cells, we treated the cystic cells without and with  $3 \times 10^{-9}$  M ouabain and explored DNA nuclear fragmentation, an important biochemical marker for cell apoptosis. For this, cells were subjected to TUNEL assays. As shown in Figure 1, ouabain induced the DNA of ADPKD cells to degrade into multiple nucleosomal units. The apoptotic effect of ouabain was abolished by the general caspase inhibitor, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoro-methylketone (z-VAD-fmk). This suggests that the hormone specifically induced programmed cell death that is mediated via caspases.

Ouabain-induced apoptosis in ADPKD cells was also revealed by following the cleavage of caspase-3. Activation of caspase-3 requires proteolytic cleavage of the inactive 35 kDa zymogen into the active p17 and p12 subunits. As shown in Figure 2, and as detected by immunoblot analysis with anti-caspase-3 antibodies, addition of  $3 \times 10^{-9}$  M ouabain to the ADPKD cell cultures resulted in cleavage of the inactive, full length, pro-caspase-3 into its active large and small fragments.

To more directly assess the activation of caspase in ADPKD cells, functional caspase levels were determined using the Caspase-3/7 Glo Assay (Promega). As shown in Figure 3, cells treated with ouabain had a significantly higher level of caspase-3/7 activity than untreated cells. Moreover, inhibition of EGFR or Src with

tyrphostin AG1478 or PP2 respectively, further enhanced caspase-3/7 activity.

Collectively, these results indicate that ouabain-induced apoptosis in ADPKD cells is independent of stimulation of the EGF receptor and Src. Because these signaling molecules are important in ouabain-induced cell growth, these data show that the apoptotic pathways induced by the hormone are separate from those that stimulate cell proliferation. Thus, although ouabain has a dual role in proliferation and apoptosis, the intracellular mechanisms for each effect are distinct.

#### *Ouabain activates the intrinsic pathway of apoptosis*

Caspase-3 is one of the final enzymes responsible for directing the cell towards self-destruction, and is the point where the intrinsic and extrinsic pathways of apoptosis converge. One event that distinguishes which of the two pathways is active in cells is the release of cytochrome c from mitochondria. Presence of cytochrome c in the cell cytoplasm is characteristic of activation of the intrinsic pathway of cell apoptosis. To further delineate which of the mechanisms of cell death are induced by ouabain, we examined localization of cytochrome c in ADPKD cells after treatment in the absence and presence of  $3 \times 10^{-9}$  M ouabain. As shown in Figure 4, analysis of mitochondrial and cytoplasmic fractions from the cells by immunoblot, using an antibody for cytochrome c, revealed that some basal levels of cytochrome c are found in the cytoplasm of the untreated cells. Interestingly, ouabain treatment significantly increased the amounts of cytochrome c in the cytoplasmic fraction of ADPKD cells over basal levels. These results indicate that the effect of ouabain in apoptotic death

of ADPKD cells is through activation of the intrinsic pathway of programmed cell death.

## DISCUSSION

Precise control of programmed cell death plays a critical role in the cellular life cycle. Defects in regulation of apoptosis can have dramatic consequences. While an increase in apoptosis could lead to degenerative autoimmune disorders, down-regulation of the process results in hyperproliferative diseases such as cancer [1].

The pathogenesis of ADPKD is believed to involve a paradoxical increase in both cell proliferation and apoptosis, and both processes are required for cystogenesis and progression of the disease [7, 8, 13, 14, 26-30]. Because apoptosis is increased in all nephron segments of the cystic kidneys, but is absent in tubules from non-PKD end-stage renal disease patients, it has been suggested that the apoptotic loss of renal tissue correlates with the progressive loss of renal function in PKD [30].

Epithelial cells cultured from polycystic kidneys have been shown to retain their apoptotic capacity, providing a valuable tool to study the mechanisms underlying the aberrant increase in cell death in ADPKD [30]. Previous work from our laboratory has shown physiological levels of ouabain to induce proliferation of ADPKD cells, activating the EGFR-Src and MAP kinase pathway [31, 32]. In this work, we show that in addition to a mitogenic effect, the same concentration of ouabain promotes ADPKD cells to undergo apoptosis. The mechanism of ouabain-stimulated cell death was independent from activation of EGFR and Src. Increased cell growth and apoptosis are pathological features of ADPKD. While activation of the MAP kinase pathway has been identified to be a key pathway of ADPKD cell proliferation, and has been pharmacologically targeted in an attempt to treat the

disease [33], the mechanisms underlying increased apoptosis are less clear. Here, we show that ouabain stimulates apoptosis via activation of the intrinsic pathway, through the release of cytochrome c from the mitochondria and the subsequent activation of caspase-3. This suggests that in ADPKD cells, ouabain functions in a manner similar to that of agents that induce stress in cells.

Our results concur with studies in a rodent model of ADPKD, the Han:SPRD rat, which revealed that the intrinsic pathway is involved in the increased programmed cell death that underlies the pathophysiology of the disease [11, 34]. Similar to our findings, Han:SPRD rats show increased levels of cytochrome c in the cytoplasm of the kidney cells. Release of cytochrome c into the cytoplasm is triggered by a variety of factors, including DNA damage, loss of cell-cell contact, deprivation of growth factors or serum from the culture media, presence of toxins, hypoxia, hyperthermia and the increase in free radicals [1, 35]. Ouabain has been shown to stimulate mitochondrial production of reactive oxygen species (ROS) in rat cardiac myocytes [36, 37]. Increases in ROS from the mitochondria can lead to damage of the mitochondrial membrane potential and integrity, allowing further release of cytochrome c into the cell cytoplasm. In this manner, ROS acts as a signal that amplifies activation of the intrinsic pathway of apoptosis to produce full activation of caspase-3 [38]. Further studies will be required to identify whether ROS is an intermediary in the intracellular cascade of events for ouabain-mediated apoptosis in ADPKD cells.



Our results also show that the pathways mediating the proliferative and apoptotic effects of ouabain in ADPKD cells are distinct. While ouabain-induced cell growth depends on activation of EGFR, Src and the MAP kinase pathway, the apoptotic effect is independent of these intracellular effectors. Rather, ouabain-induced cell death is mediated via activation of the intrinsic pathway of caspase-mediated apoptosis. Inhibition of the ouabain-induced pathways of cell proliferation with blockers of the EGFR or Src resulted in an increase in caspase-3/7 activity in ADPKD cells that is higher than that obtained when the cells were treated with ouabain alone. This suggests that the mitogenic effects of ouabain, mediated through EGFR and Src, predominate over the apoptotic effect that is mediated via the intrinsic pathway of cell death. Therefore, ouabain appears to cause an imbalance between cell proliferation and death in ADPKD cells to favor cell growth. The capacity of ouabain to increase both proliferation and apoptosis in ADPKD cells presents this hormone as an important factor that can contribute in sustaining the pathophysiology of the disease. Thus, future efforts could be directed to interfere with ouabain signaling events as an effort to attenuate renal cystic disease.

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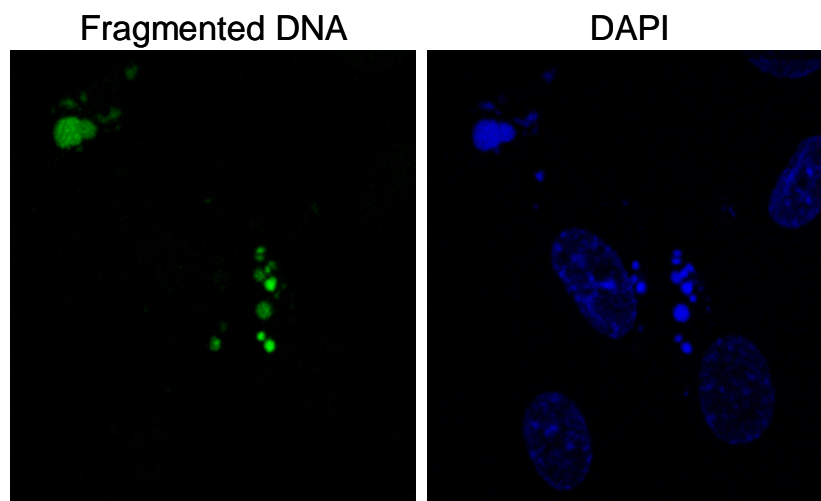
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Figure 1

A



B

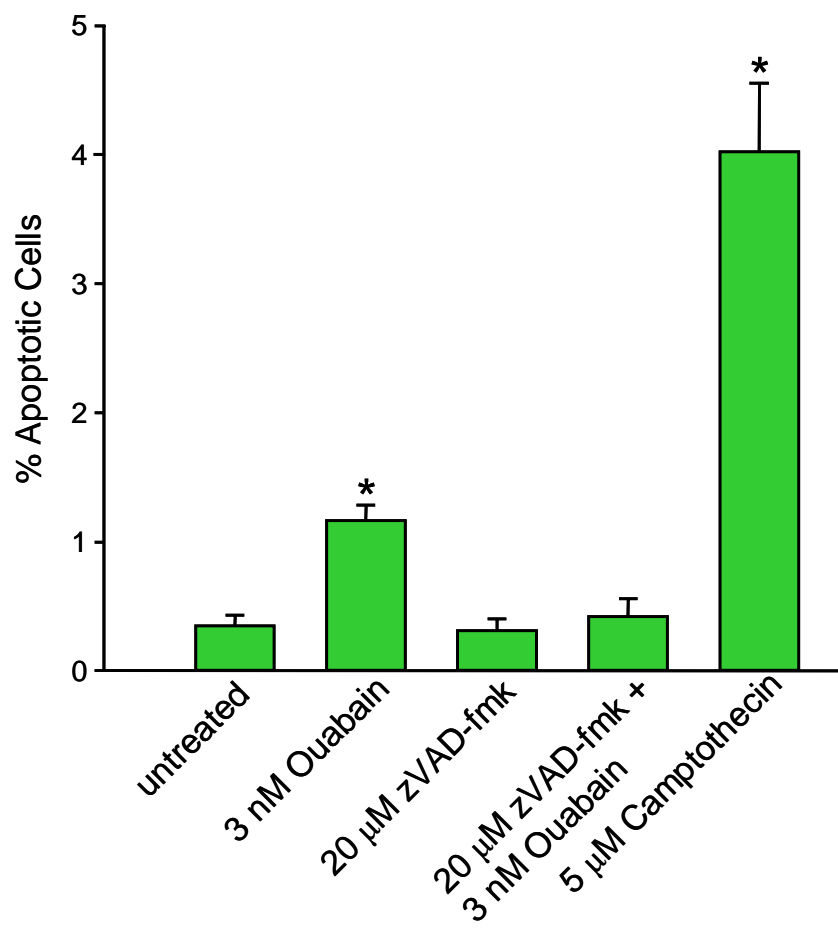


Figure 1. Ouabain induces DNA fragmentation in ADPKD cells. Cells seeded onto glass coverslips were subjected to experimental conditions for 24 h, then fixed in 10% buffered formalin. Using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI), fragmented DNA was labeled by incorporation of fluorescein-12-dUTP<sup>(a)</sup> at 3'-OH DNA ends. The nuclei were counterstained with DAPI and visualized by immunofluorescence microscopy. As a positive control, cells were treated with camptothecin. At least 10 random fields were analyzed from three different ADPKD kidneys. Bars represent the percentage of green fluorescent cells relative to the total cells per unit area. \*  $P < 0.05$  versus untreated control.



Figure 2

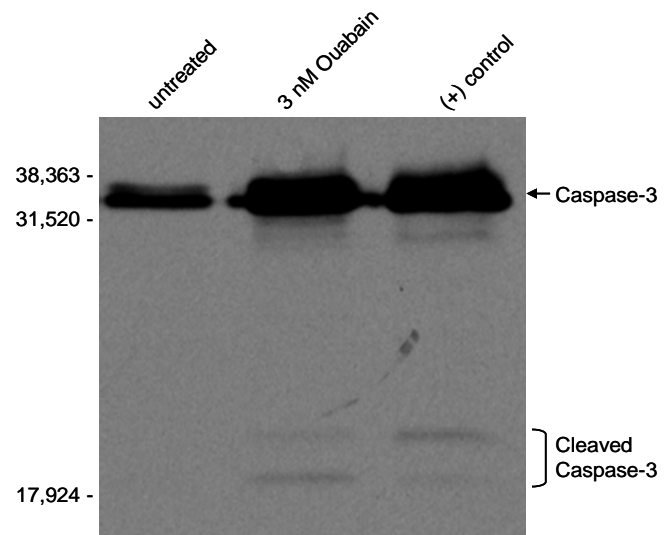


Figure 2. Caspase-3 is cleaved from its inactive pro-form into its active subunits in ADPKD after ouabain treatment. Activation of caspase-3 requires proteolytic cleavage of its inactive zymogen, detected as a 35 kDa band, into its active p17 and p12 subunits, detected at 17 and 19 kDa. ADPKD cells exposed to  $3 \times 10^{-9}$  M ouabain induced activation of caspase-3, while culture media alone had no effect. As a positive control, Jurkat cells were treated with etoposide.

Figure 3

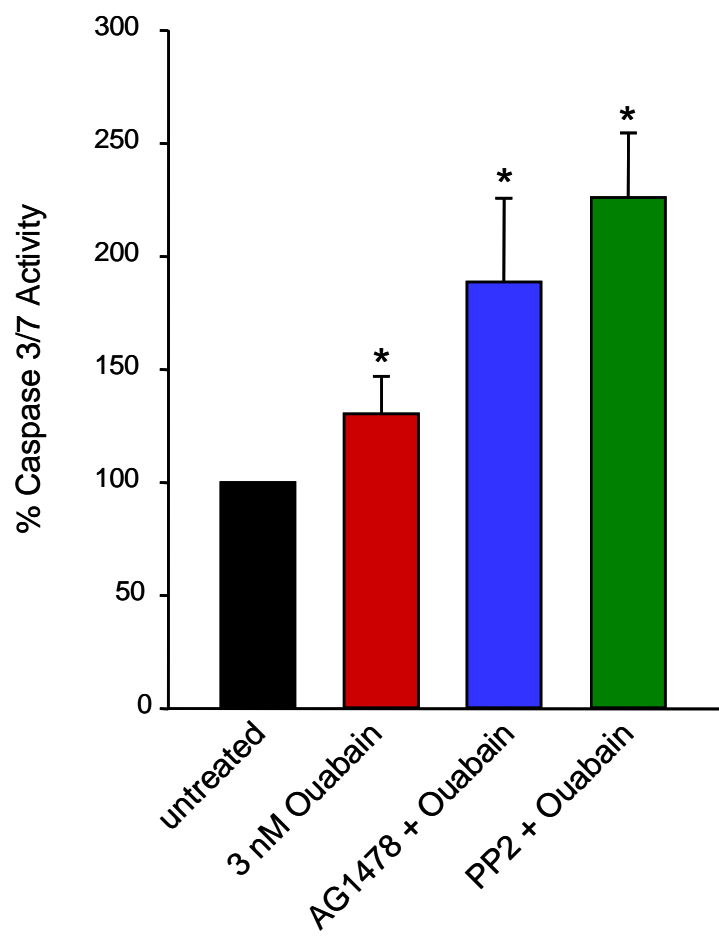


Figure 3. Ouabain-mediated increase in caspase-3/7 activity is independent of the EGFR and Src. Activity levels of caspase-3 and -7 were measured, using the Caspase-3/7 Glo Assay (Promega, Madison, WI) in cells treated without and with  $3 \times 10^{-9}$  M ouabain in the absence and presence of tyrphostin AG1478 and PP2, inhibitors of the EGFR and Src, respectively. Caspase-3 and -7 have identical peptide sequences that are cleaved upon activation, and are thus, measured simultaneously in this assay. Ouabain-stimulated increases in caspase activity were exacerbated when EGFR or Src were inhibited. Data are means  $\pm$  SE of 6 determinations on 6 different ADPKD kidneys. \*  $P < 0.05$

Figure 4

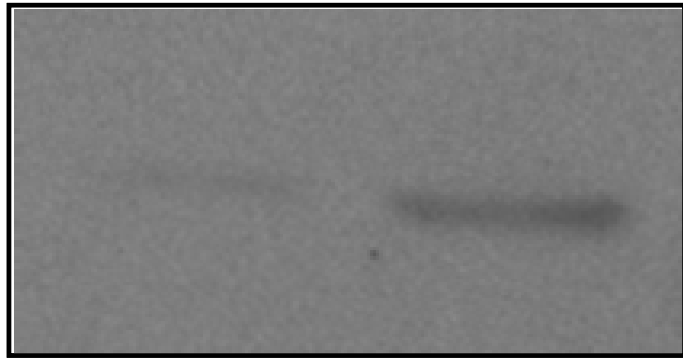


Figure 4. Ouabain stimulated translocation of cytochrome c from the mitochondria to the cytoplasm in ADPKD. Cytochrome c release from mitochondria results from an insult to the cell, activating the intrinsic pathway of apoptosis. Following treatment with  $3 \times 10^{-9}$  M ouabain, ADPKD cells were fractionated into the cytosolic and mitochondrial proteins. While a small amount of cytochrome c is observed in the cytosol of untreated cells, there is an increase after exposure to ouabain. In the mitochondria, the levels of cytochrome c are diminished following ouabain treatment, in agreement with movement of the protein into cytosol. In untreated cells, there is no difference in expression levels of cytochrome c in either compartment.

## CHAPTER 5

### OUABAIN INFLUENCES THE DEVELOPMENT OF ADPKD CYSTS

#### ABSTRACT

In autosomal dominant polycystic kidney disease (ADPKD), renal cystogenesis requires increased proliferation of the nephron tubular cells in conjunction with sustained fluid secretion through the kidney epithelium. Salt and water secretion are critical events in the expansion and successive enlargement of the cyst cavity. Several agents, such as cAMP have been identified as stimulators of fluid secretion in ADPKD, thus, accelerating the progression of the disease. We have previously shown that ouabain significantly increases proliferation of ADPKD cells. The effect is mediated through a population of the Na,K-ATPase that has an abnormally high affinity for ouabain. In this work, we have explored whether ouabain can also influence the formation and enlargement of renal cysts. For this, we have determined the effect of ouabain on microcysts of ADPKD cells grown in three dimensional collagen matrix cultures, and in metanephric organ cultures from wild type, *Pkd1<sup>m1Bei</sup>* heterozygous and homozygous mice, a well characterized model of ADPKD. We demonstrate that in both systems, ouabain in concentrations similar to those circulating in blood, has no significant effect on cyst size. However, when used in combination with conditions that increase intracellular cAMP, such as forskolin and 8-Br-cAMP, ouabain significantly enhanced the number and size of ADPKD microcysts, and increased the fractional cyst area of the metanephric organ cultures. This effect of ouabain is mediated through activation of the mitogen extracellular

kinase, MEK, indicating that it depends on the ability of the Na,K-ATPase to function as a signaling molecule that activates intracellular events in the cells. These results show that ouabain can act as a cofactor that enhances fluid secretion by the renal cystic epithelium, and provides further evidence for the role of ouabain as an agent with positive effects on the progression of ADPKD.



## INTRODUCTION

Polycystic kidney disease (PKD) includes a group of renal diseases with a common pathogenesis characterized by the development of numerous cysts that abnormally emerge from different regions of the nephron. In autosomal dominant polycystic kidney disease (ADPKD), the cysts grow over a period of years, distorting the structure of the renal parenchyma, and slowly impairing kidney function to eventually cause renal failure [1-4]. Approximately one half of ADPKD patients require dialysis or kidney replacement by the age of 60, and ADPKD is responsible for approximately 8-10% of all cases of end-stage renal failure [5-8]. Studies in ADPKD patients and a variety of experimental models of the disease implicate two major factors in the formation and maintenance of renal cysts. One is the abnormal proliferation or hyperplasia of the tubular cells; the second is the change in the salt and fluid transport properties of the renal epithelium, which modifies its reabsorptive capacity to favor secretion of fluid into the lumen of the vesicles [9, 10]. The initial event in kidney cystogenesis is the augmented cell growth, which leads to focal expansions of the tubular epithelium into “blister like” structures. As they progress, these structures increase in size, eventually losing contact with the nephron that originated them. Cysts then continue to expand as isolated fluid-filled sacs, increasing their size through the combined effect of cell proliferation and continuous fluid secretion into the growing cyst cavity. Therefore, fluid secretion plays an essential role in maintaining the shape and turgidity of the cysts.

Although the renal epithelium has been shown to secrete fluid to some extent under physiological conditions, this process is highly enhanced in ADPKD [11]. The driving force for fluid secretion in cultured epithelial cells from cysts of ADPKD kidneys is the adenosine 3',5'-cyclic monophosphate (cAMP)-dependent transepithelial transport of  $\text{Cl}^-$  [9]. This is mediated through the cystic fibrosis transmembrane conductance regulator (CFTR), present at the apical surface of cyst-lining epithelial cells. In this manner, cAMP has been shown as one of the principal humoral factors that stimulates fluid secretion in ADPKD [9, 12-16].

The molecular bases responsible for fluid secretion in ADPKD have been defined through the use of several *in vitro* approaches. For example, a useful and well-characterized model is the three dimensional collagen matrix culture, in which suspended ADPKD cells are capable of forming microcysts. Essential to the cultures is the addition of adenylyl cyclase agonists, such as forskolin, which induces the production of cAMP and enhances proliferation and fluid secretion of the cultured cells, thereby forming microcysts [17, 18]. Similar to the native renal cysts, the microcysts formed under collagen cultures are constituted by a single layer of cells that preserve their polarized properties, with the basolateral domain of the cells in contact with the collagen matrix and the luminal side facing the cyst cavity. Another approach that has been instrumental in studying the pathophysiology of ADPKD cyst development is the use of metanephric organ cultures [11, 19-21]. Cultured metanephric kidneys are largely avascular and do not support glomerular filtration. Therefore, fluid accumulation in the developing tubules derives solely from its

transport across the tubular epithelium [19]. Metanephric cultures of *Pkd1*<sup>m1Bei</sup> homozygous mice carry a point mutation on the *Pkd1* gene, express a functionally null polycystin-1 protein, exhibit massive renal cysts by day 18.5 in culture, and die *in utero* [22]. This mouse model has helped in understanding the role of cAMP and requirement of the CFTR in ADPKD cystogenesis.

In addition to cAMP, other circulating factors have been found to influence cyst growth in ADPKD. Understanding the factors involved in cyst development and progression, and their molecular mechanisms of action are essential goals in predicting the outcome of ADPKD, as well as in finding therapeutic methods to treat the disease.

We have previously shown that ouabain, a well characterized hormone that circulates in plasma of mammals, stimulates proliferation of epithelial cells from kidneys of patients with ADPKD. Ouabain exerts its effect by binding to its receptor, the Na,K-ATPase, which initiates a cascade of signaling events in the cells [23]. Interestingly, ADPKD cells have an abnormally high response to ouabain, and approximately 20% of the Na,K-ATPase expressed in the cells is sensitive to physiological concentrations of ouabain [24]. While the peculiar response of the Na,K-ATPase to ouabain is responsible for the proliferation of ADPKD cells, the effect of ouabain on the secretory function of the cells is unknown. Previous work has demonstrated that complete inhibition of activity of the Na,K-ATPase with ouabain totally abolishes fluid secretion in the ADPKD epithelium [10, 16]. However, the consequences of physiological levels of ouabain on the fluid transport

properties of ADPKD cells have not yet been evaluated. Our previous results have shown that in addition to being an ion transporter, the Na,K-ATPase of ADPKD cells functions as a signaling molecule to transmit ouabain effects into the cells [24]. Interestingly, we have shown that ouabain-Na,K-ATPase signaling in ADPKD cells includes the activation of the extracellular regulated kinase, MEK. This pathway has been implicated in the increased production of cAMP that drives fluid secretion in ADPKD cells [25-28]. These results suggest that ouabain may act as a factor that contributes to cyst enlargement, not only by promoting cell growth, but also by enhancing fluid secretion through the ADPKD epithelium. In this work, we have explored the effects of physiological levels of ouabain in cyst development of microcysts cultures of ADPKD cells and in metanephric organ cultures of *Pkd1*<sup>m1Bei</sup> mice. Our results show that ouabain acts as a co-factor that contributes to the formation and expansion of renal ADPKD cysts. This further supports the role of ouabain as an agent that exacerbates progression of ADPKD.

## METHODS

### *Microcyst cultures in hydrated collagen gels*

Cystic cells were suspended at a density of 4000 cells per well in a 96-well plate in ice cold type I collagen (Vitrogen) in Defined Medium that does not stimulate cyst growth, comprised of 1:1 DME/F12, 100U/ml penicillin, 0.1mg/mL streptomycin, ITS,  $5 \times 10^{-8}$  M hydrocortisone and  $5 \times 10^{-8}$  M triiodothyronine. After the cells/collagen suspension were seeded, the gel was polymerized by warming to 37°C. To determine if ouabain alone could induce cyst formation and growth, Defined Medium containing 3 nM ouabain was used to hydrate the gels and cells were examined daily by phase microscopy for cyst growth. To determine if ouabain acts as an agonist to cyst growth, cysts were induced to grow by pre-treatment for 2-4 days with 5  $\mu$ M forskolin and 5 ng/mL EGF. Once the cysts reached 100  $\mu$ m in diameter, EGF was removed and treated either with experimental media: 5  $\mu$ M forskolin alone;  $3 \times 10^{-9}$  M ouabain alone; ouabain + forskolin; 1  $\mu$ M U0126; forskolin + U0126; ouabain + forskolin + U0126. After 5-7 days in culture in which the cysts were allowed to grow without overlapping, they were fixed with 1% formalin in PBS and analyzed by the analySIS software (Lakewood, CO) to count and measure cysts exceeding 100  $\mu$ m in diameter. The average total surface area represents only those cysts that increased in size with experimental treatments.

### *Embryonic organ cultures*

*Pkd1*<sup>m1Be1</sup> mice obtained from the Mutant Mouse Regional Resource Center (University of North Carolina, Chapel Hill, NC) [22] were stabilized onto a C57BL/6 background [11]. Mice heterozygous for *Pkd1* were mated. Embryos from timed-pregnant females at embryonic day 15.5 (E15.5) were extracted. One front paw was removed and used to extract DNA to genotype each embryo. Under sterile conditions, metanephric kidneys were dissected and transferred to transwell filters (0.4 µm pore size; BD Biosciences, San Jose, CA) in a six-well culture plate. Metanephroi were cultured at an air-fluid interface with serum-free medium containing equal volumes of DME/F12 supplemented with 2mM L-glutamine, 10 mM HEPES, ITS, 25 ng/mL prostaglandin E<sub>1</sub>, 32 pg/mL T3, and P/S. The media was supplemented with reagents (100 µM 8-Br-cAMP and/or 3 x 10<sup>-8</sup> M ouabain) and added to the lower chamber of the culture inserts. Kidneys were refreshed daily and maintained at 37°C in a humidified chamber containing 5% CO<sub>2</sub> and 95% air. Kidneys were observed by phase contrast microscopy and images captured at 2, 3, 4, and 5 days.

#### *Quantification of dilated tubule area*

Quantification was performed on captured images using analySIS software as previously described [11]. Total kidney area was measured using the freehand polygon tool to trace the perimeter of the kidney, excluding the ureter. Cyst area was determined using the wand tool. A pixel was selected within a dilated tubule, which would automatically select all pixels of similar density. When all dilated tubules

were selected, the area was calculated by the software. Fractional cyst area was calculated as total dilated tubule area divided by total kidney area.

## RESULTS

### *Ouabain increases microcyst number and size in ADPKD cultures*

When cultured in a three dimensional matrix of polymerized collagen, ADPKD cells have been shown to form microcysts by clonal expansion of single cells with proper stimulation [16]. In these kind of cultures, the cells develop an epithelial monolayer of cells that preserves the polarized vectorial movement of salt and water that is typical of the wall surrounding the ADPKD cysts. Epidermal growth factor (EGF) is an essential requirement for the initiation of microcyst formation of these cultures, and activation of adenylyl cyclase, through agents such as forskolin, is necessary to induce fluid secretion by the epithelium [10, 16].

We have used this *in vitro* model to study the effect of ouabain on fluid secretion by the ADPKD epithelium. ADPKD cells in collagen matrices were stimulated to form microcysts with EGF and forskolin. Once the cysts reached a size of 100  $\mu\text{m}$  in diameter, EGF was removed from the medium and cultures were maintained under a variety of conditions for further analysis of microcyst growth and enlargement. Changes in cyst size were quantified and expressed as the average total surface area of cysts larger than 100  $\mu\text{m}$  in diameter and is summarized in Figure 1E.

Once the development of the microcysts was initiated, removal of EGF and forskolin from the culture media stopped further growth of the microcysts (Figure 1A). This experimental condition served as a control to further study the effect of ouabain on cyst progression. As previously reported, the addition of forskolin after EGF treatment caused a significant increase in microcyst growth (Figure 1B). This



confirmed the importance of EGF as an initiator of the process, and that activation of adenylate cyclase with forskolin alone is sufficient to stimulate ADPKD fluid secretion [10, 16]. Treatment of the cultures with only  $3 \times 10^{-9}$  M ouabain after EGF did not result in significant changes in cyst size (Figure 1C). Interestingly, however, when the pre-formed microcysts were simultaneously exposed to ouabain and forskolin, there was an increase in the size and number of cysts that was significantly higher than that observed with just forskolin alone (Figure 1D). These results support the idea that ouabain functions as a co-factor important for the enhancement of cyst growth.

We have previously shown that ouabain exerts a stimulatory effect on ADPKD cell proliferation, and that the mechanism of this effect depends on activation of the regulated mitogen kinase MEK. To explore whether ouabain-induced exacerbation of cyst formation involves activation of the mitogen activated protein kinase pathway (MAPK) in the cells, we determine the effect of MEK inhibition on the ouabain-dependent progression of ADPKD microcysts. For this, microcysts were initiated with EGF, and were subsequently stimulated with ouabain and forskolin in the presence of the MEK inhibitor, U0126. As shown in Figure 1E, U0126 did not induce changes in the non-stimulated cultures. Inhibition of MEK also had no effect on forskolin-induced growth in the cysts. In contrast, blocking MEK prevented the ability of ouabain to exacerbate microcyst growth. Altogether, these results suggest that the stimulatory effect of ouabain on cyst formation and expansion

by the ADPKD epithelium requires activation of the MAPK pathway component, MEK.

*Ouabain exacerbates cAMP-induced cystic progression in metanephric organ cultures*

Metanephric organ cultures of *Pkd1*<sup>m1Bei</sup> mice have been instrumental in the study of the mechanisms governing salt and water transport in ADPKD. Segment-specific lectin staining of sectioned metanephroi after treatment with 8-Br-cAMP for the collecting duct with *Dolichos biflorus agglutinin* (DBA), and *Lotus tetragonobulus agglutinin* (LTA) for the proximal tubule, have shown that multiple segments of the nephron have the capacity to secrete fluid when stimulated by cAMP [11]. We have used this experimental model to explore the effects of ouabain in fluid secretion by the renal cystic epithelium. Embryonic kidneys derived from *Pkd1*<sup>+/+</sup>, *+/−*, and *−/−* mice were dissected at E15.5 and cultured for 5 d as previously described [11]. Similar to cultures of ADPKD microcysts, treatment of the organs with ouabain alone did not have a noticeable effect on metanephric tubule dilation, regardless of the genotype (Figure 2).

In agreement with previous work by Magenheimer et al., treatment of kidneys derived from wild-type *Pkd1*<sup>+/+</sup> mice with the cell-permeant cAMP derivative, 8-Br-cAMP induced formation of cyst-like dilations in a series of renal tubules that continued to expand during the course of treatment (Figure 3A). At the end of day 5, cysts comprised approximately 5% of the total kidney area, expressed as fractional

cyst area (Figure 3C). Interestingly, when the wild-type metanephric cultures were treated with ouabain in the presence of 8-Br-cAMP, the cyst area doubled (Figure 3, B and C). Likewise, metanephroi from heterozygous *Pkd1*<sup>+/-</sup> mice developed cystic disease with 8-Br-cAMP treatment (Figure 4A), and the concomitant addition of ouabain significantly exacerbated cyst growth (Figure 4B), as revealed by measurements of the fractional cyst area (Figure 3C). Finally, metanephroi from homozygous, *Pkd1*<sup>-/-</sup> mice again showed a more severe response to both 8-Br-cAMP and ouabain than 8-Br-cAMP alone (Figure 5, A and B). In the kidneys from these animals, ouabain exhibited the greatest effect, with a three-fold increase in the fractional cyst area (Figure 5C).

In conclusion, in the presence of 8-Br-cAMP, physiological levels of ouabain intensified cyst development in cultured metanephroi. Interestingly, the effect of ouabain on fractional cyst area was progressively higher in the comparison of metanephroi from *Pkd1* wild-type, heterozygous and homozygous null mice (Figures 3-5, panel B). The most dramatic effect was observed in the *Pkd1*<sup>-/-</sup> kidneys, suggesting that the severity of the disease correlates with an increase in the response of the tissue to ouabain. These results suggest that the effects of ouabain on cyst progression are not limited to ADPKD cells in culture, but also occur in the native environment of the renal tissue.

## DISCUSSION

A hallmark in the development and enlargement of renal cysts in ADPKD is the secretion of salt and water through the kidney epithelium, which surpasses the reabsorptive capacity of the cells and leads to accumulation of fluid into the cyst cavity [5, 11, 29]. Circulating agents, such as cAMP, have been shown to enhance fluid secretion and the progression of ADPKD. We have previously shown that ouabain induces proliferation of ADPKD cells. Here, we demonstrate that in addition to that effect, ouabain also acts as a co-factor in the stimulation of cyst growth and expansion by the ADPKD epithelium. Thus, the cAMP-dependent growth of microcysts of ADPKD cells cultured in a collagen matrix, as well as cysts from the well-characterized ADPKD mouse model *Pkd1<sup>m1Bei</sup>*, are significantly enhanced by ouabain. This suggests that ouabain plays a dual role concomitantly enhancing cell growth and fluid secretion by the ADPKD epithelium. Importantly, this effect does not only take place in the isolated microcysts, but also in the environment of the whole renal metanephric organ, suggesting that it plays a role in cystogenesis *in vivo*.

The mechanism(s) of fluid accumulation in ADPKD cysts have been described as a disbalance between fluid reabsorption and secretion by the epithelium in favor of the latter [10, 30]. Reabsorption of Na<sup>+</sup> and water in the renal epithelium is dependent primarily on the adequate function of the Na,K-ATPase at the basolateral domain of the cells. The inward gradient of Na<sup>+</sup> that the Na,K-ATPase generates is the major force that secondarily drives the transport of Na<sup>+</sup> and other ions across the apical side of the renal epithelium [31]. Accordingly, inhibition of the

Na,K-ATPase increases the levels of intracellular Na<sup>+</sup> in the cells and affect the efficiency of the salt and water reabsorptive mechanisms across the renal tubule. Our previous work has shown that ADPKD cells have an abnormally high sensitivity for ouabain and that approximately 20% of the total Na,K-ATPase is inhibited by physiological amounts of ouabain. This ouabain sensitive phenotype is restricted to ADPKD cells, since Na,K-ATPase from normal human kidney cells is not affected by physiological ouabain concentrations. Therefore, it is possible that this partial inhibition of Na,K-ATPase activity is sufficient to impair fluid reabsorption through the epithelium, favoring the accumulation of salt and water at the luminal side of the cells, and thus, cyst enlargement.

Our results show that treatment with ouabain alone does not result in a detectable effect on fluid secretion in the ADPKD microcysts or metanephric cultures. This suggests that the role of ouabain in cysts development can not be attributed to just inhibition of the ion transport properties of the Na,K-ATPase. Studies in several cell types, and our own observations in ADPKD cells indicate that the Na,K-ATPase is involved in ouabain-induced signal transduction pathways that comprise a cascade of intracellular phosphorylating events, which start with the activation of the tyrosine kinase Src, the transactivation of the epidermal growth factor receptor (EGFR) and stimulation of the MAPK pathway [23, 32, 33]. Our results show that inhibition of MEK prevents the effects of ouabain in ADPKD microcyst formation, supporting the idea that the hormone switches on Na,K-ATPase signaling in the cells. It is interesting to postulate that the ouabain-dependent

intracellular cascade of events could increase the activity of transporters involved in fluid secretion in ADPKD cells. Ouabain has been shown to augment cAMP levels in renal cells [34], and cAMP has been implicated in the increased transport of  $\text{Cl}^-$  via the cystic fibrosis transmembrane regulator (CFTR) transporter that causes cyst fluid accumulation. It is plausible therefore, that ouabain could enhance the activity of CFTR by promoting cAMP production in the ADPKD cells. The ability of ouabain to interact with the basolaterally located Na,K-ATPase to affect transporters distantly placed in the apical side of the cells has been recently reported. Thus, in LLC-PK1 cells, a well-characterized cell line from pig renal proximal tubule, basolateral treatment with ouabain down-regulated expression and activity of the sodium/hydrogen exchanger, NHE3 at the luminal side of the cells to reduce transepithelial  $\text{Na}^+$  transport [35]. Consistent with being mediated by Na,K-ATPase signaling, ouabain-dependent regulation of NHE3 in the LLC-PK1 cells required activation of Src and the integrity of plasma membrane caveolae [23].

The chloride-dependent secretion of fluid through the ADPKD epithelium requires that  $\text{Cl}^-$  enters the cells via the basolateral membrane of the cells. This is controlled by the function of  $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$  cotransporter, NKCC1 [10, 34]. The concerted action of NKCC1 and the CFTR to favor fluid movement to the luminal side of the tubular epithelium suggests they are concomitantly regulated. Experimental evidence for this mechanism comes from studies on the effects of adenylyl cyclase agonists, such as forskolin, or cAMP in ADPKD cells and in embryonic kidney cultures [9, 11, 24]. Both the CFTR and NKCC1 are regulated by

phosphorylation and dephosphorylation processes [36-39]. Among the many enzymes regulating the activity of CFTR, Src tyrosine kinase has been identified as one modifier of the channel's activity [40]. Therefore, by acting through the Na,K-ATPase, ouabain-mediated activation of the MAPK pathway may lead to up-regulation of both the CFTR and NKCC1 function, thereby increasing fluid secretion in ADPKD cells.

Importantly, the ouabain-mediated increase in cyst fractional area of ADPKD microcysts and metanephric organ cultures takes place at nanomolar concentrations of ouabain. These ouabain amounts are similar to those normally present in blood, thus supporting the physiological importance of the effects of ouabain. Because ADPKD are normally exposed to circulating ouabain, the hormone can act as an agent that will favor cyst development to worsen progression of the disease.

In conclusion, our results demonstrates a role for ouabain-Na,K-ATPase signaling in increasing cyst formation and expansion. This evidence, added to our previous observation indicating that ouabain stimulates cystic cell growth, further supports the importance of the hormone as a factor involved in the pathophysiology of and progression of ADPKD.

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Figure 1

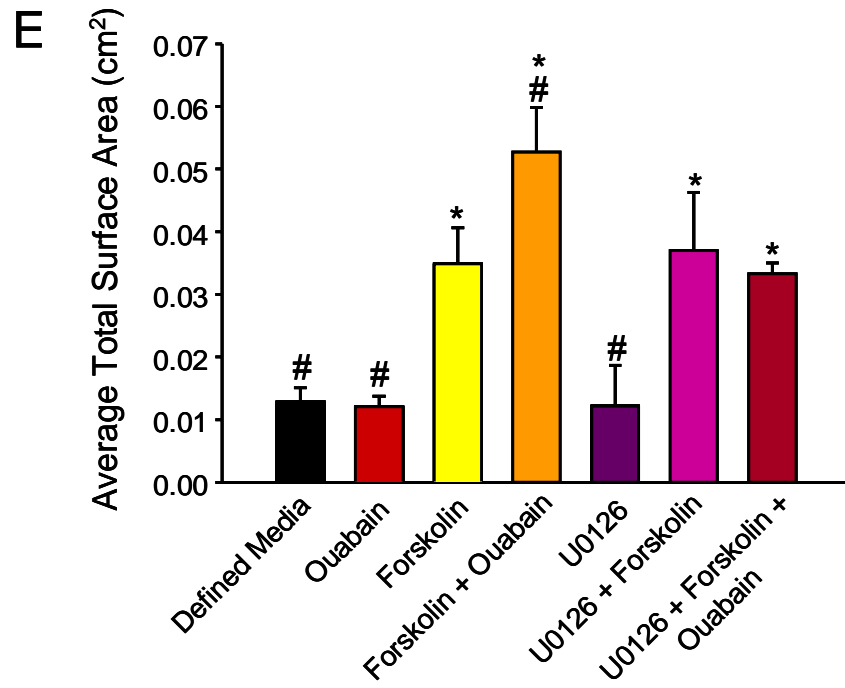
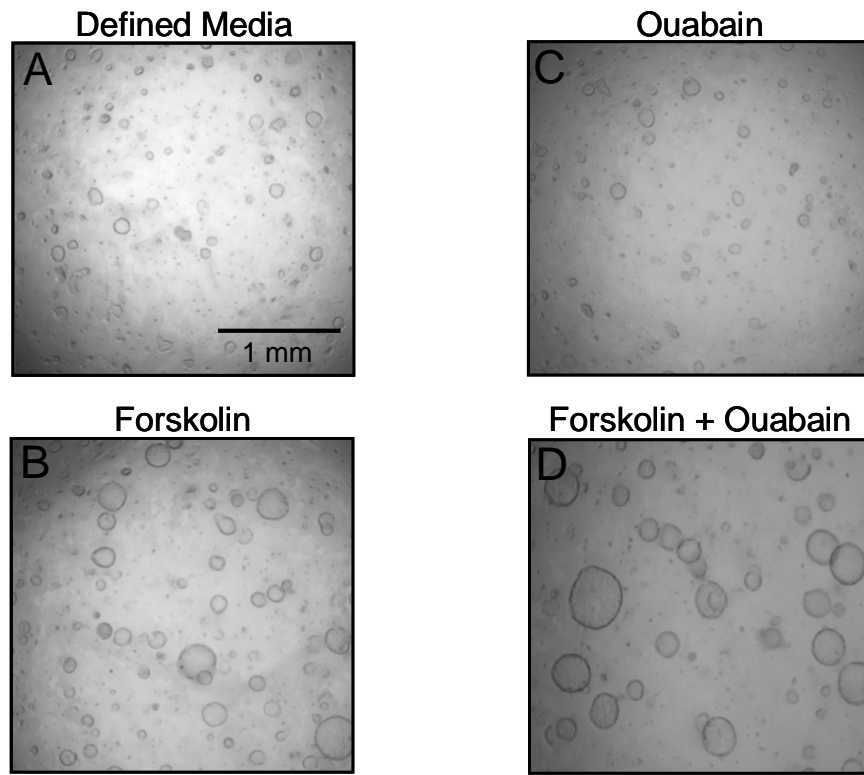


Figure 1. ADPKD microcysts cultured in polymerized collagen treated with (A) Defined Media, (B) 5  $\mu$ M forskolin, (C)  $3 \times 10^{-9}$  M ouabain, or (D) forskolin and ouabain. Images are representative of 3 experiments performed in sextuplicate in cells obtained from three different ADPKD kidneys. (E) Values represent the average total surface area of cysts exceeding 100  $\mu$ m in diameter. Bars represent the means  $\pm$  SE of two to three separate experiments in as many ADPKD kidneys, depending on the treatment regimen. \* $P < 0.05$  versus Defined Media, # $P < 0.05$  versus forskolin.

Figure 2

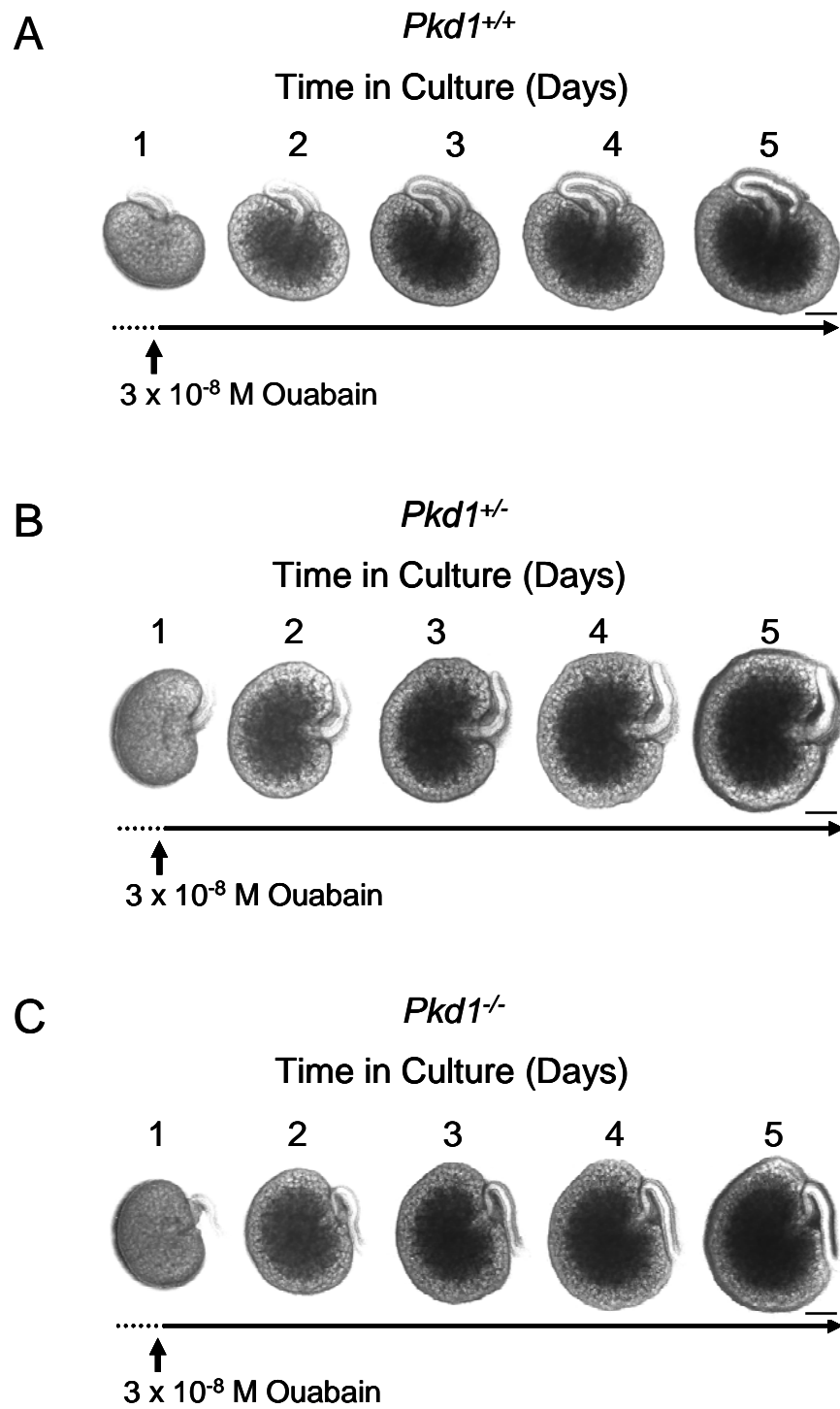




Figure 2. Metanephric cultures treated with  $3 \times 10^{-8}$  M ouabain for five days from (A) *Pkd1*<sup>+/+</sup>, (B) *Pkd1*<sup>+/-</sup>, and (C) *Pkd1*<sup>-/-</sup> mice. Media was replaced daily, and images were captured immediately afterwards. Bar, 1mm. Four experiments with 13 pairs of kidneys did not reveal a significant effect of ouabain on tubule dilation in these mice.

Figure 3

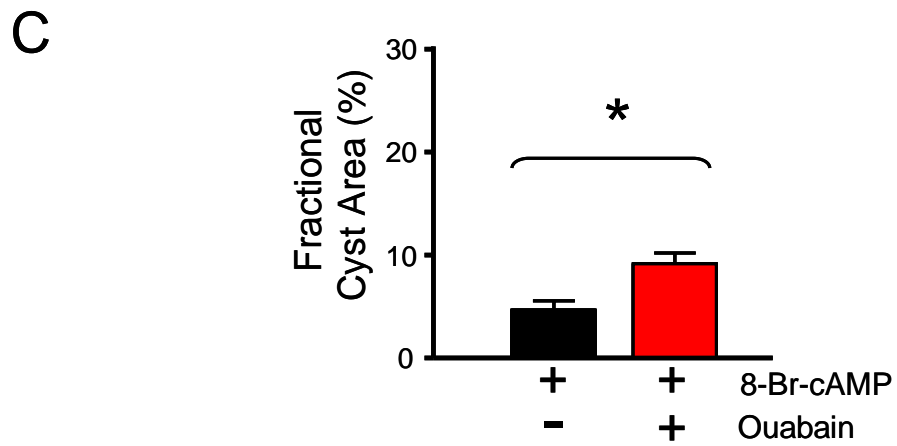
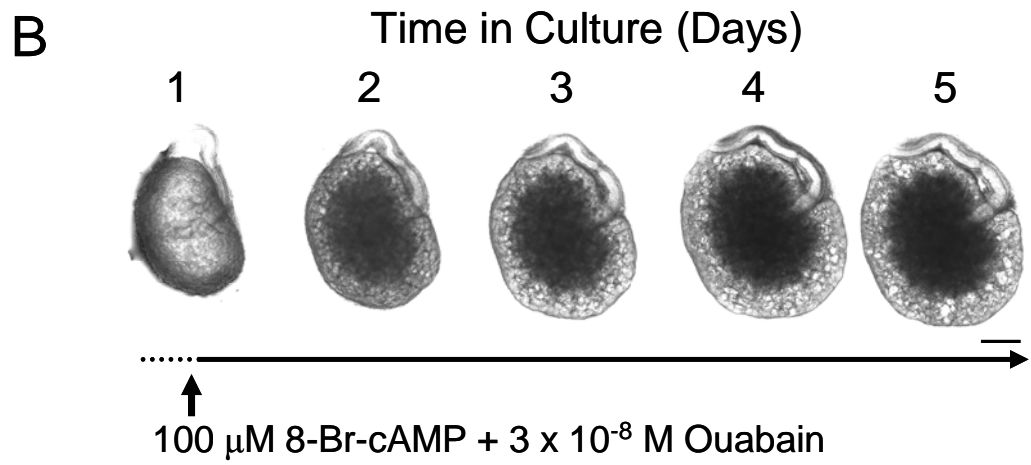
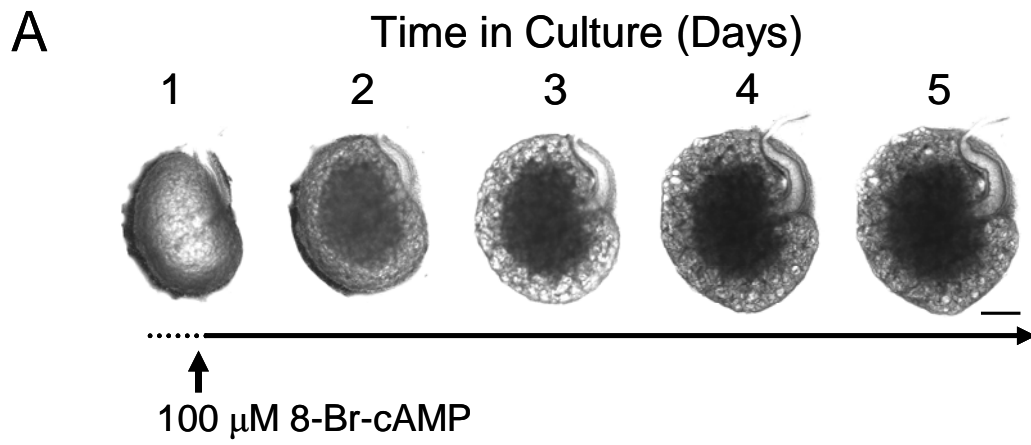


Figure 3. Metanephric cultures from *Pkd1*<sup>+/+</sup> mice treated with (A) 100  $\mu$ M 8-Br-cAMP or (B) 100  $\mu$ M 8-Br-cAMP and  $3 \times 10^{-8}$  M ouabain for five days. Media was replaced daily, and images were captured immediately afterwards. Bar, 1mm. (C) Fractional cyst area at the end of 5 days. Bars represent the means  $\pm$  SE of four experiments with 19 pairs of kidneys. \* $P < 0.05$  versus 8-Br-cAMP.

Figure 4

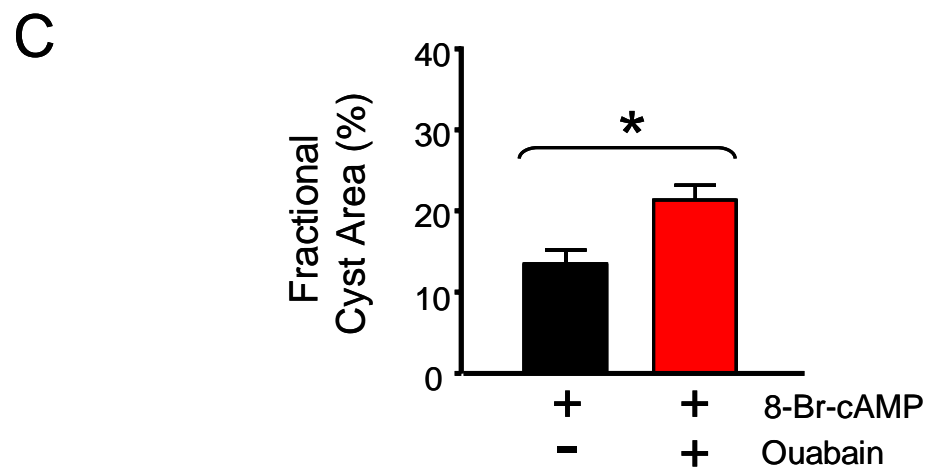
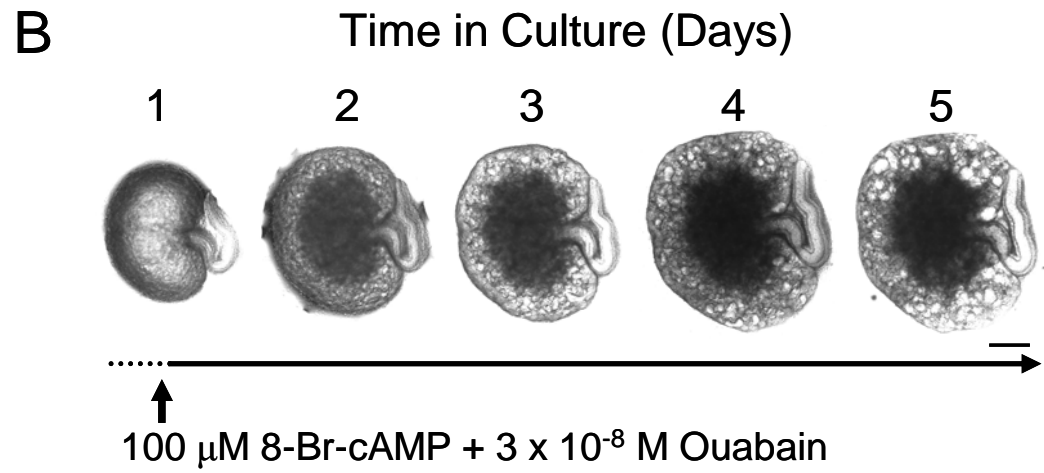
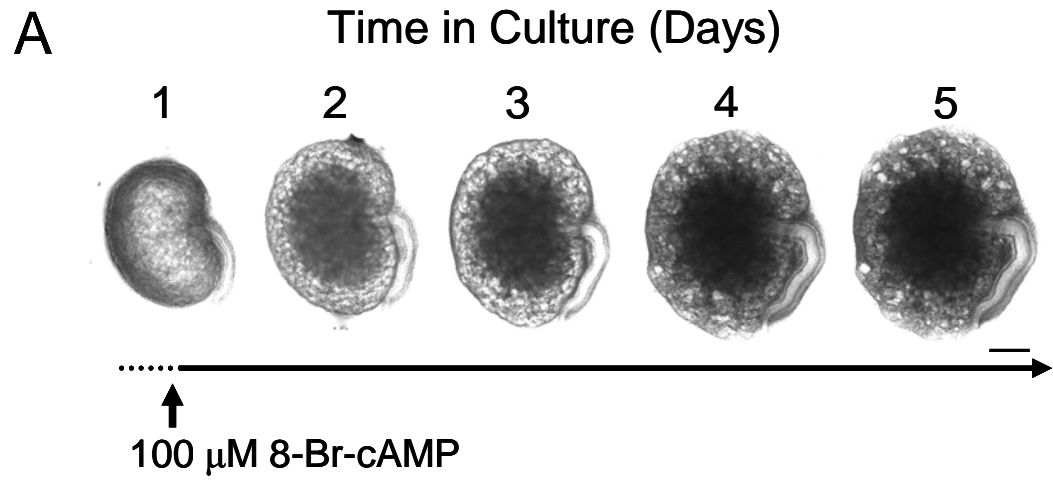


Figure 4. Metanephric cultures from *Pkd<sup>+/-</sup>* mice treated with (A) 100  $\mu$ M 8-Br-cAMP or (B) 100  $\mu$ M 8-Br-cAMP and  $3 \times 10^{-8}$  M ouabain for five days. Media was replaced daily, and images were captured immediately afterwards. Bar, 1mm. (C) Fractional cyst area at the end of 5 days. Bars represent the means  $\pm$  SE of three experiments with 12 pairs of kidneys. \* $P < 0.05$  versus 8-Br-cAMP.

Figure 5

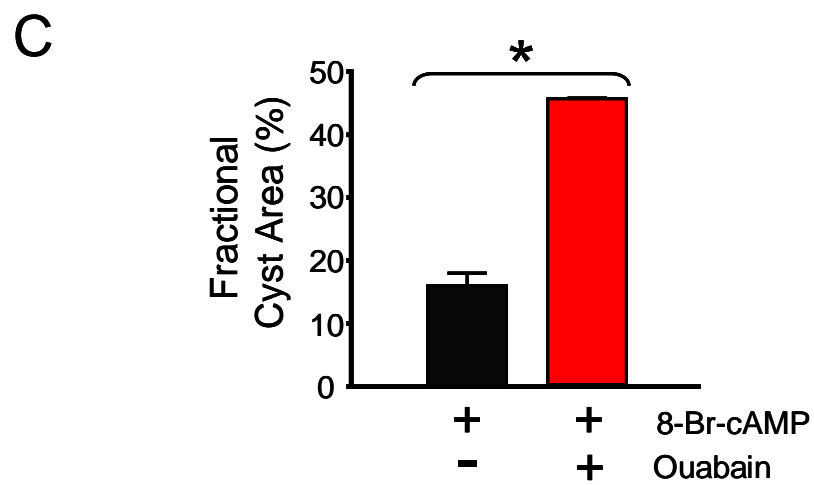
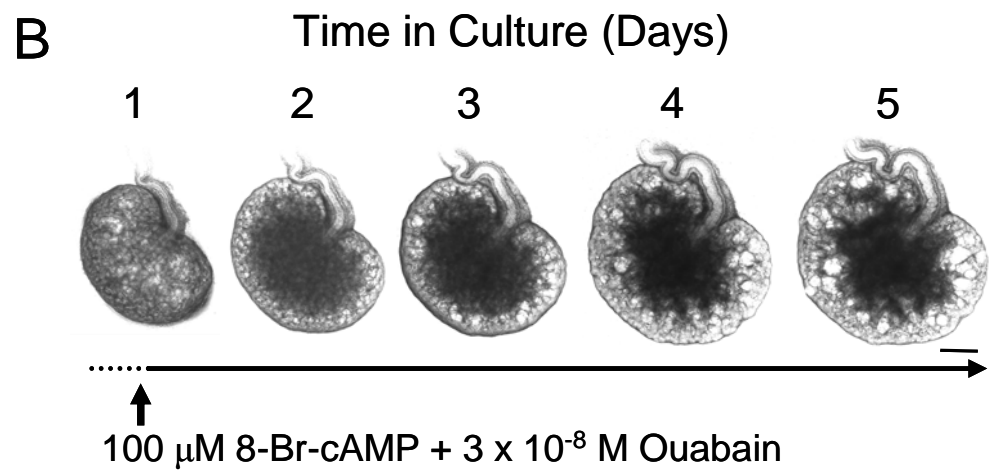
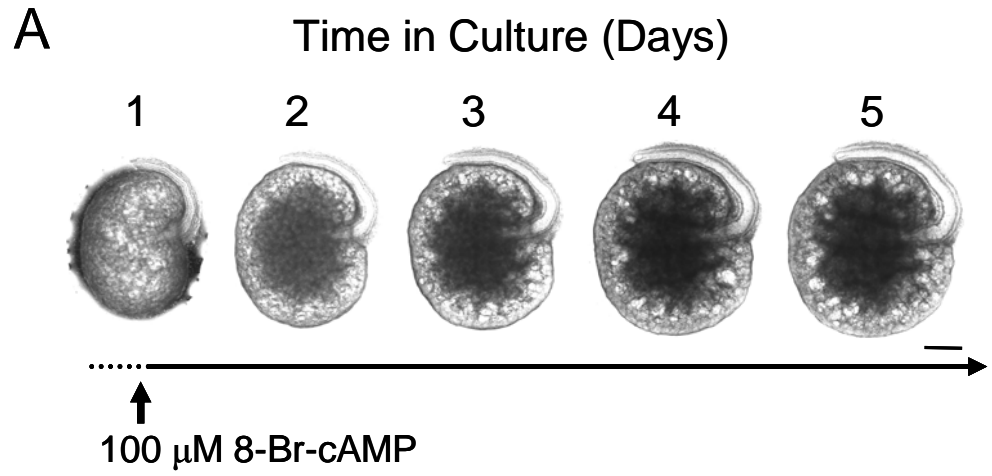


Figure 5. Metanephric cultures from *Pkd*<sup>-/-</sup> mice treated with (A) 100 μM 8-Br-cAMP or (B) 100 μM 8-Br-cAMP and 3 x 10<sup>-8</sup> M ouabain for five days. Media was replaced daily, and images were captured immediately afterwards. Bar, 1mm. (C) Fractional cyst area at the end of 5 days. Bars represent the means ± SE of three experiments with 4 pairs of kidneys. \**P* < 0.05 versus 8-Br-cAMP.

## CHAPTER 6

### ASSOCIATION WITH POLYCYSTIN-1 AFFECTS Na,K-ATPase FUNCTION

#### ABSTRACT

Experimental evidence in the last few years has shown that the Na,K-ATPase interacts with a variety of soluble and membrane-bound proteins in the cell. Interestingly, the Na,K-ATPase  $\alpha 1$  polypeptide has been shown to associate with the C-terminal tail of polycystin-1. Polycystins-1 and -2 (PC-1 and PC-2) are plasma membrane proteins that are altered in their expression patterns in autosomal polycystic kidney disease (ADPKD). To determine whether interaction with polycystins affect the function of the Na,K-ATPase, we expressed the proteins in Sf-9 insect cells using baculoviruses. When the eleven transmembrane domains and C-tail of PC-1 or the full length PC-2 were expressed with the rat Na,K-ATPase  $\alpha 1$  isoform, high levels of all polypeptides were detected at the plasma membrane and cytoplasm of the cells. Immunoprecipitation analysis indicated physical interaction between PC-1 and the Na,K-ATPase, but a negative association of the enzyme with PC-2. Analysis of the Na,K-ATPase kinetic properties showed no important changes in the apparent affinities of the enzyme to  $\text{Na}^+$  and  $\text{K}^+$ , both in the presence of PC-1 and PC-2. Dose-response curves for the inhibition of Na,K-ATPase activity by ouabain varied depending on the type of polycystin present. While PC-2 was not able to modify the normal resistance of the Na,K-ATPase  $\alpha 1$  isoform, PC-1 induced a shift in sensitivity to ouabain, with a change in  $K_i$  from  $\sim 10^{-5}$  M to  $\sim 10^{-8}$  M. Co-expression of PC-1 with the Na,K-ATPase in Sf-9 cells also increased the ability of the enzyme



to transduce the signaling effects of ouabain in the cells. Thus, in the presence of PC-1, ouabain-dependent phosphorylation of ERK was observed. The modulation of Na,K-ATPase response to ouabain by PC-1 may be important in the regulation of fluid transport and cell growth in the renal epithelium, and could contribute to the formation and maintenance of cysts in PKD.

## INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is the most common monogenic disease with an incidence of 1:1000, and is characterized by multiple fluid-filled cysts found in the kidneys, liver, pancreas and other organs. In the kidneys, the many cysts distort and damage the organ, leading to end-stage renal failure in approximately 50% of ADPKD patients [1, 2].

Approximately 85% of all cases of ADPKD result from a mutation in the *Pkd1* gene, encoding for a 450-460 kDa transmembrane protein called polycystin-1 (PC-1). This protein is predicted to span the membrane 11 times, with a large extracellular domain containing multiple motifs suggesting several roles, including cell-cell and cell-matrix interactions. The substantially smaller intracellular carboxyl-terminal region contains a G protein-binding motif and coiled-coil domain [1, 3]. The remaining cases of ADPKD are due to defects in the *Pkd2* gene, encoding for a ~110 kDa protein called polycystin-2 (PC-2). This protein spans the membrane six times, and has been shown to be a cation channel with permeability for  $\text{Ca}^{2+}$ . PC-1 and PC-2 physically interact via their coiled-coil domains in their cytoplasmic C-terminal tails, but can also function independently in various subcellular compartments. While mutations in either gene results in cystic disease, alterations in *Pkd1* lead to a more severe phenotype [1, 2, 4]. Interestingly, mutations in either gene may not necessarily result in alterations in expression levels of the protein. In primary cultures of epithelial cells derived from normal and cystic kidneys, there were similar levels of PC-1 expression with no obvious changes in the size of the protein.

However, there were differences in localization of the protein. For example, lower levels of PC-1 were found at the plasma membrane of ADPKD cells than in normal cells. Thus, it has been suggested that alterations in PC-1 localization are coincident with the disease status [3]. Thus, a key issue in understanding ADPKD is to elucidate how alterations in the functions of the polycystins can lead to cystogenesis.

The C-tail of polycystin-1 has been found to interact with the  $\alpha$  subunit of the Na,K-ATPase in yeast two-hybrid screens, *in vivo* in Madin-Darby canine kidney (MDCK) cells and in kidneys of transgenic mice overexpressing PC-1 [5, 6]. Operating at the basolateral membrane in normal kidneys, the Na,K-ATPase establishes an inward gradient of  $\text{Na}^+$  that secondarily drives the transport of other ions, glucose, amino acids and water across the renal epithelium [7]. Because of the crucial role of the Na,K-ATPase in  $\text{Na}^+$  and water absorption, alterations in its function may adversely affect renal function. In ADPKD, there is an alteration in the renal epithelium that switches it from being absorptive to secretory. Thus, alterations in the activity or regulation of the Na,K-ATPase may contribute to the pathogenesis of ADPKD.

Our studies of the kinetic properties of the Na,K-ATPase from epithelial cells of normal and ADPKD kidneys revealed that a population of the enzyme has a higher affinity for its inhibitor, ouabain [8]. In ADPKD, the altered ouabain sensitivity is not due to misexpression of isozymes of the Na,K-ATPase, since like normal cells, expression of only  $\alpha 1\beta 1$  was found in ADPKD cells. It is possible that the

functional changes of the Na,K-ATPase depend on interaction of the enzyme with PC-1 or PC-2 [9].

In addition to regulating the transport activity of the enzyme, the altered sensitivity of the Na,K-ATPase to ouabain may have other important consequences. The Na,K-ATPase is the receptor for cardiotonic steroids, such as ouabain [7]. Ouabain is a well characterized hormone that is synthesized in the adrenal glands of several mammal, including man [10]. Ouabain binding to the  $\alpha$  subunit of the Na,K-ATPase has been shown to initiate a cascade of intracellular phosphorylating events that lead to transactivation of the epidermal growth factor receptor (EGFR) and subsequent phosphorylation and activation of numerous proteins, including the mitogen activated MEK and MAP kinases [11]. These phosphorylating events result in increased expression of genes that are associated with cell growth and proliferation [11-13].

In this work, we studied the effect of the association between PC-1 and the Na,K-ATPase on the ouabain sensitivity, as well as the signaling ability of the enzyme. For this, we used the baculovirus expression system to express the Na,K-ATPase and polycystins in Sf-9 insect cells. These cells are uniquely suited for studying various aspects of Na,K-ATPase function, as they contain negligible endogenous levels of the enzyme [14]. Furthermore, they can produce abundant amounts of the transporter that are functional and retain their intrinsic ouabain binding capabilities [14]. Recent work has shown that the signaling role of the Na,K-ATPase is also retained in Sf-9 cells. Using this system, we explored the effect

of PC-1 and PC-2 on the ouabain affinity and ouabain-dependent signaling capacity of the Na,K-ATPase. Our results show an interaction between the Na,K-ATPase and a construct for PC-1 containing the 11 transmembrane domains and C-terminal tail. This interaction affects the affinity of the Na,K-ATPase for ouabain, rendering it more sensitive by several orders of magnitude; the affinity of the enzyme for the cations Na<sup>+</sup> and K<sup>+</sup> remain unchanged. In contrast, the Na,K-ATPase does not interact with an HA-tagged, full-length PC-2 and thus, its properties are not altered. This suggests that binding of PC-1 to the Na,K-ATPase is a mechanism of regulating the activity of the enzyme.

We found that the influence of PC-1 on the Na,K-ATPase not only enhanced the enzyme's affinity for ouabain, but stimulated its response to activate ERK phosphorylation with low levels of the hormone. The abnormal sensitivity of the Na,K-ATPase to ouabain in ADPKD cells may cause an alteration in the ouabain-Na,K-ATPase mediated signaling cascade, contributing to cystogenesis and maintenance.

## METHODS

### *DNA and viral constructions*

The cDNAs used in this study included: a) the  $\alpha 1$  and  $\beta 1$  subunits of the rat Na,K-ATPase, b) sIg11TM, a PC-1 construct (generously provided by Dr. R. Maser, KUMC) that included the 11 transmembrane domains and C-terminal tail of PC1 fused to a CD5 signal sequence for membrane insertion and the CH2-CH3 domain of human IgG for protein recognition, c) sIg11STOP, used as a control for sIg11TM, contained a stop codon after the first transmembrane domain, d) HA-PC2, a full-length PC-2 tagged with HA in the extracellular region of the polypeptide, between transmembrane segments 1 and 2 (generously provided by Dr. L. Tsiokas, Harvard Medical School), and e) HA-PC2STOP, used as a control for PC-2, contained HA and the first two transmembrane domains of PC-2. All constructs were subcloned into pBlueBac 4.5 baculovirus vector and recombinant baculoviruses were prepared and selected following standard procedures [15]. The resulting polypeptides of each construct are represented in a schematic diagram in Figure 1.

### *Cell and viral infections*

Sf-9 cells were grown in TNM:FH medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml fungizone. Viral infections were performed for 72 h. Cells were then scraped from the plates, centrifuged at 1,500 x g for 10 min and resuspended in 10 mM imidazole hydrochloride (pH 7.5), 1 mM EGTA. For determination of enzymatic activity, the

intact cells were used after permeabilization with the ionophore alamethicin (0.1 mg/mg of protein) for 10 min at 25 °C.

#### *Immunoblot analysis*

Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. After separation by SDS-PAGE, proteins were transferred to nitrocellulose and immunoblotted. The Na,K-ATPase  $\alpha$ 1 subunit was detected using the polyclonal anti- $\alpha$ 1 synthetic peptide antiserum or anti- $\alpha$ 1 C464/6B monoclonal antibody (provided by Dr. M. Caplan, Yale Univ.), sIg11TM and sIg11STOP were identified with an antibody against the human IgG. For HA-PC2 and HA-PC2STOP an antibody against HA was used. Phosphorylated ERK and total ERK levels were detected using antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-labeled antisera and chemiluminescence was used for detection. Quantitation of ERK activation was done by measuring band intensities of scanned images using the Gel-Pro software (Media Cybernetics, Silver Spring, MD). Levels of P-ERK and total ERK were expressed as density units relative to the untreated controls, and presented as P-ERK to total ERK ratios.

#### *Immunoprecipitation*

Sf-9 cells infected for 72h were lysed at 4°C with lysis buffer containing 0.01% SDS, 1% Triton X-100, 150mM sodium chloride and 25mM Hepes, pH 7.4. After removal of insoluble material by centrifugation (15,000 x g, 3 min), the cleared

lysates were subjected to immunoprecipitation. For PC1, the Na,K-ATPase was immunoprecipitated using 60  $\mu$ L of the anti- $\alpha$ 1, C464/6B monoclonal antibody and 50  $\mu$ L of goat anti-mouse IgG Dynabeads (DynaL Biotech, Inc., Lake Success, NY). For HA-PC2, 4  $\mu$ g of a monoclonal antibody to the HA epitope (Santa Cruz Biotechnology, Santa Cruz, CA) with 40  $\mu$ L of Goat anti-mouse IgG Dynabeads were used. In all cases, the lysates were incubated on a rotator overnight at 4°C, washed 3 times with lysis buffer, and the precipitated proteins were analyzed by SDS-PAGE (7.5% gel) and immunoblotted as described above.

#### *Immunocytochemistry and Confocal Microscopy*

Sf-9 cells were plated on 18mm glass coverslips, infected for 72h and processed for immunocytochemistry. The Na,K-ATPase was detected either with C464/6B or anti- $\alpha$ 1 synthetic peptide. sIg11TM and sIg11STOP were detected with an antibody to the human IgG conjugated to fluorescein isothiocyanate (FITC). HA-PC2 and HA-PC2STOP were detected with the anti-HA monoclonal antibody. Rhodamine-conjugated Goat anti-mouse IgG or FITC-conjugated Goat anti-rabbit IgG (Molecular Probes, Eugene, OR) were used as secondary antibodies. Samples were analysed using a Zeiss LSM 510 confocal microscope.

#### *Na,K-ATPase assays*

Maximal Na,K-ATPase activity was determined at 37°C for 30 min by measuring the initial rate of release of  $\gamma^{32}\text{P}_i$  from  $\gamma[^{32}\text{P}]\text{-ATP}$  in a final volume of



0.25 mL of medium containing 120 mM NaCl, 30 mM KCl, 3 mM MgCl<sub>2</sub>, 0.2 mM EGTA, 2 mM sodium azide, 30 mM Tris-HCl (pH 7.4) ± 1 mM ouabain, and 3 mM ATP with 0.2 µCi γ[<sup>32</sup>P]ATP. For the analysis of activation by Na<sup>+</sup> and K<sup>+</sup>, incubation media was the same as above except that for Na<sup>+</sup> dependency, Na<sup>+</sup> concentration was varied from 0 to 125 mM. For K<sup>+</sup> stimulation, K<sup>+</sup> was varied from 0 to 30 mM. Choline chloride was added to maintain the final concentration of Na<sup>+</sup> and K<sup>+</sup> plus choline at 180 mM. Ouabain dose-response was determined under saturating concentrations of all other cations at the indicated ouabain concentrations.

#### *Ouabain treatment and processing of cells for ERK analysis*

Following infection, cells were incubated for 48 h at 27°C when the complete culture media was changed to serum-free media. After an additional 24 h incubation period, cells were treated with or without 3 x 10<sup>-8</sup> M ouabain for 15 minutes. Experimental treatment was stopped by removing the media and washing the cells with ice-cold PBS. Cells were then lysed in RIPA buffer containing 1% NP-40, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 150 mM NaCl, 1 mM EDTA, 50 mM Tris and protease inhibitor cocktail (Sigma, St. Louis, MO). 30 µg of the cleared lysates were analyzed by 10% SDS-PAGE and immunoblotted as described [16].

#### *Data analysis*

Curve fitting of the experimental points was carried out using a Marquardt least-squares non linear regression computing program (Sigma Plot, Jandel Scientific, San Rafael, CA). Na<sup>+</sup> and K<sup>+</sup> activation curves were fitted according to a cooperative model for ligand binding:

$$v = (V_m [S]^n)/(K + [S]^n)$$

Where S is the concentration of the activating cation (Na<sup>+</sup> or K<sup>+</sup>) and n is the Hill coefficient. The apparent affinity,  $K_{0.5} = K_{1/n}$ .

Dose-response relations for the ouabain inhibition of Na,K-ATPase activity were fitted by the equation:  $v = 100 (1/(1 + [I]/K_i))$

Where v is the Na,K-ATPase activity corresponding to a certain concentration of the inhibitor ouabain [I], expressed as a fraction of activity in the absence of ouabain, and K<sub>i</sub> is the concentration of ouabain that gives the half-maximal inhibition.

## RESULTS

### *The Na,K-ATPase associates with polycystin-1*

To study the physical interaction and functional consequences of an association between the Na,K-ATPase and PC-1, we expressed the respective polypeptides in insect cells using baculoviruses (Figures 1A and 1B).

Immunolocalization studies revealed high levels of each polypeptide, primarily at the plasma membrane (Figure 2). Immunoblot analyses of whole lysates and co-immunoprecipitated complexes revealed that the presence of either sIg11TM or its control did not affect Na,K-ATPase expression (Figure 3A). Likewise, sIg11TM and its control were detected at high levels when co-expressed with Na,K-ATPase (Figure 3B). Thus, the baculovirus system is well suited to examine the proteins in this study.

Interaction of sIg11TM or its control with Na,K-ATPase was determined by co-immunoprecipitation with an antibody against the  $\alpha 1$  subunit of the Na,K-ATPase. As shown in Figure 3C, the immunocomplex captured by the anti- $\alpha 1$  antibody indicated only sIg11TM, and not its control, co-immunoprecipitated with the Na,K-ATPase. This suggests that the large extracellular domain of PC-1 is not required for its association with the Na,K-ATPase.

### *Association of the Na,K-ATPase with polycystin-1 affects its enzymatic activity*

As a first approach to understand the consequences of the interaction of the Na,K-ATPase with sIg11TM, we determined the kinetic properties of the enzyme in co-infected Sf-9 cells. As shown in Figure 4, the activation curves by  $\text{Na}^+$  and  $\text{K}^+$

indicated no significant differences in the presence or absence of sIg11TM. In contrast, dose-response curves of Na,K-ATPase activity showed that when co-expressed with sIg11TM, the Na,K-ATPase displays an affinity for ouabain that is higher than that of the  $\alpha 1\beta 1$  complex alone. The presence of sIg11TM produced a shift in the affinity of the enzyme for ouabain by three orders of magnitude, with a change in  $K_i$  from approximately  $3 \times 10^{-5}$  M to  $3 \times 10^{-8}$  M (Table 1). These results indicate that PC-1 is able to affect the sensitivity of the Na,K-ATPase  $\alpha 1\beta 1$  isozyme to ouabain.

*The Na,K-ATPase does not interact with polycystin-2, and its enzymatic activity is unchanged*

In addition to PC-1, we explored the interaction between the Na,K-ATPase and PC-2. To do so, two constructs were prepared: one contained the full length PC-2 tagged with HA in the extracellular region of the polypeptide, between transmembrane segments one and two (HA-PC2). Its control, HA-PC2STOP, contained only the first two transmembrane domains with the HA tag; the C-terminal tail is absent (Figure 1C). These constructs were used to produce baculoviruses for expression in Sf-9 cells in conjunction with the Na,K-ATPase. We repeated the same analyses as done with sIg11TM to examine if PC-2 affected function of the enzyme.

Similar to the expression of sIg11TM, we detected high levels of PC-2 and its control in Sf-9 cells co-expressing the  $\alpha 1\beta 1$  heterodimer of the Na,K-ATPase. Immunofluorescence imaging of the polypeptides, shown in Figure 5, found PC-2 and

its control to be found both at the plasma membrane and in the cytoplasm of Sf-9 cells, while the Na,K-ATPase was delivered to the plasma membrane. Western blot analyses of Sf-9 cells co-expressing the Na,K-ATPase with PC-2 or its control also revealed high levels of each polypeptide (Figure 6A and 6B). Immunoprecipitation using an anti-HA antibody to pull down PC-2 and anti-Na,K-ATPase  $\alpha 1$  for identification of the transporter in immunoblots showed no association of the Na,K-ATPase with PC-2 (Figure 6C). Not surprisingly, the presence of PC-2 or its control in Sf-9 cells co-expressing the  $\alpha 1\beta 1$  isozyme of the Na,K-ATPase did not affect its enzymatic properties. As shown in Figure 7 and summarized in Table 2, neither PC-2 nor its control altered the affinity of the Na,K-ATPase for its ligands,  $\text{Na}^+$  and  $\text{K}^+$ , or for the inhibitor ouabain.

These studies confirmed an interaction of the Na,K-ATPase with PC-1, but not PC-2, and this interaction only alters its sensitivity to the hormone ouabain and not for the cations.

#### *Ouabain signaling via the Na,K-ATPase is enhanced by PC-1*

Previous works have revealed that ouabain binding to the Na,K-ATPase is not just to restrict its catalytic and transport properties, but it also serves as a signal for Na,K-ATPase-mediated cellular effects. We have shown that physiological levels of ouabain leads to ERK activation in renal epithelial cells from patients with autosomal dominant polycystic kidney disease (ADPKD), while there is not a significant effect in cells from normal kidneys [16]. Furthermore, there is a population of the enzyme

that is more sensitive to ouabain, although the isozyme composition did not differ between the normal and cystic tissue. This suggests that the heightened sensitivity of the Na,K-ATPase to ouabain may be attributed to interaction with PC-1.

To determine the role of PC-1 on altering the signaling capacity of the Na,K-ATPase, we used the baculovirus expression system, and determined ouabain/Na,K-ATPase signaling by ERK activation. We found that when the Na,K-ATPase was co-expressed with sIg11TM in Sf-9 cells, treatment with  $3 \times 10^{-8}$  M ouabain stimulated phosphorylation of ERK (Figure 8). In contrast, cells expressing sIg11STOP or PC-2 with the Na,K-ATPase did not lead to ERK activation after treatment with ouabain. Therefore, the heightened sensitivity of the Na,K-ATPase to ouabain due to its interaction with PC-1 may result in enhanced ouabain/Na,K-ATPase signaling in ADPKD.

## DISCUSSION

The affinity of the Na,K-ATPase to ouabain is a key factor in regulating the "pumping" activity of the enzyme, as well as the cellular response to the hormone. Protein-protein interaction has been shown to affect some enzymatic properties of the Na,K-ATPase [17]. In this work using the heterologous baculovirus expression system, we were able to study the interaction of the Na,K-ATPase with the polycystins. We confirmed an interaction of the Na,K-ATPase with PC-1, most likely via the C-tail of PC-1, in agreement with previous reports [5, 6]. In addition, we determined the functional relevance of that association in an environment essentially free of endogenous Na,K-ATPase [14]. Interestingly, although PC-1 and PC-2 have been demonstrated both *in vitro* and *in vivo* to interact via their C-terminal tails [2, 18], PC-2 was not found to interact with the Na,K-ATPase. One explanation for this curious finding may be that the Na,K-ATPase only interacts with PC-1 in the disease state when PC-1 and PC-2 are not functioning normally.

Mutations in the genes encoding for polycystin-1 and -2 do not necessarily result in a loss of expression of these proteins. Rather, it may result in functionally defective or null proteins. It has been suggested that a disruption in the stoichiometric balance of the two polycystins can result in cyst development [3, 19, 20]. It is possible that when this balance is disrupted, PC-1 cannot interact with PC-2 as it normally would, and instead interacts with the Na,K-ATPase to affect its function. Most notably, it alters the enzyme's affinity for the steroid hormone ouabain by three orders of magnitude, similar to the  $K_i$  of the sensitive component

found in cystic kidneys and cells [16], and consistent with levels that are found in circulation. The consequences of this alteration lie in the role of the Na,K-ATPase as a signal transducer for the hormone [11, 21].

Cyst formation and enlargement have been found to be associated with activation of tyrosine kinase-mediated signaling, as well as cAMP-mediated signaling [2, 22-24]. A central pathway activated in ADPKD leading to cell growth involves the extracellular regulated kinase (MAPK/ERK) pathway, with an abnormal abundance and activation of B-Raf by cAMP-mediated PKA activation [25-27]. Because Sf-9 cells express ERK that can be modified by phosphorylation [28], we were able to study the signaling capacity of the Na,K-ATPase in the presence of PC-1 or PC-2. Our work revealed that PC-1, but not PC-2, enhanced the ouabain/Na,K-ATPase signaling.

The interaction of the Na,K-ATPase with PC-1 and subsequent alteration in the enzyme's affinity for ouabain may have important consequences in the pathophysiology of ADPKD. By inhibiting the "pumping" activities of the Na,K-ATPase, it may dissipate the Na<sup>+</sup> gradient, thereby limiting optimal water reabsorption to allow greater fluid secretion into the cyst cavity, allowing the cysts to expand. Concomitantly, the altered sensitivity of the Na,K-ATPase to ouabain may render it more susceptible to the proliferative effects of the hormone. These studies revealing that PC-1 altered the sensitivity of the Na,K-ATPase to ouabain, and enhanced ouabain/Na,K-ATPase signaling may be the mechanism by which the hormone can adversely affect the progression of ADPKD.



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Figure 1

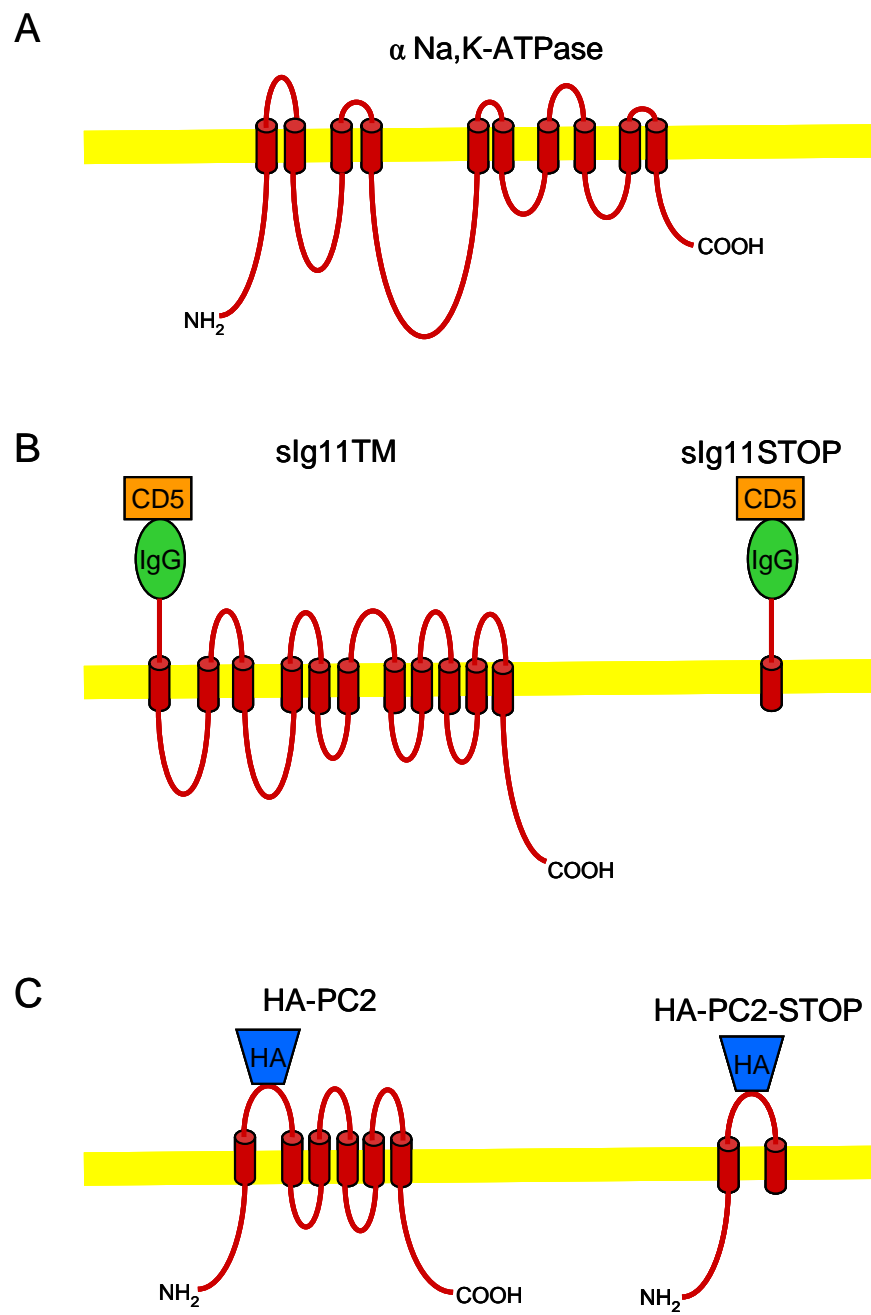


Figure 1. Schematic models of the various polypeptides used in this study. (A) The  $\alpha$  subunit of the Na,K-ATPase is represented, and its interaction with polycystin-1 is believed to occur within the N-terminal tail, the cytosolic loop between transmembrane domains 2 and 3, and/or the large intracellular loop between transmembrane segments 4 and 5 [5, 6]. (B) The construct for polycystin-1, sIg11TM, and its control, sIg11STOP. The CD5 sequence directs trafficking of the protein to the plasma membrane, while the IgG domain is for protein recognition. All 11 transmembrane domains and the full C-terminal tail comprise sIg11TM, while sIg11STOP only expresses the signal sequence, IgG and first transmembrane domains. (C) The construct for PC2, HA-PC2, contains the full-length protein with an HA tag between the first two transmembrane domains. In contrast, its control, HA-PC2STOP, does not extend beyond the first two transmembrane sequences.

Figure 2

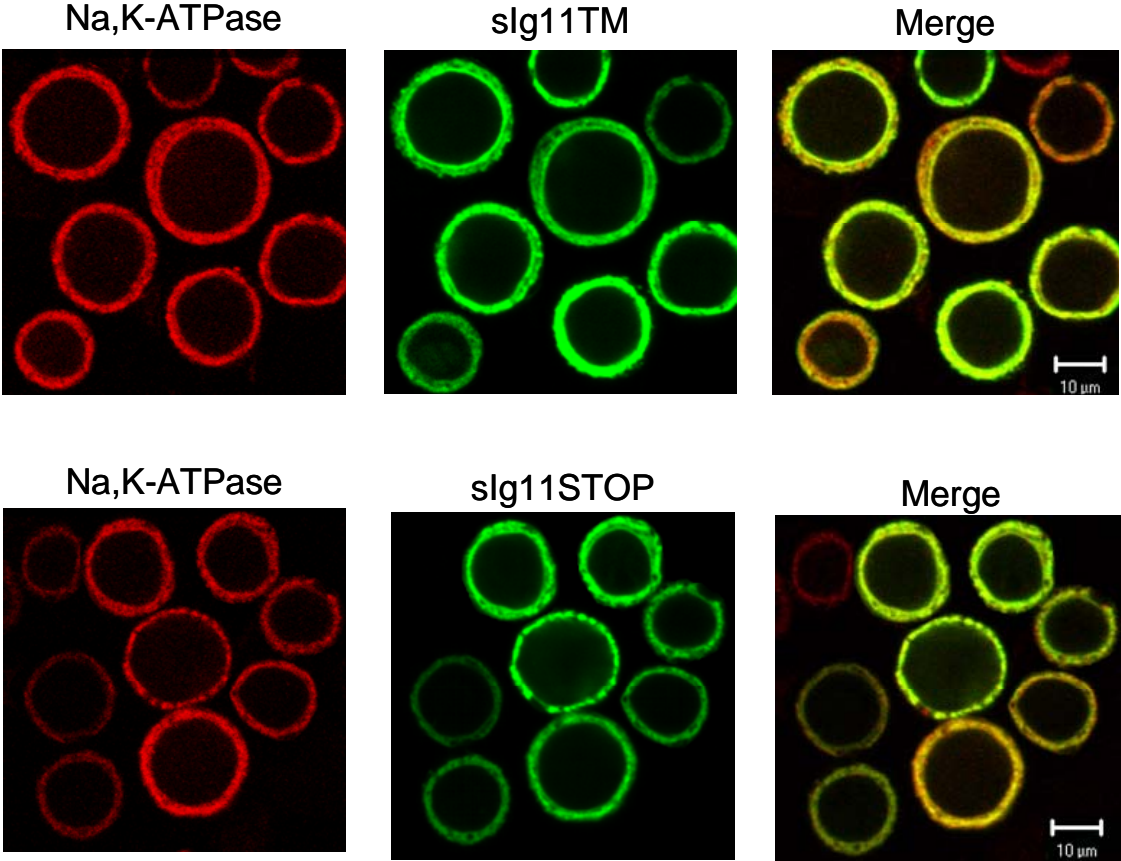


Figure 2. Immunolocalization of Na,K-ATPase and sIg11TM or sIg11STOP in Sf-9 cells. (A) Expression of the Na,K-ATPase  $\alpha$ 1 subunit and sIg11TM. (B) Cells coinfecting with Na,K-ATPase  $\alpha$ 1 and sIg11STOP. The  $\alpha$ 1 subunit was detected with the C464/6B monoclonal antibody and a rhodamine conjugated secondary antibody. sIg11TM and sIg11STOP were detected with an antibody against the human IgG domain conjugated to FITC. All polypeptides were expressed at high levels and colocalized primarily to the plasma membrane of the cells. Bar, 10  $\mu$ m.

Figure 3

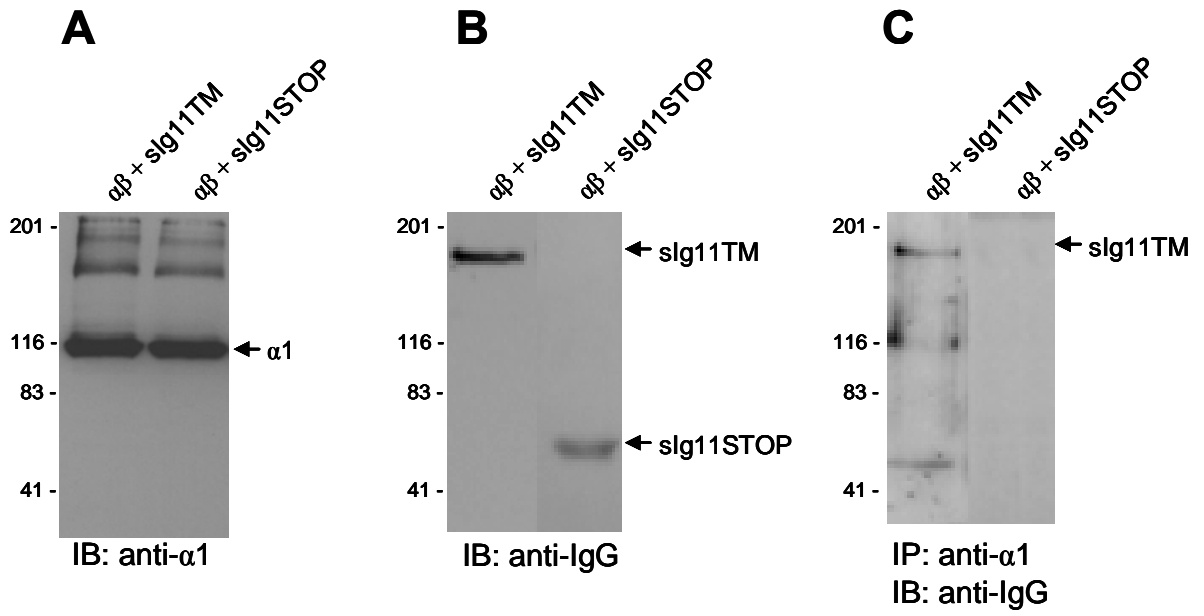




Figure 3. Immunoblot and immunoprecipitation analysis. Sf-9 cells coinfecting with baculoviruses driving expression of Na,K-ATPase  $\alpha 1\beta 1$  and sIg11TM or sIg11STOP were analyzed for expression of (A) the  $\alpha 1$  and (B) the sIg11TM or sIg11STOP polypeptides. (C) Immunoprecipitation of the Na,K-ATPase  $\alpha 1$  subunit and sIg11TM and sIg11STOP constructs. C464/6B antibody was used for immunoprecipitation and an antiserum that recognizes human IgG was used for the immunoblot. The Na,K-ATPase associates with sIg11TM, but not sIg11STOP.

Figure 4

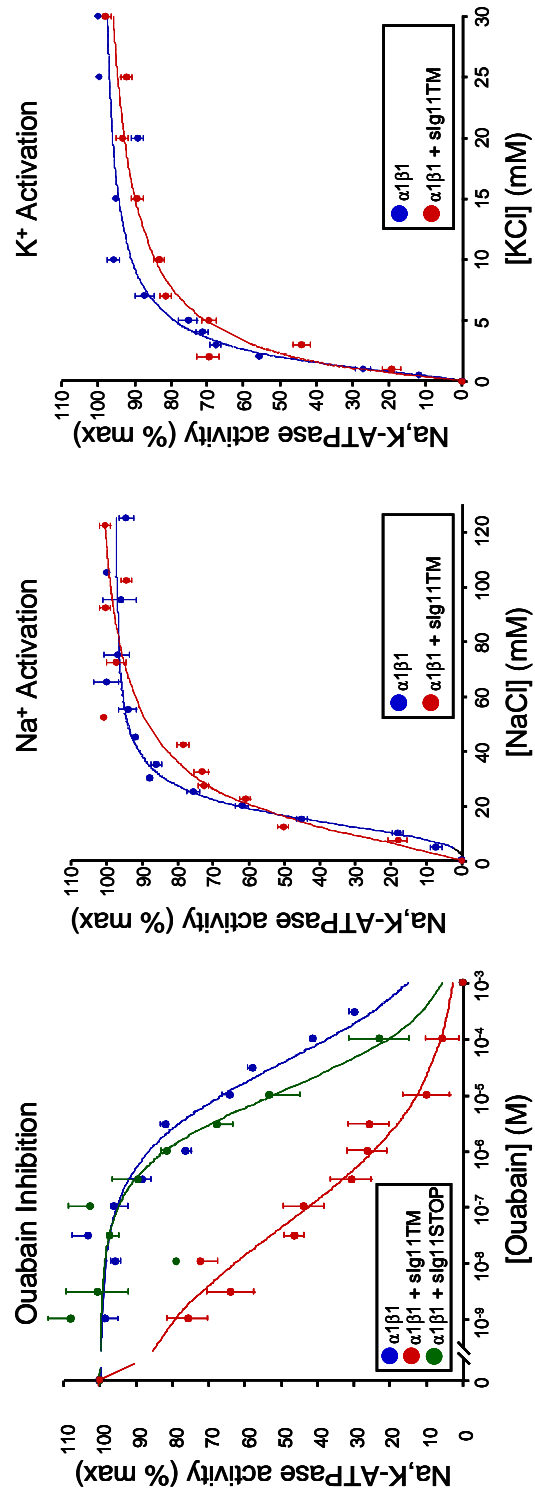


Figure 4. Na,K-ATPase kinetic properties in the absence and presence of PC-1 constructs. (A) Dose-response curves for the ouabain inhibition of Na,K-ATPase  $\alpha 1\beta 1$  activity  $\pm$  sIg11TM and sIg11STOP. (B) Activation of  $\alpha 1\beta 1$  by  $\text{Na}^+ \pm$  sIg11TM. (C) Activation of  $\alpha 1\beta 1$  by  $\text{K}^+ \pm$  sIg11TM. While sIg11TM did not affect the apparent affinities of the Na,K-ATPase to  $\text{Na}^+$  and  $\text{K}^+$ , it changed the affinity for ouabain, making the enzyme more sensitive to the compound.

Figure 5

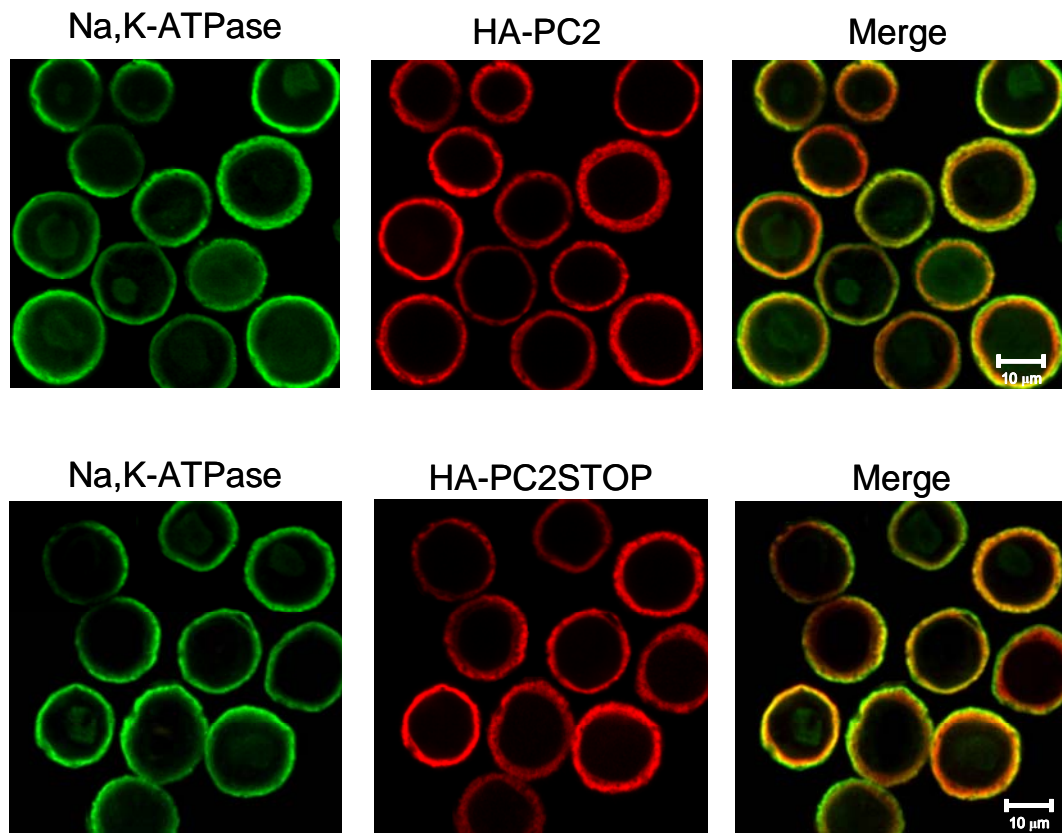


Figure 5. Immunolocalization of Na,K-ATPase and HA-PC2 or HA-PC2STOP in Sf-9 cells. (A) Expression of the Na,K-ATPase  $\alpha$ 1 subunit and HA-PC2. (B) Cells coinfecting with Na,K-ATPase  $\alpha$ 1 and HA-PC2STOP. The  $\alpha$ 1 subunit was detected using the anti- $\alpha$ 1 synthetic peptide antiserum and an anti-rabbit conjugated with FITC. HA-PC2 and HA-PC2STOP were detected by a monoclonal antibody to the HA epitope and a secondary antiserum conjugated to rhodamine. All polypeptides were expressed at high levels. While the  $\alpha$ 1 polypeptide localized primarily to the membrane, HA-PC2 and HA-PC2STOP were found both at the plasma membrane and cytoplasm. Bar, 10  $\mu$ m.

Figure 6

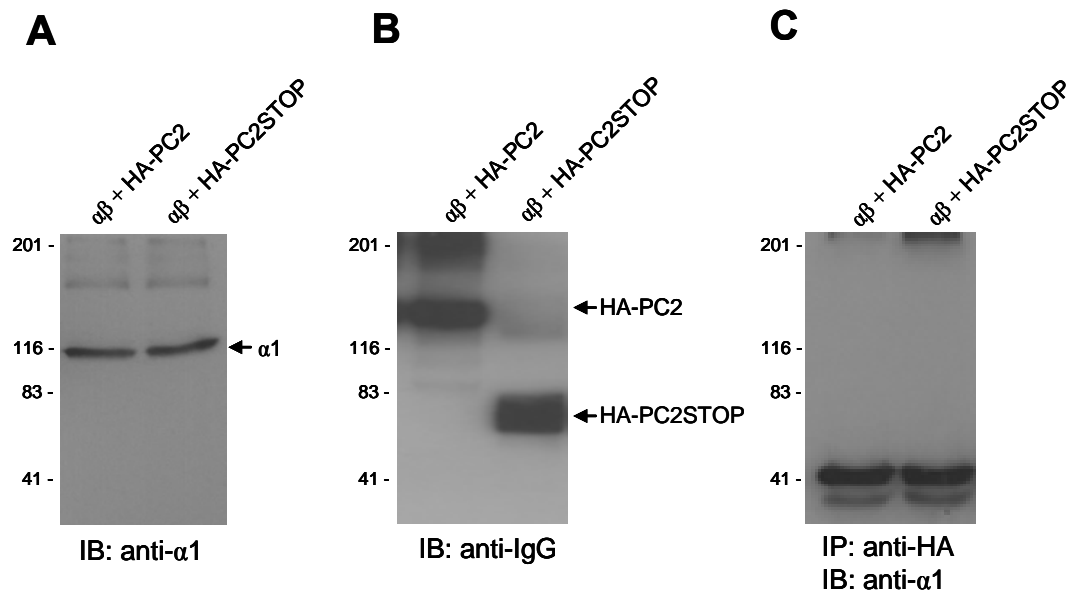


Figure 6. Immunoblot and Immunoprecipitation analysis. Sf-9 cells coinfecting with rat Na,K-ATPase  $\alpha 1\beta 1$  and HA-PC2 or HA-PC2STOP were analyzed for expression of (A) the  $\alpha 1$  and (B) HA-PC2 or HA-PC2STOP polypeptides. (C) Immunoprecipitation of PC-2 constructs and the Na,K-ATPase. An antiserum against HA was used for immunoprecipitation, and anti- $\alpha 1$  synthetic peptide was used for the immunoblot. Neither HA-PC2, nor HA-PC2STOP associate with the Na,K-ATPase.

Figure 7

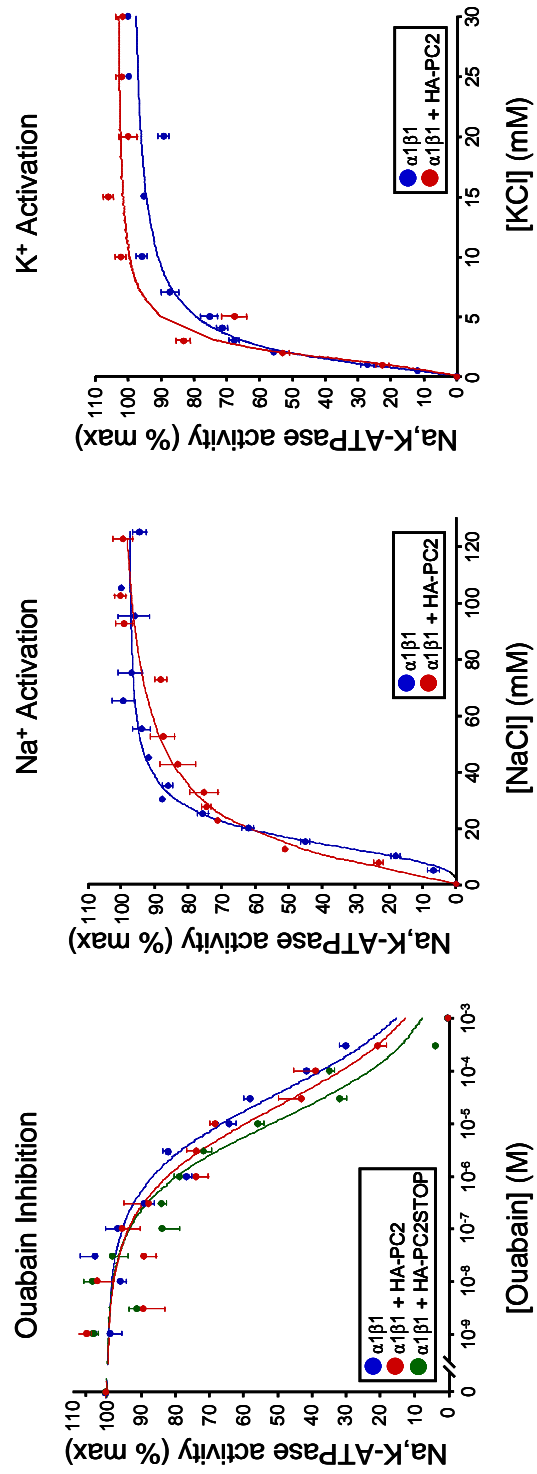




Figure 7. Na,K-ATPase kinetic properties in the absence and presence of PC2 constructs. (A) Dose-response curves for the ouabain inhibition of Na,K-ATPase  $\alpha 1\beta 1$  activity  $\pm$  HA-PC2 and its control, HA-PC2STOP. (B) Activation of  $\alpha 1\beta 1$  by  $\text{Na}^+$   $\pm$  HA-PC2. (C) Activation of  $\alpha 1\beta 1$  by  $\text{K}^+$   $\pm$  HA-PC2. PC2 does not modify the kinetic properties of Na,K-ATPase  $\alpha 1\beta 1$  to  $\text{Na}^+$ ,  $\text{K}^+$  or ouabain.

Figure 8

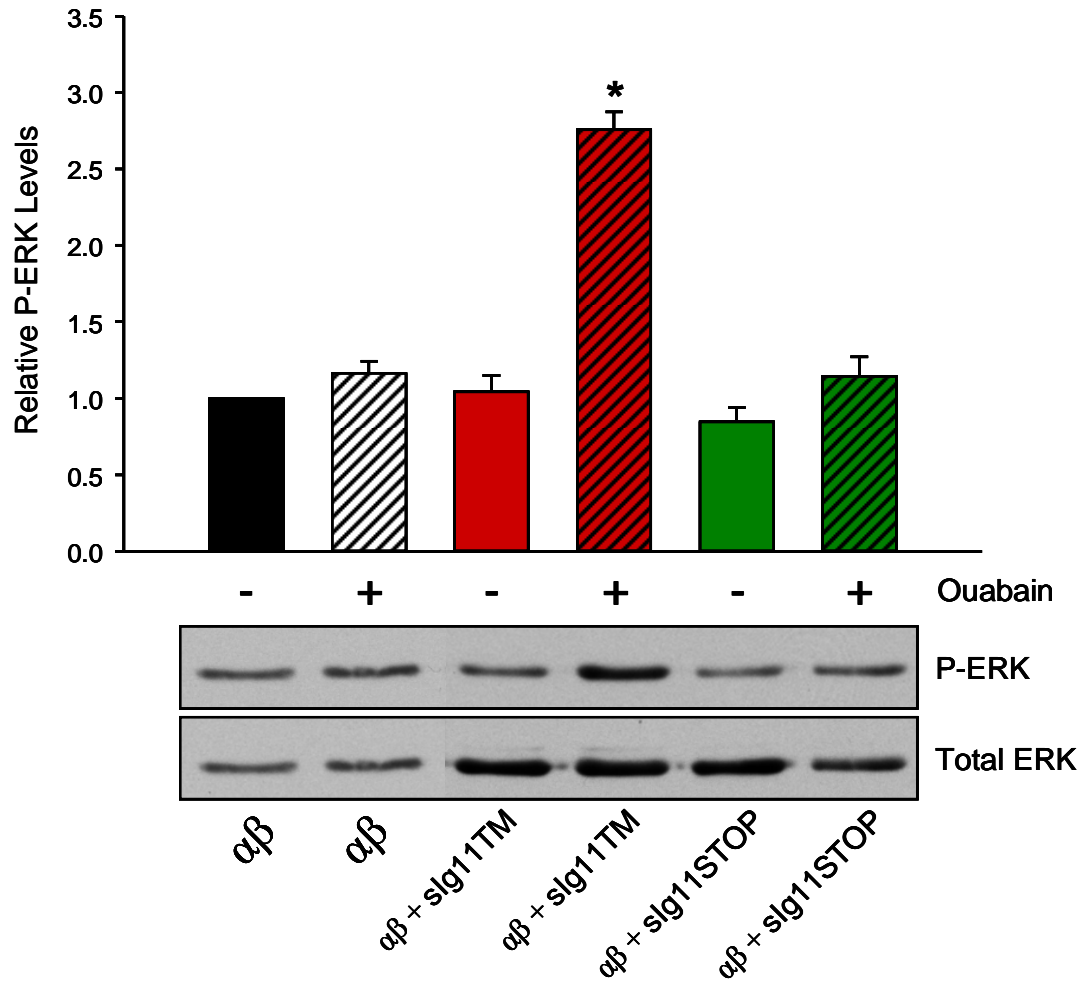


Figure 8. Ouabain-Na,K-ATPase mediated ERK phosphorylation in the presence and absence of sIg11TM in insect cells. Sf-9 cells expressing rat Na,K-ATPase  $\alpha 1\beta 1$  alone or with sIg11TM or sIG11STOP were treated with culture media alone (solid bar) or with  $3 \times 10^{-8}$  M ouabain (dashed bars) for 15 min. Phospho-ERK/total ERK ratios were normalized against the untreated control cells expressing Na,K-ATPase  $\alpha 1\beta 1$  alone. A representative blot is shown, while bars are means  $\pm$  SE of 3 independent experiments. \* $P < 0.001$  vs untreated control.

**Table 1. Kinetic characteristics of Na,K-ATPase in the presence and absence of PC-1 constructs.**

	Ouabain		Na <sup>+</sup> activation		K <sup>+</sup> activation	
	K <sub>i</sub> (M)	r <sub>H</sub>	K <sub>0.5</sub> (mM)	r <sub>H</sub>	K <sub>0.5</sub> (mM)	r <sub>H</sub>
Na,K-ATPase	3.6 ± 0.9 × 10 <sup>-5</sup>	0.5 ± 0.06	16.4 ± 0.7	2.89 ± 0.2	1.9 ± 0.2	1.42 ± 0.2
+slg11TM	2.6 ± 0.1 × 10 <sup>-8</sup>	0.3 ± 0.04	16.8 ± 2.1	1.50 ± 0.3	2.3 ± 0.8	1.2 ± 0.5
+slg11STOP	1.1 ± 0.3 × 10 <sup>-5</sup>	0.6 ± 0.10	-----	-----	-----	-----

Apparent affinities (K<sub>0.5</sub>), inhibition constants (K<sub>i</sub>) and Hill coefficients (r<sub>H</sub>) were calculated from dose-response curves for the activity of Na,K-ATPase for the indicated ligands. Values represent the mean ± standard error of the mean.

**Table 2. Kinetic characteristics of Na,K-ATPase in the presence and absence of PC-2 constructs.**

	Ouabain		Na <sup>+</sup> activation		K <sup>+</sup> activation	
	K <sub>i</sub> (M)	n <sub>H</sub>	K <sub>0.5</sub> (mM)	n <sub>H</sub>	K <sub>0.5</sub> (mM)	n <sub>H</sub>
Na,K-ATPase	3.6 ± 0.9 × 10 <sup>-5</sup>	0.5 ± 0.06	16.4 ± 0.7	2.89 ± 0.2	1.9 ± 0.2	1.42 ± 0.2
+ HA-PC2	2.1 ± 0.6 × 10 <sup>-8</sup>	0.5 ± 0.06	14.6 ± 1.4	1.40 ± 0.2	1.9 ± 0.3	1.9 ± 0.6
+ HA-PC2STOP	1.1 ± 0.3 × 10 <sup>-5</sup>	0.6 ± 0.10	-----	-----	-----	-----

Apparent affinities (K<sub>0.5</sub>), inhibition constants (K<sub>i</sub>) and Hill coefficients (n<sub>H</sub>) were calculated from dose-response curves for the activity of Na,K-ATPase for the indicated ligands. Values represent the mean ± standard error of the mean.

## OVERALL CONCLUSIONS

Cyst formation and development in autosomal dominant polycystic kidney disease (ADPKD) is characterized by increases in both cell proliferation and apoptosis, as well as in alterations in the renal epithelium such that fluid is secreted into the cyst cavity instead of being reabsorbed into the bloodstream. Understanding the factors that influence these parameters in ADPKD cells is essential in comprehending the mechanism(s) involved in cystogenesis, as well as for the future development of approaches that can be used to control the progression of the disease. Because of its central role in the vectorial movement of salt and water, the ion transport properties of the Na,K-ATPase have been the focus of investigation to understand the pathophysiology of ADPKD. Interestingly, in addition to its activity as an ion transporter, the Na,K-ATPase also functions as the receptor and signal transducer for ouabain. Ouabain is a well characterized hormone known to trigger signaling events that induce changes in metabolism, motility and growth in several cell types. At present, no information is available regarding the role of ouabain-Na,K-ATPase signaling in ADPKD. We have studied this issue in this work. Surprisingly, we have found that the Na,K-ATPase of human ADPKD cells exhibit an abnormal increase in the affinity for ouabain. Moreover, we have also found that ouabain, in amounts similar to those circulating in blood, stimulated ADPKD cell growth by approximately 40 %. In contrast, the hormone does not significantly affect proliferation of normal human kidney epithelial cells, which coincidentally have a lower affinity for the hormone.

Ouabain-induced proliferation depended on activation of EGFR, Src, B-Raf, MEK and ERK1/2. Upon activation, phosphorylated ERK1/2 translocates to the nucleus where it likely activates transcription of genes involved in cell growth and proliferation. Activation of this cascade of intracellular events required integrity of cellular caveolae, where the Na,K-ATPase has been shown to form a signaling complex, described in other cell types, as the signalosome. Our results agree with the existence of the Na,K-ATPase signalosome in ADPKD cells, and it involves pathways that are shared with those of other cell types. Importantly, our results are one of the first to establish ouabain signaling in diseased cells. More importantly, ADPKD cells have a higher affinity for ouabain, and are thus more susceptible to signaling ouabain effects into the cells. The cascade of intracellular signaling events we identified to be induced by ouabain have been shown to be of critical importance in mediating the progression of PKD. For example, targeting these signaling molecules by pharmacological intervention in rodent models of PKD have been shown to attenuate the disease, confirming the importance of this signaling pathway in ADPKD pathophysiology. Our results have therefore uncovered a new mechanism capable of influencing cyst growth. Compounds that can compete for ouabain binding to the Na,K-ATPase and block its signaling have been developed and are under clinical phase trials. This includes, for example, the drug Rostafuroxin (PST 2238) [1, 2]. Also Digibind, a Fab fragment of antisera directed against digoxin, has been shown to reverse the inhibitory effect of ouabain on the activity of the Na,K-ATPase, most likely by immunoneutralization [3]. It is expected that future use of

these compounds would be of benefit in preventing endogenous ouabain from binding to the Na,K-ATPase in cystic tissue. This could block ouabain-stimulated growth in ADPKD cells, to decrease the epithelial proliferation required for cyst expansion.

In addition to its mitogenic effect, we found that physiological levels of ouabain also promote apoptotic death of ADPKD cells, as determined by DNA fragmentation and TUNEL assay. Increased apoptosis has been reported in ADPKD, and inhibition of this process in a rodent model of ADPKD has been shown to slow progression of the disease. Ouabain-dependent increase in apoptosis does not parallel the increase the hormone causes in cell proliferation. This shows that ouabain induces a disbalance in cell proliferation and removal that favors growth of the ADPKD epithelium. This dual effect of ouabain on cell proliferation and apoptosis further supports its role in exacerbation of the disease. The disbalance in cell renewal has been identified as an important characteristic of the ADPKD epithelium. Our results have identified ouabain as one of the mechanism(s) leading to the abnormal regulation of cell growth in ADPKD. In addition, we provide evidence for the pathways through which ouabain promotes cell apoptosis. Upon ouabain exposure, caspase-3 is activated in the cells. Ouabain also stimulated the release of mitochondrial cytochrome c into the cell cytoplasm. This suggested that ouabain-mediated apoptosis takes place via the intrinsic pathway of apoptotic cell death.

Our results show that ouabain also affects cyst development. Thus, the hormone increased the number and size of microcysts formed by ADPKD cells in three dimensional collagen cultures. Also, ouabain enhanced the fractional area of



cysts developed by metanephric organ cultures from *Pkd1<sup>m1Bei</sup>* mice, an animal model of ADPKD. This suggests that ouabain may not only act by promoting cell proliferation, but also by activating the mechanisms involved in fluid secretion by the ADPKD epithelium. Although additional experiments will be conducted in the future to confirm the role of ouabain in regulating fluid secretion, it is interesting to propose that ouabain may be acting in two ways to enhance fluid movement into the cyst cavity. One involves the ion transport activity of the Na,K-ATPase, and comprises a partial inhibition of the enzyme by ouabain, which will reduce the reabsorbing capacity of the epithelium. The second involves the signaling ability of the Na,K-ATPase, which by activating intracellular signaling events that may increase cAMP levels, may induce post-translational modifications to proteins involved in fluid secretion, such as the NKCC1 or CFTR. Both of these transporters have been identified as the major routes for salt and water secretion in ADPKD that is under the influence of the MAPK pathway. The similarity in pathways activated by ouabain with those reported to cause activation of NKCC1 and CFTR supports these hypothesis.

An intriguing observation from our experiments is the alteration in the ouabain affinity phenotype of the Na,K-ATPase of ADPKD cells. We found that approximately 20% of the Na,K-ATPase of ADPKD cells is able to bind ouabain with an inhibition constant in the nanomolar range. We have demonstrated that this change in ouabain affinity does not depend on mis-expression of different isoforms of the Na,K-ATPase, as the cystic cells present the  $\alpha 1\beta 1$  isozyme normally present in

kidney cells. Interestingly we show that association of the Na,K-ATPase with PC-1 causes the phenotypic switch on ouabain affinity of the enzyme. Interaction with PC-1 had previously been reported, using overexpression of both polypeptides in CHO cells and in animal models of ADPKD. Our work is the first demonstration that Na,K-ATPase interaction with PC-1 is functionally relevant and modulates the reactivity of the enzyme to ouabain. Our results in insect cells using baculovirus-directed expression show that the increased reactivity to ouabain also causes increased ability of the Na,K-ATPase to signal ouabain effects in the cells. In ADPKD, expression of mutated and deleted forms of PC-1 has been reported to act as dominant negative forms of the polypeptide causing the disease. From the experimental evidence we gathered, we propose a model in which the affinity of the Na,K-ATPase for ouabain is increased by enhanced association with PC-1. This will make the ADPKD cells more sensitive to circulating levels of ouabain. Na,K-ATPase signaling in these cells will then be abnormally activated and the effects of ouabain in cell growth and fluid secretion enhanced. This will exacerbate cyst progression in ADPKD.

In conclusion, we have found that ouabain is a novel factor capable of stimulating the enlargement of ADPKD renal cysts. Future research should be aimed to block ouabain-induced Na,K-ATPase signaling as a way to reduce cyst development and progression in the disease.

## REFERENCES

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