NOVEL EFFECTS OF OUABAIN IN AUTOSOMAL DOMINANT

POLYCYSTIC KIDNEY DISEASE CYSTOGENESIS

By

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ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD) is caused by mutations in *Pkd1* or *Pkd2*, genes encoding for polycystin-1 (PC-1) and polycystin-2 (PC-2), respectively. ADPKD is characterized by the progressive growth of numerous fluid-filled renal cysts. Cyst formation and growth depends on proliferation of the cyst-lining epithelial cells and fluid secretion into the cyst lumen. ADPKD cystogenesis is highly influenced by non-genomic factors, many of which elicit their effects via cAMP-dependent pathways. Understanding mechanisms mediating the effects of cystogenic agents is crucial for the future development of ADPKD therapy.

Previous work has shown that cells derived from the epithelial-lining of renal cysts from patients with ADPKD (ADPKD cells) have an increased affinity for the hormone ouabain. ADPKD cells respond to ouabain by an increased rate of cell proliferation. This effect depends on binding of ouabain to the Na,K-ATPase which induces activation of Src kinase, epidermal growth factor receptor (EGFR), and the extracellular regulated kinase (ERK1/2) pathway.

The objective of the current study was to determine the role of ouabain in mechanisms of fluid secretion and cyst growth in ADPKD. Studies were carried out in human ADPKD cells, embryonic kidneys from the *Pkd1*^{m1Bei} mouse model, and M-1 mouse cortical collecting duct cells.

Results of this study show that physiologic concentrations of ouabain enhance cAMP-dependent fluid secretion and cyst growth of ADPKD cells grown in culture as monolayers or in three-dimensional structures resembling cysts. Additionally, ouabain potentiated the cAMP-dependent growth of cyst-like dilations in metanephric kidneys from the *Pkd1*^{m1Bei} mouse model. These effects were mediated via activation of the Na,K-ATPase signaling apparatus, located at the basolateral domain of ADPKD cells. Intracellular mediators of ouabain's response included the EGFR-Src-ERK pathway. Ouabain alone did not increase fluid secretion and cyst growth.

Rather, ouabain treatment altered the phenotype of ADPKD cells to allow enhanced responses to cAMP agonists. The potentiating effect of ouabain on cAMP-induced fluid secretion was associated with the capacity of ouabain to stimulate anion secretion via the apically located cystic fibrosis transmembrane conductance regulator (CFTR). Moreover, ouabain increased membrane expression of the CFTR. Finally, ouabain decreased Na,K-ATPase membrane expression and ion transport at the basolateral membrane of ADPKD cells.

The increased ouabain sensitivity of ADPKD cells depends on an abnormally high affinity of the Na,K-ATPase for ouabain. Increased ouabain affinity of the Na,K-ATPase was associated with abnormal expression of the C-terminus of PC-1 in M-1 cells.

Altogether, the study of ouabain's effects in ADPKD have uncovered a novel role for ouabain as a physiologic agent that influences renal cyst growth in ADPKD. In addition, it has identified a new mechanism in ADPKD cystogenesis, important for the progression of the disease.

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CHAPTER 1

INTRODUCTION

Structure and Function of the Na,K-ATPase

The Na,K-ATPase is a ubiquitously expressed ion transport protein present in the basolateral membrane of most mammalian cells (63). The activity of this transporter is required for maintaining the crucial sodium and potassium gradients that typically exist across cell plasma membranes (62). Specifically, the Na,K-ATPase uses energy supplied by the hydrolysis of ATP to move three Na^+ ions out and two K^+ ions into the cell. By maintaining the Na^+ and K^+ gradients, the Na,K-ATPase regulates cellular osmolarity and volume (58). The uneven exchange of Na^+ and K^+ catalyzed by the Na,K-ATPase is electrogenic and contributes to maintaining cell plasma membrane potential and the excitable properties of muscle and nervous tissue (35). Moreover, the steep transmembrane Na^+ gradient that the Na,K-ATPase generates drives the secondary transport of various solutes, including amino acids, glucose, and ions such as H^+, Ca^{2+}, and Cl^- (62). In the kidney, specifically, Na,K-ATPase activity is crucial for regulating salt and fluid reabsorption as well as regulating a variety of Na^+-dependent ion transporters (27, 62).

Structurally, the Na,K-ATPase is expressed as a heterodimer of two polypeptides, an α and β subunit (62, 63). The α subunit is a large transmembrane protein which contains binding sites for Na^+, K^+ and ATP (63) and is primarily responsible for the ion transport and enzymatic properties of the Na,K-ATPase. The α subunit contains the binding site for cardiotonic steroid hormones, such as ouabain (62). The β subunit is required for proper membrane targeting and expression of the alpha subunit at the cell plasma membrane (34). Additionally, the β subunit

has been shown to have a role in cell-cell and cell-matrix adhesion (23). Four isoforms of the α subunit (α1, α2, α3, and α4) and three isoforms of the β subunit have been described (14, 15). These isoforms vary in their distribution in mammalian cells (13). The α1 subunit is present in all cell membranes, while the other α isoforms are expressed in a more tissue-specific manner (15). The α2 isoform is expressed primarily in skeletal and cardiac muscle, adipose tissue, and the brain (68, 80, 108). The α3 isoform is found in tissues of the nervous system (85, 86). The α4 isoform of the Na,K-ATPase is expressed specifically in male germ cells (17). The Na,K-ATPase β isoforms also have tissue specific expression patterns. Similar to the α1 isoform, the β1 polypeptide is found is most tissues (13). The β2 subunit is expressed in skeletal muscle, pineal gland, and tissues in the nervous system (3, 101). The β3 subunit of the Na,K-ATPase is expressed in a variety of tissues including testis, retina, liver, and lung (8). The α and β subunits can associate in different arrangements, generating multiple Na,K-ATPase isozymes, which have unique kinetic properties (15). Expression in cells in culture and the use of transgenic mice demonstrated that Na,K-ATPase heterogeneity is not a redundant event, but rather serves to regulate Na^+ and K^+ transport according to the specific requirements of each cell (13, 15, 71, 94). A third polypeptide, known as the γ-subunit, is also associated to the α and β subunits in some tissues (33). The γ-subunit, while not required for Na,K-ATPase activity, is important in modulating the kinetic properties of Na,K-ATPase (97). In the kidney, the Na,K-ATPase is expressed as an α1β1γ heterodimer (13, 30) .

A variety of regulatory mechanisms influence Na,K-ATPase expression and activity. Na,K-ATPase expression is regulated by the rate of synthesis and degradation of the α and β subunit polypeptides (113). Additionally, expression of the Na,K-ATPase at the plasma membrane can be modulated by increasing trafficking of α and β polypeptides from intracellular stores to the plasma membrane, or through internalization of the protein and its degradation

inside the cell (14). In addition, the function of pre-existing Na,K-ATPase molecules at the plasma membrane can be regulated directly by various intracellular and extracellular conditions (113). Thus, several ions regulate Na,K-ATPase activity, with changes in intracellular Na^+ being a potent Na,K-ATPase stimulator. In addition, intracellular kinases, including protein kinase A (PKA) and protein kinase C (PKC), and extracellular hormones such as vasopressin, dopamine, and insulin can influence Na,K-ATPase activity (15, 29, 32).

The Na,K-ATPase is a signal transduction receptor

In addition to its classical role as an ion transporter, the Na,K-ATPase can function as a signal transduction receptor and scaffolding protein that mediates effects of cardiotonic steroids in the cell (7, 70, 132, 133). At the plasma membrane, the Na,K-ATPase interacts with several proteins to form a function signaling receptor, the Na,K-ATPase signalosome (93). Important interactions in the complex include the binding of the Na,K-ATPase to Src, a non-receptor tyrosine kinase, and the epidermal growth factor receptor (EGFR). In particular, the Na,K-ATPase-Src interaction is critical for the transduction of Na,K-ATPase-mediated signaling (66, 140). The protein-protein interactions that characterize the Na,K-ATPase signalosome occur within and depend on the presence of cholesterol-rich plasma membrane microdomains known as caveolae (77, 93, 125).

The Na,K-ATPase signalosome mediates the activation of a variety of cell-specific intracellular signaling events independent from alterations in intracellular ion concentrations (75). Na,K-ATPase-mediated activation of Src leads to transactivation of EGFR and downstream activation of the mitogen activated protein kinase (MAPK) pathway and phosphorylation of extracellular regulated kinase (ERK1/2) (132, 133). Additionally, stimulation of Na,K-ATPase signaling can result in activation of the PLC-γ/PKC pathway resulting in

increased IP_3-dependent release of intracellular Ca^{2+} stores (76). Studies have also shown a role for Na,K-ATPase signaling in mediating Ca^{2+}-dependent activation of nuclear factor-kappa B (NF-κB) signaling (1). Na,K-ATPase-dependent signaling can also influence mitochondrial production of reactive oxygen species (133). Thus, the Na,K-ATPase regulates important physiologic processes via ion transport mechanisms as well as intracellular signaling pathways.

Ouabain is an important physiologic hormone

Ouabain is a steroid hormone that belongs to a family of compounds known as cardiotonic steroids which are present in a variety of different plants and animals (99). Other members of this family include digoxin, proscillaridin A, and marionbufagenin (9). All of these compounds are specific ligands for the Na,K-ATPase, which contains a highly-conserved cardiotonic steroid binding site in an extracellular loop of the α subunit (72). Each α subunit of the Na,K-ATPase has a unique and specific cardiotonic steroid affinity which determines the differential, tissue-specific effect of cardiotonic steroids (15, 72). For instance, the ubiquitously expressed α1 subunit is relatively resistant to the effects of cardiotonic steroids, while the other α isoforms have higher affinities for cardiotonic steroids (13). Additionally, there is significant inter-species variability in the affinity of the Na,K-ATPase for cardiotonic steroids (14, 15).

At high, millimolar concentrations, cardiotonic steroids can inhibit Na,K-ATPase catalytic activity and ion transport and, thus, regulate Na,K-ATPase-dependent processes in the cells (96). For example, cardiotonic steroid-induced inhibition of Na,K-ATPase activity can lead to secondary alterations in Na^+-dependent transport processes within the cell. In particular, cardiotonic steroid inhibition of the Na,K-ATPase can lead to secondary inhibition of the Na^+/Ca^{2+} exchanger resulting in increased intracellular Ca^{2+} accumulation (19).This effect is particularly relevant in the heart, where it is responsible for the inotropic and chronotropic effects of cardiotonic steroids.

Ouabain is a cardiotonic steroid traditionally considered to be exclusively a plant-derived product (9). However, careful investigation by Hamlyn and colleagues showed that ouabain is indeed an endogenous substance present in human plasma (49-51, 79). Ouabain is released from the adrenal gland in response to many different physiologic and pathophysiologic stimuli leading to a variety of functional effects (49, 52, 67). Ouabain secretion can be induced by acute states of stress or exercise and in response to altered levels of sodium in the circulation (25, 36, 48). Many studies have linked endogenous ouabain to regulation of arterial blood pressure (6, 48). Acute saline loading of rodents can cause increases in circulating levels of ouabain, and ouabain infusion in rats is sufficient to induce arterial hypertension and cardiac hypertrophy (9). In addition to its role in regulation of sodium balance and blood pressure, ouabain has been shown to have roles in mood disorders, chemical addictions, and cancer (9).

At the cellular level ouabain has many diverse, cell-specific effects. Nanomolar concentrations of ouabain can increase proliferation and hypertrophic growth of different cell types and also alter apoptotic mechanisms in some cells (5, 24, 65, 87, 119). Studies have also shown an effect of ouabain on the regulation of salt transport in renal cells and epithelia. Specifically, nanomolar concentrations of ouabain can decrease Na^+ reabsorption in LLC-PK1 cells (73, 74, 137). This effect was mediated by ouabain-induced, clathrin-dependent internalization of the Na,K-ATPase from the baolateral plasma membrane. Additionally, ouabain stimulates the internalization of the apically located Na^+/H^+ exchanger, NHE3 (21, 92). The result of these combined effects appears to be involved in the natriuretic effects of ouabain in the renal proximal tubule (137). Studies in human bronchial epithelial cells have reported that ouabain modulates the expression of the cystic fibrosis transmembrane conductance regulator (CFTR) (144). In this work, nanomolar concentrations of ouabain increased expression and delivery of the CFTR to the plasma membrane. Thus, ouabain is a physiologic hormone that mediates many important, cell-specific effects. Many of these ouabain-induced effects are

mediated by ouabain-Na,K-ATPase binding and activation of Na,K-ATPase-mediated intracellular signaling events (93, 99, 100, 132).

Autosomal Dominant Polycystic Kidney Disease (ADPKD)

ADPKD is the most common, potentially fatal, inherited disease of the kidney affecting 1:400-1000 people worldwide (54). In the United States, ADPKD accounts for 4.4% of all cases of end-stage renal failure (ESRD) (54). ADPKD is a diverse, systemic pathology with significant renal manifestations, as well as defects in the liver, pancreas, arterial vasculature and brain. Symptoms of this condition include pain related to enlarged kidneys, cyst and urinary tract infection, renal stones, and arterial and cerebral aneurysms. ADPKD is most dramatically characterized by the presence of many, progressively enlarging renal cysts. These cysts, which originate from renal tubule epithelial cells, grow throughout the kidney and lead to altered renal architecture causing end-stage renal disease (ESRD) (37).

ADPKD is caused by mutations in one of two genes, *PKD1* or *PKD2*, which encode for polycystin-1 (PC-1) and polycystin-2 (PC-2), respectively (111). Mutations in PC-1 are more common, affecting approximately 85% of cases (98). Additionally, mutations in PC-1 typically cause a more severe form of the disease compared to mutations in PC-2. Thus, patients carrying PC-1 mutations reach ESRD an average of 17 years earlier than those harboring PC-2 mutations (55). PC-1 is a large, multi-pass, transmembrane protein with a long, extracellular N-terminus and a short, intracellular C-terminus (10, 60, 91, 131). PC-1 is expressed in most cells in the human body, and, in particular, PC-1 has been shown to localize to primary cilia structures in renal tubule cells (142). PC-1 has been associated with many basic properties of cell biology. The N-terminus of PC-1 has many functional domains that may be involved in cell-matrix and cell-cell adhesion and the binding of extracellular ligands. The C-terminus of PC-1

has been implicated in a variety of intracellular signaling pathways as well as protein-protein interactions. The PC-1 C-tail can be cleaved and translocate to the nucleus to influence transcriptional mechanisms. Interestingly, the C-terminus of PC-1 can interact with the intracellular C-terminus of PC-2. PC-2 is a multi-pass transmembrane protein with homology to known Ca^{2+} channels (112). Association of PC-1 with PC-2 can form a functional cation-permeable channel to regulate intracellular Ca^{2+} concentrations (53).

Cyst growth in ADPKD depends on proliferation of the epithelial cells lining the renal cysts as well as secretion of fluid into the growing cyst lumen (40, 111). Cysts begin forming when renal epithelial cells carrying germline mutations in the *PKD1* or *PKD2* gene receive a somatic mutation in the healthy allele of *PKD1* or *PKD2* (37). This causes aberrant proliferation of the mutated cells leading to the formation of blister-like structures on the sides of the renal tubules. These small cysts enlarge by the combined processes of cell proliferation and fluid secretion, and eventually the growing cyst separates from the parent tubule and grows autonomously within the renal interstitium. The nature of this somatic mutating event is currently not clear. However, it has been demonstrated that acute kidney injury can have a significant effect on the induction of cyst growth in ADPKD (110).

Proliferation of cystic epithelial cells depends on a variety of altered intracellular conditions. Specifically, abnormal intracellular Ca^{2+} concentrations and increased cAMP-dependent signaling are important pathogenic factors in ADPKD (134, 136). Cells derived from the cystic epithelium of ADPKD renal cysts (ADPKD cells) exhibit abnormally low Ca^{2+} levels (134). This may be due to the loss of PC-1 or PC-2 function. Aberrant Ca^{2+} levels allow for altered responses to cAMP. For instance, cAMP, which normally has a growth suppressing role in renal epithelia, drives enhanced proliferation in ADPKD cells (107, 136). Furthermore, studies

have shown that restoring normal intracellular Ca^{2+} levels can block the mitogenic effect of cAMP in ADPKD cells (134). Mechanistically, low concentrations of intracellular Ca^{2+} are associated with the aberrant activation of B-Raf in response to cAMP agonists (136). This activation is a crucial point in mediating the mitogenic effect of cAMP in ADPKD cells.

Fluid secretion in ADPKD is mediated by enhanced secretion of Cl^- across cystic epithelia (84, 104, 123). ADPKD epithelia regulate transepithelial Cl^- movement by a mechanism similar to that observed in other secretory epithelia (105). Specifically, Cl^- enters from the basolateral aspect of the cells via the Na^+,K^+,2-Cl^- co-transporter (NKCC1). The driving force for this transporter is provided by the Na^+ gradient generated by the basolaterally located Na,K-ATPase. Cl^- is then actively secreted across the apical membrane via the cAMP-dependent CFTR . Studies have shown that CFTR is the main mediator of apical anion secretion required for increased fluid secretion in ADPKD (20, 28, 104, 138). Increased Cl^- secretion across the apical membrane of cystic epithelial cells is followed by paracellular movement of Na^+ and water, which results in enhanced fluid accumulation in the growing cyst lumen (105). Interestingly, cAMP-dependent signals regulate the two processes required for progressive cyst growth in ADPKD, cell proliferation and fluid secretion (123, 135). Moreover, other agents affecting cAMP-dependent signaling and the downstream targets of those signals in ADPKD may stimulate the progression of the disease.

Although ADPKD is clearly a genetic condition, progression of the disease is highly variable and is influenced by circulating, non-genomic factors, such as vasopressin (AVP), prostaglandins, and insulin-like growth factor, which have been shown to influence cyst growth in ADPKD via cAMP-dependent pathways (40, 117, 121, 130). For example, it has been shown that AVP is a physiologic agent that can increase ADPKD cyst growth via cAMP-dependent

mechanisms (121). Importantly, experimental blockade of AVP results in reduced cyst growth in experimental models of ADPKD (95, 127). Moreover, recent evidence from a large clinical trial (TEMPO3:4) demonstrated the potential therapeutic benefit of targeting inhibition of AVP in ADPKD patients (114) . This offers strong support for the pathophysiologic importance of agents acting on cAMP-dependent pathways in ADPKD.

Effects of Ouabain in ADPKD

Previous work has shown that endogenous concentrations of ouabain stimulate proliferation of ADPKD cells (89, 90). ADPKD cells have an abnormally high affinity for ouabain despite normal expression of the α1β1 isozyme of the Na,K-ATPase in these cells (90). The observed increase in ouabain affinity of cystic epithelial cells may make these cells more prone to respond to circulating levels of endogenous ouabain.

Na,K-ATPase in ADPKD cells is characterized by normal basolateral localization (90). Consistent with this finding, ouabain only elicits effects in ADPKD cells when applied to the basolateral surfaces. Moreover, physiologic concentrations of ouabain elicit different effects in ADPKD cells compared to higher, non-physiologic concentrations. At physiologic concentrations, ouabain can activate intracellular signaling events in ADPKD cells. In contrast, higher, non-physiologic doses of ouabain become toxic to the cells, consistent with an inhibitory role for ouabain at high doses in Na,K-ATPase-mediated ion transport.

Treatment of ADPKD cells with nanomolar concentrations of ouabain results in a significant increase in proliferation of the cells lining the walls of renal cysts (90). This ouabain-induced proliferation is due to activation of Na,K-ATPase-mediated intracellular signaling events (89). Specifically, ouabain treatment of ADPKD cells results in activation of Src and EGFR. Ouabain-dependent activation of these kinases stimulates the MAPK pathway in ADPKD cells involving the aberrant activation of B-Raf and downstream phosphorylation of ERK1/2.

Phosphorylated ERK1/2 translocates to the nucleus in ouabain-treated ADPKD cells and may elicit transcriptional changes leading to a proliferative response in the cells. Consistent with this finding is a decrease in expression of cyclin-dependent kinase inhibitors p21 and p27 in response to ouabain treatment of ADPKD cells. Lower levels of these proteins may contribute to the proliferative response of ADPKD cells to ouabain. Taken together, these data demonstrate that ouabain is a physiologic agent that may have important cystogenic effects in ADPKD.

Signficance and Specific Aims

Due to the dependency of ADPKD cystogenesis on different circulating factors and the characteristic slow progression of this disease, understanding the non-genetic factors influencing disease progression is essential (117, 121). While several compounds are being tested for the treatment of ADPKD, there is no approved treatment yet to specifically ameliorate the disease. It is vital that basic mechanisms affecting disease progression are better understood so strategies may be developed to therapeutically intervene in these pathogenic mechanisms and stop progression of the disease.

Ouabain is a physiologic agent that may be important in the progression of ADPKD. However, the effect of ouabain on fluid secretion and cyst growth mechanisms in ADPKD remains unclear. Due to the important role the Na,K-ATPase has in regulating ion and fluid transport in normal renal epithelia, ouabain, acting through the Na,K-ATPase, may have a role in regulating ion and fluid transport in cystic epithelia. The objective of the current study was to determine the role of endogenous concentrations of ouabain in fluid secretion and cyst growth mechanisms in ADPKD. The overall hypothesis guiding this investigation is that, due to the increased ouabain affinitiy of ADPKD cells, ouabain will stimulate intracellular signaling events

that enhance fluid secretion mechanisms and increase cyst growth in ADPKD. To investigate this hypothesis the following specific aims were carried out:

1.) **Determine the effect of ouabain on fluid secretion and cyst growth in ADPKD cells and kidneys (Chapter 2).**

2.) **Determine the ion transport mechanisms involved in mediating the effect of ouabain on fluid secretion and cyst growth in ADPKD cells and kidneys (Chapter 3).**

3.) **Determine the mechanism of increased ouabain affinity observed in ADPKD cells (Chapter 4)**

As a disease that is slowly progressing over many years, there could be a great benefit for ADPKD patients if the growth trajectory of renal cysts could be lowered, potentially saving years of healthy renal function. Such therapies will not be possible without a solid, basic understanding of mechanisms mediating the effects of circulating agents affecting disease progression. This study addresses critical, mechanistic details regarding the effects of ouabain, a physiologic agent that may be influencing progression of ADPKD. Results of this study provide significant, novel advances in understanding the role of ouabain in ADPKD.

CHAPTER 2

MATERIALS AND METHODS

Antibodies used (chapters 2, 3, and 4)

Target Antigen	Antibody Name (M/P)	Source
α1-Na,K-ATPase	a6F (M)	DSHB, U. Iowa, Iowa City, IA
α1-Na,K-ATPase	C46B/CB (M)	M. Caplan, Yale University, New Haven, CT
α2-Na,K-ATPase	MCB2 (P)	K. Sweadner, Mass. Gen. Hospital, Boston, MA
α3-Na,K-ATPase	MA3-915 (M)	Affinity Bioreagents, Golden, CO
α4-Na,K-ATPase	108 (M)	Covance Immunology Services, Denver, CO
β1-Na,K-ATPase	M17-P5-F11 (M)	Affinity Bioreagents, Golden, CO
CFTR, C-terminus	clone 24-1 (M)	R&D Systems, Minneapolis, MN
α-tubulin	clone DM1A (M)	Sigma-Aldrich, St. Louis, MO
NKCC1	T4 (M)	DSHB, U. Iowa, Iowa City, IA
phospho-ERK1/2	sc-7383	Santa Cruz Biotechnology, Santa Cruz, CA
ERK1/2	sc-94	Santa Cruz Biotechnology, Santa Cruz, CA

M = monoclonal, P= polyclonal

DSHB = Developmental Studies Hybridoma Bank

Biotinylation and Western Blot Analysis (chapter 3)

ADPKD cells ($2x10^5$) were grown to confluence in 5%FBS on Transwell filters (24-mm, 0.4 μm pore size; Corning Incorporated, Corning, NY) in a 6-well culture plate. Once confluent, monolayers were serum starved overnight and then treated without and with ouabain (3 nM) for 24h or 48h. Monolayers were then washed 3x with ice-cold PBS and then incubated with 0.75 mg/ml Biotin in 1 ml of PBS, pH 8.0 for 50 min at 4°C, refreshing the biotin solution after 30 min. After washing twice with ice-cold PBS and once 100 mM Glycine, cells were lysed in 500 μL RIPA (see *Solutions*). The soluble supernatants were isolated after centrifugation at 10,000 x *g* for 10 min. Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Then lysates with equal amounts of protein were added to streptavidin coated magnetic beads and rotated overnight at 4°C. Samples were washed twice in PBS and resuspended in 6x BME-loading buffer. Samples were then subjected to SDS-PAGE and transferred to nitrocellulose. Membranes were probed and analyzed as descibed below in *Immunoblot Analysis*.

Cell culture **(chapters 2, 3, and 4)**

Primary cell cultures from NHK epithelial cells and cyst-lining renal epithelial cells from patients with ADPKD were prepared from nephrectomy specimens by the PKD Biomaterial Core at University of Kansas Medical Center. A protocol for the use of discarded human kidney tissues was approved by the Institutional Review Board at the University of Kansas Medical Center (KUMC). Cells were seeded and grown in DME/F12 supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 0.1mg/mL streptomycin, 5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL sodium selenite (ITS) as described (123).

Studies described in chapter 4 made use of two clonal cell lines, clone 17 (M-1 C17) and clone 20 (M-1 C20), developed by our collaborators (107). These cell lines were prepared from mouse cortical collecting duct cells (M-1), after stable transfection with a dexamethasone-inducible construct containing the membrane targeted C-tail fragment of PC-1. The M-1 C20 cells express the PC-1 C-tail upon induction with dexamethasone, while M-1 C17 cells do not express the construct and, therefore, serve as a negative control (107). Cells were cultured in DME/F12 supplemented with penicillin/streptomycin (P/S) and 5% heat-inactivated fetal bovine serum (FBS) as described (107). For induction of PC-1 C-tail expression, cells were treated with 1 µM dexamethasone and cultures were used 48 h later. Ouabain treatment was performed using different concentrations of ouabain on the induced cells, after 24 h starvation in medium containing 0.002% FBS and dexamethasone.

Chloride Efflux Assay **(chapter 3)**

Cl^- efflux was assessed as previously described using a Cl^--sensitive flourescent indicator, N-[ethoxycarbonylmethyl]-6-methoxyquinolinium bromide (MQAE) (Life Technologies, Grand Island, NY) (128). Briefly, cells were treated as indicated and then loaded with MQAE in a Cl^--containing Loading Buffer (5.4mM KCl, 122.6mM NaCl, 1.0mM $CaCl_2$, 1.0mM Na_2SO_4, 0.6

NaH_2PO_4, 2.4 Na_2HPO_4, 1.0mM $MgSO_4$, 10mM HEPES, 10mM D-Glucose, pH 7.4). Then, Cl^- efflux was induced by switching cells to Efflux Buffer (5.4mM KNO3, 122.6mM NaNO3, 1.0mM $Ca(NO_3)_2$, 1.0mM Na_2SO_4, 0.6 NaH_2PO4, 2.4 Na_2HPO4, 1.0mM $MgSO_4$, 10mM HEPES, 10mM D-Glucose, pH 7.4) in which Cl^- was exchanged for nitrate. In our study, ADPKD cells were plated with 1%FBS in a 96-well plate (1 x 10^4 cells/well). After 24h, cells were serum starved overnight in media containing 0.002%FBS. Cells were then treated with and without ouabain for 48h. 16h prior to the end of the treatment period, cells were loaded with MQAE (7 mM) in Loading Buffer maintaining the presence of ouabain in the appropriate wells. At the end of the treatment and dye-loading steps, cells were washed three times in Loading Buffer with and without CFTR(inh)-172 (30 μM). During the third wash, forskolin (5 μM) was added for 10 minutes to the buffer on wells with and without ouabain. Control cells received no experimental treatments in the wash buffer. For each treatment condition parallel samples were treated on the same microplate in the absence and presence of CFTR(inh)-172. Media was then quickly switched to Efflux Buffer maintaining the presence of experimental treatments. Flourescence measurments were taken using the excitation/emission pair 360nm/460nm every minute for 10 min using a microplate reader (HTS Synergy). Cl^- efflux was calculated as the difference between the measured fluorescence in each well at a given time point (F_t) and the initial fluorescence of the sample just before switching to Efflux Buffer (F_0), expressed in arbitrary flourescence units. CFTR-dependent efflux was determined as the difference in fluorescence change (F_t-F_0) between samples treated with and without CFTR(inh)-172 for a given experimental treatment.

Data analysis.

Statistical significance of the differences between means was determined by one-way ANOVA with a Student Newman-Keuls post-test for multiple comparisons or unpaired t-test for single comparisons. Statistical significance was defined as $P < 0.05$.

Embryonic organ cultures (chapters 2 and 3)

Metanephric kidneys were dissected under sterile conditions and were transferred to Transwell filters (24-mm, 0.4 μm pore size; Corning Incorporated, Corning, NY) in a 6-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_1, 32 pg/mL triiodothyronine, and penicillin/streptomycin. The medium was supplemented with 100 μM 8-Br-cAMP in the absence or presence of 30 nM ouabain, which was applied to the lower chamber of the culture inserts. The higher amounts of ouabain used for the organ culture, compared to ADPKD cultures, correspond to the known differences in ouabain affinity of the Na,K-ATPase of rodents and humans (16). Some experiments were also performed in the absence or presence of 2 μM of Tyrphostin AG1478, 10 μM of PP2 or 1 μM of U0126 (chapter 2). Metanephroi were maintained at 37°C in a humidified chamber containing 5% CO_2 and 95% air and were observed, under light microscopy, daily for four days after treatment. Images were captured at culture days 0, 1, 2, 3 and 4. Quantification of total kidney and dilated tubule area was performed on the images using analySIS software as previously described (81). Fractional cyst area was calculated as the ratio of the total dilated cystic tubule area divided by the area of the whole kidney.

Fluid secretion assay (chapters 2 and 3)

Confluent NHK and ADPKD monolayers were established on 12-mm permeable Snapwell™ inserts (Corning Incorporated, Corning, NY) in 6-well tissue culture plates as previously described (90). Cells were cultured for 1 week, until a tight epithelium was established, as determined by the achievement of a transepithelial electrical resistance across the cell monolayer (90). Then, cells were serum starved in our basal DME/F12 media for 24 h as described (90) and the basolateral side of the cell monolayers were treated with 3 nM ouabain, in the absence and presence of 5 μM forskolin or 100 μM 8-Br-cAMP. Control monolayers were incubated in basal media. Fresh medium (150 μl) was placed on the apical surface of the cells, and mineral oil was layered over the top of the medium to prevent evaporation. Cultures were incubated at 37 ^{o}C for an additional 24 h and the apical medium was collected and measured as described (124). Fluid secretion data were expressed as ul/hour/cm^2 where positive values indicate fluid secretion and negative values indicate fluid absorption. For some experiments, monolayers were treated in the absence or presence of 2 μM of the EGFR inhibitor Tyrphostin AG1478, 10 μM of the Src inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) or 1 μM of the MEK inhibitor U0126 (chapter 2). Additionally, To analyze the involvement of CFTR in any observed fluid secretion, CFTR(inh)-172 (10 μM), was added to the apical media applied to monolayers in a certain set of experiments (chapter 3)

Immunoblot Analysis (chapters 2, 3, and 4)

After the different treatment conditions, were washed twice in ice-cold PBS and lysed in RIPA buffer (see *Solutions*). Total protein content in the cleared lysates was determined using the dye-binding assay from Bio-Rad (Hercules, CA, USA). Equal amounts of protein were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were then probed for expression of the proteins of interest using specific primary antibodies (see

Antibodies). For experiments analyzing protein phoshorylation (chapter 4 – phospho-ERK1/2 analysis), membranes were first probed with phospho-secific primary antibodies. The membranes were then stripped and reprobed using a primary antibody specific for the total form of the protein of interest. Horseradish-peroxidase conjugated, species-specific secondary antibodies and chemiluminescence were used for detection. Protein loading levels were controlled for by immunoblotting for α-tubulin expression levels. Protein expression was determined by densitometry. Data were normalized against α-tubulin as a loading control and reported as values relative to expression levels in untreated control cells for each individual experiment.

Immunoflourescence (chapter 3)

ADPKD cells were grown to confluency on Snapwell™ inserts (12-mm, 0.4 μm pore size; Corning Incorporated, Corning, NY) in 6-well tissue culture plate. Following overnight serum starvation, cells were treated with and without ouabain (3 nM) for 24h. Cells were then fixed in 100% methanol for 45 min at -20 ^{o}C and were analyzed by immunocytochemistry as previously described. Expression of the Na,K-ATPase was detected using a monoclonal primary antibody that recognizes the α1 subunit of the Na,K-ATPase, a6F (see *Antibodies*) followed by exposure to an Alexa Flour 594 secondary antibody (Life Technologies, Grand Island, NY). Cells were mounted with Slow Fade ® Gold anti-fade reagent with DAPI (Life Technologies, Grand Island, NY). Samples were analyzed using a Zeiss LSM510 confocal microscope. Images were acquired in Multitrack channel mode with LSM510 (v3.2) softwre 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 μm steps.

Intracellular Sodium Measurement (chapter 3)

Changes in intracellular Na^+ were determined using the fluorescent dye Sodium Green tetra acetate (Life Technologies, Grand Island, NY) as described (56). ADPKD cells ($2x10^4$) were grown in the wells of an 8-chamber slide. Following overnight serum starvation, cells were treated with and without ouabain (3 nM) in media containing the flourescent compound for 30, 60, and 90 min. Then images of individual cells or groups of cells were analyzed using the excitation/emission pair 470/490 nm on an inverted microscope attached to a digital CCD camera. Mean flourescence intensity was measured in arbitratry flourescence units and expressed relative to mean flourescence intensity of untreated control cells. During analysis, samples were maintained at at 37 °C by use of a heating chamber regulated on-line with the system acquisition control.

Mice (chapters 2 and 3)

All experimental protocols involving mice, were approved by the KUMC Institutional Animal Care and Use Committee. *Pkd1*^{m1Bei} mice were originally obtained from the Mutant Mouse Regional Resource Center (University of North Carolina, Chapel Hill, NC) (57) and were stabilized onto a C57BL/6 background (81). Mice heterozygous for *Pkd1* were mated and embryos from timed-pregnant females were removed at embryonic day 15.5 (E15.5). Embryo genotyping showed the *Pkd1* allele was inherited with approximate expected Mendelian ratios.

Cftr^{m1UNC} (S489X) were also used in this study (chapter 3). These mice are considered to exhibit a null CFTR phenotype due to unstable CFTR expression at the mRNA or protein level (109). As previously described (81), double heterzygous mice (Pkd1+/-; CFTR+/-) were generated and bred to yield litters of mice deficient in both PC-1 and CFTR. Embryonic kidneys from these litters were used at day E15.5 for metanephric organ culture experiments.

Microcyst cultures (chapters 2 and 3)

NHK and ADPKD cells were suspended at a density of 4000 cells per well in a 96-well plate in ice cold type I collagen (PureCol®, Advanced Biomatrix, San Diego, CA) in medium comprised of 1:1 DME/F12, 100 U/ml penicillin, 0.1mg/mL streptomycin, ITS, 5 x 10^{-8} M hydrocortisone and 5 x 10^{-12} M triiodothyronine (defined medium). After the cell/collagen suspension was seeded, the gel was polymerized by warming the culture plates to 37°C. Cysts were induced to grow by pre-treatment for 2 to 4 days with 5 μM forskolin and 5 ng/mL EGF. Once the cysts began forming, the agonists were removed and cultures were treated with defined medium, in the absence and presence of 3 nM ouabain or 5 μM forskolin, alone or together. Some experiments were performed in the absence or presence of 2 μM Tyrphostin AG1478, 10 μM PP2 or 1 μM U0126 (chapter 2). Additionally, some experiments were performed in the absence or presence of a specific inhibitor of CFTR, CFTR(inh)-172 (10 μM) (chapter 3). Cysts were allowed to grow for 5 to 7 days in culture, and then they were fixed with 0.5% buffered formalin in PBS. Cultures were photographed and the diameters of individual cysts ($\geq$100 μm in diameter) were measured and used to calculate a total surface area using analySIS software (Lakewood, CO) as described previously (95). Data were expressed as average total surface area $\pm$ SEM for each culture condition.

Na,K-ATPase Activity Assays (chapter 4)

Na,K-ATPase activity was determined by measuring the initial rate of release of $^{32}P_i$ from γ[^{32}P]-ATP in homogenates from M-1 C17 and M-1 C20 cells as previously described (90). Briefly, samples were incubated at 37˚C for 30 min in a final volume of 0.25 mL of medium containing 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 2 mM sodium azide, 30 mM Tris-HCl (pH 7.4) in the absence and presence of the indicated concentrations of ouabain, and 3 mM ATP with 0.2 μCi γ[^{32}P]ATP. Specific hydrolysis of ATP dependent on the Na,K-ATPase

was determined as that sensitive to 1 mM ouabain. Curve fitting of the data for ouabain inhibition of Na,K-ATPase activity was performed using Marquardt least-squares nonlinear regression as previously described (90).

Real-time RT-PCR analysis (chapter 2):

ADPKD cells and NHK cells were treated with 8-Br-cAMP (100 μM) or ouabain (3 nM) alone or in combination, and total RNA was extracted using with the RNA easy mini kit (Qiagen Sciences, Maryland, USA). cDNA was synthesized using 1 μg of RNA with Omniscript RT Kit (Invitrogen) and oligo-dT primers. (Invitrogen). Real-time qPCR was performed using ABI Prism 7900 (Applied Biosystems, Foster City, CA, USA) and SYBR green PCR master mix (Warrington, UK) following the manufacturer's protocol. In brief, the reaction mixture (total volume, 25 μl) contained 8μl of 1:10 diluted cDNA, 5′-GTAACCCGTTGAACCCCATT-3′ (antisense) 5′-CCATCCAATCGGTAGTAGCG-3′(sense) to amplify human 18 S rRNA. NKCC1 and CFTR was amplified using the following primers: 5′-ACAATGGCGAATGGTGACT-3′ (antisense) and 5′-CATGGGGTTACTTTTTGGTTAC-3′ (sense) for NKCC1, 5′-GGAAAAGGCCAGCGTTGTC-3′ (antisense) 5′-CAGGCGCTGTCTGTATCCT-3′ (sense) for CFTR at a final concentration of 100 nM and with 12.5 μl of 2 × SYBR green PCR master mix kit. Target genes and 18sRNA gene were amplified in the same reaction. All experiments were normalized to 18s RNA. Comparative quantification is determined using the $2^{-\Delta\Delta Ct}$ method (78).

RT-PCR Analysis (chapter 4)

Total RNA from M-1 C17 and M-1 C20 cells was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA was generated by reverse transcription of total RNA using SuperScript II reverse transcriptase (Invitrogen, Grand Island, NY, USA) and oligo-dT primers (Invitrogen). Primers specific to the various α isoforms of mouse Na,K-ATPase

were used to amplify segments of the corresponding cDNAs. These primers contained the following sense (S) and antisense (AS) sequences,: For α1, 5'-GGGGTTGGACGAGACAAGTAT-3'(S), and 5'-CGGCTCAAATCTGTTCCGTAT (AS); for α2, 5'-CCACCACTGCGGAAAATGG-3'(S) and 5'-GCCCTTAGACAGATCCACTTGG-3'(AS); for α3 5'-GAAGAGGTCTGCCGGAAATAC-3'(S) and 5'- GGTGGTGTGAGGGCGTTAG(AS); and finally, for α4, 5'- CAGTGGCGCATTTGTGGTTT-3'(S) and 5'-CAGCGATTCGAGCCAGGTAAA-3'(AS). Amplification products were resolved on a 1% agarose gel containing ethidium bromide.

Rubidium (^{86}Rb) Uptake (chapter 3)

Na,K-ATPase-mediated ion transport was determined by ^{86}Rb uptake as previously described (120). ADPKD cells ($2x10^5$) were seeded in 5%FBS on Transwell filters (24-mm, 0.4 μm pore size; Corning Incorporated, Corning, NY) in a 6-well culture plate and grown to confluence. Following overnight starvation, monolayers were treated with and without ouabain (3 nM) for 30 min. Monolayers were then placed into 6-well plates containing rubidium flux medium (see Solutions)containing 5 uCi ^{86}Rb. Uptake was allowed for 5 min and was then terminated by immersing the monolayers in ice-cold, isotonic buffer containing 100 mM MgSO4 and 137 mM sucrose. Cells were lysed in RIPA buffer (see *Solutions*) and radioactivity of the obtained lysates was measured by scintillation counting. ^{86}Rb uptake was calculated as nmol ^{86}Rb/mg protein in the cell lysate.

Short Circuit Current (chapters 2 and 4)

Confluent monolayers of ADPKD cells or M-1 C17 and M-1 C20 were grown on 12-mm permeable Snapwell™ inserts (Corning Incorporated, Corning, NY) as previously described (90). Monolayers were mounted in Ussing chambers for measurement of short circuit current (I_{sc}) using a dual voltage clamp device (Warner Instruments, Hamden, CT) as previously

described (122). Prior to measurement of short circuit current, monolayers were incubated either in control media or media containing 3 nM ouabain for 24 hours. Additionally, for analysis of the involment of Src in ouabain effects on I_{sc} in M-1 cells, monolayers were pre-treated with PP2 (10 μM) for 30 min prior to the addition of ouabain (30 nM) maintaining the presence of PP2. Once monolayers were loaded in Ussing chambers and currents stabilized, benzamil (10 μM) was added to the apical media to block sodium reabsorption and ensure any increase in short circuit current was due to apical anion secretion. Forskolin (5 μM) was added basolaterally to monolayers to stimulate I_{sc}, and change in I_{sc} (ΔI_{sc}) in response to forskolin was calculated as a difference from baseline current, as previously described (4). CFTRinh-172 (10 μM) was added apically to determine the involvement of CFTR in any observed increased in I_{sc}. Data are expressed as a percentage of the maximum response to forskolin in the untreated monolayers

Solutions (chapters 2, 3, and 4)

RIPA (cell lysis buffer): 1%NP-40, 0.25% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EDTA (pH 8.0), 150 mM NaCl, 50 mM Tris (pH 7.4), 50 μM phenylarsine oxide, 1x protease inhibitor (Roche), 1x phosphatase inhibitor (Roche) .

Loading Buffer: 5.4mM KCl, 122.6mM NaCl, 1.0mM $CaCl_2$, 1.0mM Na_2SO_4, 0.6 NaH_2PO_4, 2.4 Na_2HPO_4, 1.0mM $MgSO_4$, 10mM HEPES, 10mM D-Glucose, pH 7.4

Efflux Buffer: 5.4mM KNO3, 122.6mM NaNO3, 1.0mM $Ca(NO_3)_2$, 1.0mM Na_2SO_4, 0.6 NaH_2PO4, 2.4 Na_2HPO4, 1.0mM $MgSO_4$, 10mM HEPES, 10mM D-Glucose, pH 7.4)

Rubidium flux medium: (Hank's Balanced Salt Solution + 10 mM HEPES)

CHAPTER 3

OUABAIN POTENTIATES cAMP-MEDIATED FLUID SECRETION AND CYST GROWTH IN ADPKD

INTRODUCTION

Previous studies have shown that ouabain, in concentrations similar to those circulating in blood, stimulates the proliferation of human ADPKD cyst-linning epithelial cells, but has no significant effect in normal human kidney (NHK) cells (90). Ouabain binding to the Na,K-ATPase activates the EGF receptor (EGFR), the tyrosine kinase Src, and the mitogen activated kinase-extracellular regulated kinase (MEK-ERK) pathway (90). Many cystogenic factors in ADPKD share a common mechanism of action, which involves the MEK-ERK pathway. Increased proliferation of renal tubular cells is an essential step in the development of renal cysts (22, 91, 106). However, cyst expansion also requires the concomitant and continuous addition of fluid into the cavity of the growing vesicles, which maintains the shape and turgidity of the cysts. This process depends on changes in the salt and fluid transport properties of the ADPKD cells, which favor fluid secretion over reabsorption in the cystic epithelium (104, 105). Here, studies were conducted to determine whether physiological concentrations of ouabain can affect ADPKD fluid secretion and cyst growth. Results from these studies indicate that ouabain can act as a co-factor enhancing the effects of forskolin and cAMP on fluid secretion and microcyst development of human ADPKD cells in culture and on cyst-like dilations in metanephric organ cultures of *Pkd1*^{m1Bei} mice. These effects of ouabain are mediated via the EGFR-Src-MEK pathway.

RESULTS

Ouabain enhances cAMP-dependent fluid secretion by the ADPKD epithelium

Fluid secretion is an essential event in the formation and development of ADPKD cysts (39, 40). As a first approach to determine the effect of ouabain in cystogenesis, we determined the transepithelial movement of fluid from the basal to the apical side of NHK and ADPKD cell monolayers. For this, cells were treated for 24 hours without or with 3 nM ouabain, a concentration found to be optimal for stimulating ADPKD cell growth (90). Monolayers were also treated with either 5 μM forskolin or 100 μM 8-Br-cAMP, alone or in combination with ouabain. After 24 h, the amount of fluid present in the upper chamber of the cell monolayer was measured as described (124). Untreated NHK monolayers absorbed fluid from the apical to the basolateral side (Figure 1A). This is indicated by negative values of fluid secretion. Ouabain had no significant effect on fluid transport in NHK monolayers, either when it was used alone, or in combination with forskolin or 8-Br-cAMP (Figure 1A). Addition of either forskolin or 8-Br-cAMP induced a positive fluid secretion across NHK monolayers, but this fluid transport was not significantly different from untreated controls (Figure 1A). These results agree with the small stimulatory effects that cAMP agonists have on apical fluid movement in normal dog and human kidney cells (83, 84). Similar to NHK monolayers, ADPKD monolayers absorbed fluid in the untreated condition, and ouabain alone did not significantly affect epithelial fluid transport by ADPKD monolayers (Figure 1B). Both forskolin and 8-Br-cAMP increased fluid secretion in ADPKD monolayers (Figure 1B), consistent with previous observations (84). Also, in agreement with previous observations (23), forskolin induced a higher level of fluid secretion compared to secretion induced by 8-Br-cAMP. (Figure 1B). Interestingly, the forskolin- and cAMP-dependent transepithelial fluid secretion of ADPKD cells was enhanced by the presence of ouabain (Figure

1B). These results demonstrate that in ADPKD cells, but not in normal renal cells, ouabain enhances the apical delivery of fluid that is induced by forskolin and 8-Br-cAMP.

Ouabain contributes to ADPKD microcyst development

When cultured in a three-dimensional matrix of polymerized collagen, ADPKD cells form microscopic structures resembling cysts in the presence of EGF and forskolin (105, 123). These microcysts are lined by a polarized epithelium that secretes fluid like the wall of native ADPKD cysts (139). We have used this *in vitro* model system to study the effects of ouabain on ADPKD cyst formation. For this, NHK and ADPKD cells cultured within collagen matrices were first stimulated to form microcysts with EGF and forskolin. Once the cysts began to form, the agonists were removed and the cultures were treated without or with 3 nM ouabain and 5 μM forskolin alone or combined. Microcysts were allowed to grow for 5 to 7 days. Then, changes in cyst size were quantified and expressed as average total surface area of the microcyst cultures. NHK cells showed little microcyst development in response to forskolin and ouabain had no effect on NHK microcysts, either when used alone or with forskolin (Figure 2A). ADPKD microcysts exhibited a more robust response to forskolin than NHK cells, presenting greater total cyst surface area (Figures 2B). This confirmed previous observations on the positive role of forskolin in promoting ADPKD microcyst growth (105, 123). In contrast to NHK cells, ouabain greatly enhanced cAMP-dependent growth of ADPKD microcysts, but had no effect when used alone (Figures 2B). Altogether, these results show that ouabain acts as a cofactor in a synergistic manner with forskolin to increase fluid secretion and accelerate ADPKD cyst growth.

Ouabain exacerbates cAMP-induced cystic progression in metanephric organ cultures

Metanephric organ cultures of *Pkd1*^{m1Bei} mice represent a useful tool for studying the mechanisms responsible for cystogenesis in an orthologous model of ADPKD. This mouse

model carries a mutation in the *Pkd1* gene that results in a non-functional PC1 product. Renal tubules in embryonic *Pkd1*^{m1Bei} mice secrete fluid and become dilated in response to 8-Br-cAMP (81). In the current study, we have used *Pkd1*^{m1Bei} mice to explore the effects of ouabain on cyst growth in metanephric organs of this ADPKD mouse model. Embryonic kidneys from wild type (*Pkd1*$^{+/+}$), heterozygous (*Pkd1*$^{+/-}$) or homozygous null (*Pkd1*$^{-/-}$) mice were dissected at E15.5 and cultured as previously described (81). Then, the metanephroi were treated with 30 nM ouabain in the absence or presence of 100 μM 8-Br-cAMP and the development of cystic dilations was followed for 4 days, when fractional cyst area was measured. Similar to our results in ADPKD monolayers and microcysts, ouabain by itself did not have a significant effect on metanephric tubule dilation and cyst formation in the *Pkd1*$^{+/+}$, as well as in the *Pkd1*$^{+/-}$ and *Pkd1*$^{-/-}$ embryonic kidneys (Figure 3). In agreement with previous work (81), addition of 8-Br-cAMP produced cystic dilations in the metanephroi. These cysts continued expanding during the course of the 4 day treatment of the cultures, becoming more marked in the mutated *Pkd1* than in the wild type mice (Figures 3). While ouabain alone had no effect, the concomitant addition of ouabain and 8-Br-cAMP significantly exacerbated fractional cyst area in the metanephroi. This response was greatest in the *Pkd1*$^{-/-}$ and *Pkd1*$^{+/-}$ kidneys, reaching a three-fold increase in fractional cyst area in the metanephroi from the more severe phenotype (Figure 3B, 3D and 3F). Altogether, these results indicate that, in the presence of 8-Br-cAMP, ouabain stimulated metanephric cyst development, with the most dramatic effects taking place in *Pkd1*$^{-/-}$ embryonic kidneys. This shows that the effects of ouabain on cyst development are not limited to ADPKD cells in culture but also occur in a system that maintains the architecture of the intact renal tissue.

Ouabain stimulates ADPKD fluid secretion and cyst growth through the EGFR, Src and MEK pathway.

Ouabain exerts its stimulatory effect on ADPKD cell proliferation, via the Na,K-ATPase signalosome and its downstream mediators, EGFR, Src and the mitogen activated protein kinase (MAPK) pathway (93, 133). To explore whether the stimulatory effects of ouabain on ADPKD cystogenesis involve the Na,K-ATPase signaling machinery, we tested the effects of inhibitors of EGFR (Tyrphostin AG1478), Src (PP2) and MEK (U0126) on ouabain and forskolin induced fluid secretion in ADPKD cell monolayers, ADPKD microcyst cultures, and *Pkd1*^{m1Bei} metanephric organ cultures. The doses of Tyrphostin AG1478, PP2 and U0126 chosen were based on those used previously (89). In fluid secretion experiments, ADPKD cell monolayers were grown and treated with the inhibitors mentioned above in the presence of forskolin alone or forskolin with ouabain. Inhibition of EGFR, Src and MEK, individually, blocked ouabain-dependent increases in forskolin-induced fluid secretion by ADPKD monolayers (Figure 4A). In three dimensional collagen microcyst experiments, cultures were treated with forskolin alone or forskolin with ouabain in the absence or presence of Tyrphostin AG1478, PP2 or U0126. Here, the inhibitors also prevented ouabain's enhancement of forskolin-induced ADPKD microcyst growth (Figure 4B). The effect of these inhibitors on microcyst growth was more robust than for fluid secretion in ADPKD monolayers. This could be due to the fact that cyst growth requires fluid secretion as well as cell proliferation. These signaling inhibitors have been shown to block ouabain-dependent cell proliferation (89), and the combined inhibition of proliferation and fluid secretion may account for the more complete inhibition observed in the microcyst cultures. Finally, in metanephric organ cultures, metanephroi were treated with Tyrphostin AG1478, PP2 or U0126 in the presence of 8-Br-cAMP alone or combined with ouabain. The EGFR, Src and MEK inhibitors reduced the effect of ouabain to exacerbate cAMP stimulated growth of tubular cystic dilations in embryonic kidneys from *Pkd1*$^{-/-}$ mice, the phenotype in which ouabain's effect

is most pronounced (Figure 4C). Altogether, these results show that ouabain stimulates cAMP-dependent fluid secretion and cyst growth through mediators of the Na,K-ATPase signaling pathway, involving EGFR, Src and MEK.

Ouabain increases forskolin-induced short circuit current in ADPKD cell monolayers

Fluid secretion by ADPKD cells is a key requirement for cyst growth and is regulated by apical anion secretion (84, 121). Measurement of short circuit current (I_{sc}) in monolayers of ADPKD cells has been used extensively to study regulation of anion secretion across these monolayers (4, 12, 84, 103, 104), and this work has shown that forskolin and other cAMP agonists induce anion secretion in ADPKD monolayers mainly through the function of the apically located cystic fibrosis transmembrance conductance regulator (CFTR). The stimulatory effect of ouabain on cAMP and forskolin-induced fluid secretion may be due to an effect of the hormone on forskolin-stimulated I_{sc}. To test this, monolayers of ADPKD cells were grown to confluence on permeable filter supports and treated either with control media or media containing 3 nM ouabain for 24 hours prior to measurement of short circuit current. As previously described (84, 104), forskolin induced an increase in short circuit current in monolayers of ADPKD cells (Figure 5). Interestingly, the monolayers treated with 3 nM ouabain exhibited an ~23% increase in the maximum response to forskolin treatment (Figure 5), indicating an increase in anion secretion across the monolayers. The increased I_{sc} in both the ouabain treated and untreated monolayers was dependent on CFTR function as evidenced by the inhibition of the increased current by the specific CFTR inhibitor, CFTRinh172 (Figure 5). This effect of ouabain to increase anion secretion across ADPKD monolayers may contribute to the hormone's effect to exacerbate cAMP- mediated fluid secretion and cyst growth in ADPKD.

Ouabain does not affect expression of CFTR or NKCC1 mRNA transcripts

Fluid secretion and cyst growth in ADPKD depend on Cl^- transport regulated by the apical CFTR and the basolateral $N^+,K^+,2Cl^-$-cotransporter (NKCC1) (84). To determine the effect of ouabain on expression of these two required ion transport proteins, levels of mRNA for CFTR and NKCC1 were measured by RT-PCR in ADPKD and NHK cells treated with ouabain or 8-Br-cAMP alone or in combination. In both ADPKD and NHK cells, ouabain alone or in combination with cAMP had no effect on CFTR or NKCC1 mRNA expression (Figure 6). There was a slight increase in CFTR transcript expression in ADPKD cells treated with 8-Br-cAMP that was insignificant ($p=0.314$ vs. control). Additionally, the presence of ouabain with 8-Br-cAMP did not further increase the expression of CFTR transcripts in ADPKD cells ($p=0.428$ vs. control). This indicates that ouabain-induced increases in fluid secretion and cyst growth in ADPKD do not depend on a direct increase in expression of either CFTR or NKCC1. Future work will further define the specific molecular mechanism responsible for ouabain's effect on fluid secretion and cyst growth in ADPKD.

DISCUSSION

Previous studies have demonstrated that ouabain stimulates proliferation of ADPKD cells through activation of the Na,K-ATPase signalosome (89, 90). Here, we show that ouabain also affects fluid secretion by the ADPKD epithelium. Ouabain enhances the vectorial movement of fluid from the basolateral to the apical side of ADPKD monolayers treated with either 8-Br-cAMP or forskolin. In addition, ouabain stimulates the forskolin-dependent growth of microcysts in ADPKD three-dimensional collagen cultures, which is an indicator of increased fluid delivery by the cells surrounding the lumen of the microcysts (44). Ouabain also promotes cAMP-dependent tubular dilations in metanephric organ cultures of *Pkd1*^{m1Bei} mice. These findings

highlight the importance of ouabain not only as a cell proliferation agent in ADPKD, but also as a factor that increases cyst volume content and growth. Additionally, ouabain increased the I_{sc} response of ADPKD monolayers to forskolin treatment indicating that ouabain can augment the cAMP-dependent anion secretion regulated by CFTR that is a key requirement for fluid secretion and cyst growth in ADPKD. Therefore, ouabain influences two renal epithelial processes that are fundamental mechanisms for ADPKD cyst development.

Exogenous and endogenous ouabain have been shown to elicit a variety of effects on kidney cells and tissues. These include changes in cell proliferation (65), cell detachment and death (2, 26), regulation of sodium reabsorption (18, 21, 88) and protection of the kidney from the adverse effects of malnutrition and serum deprivation (69). In the current study we show that ouabain modulates the amplitude of cAMP-dependent fluid secretion and cyst growth of ADPKD microcysts and metanephric organ cultures, providing evidence for a novel effect of ouabain on renal cells. These effects take place in response to nanomolar concentrations of ouabain, which are similar to those normally found in blood (100). This supports the pathophysiological relevance of ouabain as a modulating factor that contributes to progression of ADPKD cyst growth and the hypothesis that endogenous ouabain may contribute to cyst enlargement in ADPKD patients.

While ouabain helps enhance fluid secretion and microcyst growth in ADPKD cells, it has no significant effect on normal human kidney cells, both in the absence or presence of 8-Br-cAMP or forskolin. Comparison of the fluid transport rates in the untreated NHK and ADPKD monolayers (Figure 1) show that both cell types absorb fluid in the absence of secretory agonsits. This is consistent with the normal function of most renal epithelia to reabsorb the renal plasma filtrate. However, upon addition of 8-Br-cAMP or forskolin, ADPKD cells and NHK

cells can be induced to secrete fluid. This secretory response is greatly enhanced in ADPKD cells compared to NHK cells, and ouabain is augments this cAMP-dependent fluid secretion specifically in ADPKD cells. Similarly, ouabain differentially affects metanephric kidneys, causing only a small increase in fractional cyst area in wild type metanephroi, and a more pronounced, and progressively larger increase in heterozygous and homozygous *Pkd1* mutated metanephric kidneys. Therefore, cystic dilations induced by ouabain appear to correlate with the severity of the *Pkd1*^{m1Bei} mouse phenotype. In agreement with these data, we have previously shown that ouabain differentially impacts the growth of NHK and ADPKD cells (90). We speculate that the difference in the effect of ouabain may depend on the higher affinity that the Na,K-ATPase of ADPKD cells has for ouabain compared to NHK cells (90). This characteristic property of ADPKD cells may make the cystic cells more susceptible to circulating levels of ouabain, biasing them to respond to ouabain in an exacerbated manner compared to normal cells.

Our previous and current observations suggest that the dual roles ouabain plays on the ADPKD epithelium, i.e. cell proliferation and fluid secretion, are affected in different ways. Thus, while ouabain enhances cell growth by itself (89, 90), it functions as an agonist together with other cyst inducing factors, such as cAMP and forskolin, to stimulate fluid secretion by the ADPKD epithelium. These effects produced by ouabain, acting either alone, or as a cofactor, suggest that different thresholds for the actions of ouabain may exist in the pathways or effector mechanisms leading to cell proliferation and fluid secretion in ADPKD cells. Although further experiments are needed to prove these possibilities, our results show that ouabain has complementary effects on cell proliferation and fluid secretion to promote ADPKD cyst development.

Regarding its mechanisms of action, we have found that ouabain's contribution to ADPKD fluid secretion and cystogenesis involves essential components of the Na,K-ATPase signaling machinery. In this manner, Tyrphostin AG1478, PP2 or U0126 prevent the synergistic effect of ouabain on 8-Br-cAMP- and forskolin-induced cystogenesis, suggesting that activation of EGFR, Src, and the MAPK pathway are necessary for ouabain's action. As previously reported, phosphorylation and stimulation of activity of these intracellular mediators is also required for the ouabain-dependent proliferation of ADPKD cells (89). Therefore, both the proliferative and secretory effects of ouabain are mediated through some common components of the Na,K-ATPase signalosome. Interestingly, forskolin and cAMP mediate their effects by direct activation of the MAPK pathway (104, 121). Therefore, it is possible that, acting through EGFR and Src, ouabain can provide another route for activation of the MAPK pathway, with ouabain, cAMP and forskolin merging their effects at the level of the MAPK pathway.

Ouabain and cAMP may also have common effects on the final effectors that regulate fluid secretion and cyst growth in ADPKD. Fluid secretion induced by cAMP in the ADPKD epithelium is mediated by activation of Cl^- transport involving the apical CFTR and the basolateral NKCC1 (84). The enhancement of 8-Br-cAMP-dependent fluid secretion and of cyst growth induced by ouabain may be due to its combined effects with cAMP on CFTR and NKCC1. Our I_{sc} results show that ouabain-treated monolayers exhibit an increased responsiveness to forskolin stimulation resulting in an increased cAMP-dependent Cl^- current mediated by activation of CFTR. Factors favoring increased Cl^- secretion in ADPKD monolayers are crucial regulators of increased fluid secretion and cyst growth (84, 123). While our results show ouabain does not directly increase the expression of either CFTR or NKCC1 mRNA, the glycoside may be influencing the effect of cAMP on these transporters by modulating phosphorylation or localization of CFTR or NKCC1 in ADPKD cells in a way that favors increased Cl^- secretion. The

full mechanism governing ouabain-dependent increases in Cl^- secretion requires further investigation, but the observation that ouabain-treated monolayers exhibit an augmented I_{sc} response to forskolin indicates that physiologic concentrations of ouabain could exacerbate a key mechanism regulating fluid secretion in ADPKD. Furthermore, as noted previously (82), fluid secretion contributing to ADPKD cyst growth is a slow, chronic process. Therefore even modest changes in this secretory mechanism, when accumulated over many years, could account for substantial changes in cyst growth. The possibility that ouabain via the Na,K-ATPase could influence other ion transport systems distantly located on the cell plasma membrane is supported by previous work, which has shown that Na,K-ATPase-mediated ouabain signaling regulates the Na^+/H^+ exchanger (NHE3) at the apical membrane of pig kidney epithelial cell monolayers (21, 137). Additional studies in our laboratory will be directed to understanding the molecular basis through which ouabain augments transepithelial apical Cl^-secretion and fluid transport in ADPKD.

In conclusion, our results provide novel information for the role of ouabain on ADPKD cystogenesis. The stimulating action of ouabain on fluid secretion, in addition to the effect of this hormone to induce cystic cell proliferation, supports the important role of ouabain as an agent which can affect the pathophysiology and progression of ADPKD.

FIGURES

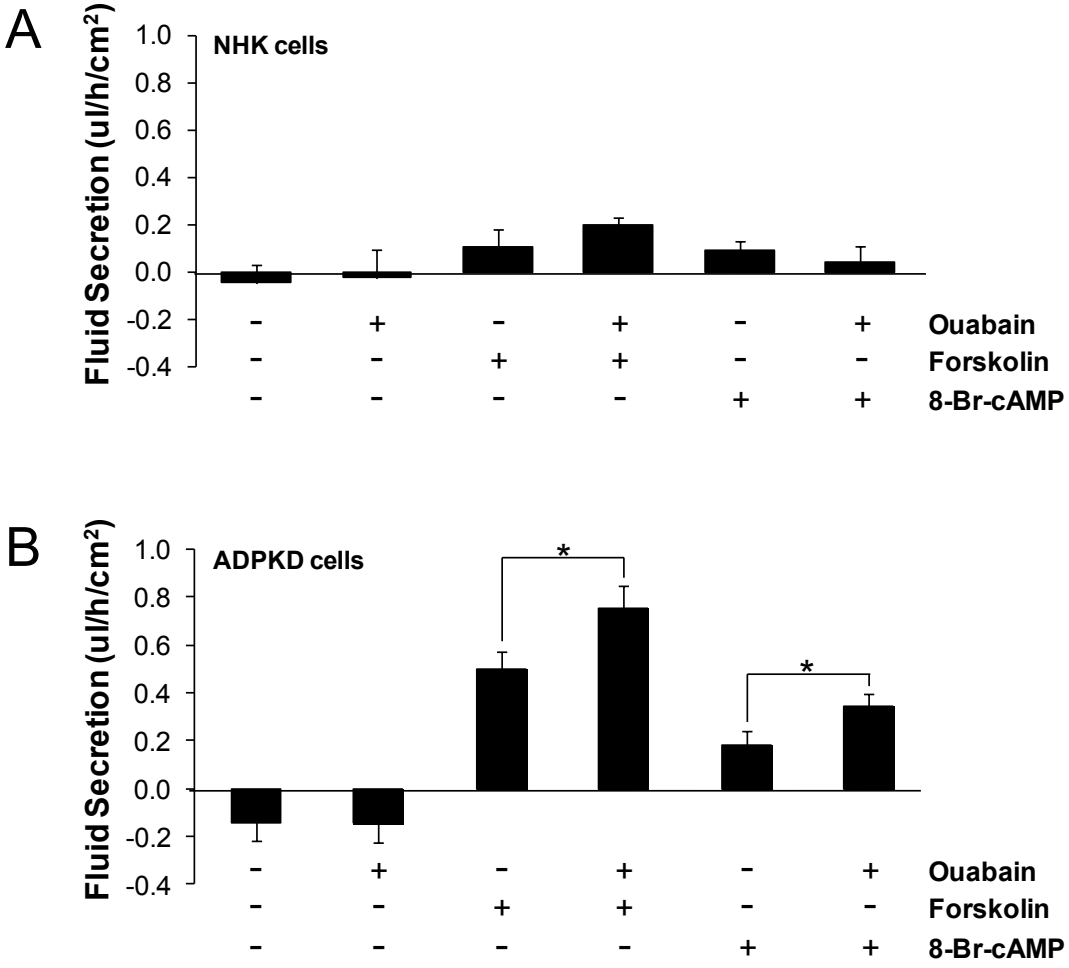


FIGURE 1 Ouabain increases forskolin- and cAMP-dependent fluid secretion in ADPKD cells. Confluent cell monolayers were treated with 3 nM ouabain, 5 μM forskolin or 100 μM 8-Br-cAMP alone, or ouabain combined with each of these agents. Control monolayers were incubated in media with no treatments added. After 24 h, the fluid volume on the apical side of A) NHK and B) ADPKD cells monolayers was measured. Data are expressed as a fluid secretion rate, ul/h/cm^2. Values are the mean $\pm$ SEM of three (NHK) or four (ADPKD) experiments performed in cells obtained from different kidneys. (*) $P < 0.01$.

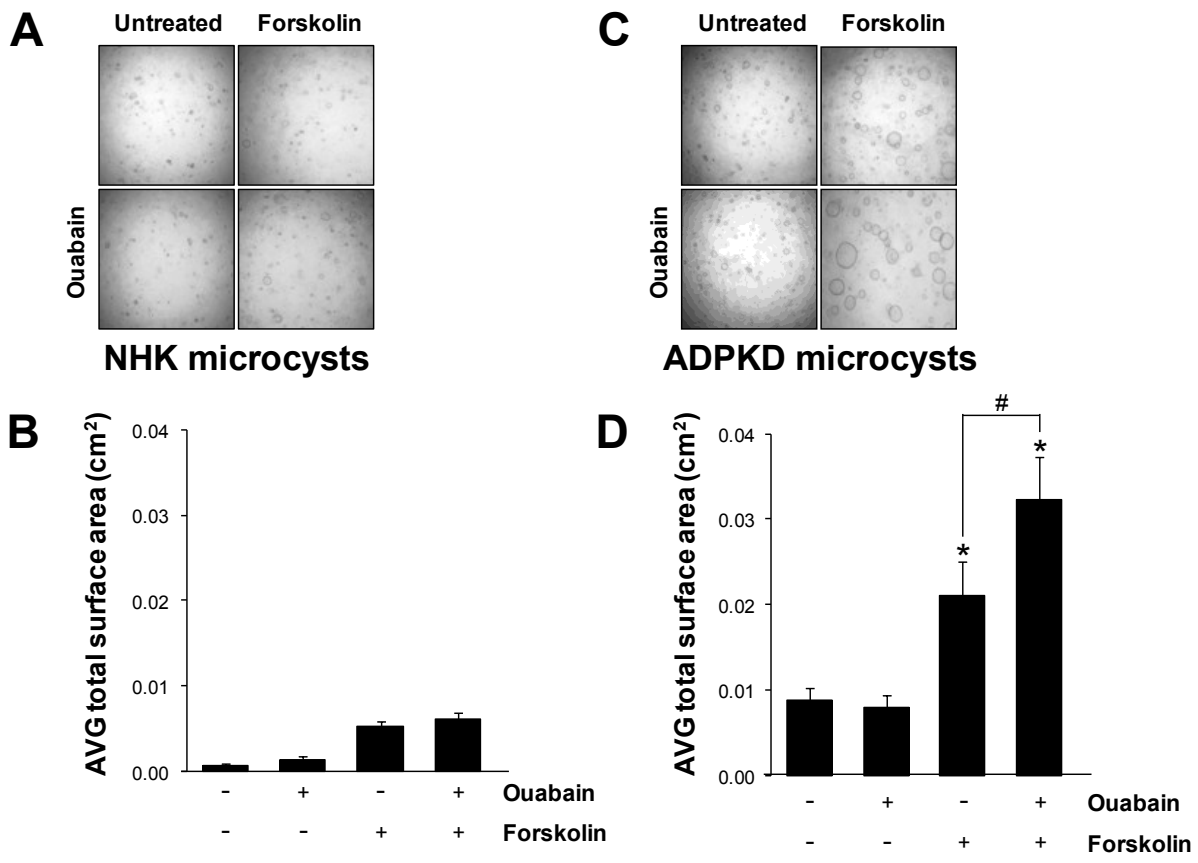


FIGURE 2 Ouabain increases forskolin-mediated microcyst growth in ADPKD cells.

A,B) NHK and C,D) ADPKD cells were cultured within a three dimensional collagen gel and treated in the absence and presence of 3 nM ouabain, 5 μM forskolin, or both. Microcysts were maintained for 5-7 days after treatment and average total surface area of the formed microcysts per well was measured. Data are expressed as microcyst average total surface area in cm^2. Bars represent the means $\pm$ SEM of six experiments performed in sextuplicate in cells obtained from six different ADPKD kidneys. (*) $P < 0.05$ vs control, (#) $P < 0.05$.

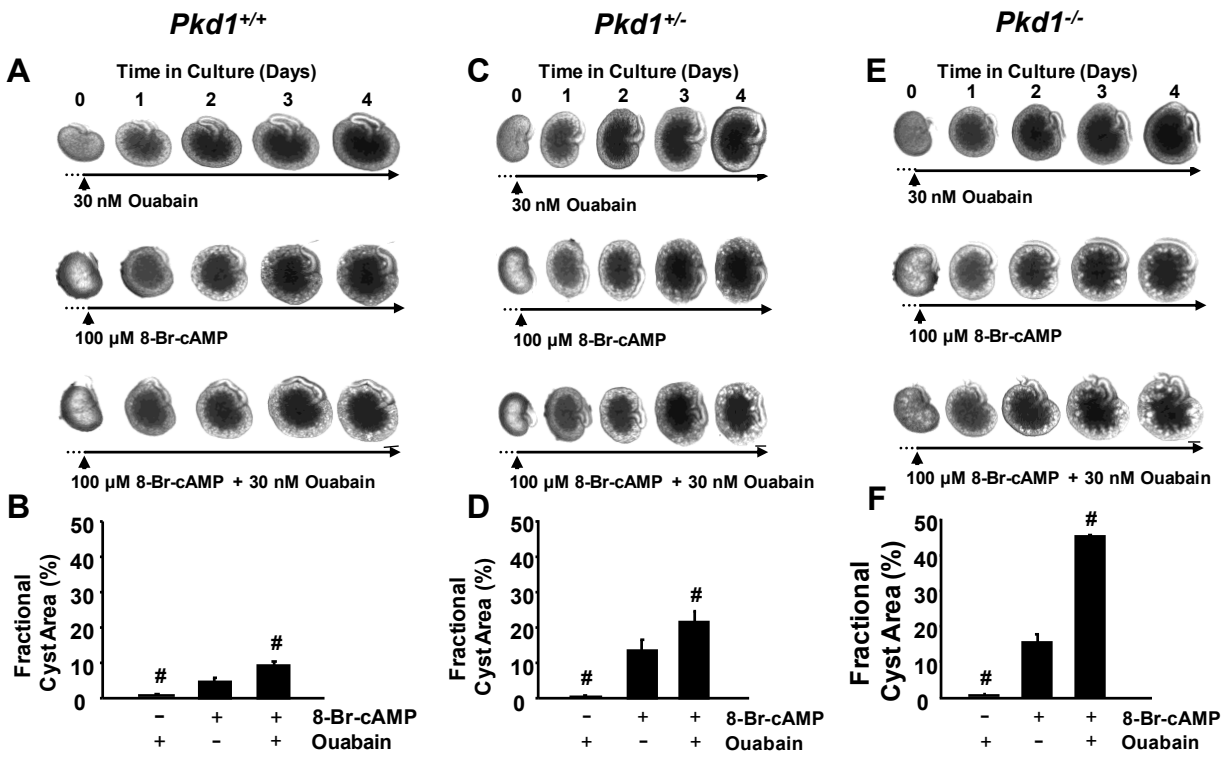


FIGURE 3 **Ouabain increases cAMP-dependent cyst growth in metanephric organ cultures from *Pkd1*^{m1Bei} mice.** Metanephric cultures were treated with 30 nM ouabain or 100 µM 8-Br-cAMP added separate or together. Images from the samples were captured and cyst area was measured. A,B) wild type *Pkd1*$^{+/+}$ mice, (C,D) *Pkd1*$^{+/-}$ mice and (E,F) *Pkd1*$^{-/-}$ mice. Top panels (A,C and E) show representative images of metanephroi taken at days 0 to 4 after treatment. Bottom panels (B,D,F) depict the average fractional cyst area captured at day 4 for the different experimental conditions on the indicated *Pkd1* phenotypes. Bars represent the means $^{\pm}$ SEM of three to eight kidneys depending on the treatment. (*) $P < 0.05$ vs. 8-Br-cAMP.

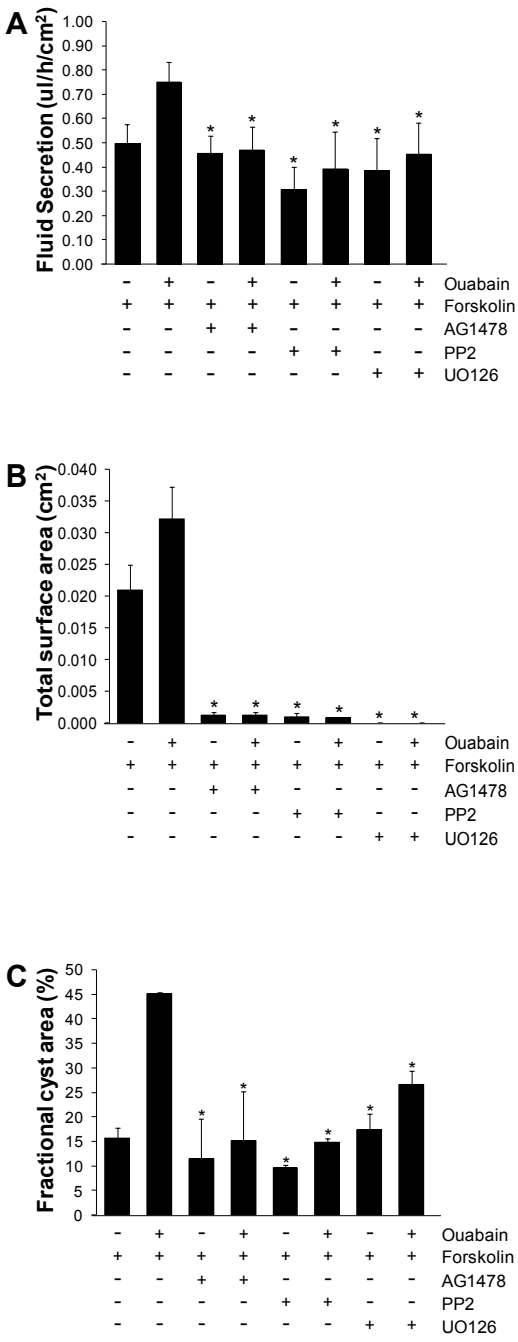


FIGURE 4 **The cystogenic effect of ouabain is mediated via the EGFR-Src-MEK pathway.**

A) Monolayers of ADPKD cells were treated with the indicated inhibitors in the presence of forskolin (5 μM) or foskolin with ouabain (3 nM). Following 24h treatment, apical fluid was collected and measured. Bars represent the means ± SEM of four to seven determinations on samples from cells obtained from two to four different ADPKD kidneys. Values different from samples treated with forskolin plus ouabain are indicated with an asterisk, with $P < 0.05$. B) ADPKD microcysts treated with the indicated inhibitors in the presence of forskolin (5 μM), with or without ouabain. After 5-7 days of growth, the average total surface area of all of the microcyst per well was determined. Bars represent the means ± SEM of six determinations in cells obtained from two or three ADPKD kidneys. Values different from samples treated with forskolin + ouabain are indicated with an asterisk, with $P < 0.05$. C) *Pkd1*^{m1Bei} mice organ cultures were treated with the indicated inhibitors in the presence of 8-Br-cAMP (100 μM), with or without ouabain. At day 4 after treatment, images from the cultures were captured and cyst surface area was measured for each condition. Bars depict mean ± SEM for average fractional cyst area for the different experimental conditions, using two to three different kidneys. (*) $P < 0.05$ vs. 8-Br-cAMP plus ouabain.

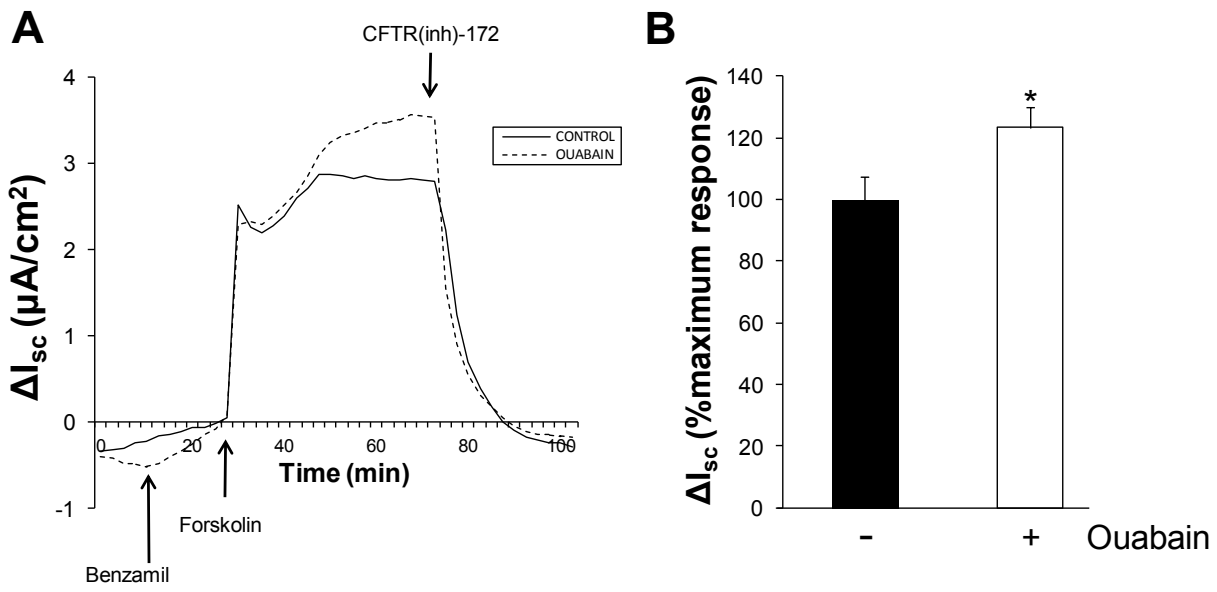


FIGURE 5 Ouabain increases forskolin-induced short circuit current (I_{sc}) in ADPKD monolayers. Monolayers of ADPKD cells, treated in the absence and presence of 3 nM for 24h, were mounted in Ussing chambers and I_{sc} was measured by a dual voltage clamp device. Forskolin (5 μM) was added to media on the basolateral side of the monolayers to stimulate I_{sc}, and CFTR(inh)-172 (10 μM), a specific CFTR inhibitor, was added apically to determine the contribution of CFTR. (A) Change in I_{sc} (ΔI_{sc}) from baseline for monolayers incubated in either control media or ouabain (3 nM) for 24 hours prior to measurement of I_{sc}. Baseline currents were calculated as an average of the I_{sc} reading for 1 to 2 minutes prior to the addition of forskolin. (B) I_{sc} response of ADPKD monolayers to forskolin in the absence and presence of ouabain. Data are expressed as a percentage of the maximum forskolin response in untreated monolayers. Bars represent mean ± SEM for 9 control monlayers and 8 ouabain treated monolayers from different ADPKD kidneys. $P < 0.05$ compared to control.

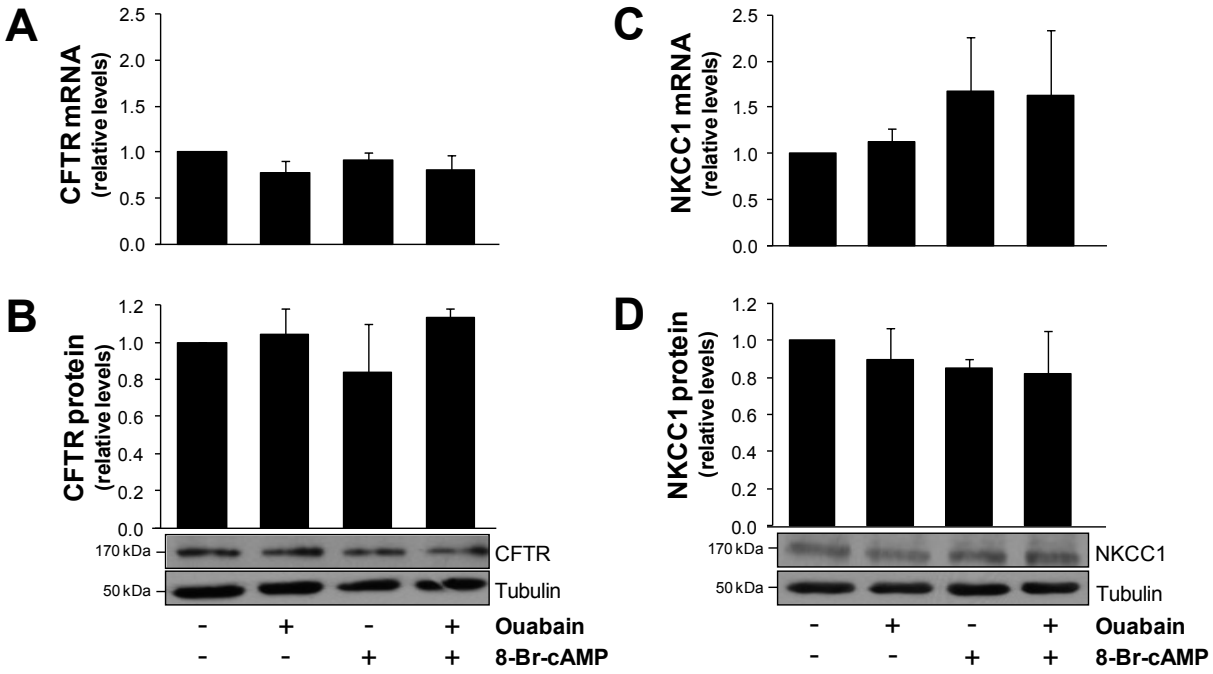


FIGURE 6 Ouabain does not affect the expression of CFTR or NKCC1 mRNA in ADPKD or NHK cells. ADPKD cells and NHK cells were treated with 8-Br-cAMP (100 μM) or ouabain (3 nM) alone or in combination. RNA was extracted from these cells, and real time quantitative PCR (RT-qPCR) and immunoblot were used to measure expression of CFTR (A, B) and NKCC1 (C,D) mRNA and protein levels, respectively. Transcript expression was normalized against expression of 18s RNA for each sample. Protein levels were normalized to corresponding untreated controls. Bars represent the mean ± SEM for 3 experiments each run with triplicate samples.

CHAPTER 4

OUABAIN ALTERS ION TRANSPORT MECHANISMS TO ENHANCE FLUID SECRETION AND CYST GROWTH IN ADPKD

INTRODUCTION

The hormone ouabain has been shown to influence mechanisms that are essential for the ADPKD cystogenesis. Ouabain not only increases ADPKD cell proliferation, it also potentiates cAMP-dependent fluid secretion across ADPKD monolayers as well as cAMP-dependent cyst growth of *in vitro* models of ADPKD (61). These effects requires the activation of the kinase Src, EGFR and the MAPK pathway (61, 89).

Fluid secretion is a key component of ADPKD cystogenesis (37, 44). In ADPKD, fluid secretion depends on transepithelial secretion of Cl- across the apical membrane of cystic epithelia (104, 123). This Cl- transport provides a electrochemical stimulus to drive Na^+ and, thus fluid, into the cystic lumen (105). The main apical regulator of this transepithelial Cl- tranport is the cystic fibrosis transmembrane conductance regulator (CFTR) (28, 104). Several studies have shown this transporter to be a crucial factor contributing to ADPKD fluid secretion and cystogenesis (20, 28, 81, 138).

Experiments described here were carried out to determine the effectors mediating ouabain-induced ADPKD fluid secretion and cyst growth. We show that ouabain-dependent fluid secretion and cyst growth require the presence and activity of CFTR. Moreover, ouabain specifically augments forskolin-induced activation of CFTR. This is associated with ouabain-dependent increases in membrane expression of CFTR as well as an increase in expression of an accessory protein, PDZK1, a potentiator of CFTR activity. Finally, ouabain treatment resulted

in decreased Na,K-ATPase membrane expression and ion transport which may have impacts on the fluid reabsorption potential of ADPKD epithelia.

RESULTS

Ouabain-induced cyst growth requires the presence and activity of CFTR

Cyst growth in ADPKD requires fluid secretion driven by transepithelial Cl^- transport (104, 123). This transport mechanism is regulated by the cAMP-dependent Cl^- transporter, CFTR, at the apical membrane of cystic epithelial cells (28). Our previous work has shown that ouabain can potentiate cAMP- and forskolin-induced cyst growth as well as forskolin-stimulated fluid secretion (61). Additionally, ouabain can augment cAMP-dependent growth of cystic dilations in Pkd1m1Bei metanephric organ cultures from wild type (Pkd1+/+), heterozygous (Pkd1+/-), and homozygous null (Pkd1-/-) embryos. To determine the involvement of CFTR in ouabain-stimulated cyst growth, $Pkd1^{m1Bei}$+/- mice were crossed with Cftr+/- mice to obtain embryos deficient in PC-1 and CFTR. Magenheimer et. al. showed these mice fail to develop cystic dilations in response to 8-Br-cAMP treatment (81). Metanephric organ cultures were established at day E15.5 from Pkd^{m1Bei}+/- mice with wild type (Pk1+/-; CF+/+) or null (Pkd1+/-; CF-/-) CFTR expression. Embryonic kidneys were treated with 100 μM 8-Br-cAMP in the presence and absence of 30 nM ouabain. Cultures were observed for 4 days, and cystic area was measured after 4 days of treatment. In agreement with previous studies (81), embryonic kidneys lacking CFTR showed no cyst growth in response to 8-Br-cAMP (Figure 1). Furthermore, in contrast to kidneys with wild type levels of CFTR, there was no ouabain-dependent induction of cystic dilations in the metanephric cultures lacking CFTR expression (Figure 1). These data suggest that ouabain increases growth of cystic dilations in the $Pkd1^{m1Bei}$ embryonic kidneys via a mechanism requiring the presence of CFTR.

Several studies have shown that ADPKD cells grown in a three dimensional collagen matrix will form individual cysts lined by a polarized epithelium, and this method is commonly used to investigate mechanisms influencing growth of ADPKD renal cysts (89, 104). Previously we showed that endogenous concentrations of ouabain can augment forskolin-induced cyst growth in this *in vitro* setting (61). To further investigate the requirement for CFTR in ouabain-induced cyst growth, microcyst cultures of ADPKD cells were established, as previously described (61, 89). Microcyst cultures were treated with and without ouabain (3 nM) in the presence and absence of forskolin (5 μM). Additionally, some cultures were treated with a specific CFTR inhibitor, CFTR(inh)-172 (10 μM), and forskolin (5 μM) in the absence and presence of ouabain (3 nM). CFTR(inh)-172 has been shown previously to block growth of microcysts derived from a canine cell line model of ADPKD (138). Figure 2 shows that ouabain itself has no effect on microcyst formation and growth. In contrast, ouabain augmented forskolin-induced microcyst. The presence of CFTR(inh)-172 abolished mycrocyst formation. These data, along with results from the metanephric organ cultures, indicate that the potentiating effect that ouabain has on cAMP- and forskolin-induced cyst growth requires the presence and activity of CFTR.

Ouabain-enhanced fluid secretion requires CFTR activity

Our previous work has shown that physiologic concentrations of ouabain can augment forskolin-induced fluid secretion across ADPKD monolayers (61). To determine the involvement of CFTR in this pro-secretory effect of ouabain, fluid secretion by ADPKD monolayers was analyzed in the presence of the specific CFTR inhibitor, CFTRinh-172. ADPKD cells were grown on permeable membrane supports until a tight monolayer was formed, as previously described (61). Following overnight starvation in 0.002% FBS, monolayers were treated for 24

hours with and without ouabain (3 nM) in the absence and presence of forskolin (5 μM). Additionally, CFTR(inh)-172 was added to the apical media of some monolayers treated with forskolin (5 μM) alone or in the presence of ouabain (3 nM). Following treatment, media on the apical side of each monolayer was collected and measured. In agreement with our previous results (61), ouabain augmented forskolin-induced fluid secretion across monolayers of ADPKD cells (Figure 2). Fluid secretion in the presence of CFTR(inh)-172 was not significantly increased above baseline values. Importantly, ouabain had no augmenting effect on fluid secretion observed in the presence of CFTR(inh)-172 (Figure 2). These results indicate that, in the setting of CFTR inhibition, ouabain does not enhance a secretory response in ADPKD monolayers. Moreover, ouabain-dependent potentiation of forskolin-induced fluid secretion requires the presence of an active CFTR.

Ouabain enhances forskolin-dependent Cl^- transport in ADPKD cells

Fluid secretion across ADPKD monolayers is regulated by the transepithelial transport of Cl^- (104). Previously, we showed that chronic treatment of ADPKD monolayers with physiologic concentrations of ouabain enhanced forskolin-induced increases in short circuit current of the cells indicating a ouabain-dependent increase in anion secretion (61). This increase was blocked by the application of CFTR(inh)-172. To further examine the effect ouabain has on the activity of CFTR in ADPKD cells, Cl^- efflux assays were performed. Cells were plated in 96-well plates and grown for 24 h in media containing 1.0% FBS. Following overnight serum starvation, cells were treated with and without ouabain (3 nM) for 48 hours. Cells were then loaded with the fluorescent indicator dye, MQAE (7 mM), in a Cl^- containing buffer, maintaining the presence of ouabain. Following treatment and dye loading, cells were washed with dye-free, Cl^--containing buffer, and forskolin was added for 10 min during the final wash to cells with and without ouabain. For all treatment conditions, parallel samples were analyzed in the absence and

presence of CFTR(inh)-172. To induce Cl^- efflux, cells were switched from a Cl^--containing buffer to a Cl^- free buffer maintaining the presence of experimental treatment. The change in flourescence was monitored in a plate reader taking measurements every minute for 10 minutes. Cl^- efflux was determined as the change in fluorescence of a sample at a given time point (F_t) from the initial flouresecnce (F_0) prior to inducing Cl^- efflux. The portion of fluorescence change (F_t-F_0) sensitive to CFTR inhibition was calculated to determine the CFTR-dependent Cl^- efflux. Representative traces of the CFTR-dependent fluorescence change are shown in Figure 4A. Average F_t-F_0 values were determined to analyze the effect of the various treatments on the activation state of CFTR (Figure 4B). CFTR-dependent Cl^- efflux was not affected by ouabain treatment alone. Forskolin caused a significant increase in CFTR-dependent Cl^- efflux, consistent with the role of forskolin as a cAMP-agonist in ADPKD cells. Interestingly, in cells treated with ouabain for 48 h, forskolin had an enhanced effect on CFTR-dependent Cl^- efflux. This is in agreement with our previous findings in short-circuit current analysis. These results indicate that ouabain treatment alone has no effect on CFTR-dependent Cl^- efflux. However, treatment with nanomolar concentrations of ouabain alters ADPKD cells in way that allows enhanced forskolin-stimulated Cl^- secretion via the CFTR.

Ouabain alters expression of CFTR and PDZK1in ADPKD cells

Previous work has shown a correlation between the expression level of CFTR and the extent of cyst growth in ADPKD (28). Our past work has shown that ouabain has no effect on the total cellular expression of CFTR in ADPKD cells (61). However, studies from Zhang et. al. showed that low doses of ouabain were sufficient to increase membrane delivery of mutant CFTR in a bronchial epithelial cell line (144). To determine if ouabain may be affecting the expression of CFTR at the plasma membrane in ADPKD cells, biotinylation studies were conducted. Monolayers of ADPKD cells were established on permeable membrane supports.

Following serum starvation, monolayers were treated without and with 3 nM ouabain in the absence and presence of 5 μM forskolin for 24h-48h. Monolayers were then labeled with biotin and lysed in RIPA buffer. The biotinylated lysates were immunoprecipated overnight with streptavidin-conjugated magnetic beads. The immunoprecipitated products were separated by SDS-PAGE (7.5% gel) and electrotransferred to nitrocellulose membranes. An antibody to the C-terminus of human CFTR was used to analyze the plasma membrane expression of CFTR. Figure 5 shows that ouabain alone was sufficient to increase membrane expression of CFTR in ADPKD monolayers at 48h. These results indicate that chronic ouabain exposure leads to increased expression of CFTR at the membrane of ADPKD cells. Enhancing the level of CFTR membrane expression may create an altered cellular phenotype allowing for augmented responses to secretory agonists that target CFTR activation.

Expression and activity of CFTR can be regulated by protein-protein interactions (47). Many proteins involved in regulation of CFTR act by binding to a PDZ-domain in the C-terminus of CFTR (46, 47, 102). PDZ-domains are amino acid sequences that mediate protein-protein interactions (47). Previous studies uncovered a particular PDZ-domain containing protein, PDZK1, that may play an important role in regulation of CFTR activity (126). In particular, coexpression of CFTR with PDZK1, caused potentiation of PKA-dependent activity of CFTR. To determine whether PDZK1 may be associated with ouabain-induced changes in Cl^- transport in ADPKD cells, expression levels of PDZK1 were analyzed by western blot in ADPKD cells treated with and without 3 nM ouabain for 24h. Ouabain treatment of ADPKD cells caused a significant increase in the expression levels of PDZK1 (Figure 6). This increase in PDZK1 expression is consistent with a ouabain-induced potentiation of cAMP-dependent Cl^- transport in ADPKD cells. Taken together these results indicate that nanomolar concentrations of ouabain

can elicit changes in protein expression consistent with ouabain-induced augmentation of cAMP-dependent anion and fluid secretion.

Ouabain decreases membrane expression of Na,K-ATPase

The Na,K-ATPase is responsible for regulating sodium and water reabsorption in normal renal epithelia. While fluid secretion in ADPKD epithelia has been extensively studied, there is little information regarding fluid reabsorption mechanisms in this epithelium. It is possible that alterations leading to increased accumulation of fluid in the cystic lumen may result from a combination of increased fluid secretion and decreased fluid reabsorption. To determine the effect of ouabain on factors that may be regulating fluid reabsorption in ADPKD epithelia, ouabain-dependent Na,K-ATPase expression was determined by biontinylation and immunocytochemistry analysis of monolayers of ADPKD cells. Ouabain treatment of ADPKD monolayers resulted in a significant decrease in membrane expression of Na,K-ATPase (Figure 7). This decreased membrane expression could be visualized by immunocytochemistry (Figure 7A) and also quantified by immunoblot analysis of biotinylated monolayers using an antibody specific for α1-Na,K-ATPase (Figure 7B). This is consistent with results from other studies in LLC-PK1 cells (73, 74, 137). These results indicate that physiologic concentrations of ouabain can influence the membrane expression of the Na,K-ATPase. Specifically, ouabain treatment leads to decreased levels of Na,K-ATPase at the plasma membrane of ADPKD cells.

Ouabain decreases Na,K-ATPase-mediated ion transport

Decreased membrane expression of the Na,K-ATPase could have significant effects on the ion transport directly and indirectly regulated by the Na,K-ATPase. To determine the effect of physiologic concentrations of ouabain on Na,K-ATPase-dependent ion transport rubidium uptake assays were performed. ADPKD cells were grown to confluence on permeable

membrane supports. Following serum starvation, cells were treated with and without 3 nM ouabain for 30 min. Rubidium uptake was then analyzed in the monolayers. Figure 8A shows there was approximately 20% less rubidium uptake in ouabain-treated monolayers compared to control monolayers. This is consistent with a ouabain-dependent reduction in membrane expression of the Na,K-ATPase causing reduced Na,K-ATPase-mediated ion transport.

Na,K-ATPase-mediated ion transport is responsible for maintaining a low intracellular concentration of sodium required for normal cellular function. Additionally, by regulating intracellular sodium concentrations in renal epithelial cells, the Na,K-ATPase can regulate sodium-dependent fluid reabsortion. Assays were performed to analyze the effect of low concentrations of ouabain on the intracellular concentration of sodium in ADPKD cells. Cells were incubated in media containing the fluorescent dye, sodium green tetra acetate, with and without 3 nM ouabain for 30, 60, and 90 minutes. At each time point analyzed, ouabain caused a significant increase in the intracellular sodium concentration. This result is in agreement with our other results showing a ouabain-induced decrease in Na,K-ATPase membrane expression and ion transport (137). Altogether, these data suggest that oubain may be altering the ion transport systems regulating fluid reabsorption in cystic epithelia in a manner that favors less fluid reabsorption.

DISCUSSION

Investigation of the mechanisms by which humoral factors affect progression of ADPKD yields important pathophysiologic information that can be used for the potential development of therapeutic approaches to treat ADPKD. Previously, we demonstrated that ouabain is an agent that augments cystogenic mechanisms in ADPKD. Specifically, physiologic concentrations of

ouabain can induce proliferation of ADPKD cells and enhance cAMP-dependent fluid secretion and cyst growth of *in vitro* models of ADPKD. Results from the current study provide important information on the mechanisms by which ouabain stimulates fluid secretion and cyst growth in ADPKD. Ouabain enhanced forskolin-induced cyst growth and fluid secretion via CFTR-dependent mechanisms in metanephric organ cultures from $Pkd1^{m1Bei}$ mice as well as ADPKD microcyst cultures and in monolayers of ADPKD cells. Ouabain-dependent potentiation of forskolin-stimulated cyst growth and fluid secretion required the presence and activity of CFTR. Cells treated with ouabain exhibited an increased CFTR-dependent Cl^- efflux in response to forskolin. This ouabain-induced potentiation of Cl^- efflux may be due to changes at the level of protein expression. Ouabain treatment of ADPKD monolayers led to an increased membrane expression of CFTR. A ouabain-induced increase in CFTR membrane expression is consistent with the enhanced forskolin-induced Cl^- efflux observed in ADPKD cells treated with ouabain.

A role for ouabain in affecting membrane expression of CFTR has been reported by Zhang, et al using a human bronchial epithelial cell line expressing a mutated form of CFTR (144). In this study, treatment with nanomolar concentrations of ouabain resulted in increased total expression and membrane expression of CFTR which was correlated with increased CFTR function. Mechanistically, this study showed that ouabain-dependent increases in CFTR membrane expression were associated with altered mechanisms of protein trafficking in the cells. In particular, ouabain treatment led to a decrease in chaperone proteins (HSP8/Hsc70 and HSPA1L/Hsp70) that are involved in protein folding and regulation of ER-associated protein degradation. Additionally, ouabain decreased expression of components of the COPII complex which is involved in the export of proteins from the Golgi. Altogether, these results demonstrate that ouabain exerts important effects on the intracellular trafficking mechanisms responsible for the targeting of CFTR to the cell plasma membrane. Further studies are required to confirm the involvement of the effect of ouabain in the cell biology of CFTR trafficking in ADPKD cells .

ADPKD cells, derived from the epithelium lining renal cysts express normal, non-mutated CFTR. CFTR is known to be expressed in the mammalian kidney, and several studies have shown it to be a crucial factor in ADPKD cystogenesis (20, 28, 81, 138). Cyst growth and formation in ADPKD depend on the continuous secretion of fluid into the growing cyst lumen (37). This fluid secretion is mainly regulated by transepithelial Cl^- secretion which depends on the activity of CFTR (28, 123). By mediating the cAMP-dependent apical secretion of Cl^-, CFTR generates the stimulus driving Na^+ and fluid into the growing cyst lumen (40, 104).

Regulation of CFTR expression is complex and depends on the interaction of a variety of chaperones and scaffolding proteins (47). These interactions are associated with regulation of CFTR protein trafficking, membrane localization, and intermolecular interaction with other ion transporters. In particular, several PDZ-domain containing proteins have been demonstrated to be involved CFTR regulation (102, 126). Our results show ouabain treatment of ADPKD cells led to an increase in the expression of a particular PDZ-domain containing protein, PDZK1. This protein has been implicated in increasing dimerization of membrane expressed CFTR leading to increases in PKA-dependent activation(126). Importantly, in these co-expression studies, PDZK1 alone caused no increase in CFTR activity. Rather, expression of PDZK1 with CFTR facilitated increased CFTR activation in response to PKA signals. Such a mechanism is consistent with our observations in ADPKD cells that ouabain, which alone causes no change in fluid secretion or anion transport, allows for increased forskolin- and cAMP-induced fluid secretion, anion secretion, and *in vitro* cyst growth. Additionally, the results of our Cl^- efflux assay demonstrate that ouabain treatment alone does not activate CFTR-dependent Cl^- transport. Rather, cells treated with ouabain have an increased response to forskolin-induced activation of CFTR-mediated Cl^- efflux. This ouabain-dependent increase in responsiveness to cAMP-dependent signals in ADPKD cells may be elicited by alterations at the protein expression level. By increasing expression of PDZK1 and membrane expression of CFTR,

ouabain may be inducing a phenotype in ADPKD cells with augmented responses to cAMP-dependent signals resulting in increased cAMP-induced fluid secretion and cyst growth.

Other important physiologic agents affecting ADPKD progression have been shown to affect cAMP-dependent pathways in ADPKD cells (117, 121). For example, arginine vasopressin (AVP), can activate cAMP-dependent pathways in ADPKD cells leading to increased Cl^- transport and, ultimately, increased cyst growth (11, 89). These effects of AVP in ADPKD cells can be abolished by treatment with an antagonist of the AVP receptor (89). This highlights the pathophysiologic importance of agents affecting cAMP-dependent mechanisms in ADPKD. A direct effect of ouabain on the expression of CFTR at the plasma membrane identifies a downstream effector where ouabain-induced effects may be converging with cAMP-dependent mechanisms known to be important for ADPKD progression. Previous work in our laboratory demonstrated a role for ouabain in activating cell proliferation in ADPKD cells (89, 90). This proliferative effect of ouabain was mediated by activation of Src, EGFR, and the MAPK signaling pathway. Similarly, we have shown that ouabain-induced changes in fluid secretion and cyst growth depend on these same intracellular signaling events. However, the proliferative effect of ouabain is induced by ouabain alone, while the effects of ouabain on fluid secretion and cyst growth are only observed in conjunction with treatments increasing intracellular cAMP. It is likely that ouabain-induced signals which lead to inceased fluid secretion and cyst growth in ADPKD compliment the cAMP-dependent signals already activated in the cells. Our results here identify CFTR as a potential downstream effector where ouabain and cAMP-dependent signaling may converge leading to a potentiation of ADPKD fluid secretion and cyst growth.

Fluid secretion in ADPKD has been shown to be mediated by active anion secretion across the apical membrane creating a stimulus for Na^+ and water to follow resulting in increased intraluminal fluid accumulation (104). Normal renal tubules reabsorb most of the fluid in the glomerular filtrate, largely by sodium-dependent mechanisms regulated by the Na,K-

ATPase. However, reabsorption mechanisms have not been widely studied in cystic epithelial cells. Our results suggest that ouabain may be decreasing fluid reabsorption across cystic epithelia by decreasing Na,K-ATPase membrane expression and Na,K-ATPase mediated ion transport. While physiologic concentrations of ouabain do not completely abolish Na,K-ATPase activity, we have observed a slight decrease in Na,K-ATPase activity in response to nanomolar concentrations of ouabain. Decreases in this ion transport mechanism are associated with an increase in intracellular Na^+ concentrations in ADPKD cells exposed to ouabain. By altering mechanisms governing Na^+ transport in ADPKD cells, oubain may be reducing the ability of these cells to reabsorb fluid. Such an effect would likely exacerbate the secretory phenotype of cystic epithelia leading to increased fluid accumulation in the growing cysts.

Studies conducted in normal porcine epithelial cells have demonstrated a similar effect of nanomolar concentrations of ouabain on Na,K-ATPase membrane expression and ion transport (73, 74, 137). These studies have shown that ouabain can regulate transcellular sodium transport mechansims by a direct effect on the apical expression of the sodium/hydrogen exchanger (NHE3) and the Na,K-ATPase in proximal tubule cells (21, 92). More investigation is needed to define the specific role of ouabain in regulating sodium transport and fluid reabsorption in ADPKD epithelia. However, results from our study and from other laboratories support the hypothesis that ouabain is a physologic factor that may decrease fluid reabsorption mechanisms in ADPKD epithelia to further favor fluid secretion over reabsorption and lead to enhanced accumulation of fluid in growing renal cysts.

In conclusion, our current results identify specific ouabain-dependent mechanisms that may be responsible for ouabain's effect on fluid secretion and cyst growth in ADPKD. We suggest that ouabain acts on ADPKD cells to confer a phenotype more responsive to cAMP-dependent secretory agonists while also reducing mechanisms of fluid reabsorption to further enhance fluid secretion across ADPKD epithelia. Our current results support the important role

ouabain may have as a physiologic agent affecting the progression of renal cyst growth in ADPKD.

FIGURES

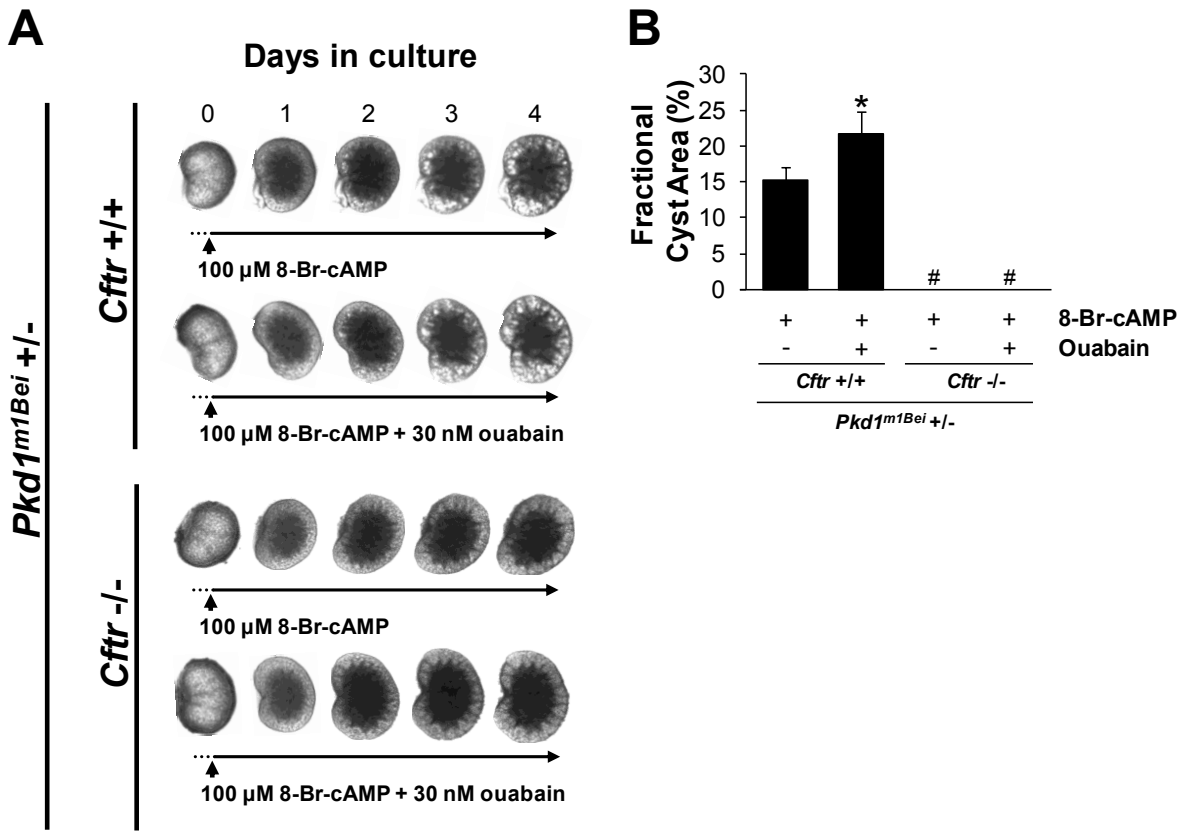


FIGURE 1 Ouabain-enhanced cyst growth in metanephric kidneys requires CFTR. Embryonic kidneys (E15.5) from *Pkd1*^{m1Bei}+/-; *Cftr*+/+ and *Pkd1* ^{m1Bei} +/-;*Cftr*-/- mice were isolated and placed into culture in media containing 100 µM 8-Br-cAMP in the absence and presence of 30 nM ouabain. (A) Representative images of metanephic kidneys in culture. (B) Average fractional cyst area was calculated after four days of treatment. Bars represent the means ± SEM for 5 to 8 kidneys depending on the treatment. (*) $P < 0.05$ vs. 8-Br-cAMP treatment alone in the *Cftr*+/+ kidneys; (#) $P < 0.05$ vs. corresponding treatments in *Cftr*+/+ kidneys.

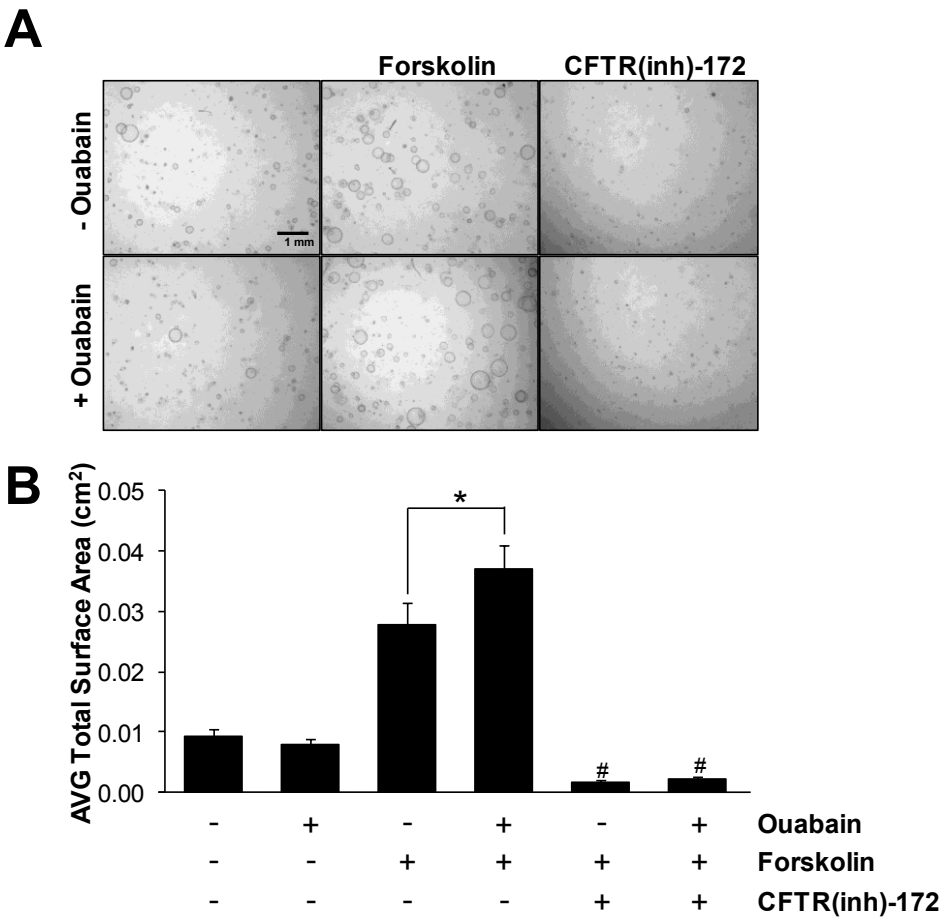


FIGURE 2 Ouabain-enhanced ADPKD microcyst growth requires CFTR. Microcyst cultures were established by seeding ADPKD cells within a three-dimensional collagen matrix. Cultures were treated without and with 5 μM forskolin in the absence and presence of 3 nM ouabain. Additionally, some cultures were treated with 10 μM CFTR(inh)-172 and forskolin (5 μM) in the absence and presence of ouabain. Untreated, control cultures remained in defined media alone during the treatment period. After 5-7 days total cyst surface area of the microcyst cultures was measured. (A) Representative images of microcyst cultures for each of the indicated treatments. (B) Average total surface area of microcyst cultures was measured for each treatment condition. Bars represent means ± SEM for microcyst cultures grown in sextuplicate with cells from different ADPKD kidneys. (*) $P < 0.05$ vs. forskolin alone; (#) $P < 0.01$ vs. forskolin+ouabain treatment.

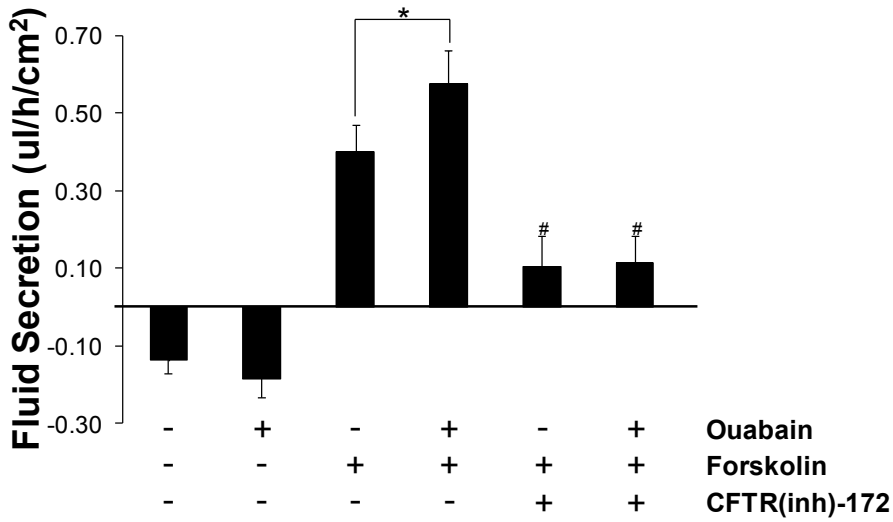


FIGURE 3 Ouabain-enhanced fluid secretion in ADPKD monolayers requires CFTR. Confluent monolayers of ADPKD cells were treated without and with 5 μM forskolin in absence and presence of 3 nM ouabain. Monolayers were also treated with 10 μM CFTR(inh)-172 and 5 μM forskolin in the absence and presence 3 nM ouabain. Untreated, control monolayers were incubated in media containing no experimental agents. After 24h, the volume of fluid on the apical side of each monolayer was removed and measured. Data are expressed as fluid secretion rate, ul/h/cm^2. Bars represent the fluid secretion rate mean ± SEM for monolayers grown in triplicate from different ADPKD kidneys. (*) $P < 0.05$; (#) $P < 0.01$ vs. forskolin+ouabain treatment.

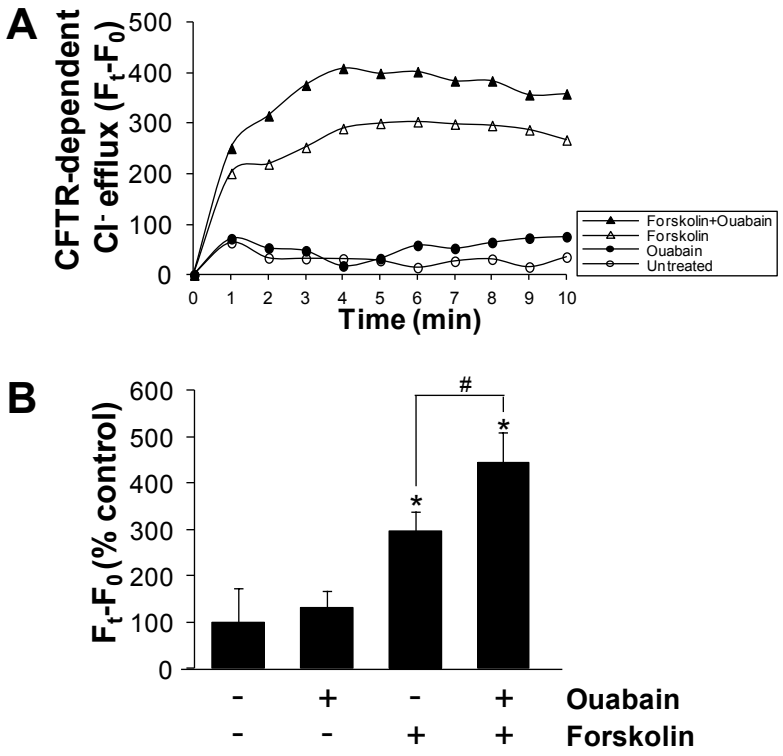


FIGURE 4 Ouabain augments forskolin-induced, CFTR-dependent Cl^- efflux in ADPKD cells. ADPKD cells grown in 96-well plates were treated without and with 3 nM ouabain for 48 hours. Cells were loaded with MQAE, and then Cl^- efflux was induced by swithching to a Cl^--free buffer. Prior to Cl^- efflux induction, forskolin (5 μM) was added to cells with and without Flourescent measurements were taken every minute for 10 minutes. Cl^- efflux was determined from the difference between the fluorescence of a sample at a give time (F_t) and the initial fluorescence of that sample prior to inducing Cl^- efflux (F_0). The portion of the flourescence change (Ft-F0) sensitive to CFTR(inh)-172 was determined to be CFTR-dependent Cl^- efflux. (A) Representative trace of CFTR-dependent Cl^- efflux for samples exposed to the indicated treatments. (B) F_t-F_0 at the plateau of each efflux curve was calculated for each treatment condition and expressed relative to control flouresence change. Bars represent means ± SEM for 6 different experiments using ADPKD cells from different kidneys. (*) $P < 0.05$ vs. untreated control; (#) $P < 0.01$.

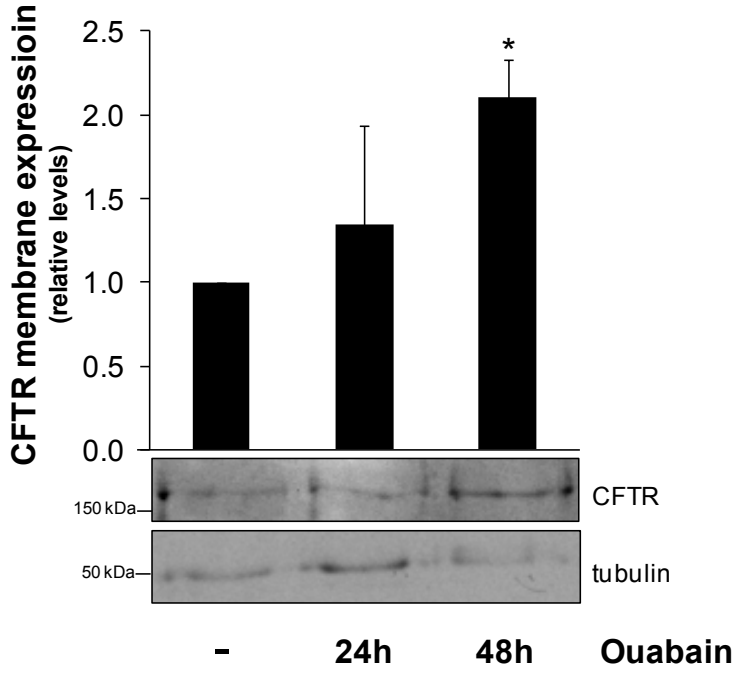


FIGURE 5 Ouabain increases CFTR membrane expression in ADPKD monolayers. Confluent monolayers of ADPKD cells were treated without and with ouabain (3 nM) for 24h-48h. Following treatment, membrane proteins from the monolayers were isolated by biotinylation and streptavidin immunoprecipitation. These samples were analyzed by SDS-PAGE and immunoblot using an antibody specific for human CFTR. Bars represent CFTR membrane expression for each treatment relative to untreated control levels and normalized to tubulin expressed as means ± SEM for 4 determinations using cells from different ADPKD kidneys . A representative immunoblot is shown below the graph. (*) $P < 0.05$ vs. untreated control.

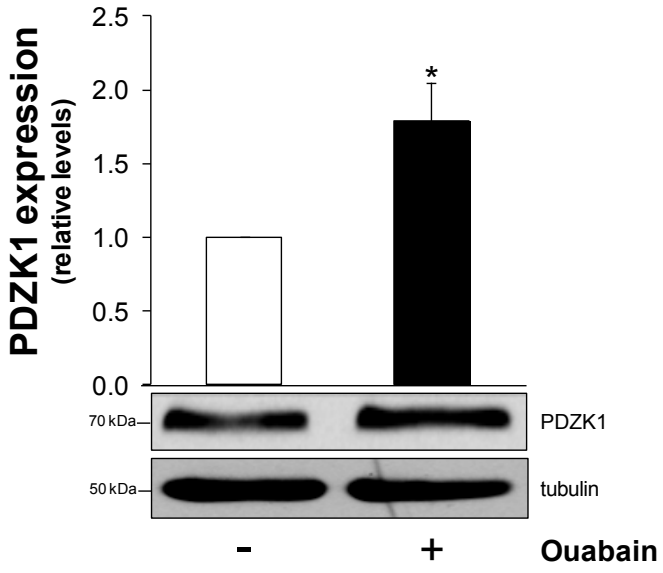


FIGURE 6 Ouabain increases PDZK1 expression in ADPKD cells. Cells were treated without and with ouabain (3 nM) for 24h. Following treatment, cells were lysed and equal amounts of cell lysate were separated by SDS-PAGE and then immunoblotted using an antibody specific for human PDZK1. Protein expression was determined by densitometry. Bars represent PDZK1 expression relative to untreated control levels and normalized to tubulin. Data are expressed as means ± SEM for 3 determinations using cells from different ADPKD kidneys. A representative immunoblot is displayed below the graph. (*) $P < 0.05$ vs. untreated control.

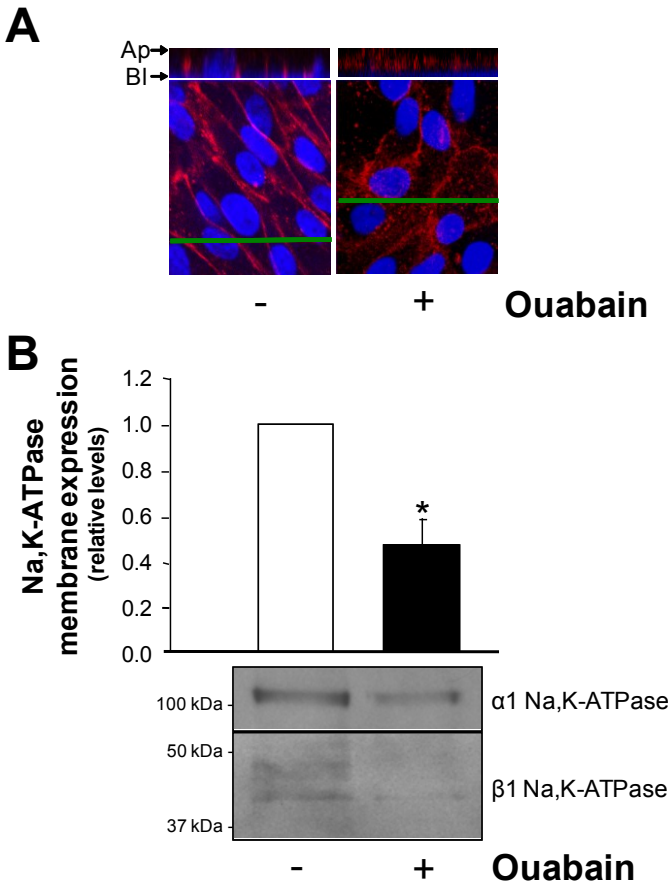


FIGURE 7 Ouabain reduces membrane expression of Na,K-ATPase in ADPKD cells. Na,K-ATPase membrane expression was analyzed by immunocytochemistry and biotinylation. (A) Immunofluorescence microscopy of confluent cultures grown on filter supports treated with ouabain for 24 h and labeled for the α1 subunit 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. (B) Western blot analysis following biotinylation of surface proteins. Na,K-ATPase membrane expression was determined using antibodies specific for the α1 and β1 subunits of the Na,K-ATPase. Bars represent the mean ± SEM of 3 determinations performed on cells obtained from different ADPKD kidneys. (*) $P < 0.01$ vs untreated control.

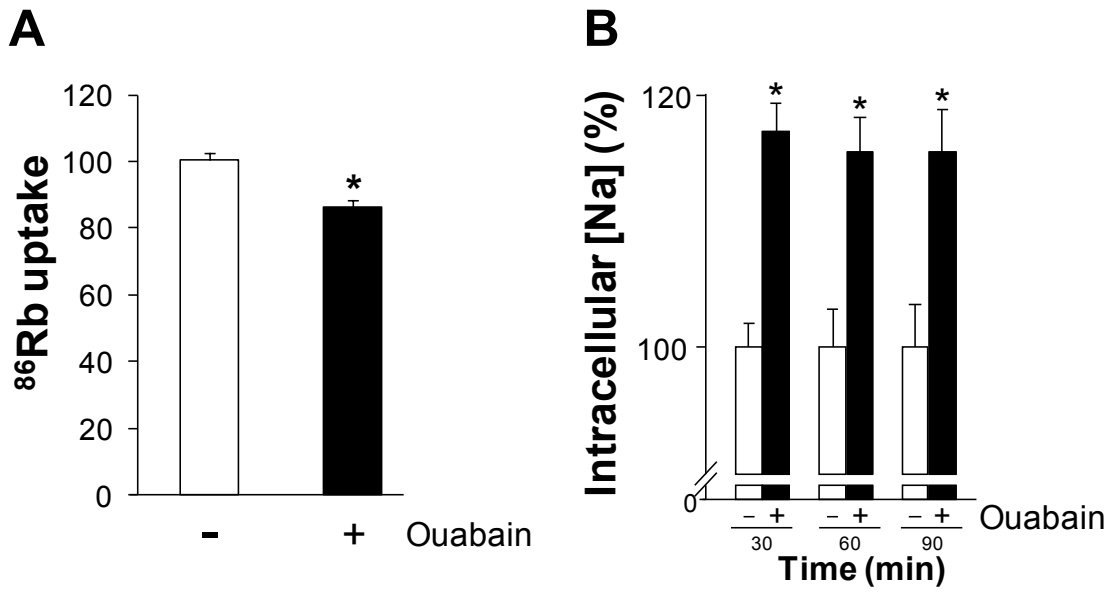


FIGURE 8 Ouabain reduces Na,K-ATPase-mediated ion transport in ADPKD cells. (A) ^{86}Rb uptake in ADPKD cells. Confluent monolayers of ADPKD cells were treated with and without ouabain (3 nM) for 24h. ^{86}Rb uptake was measured and data are expressed as ^{86}Rb uptake relative to uptake in untreated control monolayers. Bars represent means ± SEM of 6 determinations performed on cells obtained from different ADPKD kidneys. (*) $P < 0.05$ vs untreated control. (B) Intracellular sodium, $[Na]_i$, was determined using sodium green tetra acetate. ADPKD cells were incubated in media containing the flourescent dye with and without ouabain (3 nM) for the indicated times. Mean flourescence intensity of each sample was then analyzed and expressed relative to the untreated control for each time point. Bars represent mean ± SEM for experiments performed on cells obtained from different ADPKD kidneys. (*) $P < 0.05$ vs untreated control for each corresponding time point.

CHAPTER 5

ABNORMAL EXPRESSION OF THE POLYCYSTIN-1 C-TAIL ALTERS THE RESPONSE OF M-1 COLLECTING DUCT CELLS TO OUABAIN

INTRODUCTION

A great amount of work has been devoted to understanding the pathophysiology of ADPKD; however, the relationship between defects in the polycystins (PC-1 and PC-2) and the cystic phenotype remains unclear. Different *in vitro* models and mouse models have been generated in which deletion, mutation, and overexpression of the *Pkd1* gene have been performed. These have provided evidence connecting PC-1 function to known signaling pathways (116). Studies reported here utilized a mouse cortical collecting duct cell model, in which inducible overexpression of the C-terminal cytosolic domain of PC-1 confers the cells with an ADPKD proliferative phenotype in response to cAMP. This behavior, typical of ADPKD cells, is not observed in normal kidney cells, or in collecting duct cells lacking PC-1 C-tail overexpression, which respond to cAMP by decreasing cell growth (107). The effect of PC-1 C-tail overexpression can be explained by a dominant-negative loss-of-function mechanism that causes disruption of the normal polycystin signaling pathway in the cells (107).

The following series of experiments examined whether the ADPKD phenotype of these M-1 cortical collecting duct cells, which overexpress the PC-1 C-tail, affects the response of the cells to ouabain. Results of this investigation indicate that acquisition of the cystic phenotype by PC-1 C-tail overexpression increases the affinity of M-1 cortical collecting duct cells to ouabain,

induces ouabain-stimulated intracellular signaling events in these cells and stimulates their ability to proliferate and secrete anions, all features typical of ADPKD cells.

RESULTS

Overexpression of polycystin-1 C-tail enhances ouabain proliferative effects of M1 cells

Previous work in our laboratory had shown that human ADPKD epithelial cell cultures derived from the cystic epithelium of ADPKD kidneys, respond to nanomolar concentrations of ouabain by increasing cell proliferation. In contrast, human normal kidney cell growth is not significantly affected by ouabain. (89, 90). It has been shown that overexpression of the C-tail portion of PC-1 in M-1 cortical collecting duct cells provides these cells with changes in their proliferative response to agonists that increase intracellular cAMP levels, reminiscent of the behavior observed in ADPKD cystic cells (107). We investigated if M-1 cells expressing the C-tail of PC-1 also responded to ouabain with exacerbated cell growth. For this, we treated M-1 C20 and M-1 C17 cells with dexamethasone and then incubated them in the absence or presence of various concentrations of ouabain for 24 hours. While both M-1 C20 and M-1 C17 cells have been transfected with a dexamethasone-inducible PC-1 C-tail construct, only the M-1 C20 clone expresses the PC-1 construct after induction. The lack of PC-1 C-tail expression in the M-1 C17 clone makes these cells an adequate control for studying the effects of the PC-1 construct. This property of the M-1 clones has been previously described (107). Following ouabain treatment, cell proliferation was measured after 24 h. As shown in Figure 1, ouabain stimulated proliferation of M-1 C20 cells in a dose-dependent manner. The maximal proliferative effect of ouabain was observed at ouabain concentrations between 3×10^{-9} M and 10^{-7} M. At doses above 10^{-7} M, cell growth was progressively inhibited, agreeing with the notion that relatively high amounts of ouabain are toxic for the cells. In contrast to M-1 C20 cells, ouabain

did not significantly affect proliferation of M-1 C17 cells. These results demonstrate that overexpression of the C-terminal portion of PC-1 confers a ouabain-dependent proliferative phenotype to M-1 cells.

M-1 C20 cells respond to ouabain by activating the ERK1/2 pathway

ADPKD cells have been shown to respond to nanomolar concentrations of ouabain by activating the Na,K-ATPase signaling apparatus (89). One of the main downstream intracellular intermediates of this signaling system is the kinase ERK1/2. To determine whether ouabain utilizes signaling pathways in M-1 C20 cells common to those of ADPKD cells, we studied the ouabain-dependent phosphorylation of ERK1/2 in M-1 C20 and M-1 C17 cells. After induction of PC-1 C-tail expression with dexamethasone, cells were treated with different amounts of ouabain for 30 min and the total and phosphorylated forms of ERK1/2 were analyzed by immunoblot using lysates from the cells. As shown in Figure 2A, the levels of phosphorylated ERK1/2 were not significantly changed by ouabain in M-1 C17 cells. In contrast, ouabain caused a dose-dependent increase in ERK1/2 phosphorylation in M-1 C20 cells, with a maximal level of phosphorylation induced at 3×10^{-8} M ouabain (Figure 2B). These results show that ouabain exerts effects on the cells expressing the PC-1 C-tail by activating the ERK1/2 pathway.

Ouabain activates the EGFR-Src-MEK-ERK pathway in M-1 C20 cells

Important intermediates upstream of ERK1/2 in the ouabain-induced, Na,K-ATPase-mediated pathway of ADPKD cells include Src, EGFR and MEK. To investigate whether this signaling pathway is activated by ouabain in the M-1 C20 cells induced to express PC-1 C-tail, we studied the phosphorylation of ERK1/2 as a downstream indicator of ouabain signaling, in the absence and presence of EGFR, Src and MEK inhibitors. M-1 C20 cells were treated with

and without 3 x 10^{-8} M ouabain, in the absence and presence of tyrphostin AG1478, PP2, and U0126 and 30 min later, ERK phosphorylation was determined. The inhibitors were used at concentrations that have been shown to be optimal for blocking EGFR, Src, and MEK activation of ADPKD cells (89). Figure 3A shows that ouabain-dependent phosphorylation of ERK1/2 in M-1 C20 cells is prevented by inhibition of EGFR, Src, and MEK.

To further determine if activation of the EGFR-Src-MEK-ERK signaling pathway is responsible for ouabain-stimulated proliferation of M-1 C20 cells, proliferation assays were performed on these cells after treatment with and without 3 x 10^{-8} M ouabain in the absence and presence of tyrphostin AG1478, PP2, or U0126 for 24 hours. Figure 3B shows that inhibition of EGFR, Src, and MEK prevented the proliferation that ouabain induced in M-1 C20 cells. These data indicate that in M-1 C20 cells, ouabain-dependent activation of the EGFR-Src-MEK-ERK signaling pathway is necessary to cause cell proliferation.

Ouabain enhances forskolin-dependent anion secretion in M-1 C20 monolayers

Previous results indicated that overexpression of the C-terminal domain of PC-1 alters the ion transport properties of M-1 C20 cells, enhancing the ATP-stimulated apical Cl^- conductance of the cells (59, 129). Measurement of short circuit current, Isc, has been used extensively as an indicator of anion fluid secretion in cystic cell monolayers (44, 61, 84, 123). We previously used this method and showed that ouabain augments a forskolin-induced increase of short circuit current in ADPKD monolayers (61). Here, we determined if expression of the PC-1 C-tail endows the cells with modified ion transport properties in response to ouabain. For this, we studied the effect of ouabain on forskolin-induced anion secretion of M-1 C20 and M-1 C17 cells. Cells were grown on permeable filter supports until a tight monolayer was formed. Following dexamethasone treatment and overnight starvation in 0.002% FBS, the

monolayers were treated with and without 3 x 10^{-8} M ouabain for 24 hours and short circuit currents were measured. Ouabain had no significant effect on the forskolin-induced Isc of M-1 C17 cells (Figure 4A and 4C). In contrast, ouabain treatment of M-1 C20 monolayers resulted in an approximately 40% augmentation of the forskolin-induced increase in Isc (Figure 4B and 4D). The forskolin-induced increase in Isc of M-1 C20 cells was inhibited by addition of CFTR(inh)-172, a specific inhibitor of CFTR. To assess whether the effect of ouabain on forskolin-induced anion secretion was mediated via the Na,K-ATPase signaling cascade, we tested the sensitivity of the ouabain-dependent increase of short circuit currents to the Src kinase inhibitor, PP2. PP2 prevented the ouabain-dependent increases in forskolin-stimulated anion secretion of M-1 C20 cells. In contrast PP2 did not significantly affect the basal Isc in the absence of ouabain, indicating that this inhibitor specifically interfered with ouabain-Na,K-ATPase signaling (Figure 4B and 4D). Altogether, these data demonstrate that ouabain enhances forskolin-induced, CFTR-dependent Isc in cells overexpressing the C-tail of PC-1. This effect is mediated by activation of the intracellular tyrosine kinase, Src.

Overexpression of the PC-1 C-tail increases the ouabain affinity of M1 cells

In ADPKD cells, a fraction of Na,K-ATPase has an abnormally high sensitivity to ouabain. This is indicated by a biphasic response to ouabain inhibition, with approximately 20% of the Na,K-ATPase of ADPKD cells presenting a ouabain inhibition constant in the nanomolar range. The remaining Na,K-ATPase activity of ADPKD cells retains a relatively lower sensitivity to ouabain inhibition, which is characteristic of normal kidney cells (90). We determined whether overexpression of the PC-1 C-tail in cortical collecting duct cells, which confers an ADPKD phenotype, affected the sensitivity of these cells to ouabain. For this, the ouabain inhibition profile of Na,K-ATPase activity was tested on homogenates of M-1 C20 cells after induction of overexpression of PC-1 C-tail with dexamethasone. As a control, M-1 C17 cells which do not

express the PC-1 construct were used. As shown in Figure 5A, Na,K-ATPase from M-1 C20 cells exhibited a bimodal dose-response curve to ouabain, presenting components with high and low sensitivity to ouabain. In contrast, M-1 C17 cells only presented the Na,K-ATPase fraction with low sensitivity to ouabain, which is typical for normal rodent kidney cells (15). The calculated K_i values for ouabain inhibition of Na,K-ATPase activity and relative amounts of each Na,K-ATPase population are depicted in Table 1. As shown, approximately three-fourths of the Na,K-ATPase of M-1 C20 cells had an inhibition constant (K_i) for ouabain in the millimolar range, similar to that of M-1 C17 cells. The remaining one-fourth of the Na,K-ATPase of M-1 C20 cells had a K_i for ouabain in the nanomolar range. These results indicate that a portion of the Na,K-ATPase of M-1 C20 cells exhibits an abnormally increased sensitivity to ouabain, which is not found in the M-1 C17 cells not expressing PC-1 C-tail.

One of the mechanisms that dictates the response of Na,K-ATPase to ouabain is the presence of different isoforms of its catalytic α subunit. Four different Na,K-ATPase α polypeptides (α1, α2, α3 and α4), are expressed in mammalian cells. These isoforms are characterized by distinct kinetic properties, among which is their particular response to ouabain (15). Normally, renal epithelial cells largely express the α1 isoform of the Na,K-ATPase, which is relatively insensitive to ouabain. The presence of this population of Na,K-ATPase with high sensitivity to ouabain suggests that M-1 C20 cells may be mis-expressing one of the Na,K-ATPase isoforms that has a high affinity for ouabain. To investigate this, expression of the different Na,K-ATPase α isoforms was determined at the mRNA and protein levels. As shown in Figures 5B and 5C, both M-1 C20 and M-1 C17 cells only expressed the α1 isoform of Na,K-ATPase. This demonstrates that the increased ouabain-sensitivity observed in M-1 C20 cells is not due to an altered expression of α isoforms of the Na,K-ATPase.

Discussion

Results from our studies show that overexpression of a PC-1 C-tail construct in M-1 cells induces a phenotypic change with respect to the affinity and response of the cells to ouabain. This was reflected by increased ouabain-dependent cell proliferation and enhancement of forskolin-induced transepithelial anion secretion, as well as an augmented sensitivity of Na,K-ATPase activity to ouabain. Overexpression of the C-terminal domain of PC-1 has been shown to confer M-1 cells with phenotypic characteristics of ADPKD cells, likely through a dominant-negative mechanism (107). In particular, M-1 cells expressing an excess of the PC-1 cytosolic segment, exhibit aberrant cell proliferation and enhanced transepithelial Cl^- secretion (59, 107). Both of these characteristics are typical of the ADPKD phenotype. Our data here suggest that an increased ouabain sensitivity is another distinctive property of these cells. This ouabain sensitive phenotype mimics our previous findings in human ADPKD cells and allows ouabain to stimulate both cell proliferation and apical anion secretion, which are two essential components of ADPKD cystogenesis (61, 89, 90). Moreover, these effects take place at ouabain concentrations that are similar to those normally circulating in plasma. Therefore, our current findings further support the importance of ouabain as a cystogenic factor in ADPKD. Additionally, the ouabain-responsive phenotype makes the cells more prone to respond to ouabain and may be relevant to the progression of renal ADPKD cysts.

In M-1 cells expressing a PC-1 C-tail construct, ouabain induced cell proliferation through activation of the Na,K-ATPase signaling pathway. Similar to ADPKD cells, the effects of ouabain on M-1 C20 cell proliferation are abolished by tyrphostin AG1478, PP2 and U0126, suggesting that the mechanism of action of ouabain in M-1 C20 cells requires activation of EGFR, Src, MEK and ERK1/2. Therefore, ouabain-induced signaling in M-1 C20 cells is

mediated through components that are common to those of the Na,K-ATPase signalosome of other cells. In addition, these intracellular messengers constitute important mediators of the cystic ADPKD phenotype in response to cAMP (121). Moreover, aberrant overexpression of the C-terminal cytosolic tail of PC-1 has been implicated in dysregulation of several signaling pathways, including the response of the MAPK pathway to cAMP stimulation (107). In this manner, it is possible that ouabain, acting independently through EGFR-Src-MEK-ERK, converges on the same, already hyperactive, cAMP-stimulated pathway of cystic cells.

While previous work has shown that overexpression of the PC-1 C-tail enhances ATP-stimulated Cl^- secretion (59), our work is the first to investigate the effect of the C-tail construct on forskolin-induced anion secretion. In our studies, forskolin-stimulated increases in Isc were comparable between M-1 C17 and M-1 C20 cells, however, ouabain treatment enhanced the forskolin-induced anion secretion only in M-1 C20 cells. Additionally, the ouabain enhanced, forskolin-stimulated Isc resulting from PC-1 C-tail overexpression in M-1 C20 cells, was sensitive to the specific CFTR inhibitor, CFTR(inh)-172. This indicates that the acquired cystic phenotype of the cells follows molecular mechanisms for anion secretion that are similar to those described in native ADPKD epithelium. However, in contrast to our studies in human ADPKD cells (61), inhibition of CFTR did not completely abolish the ouabain-dependent and forskolin-induced Cl^- current in these monolayers. The small remaining Isc fraction, resistant to CFTR(inh)-172, suggests that M-1 C20 cells have an additional pathway for the apical movement of Cl^- that is independent from CFTR. On the other hand, the similar degree of Isc inhibition achieved by CFTR(inh)-172 in M-1 C20 monolayers treated with and without ouabain, suggests that the component of the forskolin-induced increase in Isc that was augmented by ouabain treatment is due to specific activation of the CFTR Cl^- channel. The dependency of Isc current on the activity of CFTR agrees with that previously reported in ADPKD cells and

supports the role of ouabain in the regulation of ion transport systems of the cell plasma membrane (137). The ouabain enhanced, forskolin-stimulated Cl^- current in the M-1 C20 cells overexpressing the PC-1 C-tail was blocked by PP2. This suggests that both proliferation and Cl^- secretion induced by ouabain in M-1 C20 cells involves Src, a key component of the Na,K-ATPase signaling machinery.

At present the link between abnormalities in expression of PC-1 and the final effectors involved in ADPKD cystogenesis are not clear. The exacerbated ouabain-responsive phenotype resulting from overexpression of PC-1 C-tail in M1 cells is also poorly understood. However, we found that M-1 C20 cells have a population of Na,K-ATPase that has an increased sensitivity to ouabain. Our observation that M-1 C20 cells, similar to M-1 C17 cells, only express the α1 isoform of the Na,K-ATPase indicates that the molecular basis for the change in ouabain sensitivity is not based on mis-expression of one of the ouabain sensitive α isoforms of the Na,K-ATPase. Similar results were obtained in ADPKD cells, which express the Na,K-ATPase α1 subunit that is normally found in kidney epithelial cells. Alternatively, the increase in ouabain affinity may depend on the interaction of Na,K-ATPase with other proteins. Through the use of the yeast two-hybrid system, the cytosolic tail of PC-1 has been shown to interact with the Na,K-ATPase (143). We previously found that exogenous co-expression of the transmembrane and C-tail domains of PC-1 and the Na,K-ATPase, in Sf-9 insect cells, augments the sensitivity of the Na,K-ATPase to ouabain (90). Currently, additional experiments are being conducted to confirm this possibility.

In conclusion, we show that, overexpression of a membrane anchored C-tail domain of PC-1 induces a ouabain-responsive phenotype in mouse cortical collecting duct cells. The similarity of the ouabain effects in M-1 C20 cells with those of primary ADPKD cells is important,

since M-1 C20 cells represent a valuable system to study the role and mechanisms of action of ouabain in a cell model for ADPKD in which PC-1 function, specifically, has been rendered abnormal.

FIGURES

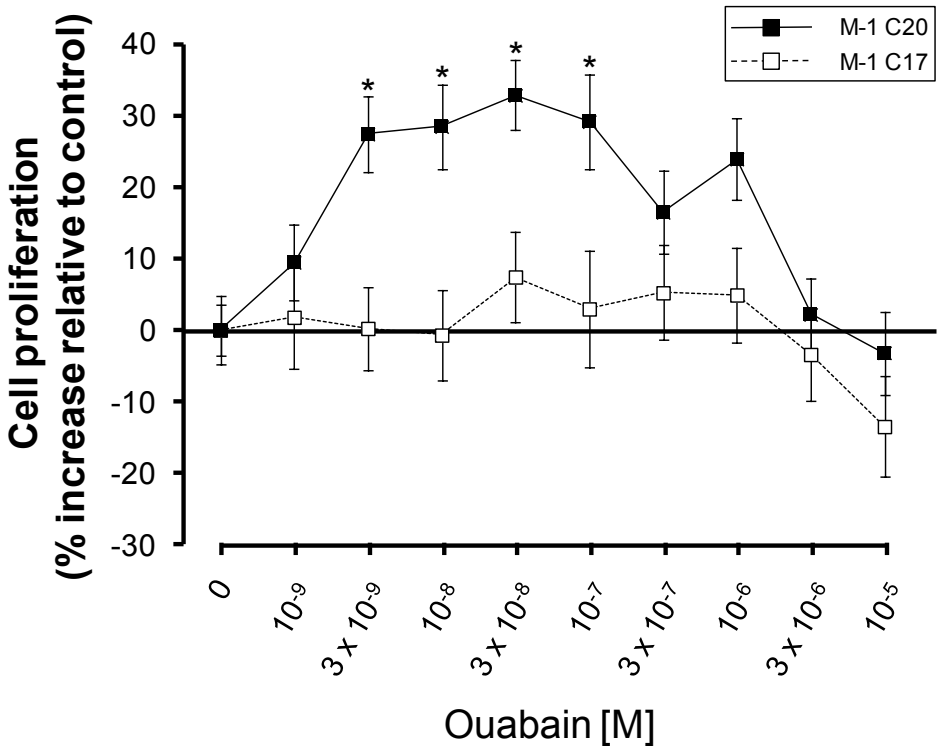


FIGURE 1 Ouabain increases proliferation of M-1 C20 cells. Cells were plated in 96-well culture dishes. After 24 hours in culture, cells were treated with 1 μM dexamethasone for 48 hours. Following an overnight starvation in medium with 0.002% FBS, the indicated concentrations of ouabain were added for 24 hours. Proliferation was measured using the CellTiter 96 assay. Points represent the changes in cell proliferation relative to untreated controls. Symbols are the mean ± SEM (n=5). Asterisks indicate values statistically different from the respective controls not treated with ouabain, with $P < 0.05$.

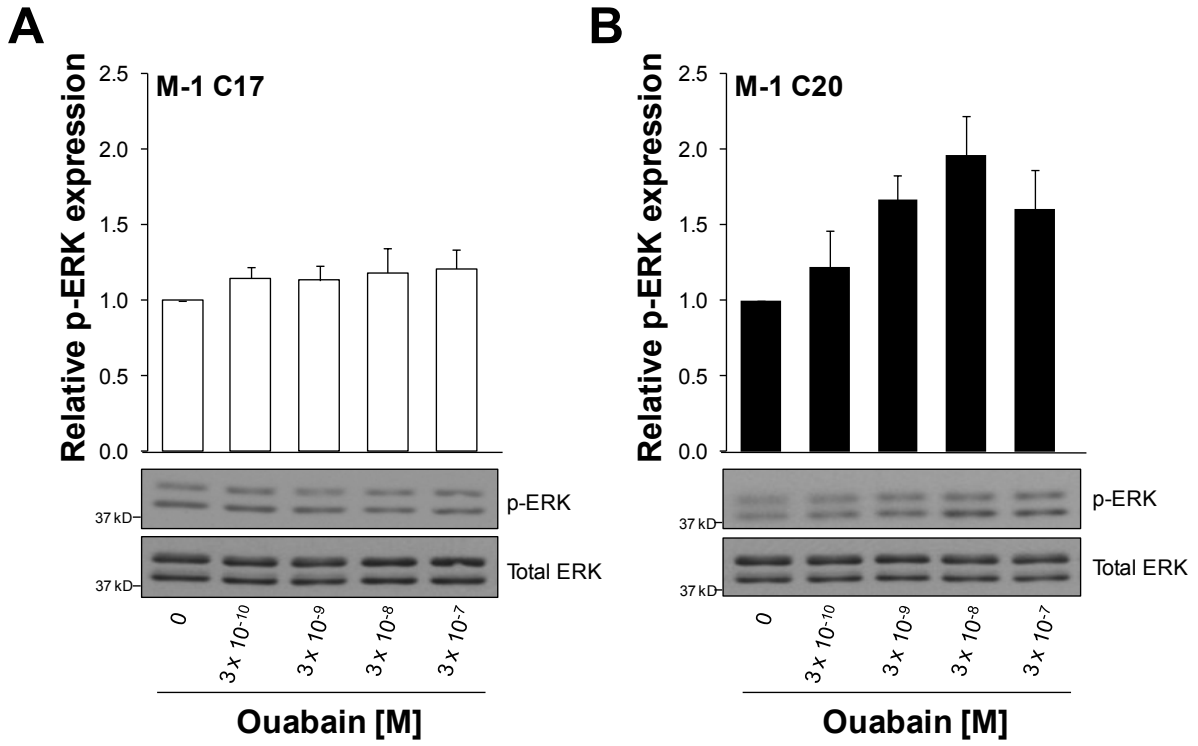
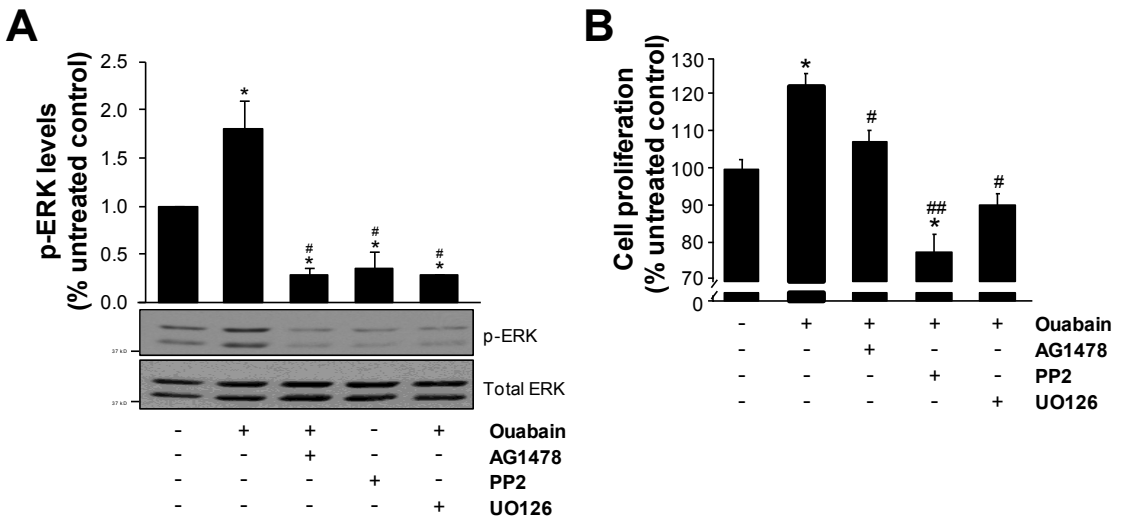


FIGURE 2 Ouabain increases ERK1/2 phosphorylation in M-1 C20 cells. A) M1-C17 and B) M-1 C20 cells (1×10^5) were plated in 6-well culture dishes. After 24 hours, expression of PC-1 C-tail was induced with 1 μM dexamethasone for 48 hours. Following overnight starvation in media supplemented with 0.002% FBS, cells were treated with the indicated concentrations of ouabain for 30 minutes. Samples were lysed and ERK1/2 phosphorylation (p-ERK) was analyzed by immunoblot. Bands in the blots were quantified by densitometric analysis. Bars in the graphs represent the ratio of p-ERK to total ERK levels for each ouabain concentration, normalized to the untreated controls. Representative immunoblots are shown under each graph. Data represent the mean ± SEM (n = 7). The asterisk indicates statistically significant values, with $P < 0.05$ vs. untreated control.



<u>FIGURE 3</u> Ouabain increases ERK1/2 phosphorylation and proliferation of M-1 C20 cells via the EGFR-Src-MEK pathway. A) Dependency of ERK phosphorylation on the EGFR-Src-MEK pathway. Expression of PC-1 in M-1 C20 cells was induced with 1 μM dexamethasone for 48 h and then, after overnight starvation in medium with 0.002% FBS, cells were treated with 3 X 10^{-8} M ouabain in the absence and presence of the indicated inhibitors for 30 minutes. Samples were lysed and ERK1/2 phosphorylation was analyzed by immunoblot. Bars represent the ratio of p-ERK to total ERK for each treatment condition normalized to the untreated control. A representative blot is shown under the graph. Data are the mean ± SEM (n = 4). Symbols indicate statistically significant values, with (*) $P < 0.05$ vs. untreated control and (#) $P < 0.05$ vs. ouabain treatment. B) Dependency of M-1 C20 cell proliferation on the EGFR-Src-MEK pathway. Cells were treated as described in A, except that cell proliferation was measured 24 h later, using the CellTiter 96 assay kit. Bars represent cell proliferation relative to the untreated control. Bars are the mean ± SEM (n=3). Symbols indicate statistically significant values, with (*) representing $P< 0.001$, compared to untreated controls and (#) and (##) indicating $P<0.01$ and $P<0.001$ respectively, compared to ouabain treated samples. AG1478, PP2 and UO126 refer to the EGFR, Src kinase and MEK inhibitors, respectively.

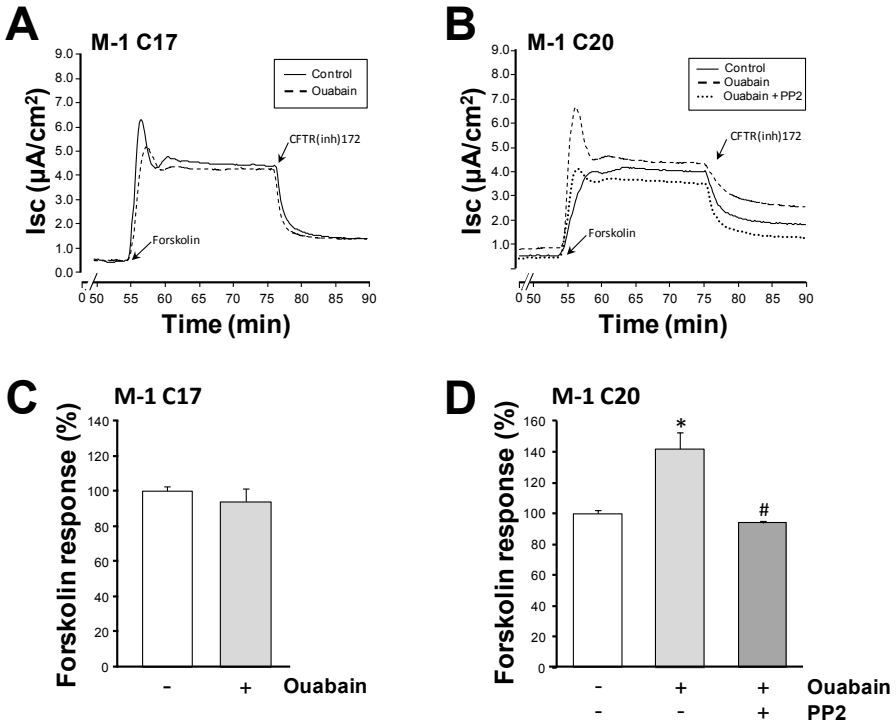


FIGURE 4 Ouabain augments forskolin-induced increases in short circuit current of M-1 C20 cell monolayers. Confluent cell cultures were grown on permeable filter supports and PC-1 C-tail expression was induced with 1 μM dexamethasone for 48 h. After overnight starvation, cells were treated with and without 3 x 10^{-8} M ouabain for 24 hours. To determine if ouabain-induced potentiation of anion secretion in M-1 C20 cells involves Src, the effect of PP2 was tested. Short-circuit currents were then analyzed in Ussing chambers. Benzamil (10 μM) was applied to the apical side of the monolayer to block sodium transport across the epithelium. A - B) Representative traces of M-1 C17 and M-1 C20 monolayers, treated in the absence or presence of ouabain and with or without the presence of PP2 for M-1 C20 cells. C and D) Quantification of the forskolin-induced increase in short circuit current in M-1 C17 and M-1 C20 monolayers respectively. Bars represent maximal forskolin responses as a percent of the untreated controls. Bars are the mean ± SEM (n=3-9 monolayers for each condition). The asterisk indicates statistically significant values compared to untreated controls. In M-1 C20 cells, (#) indicates differences vs. forskolin + ouabain, $P < 0.05$.

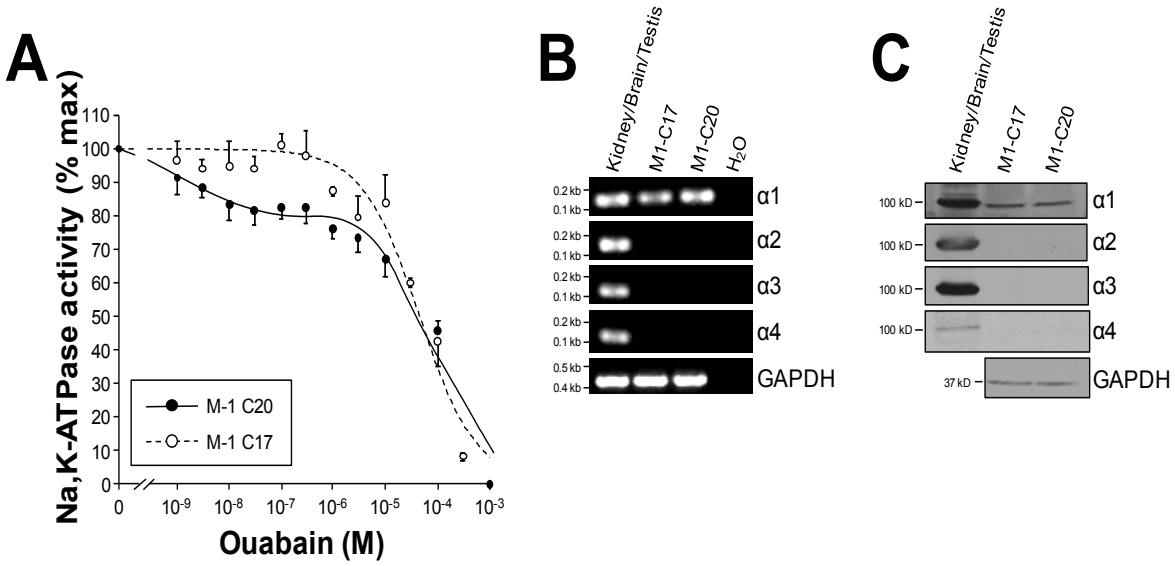


FIGURE 5 Na,K-ATPase from M-1 C20 cells exhibits increased sensitivity to ouabain, despite a normal expression pattern of Na,K-ATPase α isoforms. A) Dose-response curves for the inhibition of Na,K-ATPase by ouabain. Cells were treated with 1 μM dexamethasone for 48 hours and were harvested and homogeneized. Na,K-ATPase activity was measured in the absence and presence of the indicated concentrations of ouabain. Data are expressed as percent of the Na,K-ATPase activity in the absence of ouabain. Curves represent the best fit of the data, which favored one (M-1 C17) versus two (M-1 C20) Na,K-ATPase populations with different affinities for ouabain. Values are the mean ± SEM of five experiments. B-C) Expression profile of Na,K-ATPase α isoforms in M-1 C20 and M-1 C17 cells. Cells were harvested after 48 hr induction with 1 μM dexamethasone. (B) PCR amplification was performed using primers to specific regions of each isoform. Amplified cDNA fragments were run in agarose gels. The sizes of the amplified fragments were as follows: α1 isoform, 173 bp; α2 isoform, 143 bp; α3 isoform, 104 bp; α4 isoform, 120 bp. (C) Analysis of Na,K-ATPase α polypeptides was performed by immunoblot, using antibodies specific to each α isoform. As positive controls, cDNA and protein lysates from mouse kidney (for α1), brain (for α2 and α3) and testis (for α4) were used.

Table 1 **Kinetic parameters for the interaction of Na,K-ATPase from M-1 C20 and M-1 C17 cells with ouabain**

	Inhibition constant, K_i (M)		Relative amounts (%)	
Cell type	**High affinity**	**Low affinity**	**High affinity**	**Low affinity**
M-1 C20	$1.4 \pm 3.2 \times 10^{-9}$	$1.4 \pm 0.3 \times 10^{-4}$	24.6 ± 4.3	75.4 ± 2.1
M-1 C17	——	$6.3 \pm 3.7 \times 10^{-5}$	——	100.0 ± 0.0

CHAPTER 6

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

ADPKD is a systemic disorder characterized most dramatically by the progressive growth of renal cysts (37). Several non-genomic factors influence ADPKD cyst formation and growth (117, 121). The results of the current study have discovered, through the study of ADPKD, a novel role for ouabain. Specifically, physiologic concentrations of ouabain influence ADPKD cystogenesis by concomitantly inducing cell proliferation and potentiating cAMP-dependent mechanisms of ADPKD fluid secretion. These effects of ouabain are exerted through the Na,K-ATPase and involve activation of Na,K-ATPase-signaling apparatus, including the Src-EGFR-MEK pathway. Moreover, ouabain-dependent enhancement of fluid secretion and cyst growth takes place at concentrations similar to those seen in human plasma. This supports the role of ouabain as a physiologic agent that influences renal cyst growth and progression of ADPKD.

Previous work has shown that ouabain alone is sufficient to enhance proliferation of ADPKD cells (89, 90). However, results of the current study showed no effect of ouabain treatment alone in ADPKD fluid secretion or cyst growth. However, ouabain was sufficient to augment forskolin- and cAMP-dependent increases in fluid secretion and growth of ADPKD microcysts as well as growth of cyst-like dilations in metanephric kidneys from the *Pkd1*^{m1Bei} mouse model. These effects required the presence and activity of CFTR. Additionally, the potentiating effect of ouabain on fluid secretion and cyst growth required long-term treatment, suggesting an effect of ouabain on protein expression mechanisms in ADPKD cells. Further investigation into a potential effect of ouabain on mechanisms regulating ADPKD fluid secretion

revealed that ouabain increased membrane expression of CFTR in monolayers of ADPKD cells. Furthermore, ouabain treatment increased the expression of PDZK1, an accessory protein associated with potentiation of PKA-dependent activation of CFTR. Both of these mechanisms may lead to increased CFTR-mediated effects in ADPKD cells. Consistent with this possibility, cells treated with ouabain exhibited enhanced CFTR-dependent increases in short-circuit current and Cl^- efflux. These results indicate an important mechanism mediating ouabain-induced augmentation of fluid secretion and cyst growth in ADPKD.

Fluid secretion in ADPKD is dependent on active Cl^- secretion via the apically located CFTR (28, 104, 123). CFTR-dependent Cl^- transport in ADPKD cells is regulated by cAMP. By directly affecting expression and function of CFTR, ouabain induces a phenotypic alteration in ADPKD cells characterized by enhanced sensitivity to cAMP agonists. This results in an augmented secretory potential of cystic epithelial cells exposed to physiologic concentrations of ouabain. Furthermore, oubain treatment of ADPKD cells resulted in decreased Na,K-ATPase membrane expression and ion transport. Given the important role for the Na,K-ATPase in regulating fluid reabsorption mechanisms in renal epithelia, a decreased expression of this transporter at the plasma membrane may cause a reduction in fluid reabsorption across ADPKD epithelia. Taken together, these effects on CFTR and the Na,K-ATPase demonstrate ouabain-dependent mechansisms influencing crucial effectors regulating progression of renal cyst growth in ADPKD.

Fluid secretion and cyst growth in ADPKD are significantly influenced by intracellular cAMP (121). The potentiating effect of ouabain on cAMP-dependent fluid secretion and cyst growth suggests that intracellular events activated by ouabain and cAMP converge at some downstream target in the cells. Effects of ouabain on fluid secretion and cyst growth in this study

were blocked by inhibitors of Src, EGFR, and MEK. These intracellular signaling molecules are known to be important mediators of ouabain-induced, Na,K-ATPase-mediated signaling involved in ADPKD cell proliferation. The mitogenic effect of cAMP in ADPKD is also mediated by activation of the MEK-ERK pathway. Thus, activation of ERK1/2 is a potential downstream site where ouabain-induced and cAMP-dependent signals may converge. Results from the current study also indicate that the CFTR in ADPKD cells may be another important downstream effector where ouabain-induced and cAMP-dependent signals converge resulting in increased fluid secretion and cyst growth. Future investigations will be required to more fully analyze the interaction of signals induced by ouabain and cAMP agonists in ADPKD cells.

CFTR-dependent Cl^- secretion represents the main cAMP-dependent mechanism that regulates fluid secretion and cyst growth mechanisms in ADPKD (121). Many circulating factors, such as vasopression (AVP), epidermal growth factor (EGF), and prostaglandins, can increase cAMP levels in ADPKD cells and cause activation of CFTR-dependent Cl^- secretion (83, 104, 117, 121). AVP is of particular importance to renal cyst growth since renal epithelia are continuously exposed to this hormone as the kidneys work to concentrate urine and maintain proper serum osmolality. This continuous supply of AVP likely creates a state of constant activation of CFTR in ADPKD epithelia resulting in ongoing fluid secretion in renal cystic epithelia. In a similar manner, the abnormally high ouabain affinity of cystic epithelial cells, may be causing circulating concentrations of ouabain to preferentially, and continuously, stimulate Na,K-ATPase-mediated signaling events. This may provide an ongoing potentiation of mechanisms of cyst growth and progression of renal disease in ADPKD.

Results of the current study raise the interesting possibility that a balance between fluid secretion and fluid reabsorption exists across ADPKD epithelia. Intraluminal fluid accumulation

is undoubtedly required for progressive renal cyst growth (44), and many studies have defined in detail the secretory mechanisms regulating this fluid accumulation (104, 105). However, mechanisms of fluid reabsorption across ADPKD epithelia have not been studied as extensively. Normal renal tubules reabsorb most of the fluid and electrolytes present in the glomerular filtrate, and for many years fluid secretion by renal tubules was not recognized as an important physiologic process in the kidney (45). However, pioneering studies by Grantham and colleagues clearly showed the potential for normal renal tubules to secrete fluid and electrolytes (38, 41, 42). This important discovery lead to a more complete understanding of renal tubular physiology in which a dynamic balance of reabsorptive and secretory processes modifies the glomerular filtrate along the length of the nephron. In the same way, important mechanistic information regarding fluid transport in ADPKD will likely be uncovered by further examining the role of fluid reabsorption mechanisms in cystic epithelia.

This also leads to questions regarding the basic pathophysiologic switch that induces ADPKD epithelia to have such a dramatic secretory phenotype. ADPKD cysts arise from cells lining normal renal tubules (54). These tubules have the capability to reabsorb enormous quantities of fluid on an ongoing basis. However, in ADPKD, cells with disrupted polycystin function, switch to a secretory phenotype. Future studies could uncover important mechanistic information concerning ADPKD pathogenesis by investigating the nature of this switch from a reabsorptive to a secretory phenotype observed in ADPKD epithelia.

The potentiating effect of ouabain on ADPKD fluid secretion and cyst growth was specific for ADPKD cells and microcysts. Nanomolar concentrations of ouabain failed to augment cAMP-dependent effects on fluid secretion or cyst growth analyzed using normal human renal epithelial cells. These results, in conjunction with previous results showing

ADPKD-specific effects of ouabain, emphasize the need to understand the nature of the increased ouabain affinity of ADPKD cells.

Results from studies using M-1 cells indicate a specific, novel link between PC-1 and the ouabain affinity of M-1 mouse cortical collecting duct cells. M-1 cells overexpressing a dominant-negative construct of the PC-1 C-tail (M-1 C20 cells) exhibited an abnormally high affinity for ouabain despite normal expression of the α1β1 Na,K-ATPase isozyme. In M-1 C20 cells, ouabain caused cell proliferation as well potentiation of forskolin-stimulated anion secretion. Ouabain elicited these effects via activation of the Src-EGFR-MEK-ERK pathway, similar to mechanisms demonstrated in human ADPKD cells. These effects were not seen in a control cell line which did not express the abnormal PC-1 C-tail construct. This suggessts that loss of PC-1 function is sufficient to induce a ouabain sensitve phenotype in murine renal epithelial cells. These results demonstrate a novel, functional link between PC-1 and the ouabain affinity of the renal Na,K-ATPase. Furthermore, the results of these studies indicate that M-1 cells overexpressing the PC-1 C-tail offer an effective *in vitro* model to further investigate ouabain-dependent mechanisms in cystic epithelial cells.

The molecular explanation for a functional link between PC-1 and Na,K-ATPase signaling is unclear. The increased ouabain affinity of the Na,K-ATPase in M-1 C20 may be due to altered intracellular calcium levels in these cells. The intracellular ionic composition can have significant impacts on functional properties of the Na,K-ATPase, and studies have shown that intracellular Ca^{2+} concentrations, in particular, can influence the ouabain affinity of the Na,K-ATPase (141) . This is an intriguing possibility given the known pathogenic importance of abnormally low Ca^{2+} concentrations in ADPKD cells (134, 136). Increased ouabain affinity of cystic epithelial cells may be another consequence of disrupted Ca^{2+} levels in ADPKD cells.

Another related possibility is that alteration in signals derived from the C-tail of PC-1 are causing an altered ouabain affinity in M-1 C20 and ADPKD cells. The M-1 C20 cells overexpress a dominant-negative construct of the PC-1 C-tail. This portion of the PC-1 protein has been implicated in numerous different signaling pathways, some of which regulate intracellular Ca^{2+} concentrations (91). Investigation of a potential link between the effect of PC-1 C-tail derived signaling events and ouabain affinity of epithelial cells may uncover important basic information regarding PC-1 function and factors regulating Na,K-ATPase function.

Growth of renal cysts in ADPKD is an exponential process occuring over many years (43). Any alteration that may increase the slope of the growth curve of renal cystogenesis, no matter how slight, is likely to have an extremely important effect on cystic disease that is compounded over the lifetime of a patient affected by ADPKD. Since ouabain can influence both proliferative and secretory responses in cystic epithelial cells, this hormone emerges as a physiologic factor that may potentially have a large influence on the growth of ADPKD cysts. The possible pathophysiologic role of endogenous ouabain in ADPKD is supported by data from pre-clinical and clinical studies. High-salt diets can increase circulating levels of endogenous ouabain (9). A high-salt diet fed to Han:SPRD rats, a model of polycystic kidney disease, led to increased renal disease in the rats (64). Additionally, analysis of data from The Consortium for Radiologic Imaging Studies of Polycystic Kidney Disease (CRISP) has found baseline urinary sodium exretion (a marker of sodium intake) to be an independent predictor of increased renal cyst growth in ADPKD (115, 118). This supports the possibility that ouabain may be a circulating factor contributing to increased progression of renal disease in ADPKD.

The importance of therapeutically targeting physiologic factors that affect progression of ADPKD cyst growth is underscored by the results of the TEMPO3:4 trial. This study evaluated

the effectiveness of tolvaptan, an antagonist of the renal vasopressin receptor (V_2R), in ADPKD patients. Tolvaptan treatment resulted in slower rates of cyst growth and renal functional decline for patients receiving the intervention (114). The results of this trial emphasize the importance and confirm the rationale for targeting physiologic agents influencing mechanisms of cyst growth in ADPKD.

By this rationale, further investigation into ouabain-induced mechanisms in ADPKD is clearly warranted. Moreover, future work should address strategies to inhibit ouabain-dependent effects. The ability to therapeutically target ouabain has been demonstrated by studies using Rostafuroxin (17β-[3-furyl]-5β-androstan-3β,14 β,17α-triol; PST2238) as a therapy for essential hypertention (31). Rostafuroxin selectively displaces ouabain from its binding site on the Na,K-ATPase, and, thus, acts as an inhibitor of endogenous ouabain. Rostafuroxin has been shown to lower blood pressure in rats and humans in some studies. It would be interesting to investigate the effectiveness of Rostafuroxin in antagonizing ouabain-dependent potentiation of ADPKD cyst growth.

In conclusion, the results of this study demonstrate important, novel effects of ouabain on mechansisms of fluid secretion and cyst growth in ADPKD. Ouabain is an endogenous factor that can affect the two processes required for enhanced ADPKD cyst growth, cell proliferation and fluid secretion. Ouabain mediates an effect on mechanisms of fluid secretion by influencing expression and activity CFTR to induce a phenotypic change in ADPKD cells characterized by enhanced responsiveness to cAMP agonists. Additionally, these results raise that possibility that fluid reabsorption mechanisms may be contributing to the aberrant fluid secretion observed in ADPKD cystic epithelia. Ouabain may be modifying mechanisms of reabsorption in a manner that favors enhanced fluid secretion by further augmenting the disbalance between fluid

secretion and reabsorption in ADPKD epithelia. Altogether, the results of the current study provide a significant advancement in our understanding of ouabain's mechanisms of action in ADPKD and support the role of ouabain as a physiologic agent that may be influencing the progression of renal disease in ADPKD.

REFERENCE LIST

1. **Aizman O, Uhlen P, Lal M, Brismar H, and Aperia A**. Ouabain, a steroid hormone that signals with slow calcium oscillations. *Proceedings of the National Academy of Sciences of the United States of America* 98: 13420-13424, 2001.
2. **Akimova OA, Poirier M, Kotelevtsev SV, Hamet P, and Orlov SN**. The death of ouabain-treated renal epithelial cells: evidence against anoikis occurrence. *Apoptosis* 13: 670-680, 2008.
3. **Akopyanz NS, Broude NE, Bekman EP, Marzen EO, and Sverdlov ED**. Tissue-specific expression of Na,K-ATPase beta-subunit. Does beta 2 expression correlate with tumorigenesis? *FEBS letters* 289: 8-10, 1991.
4. **Albaqumi M, Srivastava S, Li Z, Zhdnova O, Wulff H, Itani O, Wallace DP, and Skolnik EY**. KCa3.1 potassium channels are critical for cAMP-dependent chloride secretion and cyst growth in autosomal-dominant polycystic kidney disease. *Kidney international* 74: 740-749, 2008.
5. **Allen JC, Abramowitz J, and Koksoy A**. Low concentrations of ouabain activate vascular smooth muscle cell proliferation. *Annals of the New York Academy of Sciences* 986: 504-508, 2003.
6. **Anderson DE, Fedorova OV, Morrell CH, Longo DL, Kashkin VA, Metzler JD, Bagrov AY, and Lakatta EG**. Endogenous sodium pump inhibitors and age-associated increases in salt sensitivity of blood pressure in normotensives. *American journal of physiology Regulatory, integrative and comparative physiology* 294: R1248-1254, 2008.
7. **Aperia A**. New roles for an old enzyme: Na,K-ATPase emerges as an interesting drug target. *Journal of internal medicine* 261: 44-52, 2007.
8. **Arystarkhova E, and Sweadner KJ**. Tissue-specific expression of the Na,K-ATPase beta3 subunit. The presence of beta3 in lung and liver addresses the problem of the missing subunit. *The Journal of biological chemistry* 272: 22405-22408, 1997.
9. **Bagrov AY, Shapiro JI, and Fedorova OV**. Endogenous cardiotonic steroids: physiology, pharmacology, and novel therapeutic targets. *Pharmacological reviews* 61: 9-38, 2009.
10. **Basavanna U, Weber KM, Hu Q, Ziegelstein RC, Germino GG, and Sutters M**. The isolated polycystin-1 COOH-terminal can activate or block polycystin-1 signaling. *Biochemical and biophysical research communications* 359: 367-372, 2007.
11. **Belibi FA, Reif G, Wallace DP, Yamaguchi T, Olsen L, Li H, Helmkamp GM, Jr., and Grantham JJ**. Cyclic AMP promotes growth and secretion in human polycystic kidney epithelial cells. *Kidney international* 66: 964-973, 2004.
12. **Belibi FA, Wallace DP, Yamaguchi T, Christensen M, Reif G, and Grantham JJ**. The Effect of Caffeine on Renal Epithelial Cells from Patients with Autosomal Dominant Polycystic Kidney Disease. *J Am Soc Nephrol* 13: 2723-2729, 2002.
13. **Blanco G**. Na,K-ATPase subunit heterogeneity as a mechanism for tissue-specific ion regulation. *Seminars in nephrology* 25: 292-303, 2005.
14. **Blanco G**. The NA/K-ATPase and its isozymes: what we have learned using the baculovirus expression system. *Frontiers in bioscience : a journal and virtual library* 10: 2397-2411, 2005.
15. **Blanco G, and Mercer RW**. Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *Am J Physiol* 275: F633-650, 1998.
16. **Blanco G, and Mercer RW**. Isozymes of the Na,K-ATPase: heterogeneity in structure. *Am J Physiol* 275 F633-650, 1998.

17. **Blanco G, Sanchez G, Melton RJ, Tourtellotte WG, and Mercer RW**. The alpha4 isoform of the Na,K-ATPase is expressed in the germ cells of the testes. *J Histochem Cytochem* 48: 1023-1032, 2000.
18. **Blaustein MP, and Hamlyn JM**. Signaling mechanisms that link salt retention to hypertension: endogenous ouabain, the Na(+) pump, the Na(+)/Ca(2+) exchanger and TRPC proteins. *Biochimica et biophysica acta* 1802: 1219-1229.
19. **Blaustein MP, Juhaszova M, and Golovina VA**. The cellular mechanism of action of cardiotonic steroids: a new hypothesis. *Clin Exp Hypertens* 20: 691-703, 1998.
20. **Brill SR, Ross KE, Davidow CJ, Ye M, Grantham JJ, and Caplan MJ**. Immunolocalization of ion transport proteins in human autosomal dominant polycystic kidney epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America* 93: 10206-10211, 1996.
21. **Cai H, Wu L, Qu W, Malhotra D, Xie Z, Shapiro JI, and Liu J**. Regulation of apical NHE3 trafficking by ouabain-induced activation of the basolateral Na,K-ATPase receptor complex. *American journal of physiology Cell physiology* 294: C555-563, 2008.
22. **Calvet JP, and Grantham JJ**. The genetics and physiology of polycystic kidney disease. *Seminars in nephrology* 21: 107-123, 2001.
23. **Cereijido M, Contreras RG, Shoshani L, and Larre I**. The Na+-K+-ATPase as self-adhesion molecule and hormone receptor. *American journal of physiology Cell physiology* 302: C473-481, 2012.
24. **Chueh SC, Guh JH, Chen J, Lai MK, and Teng CM**. Dual effects of ouabain on the regulation of proliferation and apoptosis in human prostatic smooth muscle cells. *The Journal of urology* 166: 347-353, 2001.
25. **Clerico A, Cambi A, Del Chicca MG, Cecchini L, and Giaconi S**. Urinary excretion of digoxin-like immunoreactivity after physical exercise. *Clinical chemistry* 34: 215, 1988.
26. **Contreras RG, Shoshani L, Flores-Maldonado C, Lazaro A, and Cereijido M**. Relationship between Na(+),K(+)-ATPase and cell attachment. *J Cell Sci* 112 (Pt 23): 4223-4232, 1999.
27. **Dantzler WH**. Regulation of renal proximal and distal tubule transport: sodium, chloride and organic anions. *Comparative biochemistry and physiology Part A, Molecular & integrative physiology* 136: 453-478, 2003.
28. **Davidow CJ, Maser RL, Rome LA, Calvet JP, and Grantham JJ**. The cystic fibrosis transmembrane conductance regulator mediates transepithelial fluid secretion by human autosomal dominant polycystic kidney disease epithelium in vitro. *Kidney international* 50: 208-218, 1996.
29. **Feraille E, Carranza ML, Buffin-Meyer B, Rousselot M, Doucet A, and Favre H**. Protein kinase C-dependent stimulation of Na(+)-K(+)-ATP epsilon in rat proximal convoluted tubules. *Am J Physiol* 268: C1277-1283, 1995.
30. **Feraille E, and Doucet A**. Sodium-potassium-adenosinetriphosphatase-dependent sodium transport in the kidney: hormonal control. *Physiological reviews* 81: 345-418, 2001.
31. **Ferrari P**. Rostafuroxin: an ouabain-inhibitor counteracting specific forms of hypertension. *Biochimica et biophysica acta* 1802: 1254-1258, 2010.
32. **Fisone G, Cheng SX, Nairn AC, Czernik AJ, Hemmings HC, Jr., Hoog JO, Bertorello AM, Kaiser R, Bergman T, Jornvall H, and et al.** Identification of the phosphorylation site for cAMP-dependent protein kinase on Na+,K(+)-ATPase and effects of site-directed mutagenesis. *The Journal of biological chemistry* 269: 9368-9373, 1994.
33. **Geering K**. Function of FXYD proteins, regulators of Na, K-ATPase. *Journal of bioenergetics and biomembranes* 37: 387-392, 2005.

34. **Geering K**. The functional role of beta subunits in oligomeric P-type ATPases. *Journal of bioenergetics and biomembranes* 33: 425-438, 2001.
35. **Gloor SM**. Relevance of Na,K-ATPase to local extracellular potassium homeostasis and modulation of synaptic transmission. *FEBS letters* 412: 1-4, 1997.
36. **Goto A, Yamada K, Nagoshi H, Terano Y, and Omata M**. Stress-induced elevation of ouabainlike compound in rat plasma and adrenal. *Hypertension* 26: 1173-1176, 1995.
37. **Grantham JJ**. Clinical practice. Autosomal dominant polycystic kidney disease. *The New England journal of medicine* 359: 1477-1485, 2008.
38. **Grantham JJ**. Fluid secretion in the nephron: Relation to renal failure. *Physiological reviews* 56: 248-258, 1976.
39. **Grantham JJ**. Mechanisms of progression in autosomal dominant polycystic kidney disease. *Kidney Int Suppl* 63: S93-97, 1997.
40. **Grantham JJ**. Pathogenesis of autosomal dominant polycystic kidney disease: recent developments. *Contrib Nephrol* 122: 1-9, 1997.
41. **Grantham JJ, Irwin RL, Qualizza PB, Tucker DR, and Whittier FC**. Fluid secretion in isolated proximal straight renal tubules. Effect of human uremic serum. *The Journal of clinical investigation* 52: 2441-2450, 1973.
42. **Grantham JJ, Qualizza PB, and Irwin RL**. Net fluid secretion in proximal straight renal tubules in vitro: role of PAH. *Am J Physiol* 226: 191-197, 1974.
43. **Grantham JJ, Torres VE, Chapman AB, Guay-Woodford LM, Bae KT, King BF, Jr., Wetzel LH, Baumgarten DA, Kenney PJ, Harris PC, Klahr S, Bennett WM, Hirschman GN, Meyers CM, Zhang X, Zhu F, and Miller JP**. Volume progression in polycystic kidney disease. *The New England journal of medicine* 354: 2122-2130, 2006.
44. **Grantham JJ, Uchic M, Cragoe EJ, Jr., Kornhaus J, Grantham JA, Donoso V, Mangoo-Karim R, Evan A, and McAteer J**. Chemical modification of cell proliferation and fluid secretion in renal cysts. *Kidney international* 35: 1379-1389, 1989.
45. **Grantham JJ, and Wallace DP**. Return of the secretory kidney. *American journal of physiology Renal physiology* 282: F1-9, 2002.
46. **Guggino WB**. The cystic fibrosis transmembrane regulator forms macromolecular complexes with PDZ domain scaffold proteins. *Proceedings of the American Thoracic Society* 1: 28-32, 2004.
47. **Guggino WB, and Stanton BA**. New insights into cystic fibrosis: molecular switches that regulate CFTR. *Nature reviews Molecular cell biology* 7: 426-436, 2006.
48. **Hamlyn JM, and Blaustein MP**. Salt sensitivity, endogenous ouabain and hypertension. *Current opinion in nephrology and hypertension* 22: 51-58, 2013.
49. **Hamlyn JM, Blaustein MP, Bova S, DuCharme DW, Harris DW, Mandel F, Mathews WR, and Ludens JH**. Identification and characterization of a ouabain-like compound from human plasma. *Proceedings of the National Academy of Sciences of the United States of America* 88: 6259-6263, 1991.
50. **Hamlyn JM, Harris DW, Clark MA, Rogowski AC, White RJ, and Ludens JH**. Isolation and characterization of a sodium pump inhibitor from human plasma. *Hypertension* 13: 681-689, 1989.
51. **Hamlyn JM, Harris DW, and Ludens JH**. Digitalis-like activity in human plasma. Purification, affinity, and mechanism. *The Journal of biological chemistry* 264: 7395-7404, 1989.
52. **Hamlyn JM, Lu ZR, Manunta P, Ludens JH, Kimura K, Shah JR, Laredo J, Hamilton JP, Hamilton MJ, and Hamilton BP**. Observations on the nature, biosynthesis, secretion and significance of endogenous ouabain. *Clin Exp Hypertens* 20: 523-533, 1998.

53. **Hanaoka K, Qian F, Boletta A, Bhunia AK, Piontek K, Tsiokas L, Sukhatme VP, Guggino WB, and Germino GG**. Co-assembly of polycystin-1 and -2 produces unique cation-permeable currents. *Nature* 408: 990-994, 2000.
54. **Harris PC, and Torres VE**. Polycystic kidney disease. *Annual review of medicine* 60: 321-337, 2009.
55. **Hateboer N, v Dijk MA, Bogdanova N, Coto E, Saggar-Malik AK, San Millan JL, Torra R, Breuning M, and Ravine D**. Comparison of phenotypes of polycystic kidney disease types 1 and 2. European PKD1-PKD2 Study Group. *Lancet* 353: 103-107, 1999.
56. **Hernandez-Gonzalez EO, Sosnik J, Edwards J, Acevedo JJ, Mendoza-Lujambio I, Lopez-Gonzalez I, Demarco I, Wertheimer E, Darszon A, and Visconti PE**. Sodium and epithelial sodium channels participate in the regulation of the capacitation-associated hyperpolarization in mouse sperm. *The Journal of biological chemistry* 281: 5623-5633, 2006.
57. **Herron BJ, Lu W, Rao C, Liu S, Peters H, Bronson RT, Justice MJ, McDonald JD, and Beier DR**. Efficient generation and mapping of recessive developmental mutations using ENU mutagenesis. *Nature genetics* 30: 185-189, 2002.
58. **Hoffmann EK, and Simonsen LO**. Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiological reviews* 69: 315-382, 1989.
59. **Hooper KM, Unwin RJ, and Sutters M**. The isolated C-terminus of polycystin-1 promotes increased ATP-stimulated chloride secretion in a collecting duct cell line. *Clin Sci (Lond)* 104: 217-221, 2003.
60. **Hughes J, Ward CJ, Peral B, Aspinwall R, Clark K, San Millan JL, Gamble V, and Harris PC**. The polycystic kidney disease 1 (PKD1) gene encodes a novel protein with multiple cell recognition domains. *Nature genetics* 10: 151-160, 1995.
61. **Jansson K, Nguyen AN, Magenheimer BS, Reif GA, Aramadhaka LR, Bello-Reuss E, Wallace DP, Calvet JP, and Blanco G**. Endogenous concentrations of ouabain act as a cofactor to stimulate fluid secretion and cyst growth of in vitro ADPKD models via cAMP and EGFR-Src-MEK pathways. *American journal of physiology Renal physiology* 303: F982-990, 2012.
62. **Jorgensen PL, Hakansson KO, and Karlish SJ**. Structure and mechanism of Na,K-ATPase: functional sites and their interactions. *Annual review of physiology* 65: 817-849, 2003.
63. **Kaplan JH**. Biochemistry of Na,K-ATPase. *Annual review of biochemistry* 71: 511-535, 2002.
64. **Keith DS, Torres VE, Johnson CM, and Holley KE**. Effect of sodium chloride, enalapril, and losartan on the development of polycystic kidney disease in Han:SPRD rats. *American journal of kidney diseases : the official journal of the National Kidney Foundation* 24: 491-498, 1994.
65. **Khundmiri SJ, Metzler MA, Ameen M, Amin V, Rane MJ, and Delamere NA**. Ouabain induces cell proliferation through calcium-dependent phosphorylation of Akt (protein kinase B) in opossum kidney proximal tubule cells. *American journal of physiology Cell physiology* 291: C1247-1257, 2006.
66. **Lai F, Madan N, Ye Q, Duan Q, Li Z, Wang S, Si S, and Xie Z**. Identification of a Mutant alpha1 Na/K-ATPase That Pumps but Is Defective in Signal Transduction. *The Journal of biological chemistry* 288: 13295-13304, 2013.
67. **Laredo J, Hamilton BP, and Hamlyn JM**. Secretion of endogenous ouabain from bovine adrenocortical cells: role of the zona glomerulosa and zona fasciculata. *Biochemical and biophysical research communications* 212: 487-493, 1995.

68. **Lavoie L, Levenson R, Martin-Vasallo P, and Klip A**. The molar ratios of alpha and beta subunits of the Na+-K+-ATPase differ in distinct subcellular membranes from rat skeletal muscle. *Biochemistry* 36: 7726-7732, 1997.
69. **Li J, Zelenin S, Aperia A, and Aizman O**. Low doses of ouabain protect from serum deprivation-triggered apoptosis and stimulate kidney cell proliferation via activation of NF-kappaB. *J Am Soc Nephrol* 17: 1848-1857, 2006.
70. **Liang M, Tian J, Liu L, Pierre S, Liu J, Shapiro J, and Xie ZJ**. Identification of a pool of non-pumping Na/K-ATPase. *The Journal of biological chemistry* 282: 10585-10593, 2007.
71. **Lingrel J, Moseley A, Dostanic I, Cougnon M, He S, James P, Woo A, O'Connor K, and Neumann J**. Functional roles of the alpha isoforms of the Na,K-ATPase. *Annals of the New York Academy of Sciences* 986: 354-359, 2003.
72. **Lingrel JB**. The physiological significance of the cardiotonic steroid/ouabain-binding site of the Na,K-ATPase. *Annual review of physiology* 72: 395-412, 2010.
73. **Liu J, Kesiry R, Periyasamy SM, Malhotra D, Xie Z, and Shapiro JI**. Ouabain induces endocytosis of plasmalemmal Na/K-ATPase in LLC-PK1 cells by a clathrin-dependent mechanism. *Kidney international* 66: 227-241, 2004.
74. **Liu J, Liang M, Liu L, Malhotra D, Xie Z, and Shapiro JI**. Ouabain-induced endocytosis of the plasmalemmal Na/K-ATPase in LLC-PK1 cells requires caveolin-1. *Kidney international* 67: 1844-1854, 2005.
75. **Liu J, Tian J, Haas M, Shapiro JI, Askari A, and Xie Z**. Ouabain interaction with cardiac Na+/K+-ATPase initiates signal cascades independent of changes in intracellular Na+ and Ca2+ concentrations. *The Journal of biological chemistry* 275: 27838-27844, 2000.
76. **Liu J, and Xie ZJ**. The sodium pump and cardiotonic steroids-induced signal transduction protein kinases and calcium-signaling microdomain in regulation of transporter trafficking. *Biochimica et biophysica acta* 1802: 1237-1245, 2010.
77. **Liu L, Mohammadi K, Aynafshar B, Wang H, Li D, Liu J, Ivanov AV, Xie Z, and Askari A**. Role of caveolae in signal-transducing function of cardiac Na+/K+-ATPase. *American journal of physiology Cell physiology* 284: C1550-1560, 2003.
78. **Livak KJ, and Schmittgen TD**. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method. *Methods* 25: 402-408, 2001.
79. **Ludens JH, Clark MA, DuCharme DW, Harris DW, Lutzke BS, Mandel F, Mathews WR, Sutter DM, and Hamlyn JM**. Purification of an endogenous digitalislike factor from human plasma for structural analysis. *Hypertension* 17: 923-929, 1991.
80. **Lytton J, Lin JC, and Guidotti G**. Identification of two molecular forms of (Na+,K+)-ATPase in rat adipocytes. Relation to insulin stimulation of the enzyme. *The Journal of biological chemistry* 260: 1177-1184, 1985.
81. **Magenheimer BS, St John PL, Isom KS, Abrahamson DR, De Lisle RC, Wallace DP, Maser RL, Grantham JJ, and Calvet JP**. Early embryonic renal tubules of wild-type and polycystic kidney disease kidneys respond to cAMP stimulation with cystic fibrosis transmembrane conductance regulator/Na(+),K(+),2Cl(-) Co-transporter-dependent cystic dilation. *J Am Soc Nephrol* 17: 3424-3437, 2006.
82. **Mangoo-Karim R, Uchic M, Grant M, Shumate WA, Calvet JP, Park CH, and Grantham JJ**. Renal epithelial fluid secretion and cyst growth: the role of cyclic AMP. *FASEB J* 3: 2629-2632, 1989.
83. **Mangoo-Karim R, Uchic ME, Grant M, Shumate WA, Calvet JP, Park CH, and Grantham JJ**. Renal epithelial fluid secretion and cyst growth: the role of cyclic AMP. *FASEB J* 3: 2629-2632, 1989.

84. **Mangoo-Karim R, Ye M, Wallace DP, Grantham JJ, and Sullivan LP**. Anion secretion drives fluid secretion by monolayers of cultured human polycystic cells. *Am J Physiol* 269: F381-388, 1995.
85. **McGrail KM, Phillips JM, and Sweadner KJ**. Immunofluorescent localization of three Na,K-ATPase isozymes in the rat central nervous system: both neurons and glia can express more than one Na,K-ATPase. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 11: 381-391, 1991.
86. **McGrail KM, and Sweadner KJ**. Complex Expression Patterns for Na+,K+-ATPase Isoforms in Retina and Optic Nerve. *The European journal of neuroscience* 2: 170-176, 1990.
87. **Murata Y, Matsuda T, Tamada K, Hosoi R, Asano S, Takuma K, Tanaka K, and Baba A**. Ouabain-induced cell proliferation in cultured rat astrocytes. *Japanese journal of pharmacology* 72: 347-353, 1996.
88. **Nesher M, Dvela M, Igbokwe VU, Rosen H, and Lichtstein D**. Physiological roles of endogenous ouabain in normal rats. *Am J Physiol Heart Circ Physiol* 297: H2026-2034, 2009.
89. **Nguyen AN, Jansson K, Sanchez G, Sharma M, Reif G, Wallace DP, and Blanco G**. Ouabain activates the Na,K-ATPase signalosome to induce autosomal dominant polycystic kidney disease cell proliferation. *American journal of physiology Renal physiology* 301: F897-906, 2011.
90. **Nguyen AN, Wallace DP, and Blanco G**. Ouabain binds with high affinity to the Na,K-ATPase in human polycystic kidney cells and induces extracellular signal-regulated kinase activation and cell proliferation. *J Am Soc Nephrol* 18: 46-57, 2007.
91. **Ong AC, and Harris PC**. Molecular pathogenesis of ADPKD: the polycystin complex gets complex. *Kidney international* 67: 1234-1247, 2005.
92. **Oweis S, Wu L, Kiela PR, Zhao H, Malhotra D, Ghishan FK, Xie Z, Shapiro JI, and Liu J**. Cardiac glycoside downregulates NHE3 activity and expression in LLC-PK1 cells. *American journal of physiology Renal physiology* 290: F997-1008, 2006.
93. **Pierre SV, and Xie Z**. The Na,K-ATPase receptor complex: its organization and membership. *Cell biochemistry and biophysics* 46: 303-316, 2006.
94. **Radzyukevich TL, Neumann JC, Rindler TN, Oshiro N, Goldhamer DJ, Lingrel JB, and Heiny JA**. Tissue-specific role of the Na,K-ATPase alpha2 isozyme in skeletal muscle. *The Journal of biological chemistry* 288: 1226-1237, 2013.
95. **Reif G, Yamaguchi T, Nivens E, Fujiki H, Pinto CS, and Wallace DP**. Tolvaptan inhibits ERK-dependent cell proliferation, Cl- secretion, and in vitro cyst growth of human ADPKD cells stimulated by vasopressin. *American journal of physiology Renal physiology* 301: F1005-1013, 2011.
96. **Riganti C, Campia I, Kopecka J, Gazzano E, Doublier S, Aldieri E, Bosia A, and Ghigo D**. Pleiotropic effects of cardioactive glycosides. *Current medicinal chemistry* 18: 872-885, 2011.
97. **Rivard CJ, Almeida NE, Berl T, and Capasso JM**. The gamma subunit of Na/K-ATPase: an exceptional, small transmembrane protein. *Frontiers in bioscience : a journal and virtual library* 10: 2604-2610, 2005.
98. **Rossetti S, Consugar MB, Chapman AB, Torres VE, Guay-Woodford LM, Grantham JJ, Bennett WM, Meyers CM, Walker DL, Bae K, Zhang QJ, Thompson PA, Miller JP, and Harris PC**. Comprehensive molecular diagnostics in autosomal dominant polycystic kidney disease. *J Am Soc Nephrol* 18: 2143-2160, 2007.
99. **Schoner W**. Endogenous cardiac glycosides, a new class of steroid hormones. *European journal of biochemistry / FEBS* 269: 2440-2448, 2002.

100. **Schoner W, and Scheiner-Bobis G**. Endogenous cardiac glycosides: hormones using the sodium pump as signal transducer. *Seminars in nephrology* 25: 343-351, 2005.
101. **Shyjan AW, Gottardi C, and Levenson R**. The Na,K-ATPase beta 2 subunit is expressed in rat brain and copurifies with Na,K-ATPase activity. *The Journal of biological chemistry* 265: 5166-5169, 1990.
102. **Singh AK, Riederer B, Krabbenhoft A, Rausch B, Bonhagen J, Lehmann U, de Jonge HR, Donowitz M, Yun C, Weinman EJ, Kocher O, Hogema BM, and Seidler U**. Differential roles of NHERF1, NHERF2, and PDZK1 in regulating CFTR-mediated intestinal anion secretion in mice. *The Journal of clinical investigation* 119: 540-550, 2009.
103. **Sullivan LP, Wallace DP, Gover T, Welling PA, Yamaguchi T, Maser R, Eppler JW, and Grantham JJ**. Sulfonylurea-Sensitive K^+ Transport is Involved in Cl^- Secretion and Cyst Growth by Cultured ADPKD Cells. *J Am Soc Nephrol* 13: 2619-2627, 2002.
104. **Sullivan LP, Wallace DP, and Grantham JJ**. Chloride and fluid secretion in polycystic kidney disease. *J Am Soc Nephrol* 9: 903-916, 1998.
105. **Sullivan LP, Wallace DP, and Grantham JJ**. Epithelial transport in polycystic kidney disease. *Physiological reviews* 78: 1165-1191, 1998.
106. **Sutters M**. The pathogenesis of autosomal dominant polycystic kidney disease. *Nephron* 103: e149-155, 2006.
107. **Sutters M, Yamaguchi T, Maser RL, Magenheimer BS, St John PL, Abrahamson DR, Grantham JJ, and Calvet JP**. Polycystin-1 transforms the cAMP growth-responsive phenotype of M-1 cells. *Kidney international* 60: 484-494, 2001.
108. **Sweadner KJ, Herrera VL, Amato S, Moellmann A, Gibbons DK, and Repke KR**. Immunologic identification of Na+,K(+)-ATPase isoforms in myocardium. Isoform change in deoxycorticosterone acetate-salt hypertension. *Circulation research* 74: 669-678, 1994.
109. **Takacs-Jarrett M, Sweeney WE, Avner ED, and Cotton CU**. Generation and phenotype of cell lines derived from CF and non-CF mice that carry the H-2K(b)-tsA58 transgene. *American journal of physiology Cell physiology* 280: C228-236, 2001.
110. **Takakura A, Contrino L, Zhou X, Bonventre JV, Sun Y, Humphreys BD, and Zhou J**. Renal injury is a third hit promoting rapid development of adult polycystic kidney disease. *Human molecular genetics* 18: 2523-2531, 2009.
111. **Takiar V, and Caplan MJ**. Polycystic kidney disease: pathogenesis and potential therapies. *Biochimica et biophysica acta* 1812: 1337-1343, 2011.
112. **Tan YC, Blumenfeld J, and Rennert H**. Autosomal dominant polycystic kidney disease: genetics, mutations and microRNAs. *Biochimica et biophysica acta* 1812: 1202-1212, 2011.
113. **Therien AG, and Blostein R**. Mechanisms of sodium pump regulation. *American journal of physiology Cell physiology* 279: C541-566, 2000.
114. **Torres VE, Chapman AB, Devuyst O, Gansevoort RT, Grantham JJ, Higashihara E, Perrone RD, Krasa HB, Ouyang J, and Czerwiec FS**. Tolvaptan in patients with autosomal dominant polycystic kidney disease. *The New England journal of medicine* 367: 2407-2418, 2012.
115. **Torres VE, Grantham JJ, Chapman AB, Mrug M, Bae KT, King BF, Jr., Wetzel LH, Martin D, Lockhart ME, Bennett WM, Moxey-Mims M, Abebe KZ, Lin Y, and Bost JE**. Potentially modifiable factors affecting the progression of autosomal dominant polycystic kidney disease. *Clinical journal of the American Society of Nephrology : CJASN* 6: 640-647, 2011.
116. **Torres VE, and Harris PC**. Autosomal dominant polycystic kidney disease: the last 3 years. *Kidney international* 76: 149-168, 2009.

117. **Torres VE, and Harris PC**. Polycystic kidney disease: genes, proteins, animal models, disease mechanisms and therapeutic opportunities. *Journal of internal medicine* 261: 17-31, 2007.
118. **Torres VE, King BF, Chapman AB, Brummer ME, Bae KT, Glockner JF, Arya K, Risk D, Felmlee JP, Grantham JJ, Guay-Woodford LM, Bennett WM, Klahr S, Meyers CM, Zhang X, Thompson PA, and Miller JP**. Magnetic resonance measurements of renal blood flow and disease progression in autosomal dominant polycystic kidney disease. *Clinical journal of the American Society of Nephrology : CJASN* 2: 112-120, 2007.
119. **Trevisi L, Pighin I, and Luciani S**. Vascular endothelium as a target for endogenous ouabain: studies on the effect of ouabain on human endothelial cells. *Cell Mol Biol (Noisy-le-grand)* 52: 64-70, 2006.
120. **Wagoner K, Sanchez G, Nguyen AN, Enders GC, and Blanco G**. Different expression and activity of the alpha1 and alpha4 isoforms of the Na,K-ATPase during rat male germ cell ontogeny. *Reproduction* 130: 627-641, 2005.
121. **Wallace DP**. Cyclic AMP-mediated cyst expansion. *Biochimica et biophysica acta* 1812: 1291-1300, 2011.
122. **Wallace DP, Christensen M, Reif G, Belibi F, Thrasher JB, Herrell D, and Grantham JJ**. Electrolyte and fluid secretion by cultured human inner medullary collecting duct cells. *American journal of physiology Renal physiology* 283: F1337-F1350, 2002.
123. **Wallace DP, Grantham JJ, and Sullivan LP**. Chloride and fluid secretion by cultured human polycystic kidney cells. *Kidney international* 50: 1327-1336, 1996.
124. **Wallace DP, Reif G, Hedge AM, Thrasher JB, and Peitrow P**. Adrenergic regulation of salt and fluid secretion in human medullary collecting duct cells. *American journal of physiology Renal physiology* 287: F639-648, 2004.
125. **Wang H, Haas M, Liang M, Cai T, Tian J, Li S, and Xie Z**. Ouabain assembles signaling cascades through the caveolar Na+/K+-ATPase. *The Journal of biological chemistry* 279: 17250-17259, 2004.
126. **Wang S, Yue H, Derin RB, Guggino WB, and Li M**. Accessory protein facilitated CFTR-CFTR interaction, a molecular mechanism to potentiate the chloride channel activity. *Cell* 103: 169-179, 2000.
127. **Wang X, Gattone V, 2nd, Harris PC, and Torres VE**. Effectiveness of vasopressin V2 receptor antagonists OPC-31260 and OPC-41061 on polycystic kidney disease development in the PCK rat. *J Am Soc Nephrol* 16: 846-851, 2005.
128. **West MR, and Molloy CR**. A microplate assay measuring chloride ion channel activity. *Analytical biochemistry* 241: 51-58, 1996.
129. **Wildman SS, Hooper KM, Turner CM, Sham JS, Lakatta EG, King BF, Unwin RJ, and Sutters M**. The isolated polycystin-1 cytoplasmic COOH terminus prolongs ATP-stimulated Cl- conductance through increased Ca2+ entry. *American journal of physiology Renal physiology* 285: F1168-1178, 2003.
130. **Wilson PD**. Polycystic kidney disease: new understanding in the pathogenesis. *The international journal of biochemistry & cell biology* 36: 1868-1873, 2004.
131. **Wilson PD, Geng L, Li X, and Burrow CR**. The PKD1 gene product, "polycystin-1," is a tyrosine-phosphorylated protein that colocalizes with alpha2beta1-integrin in focal clusters in adherent renal epithelia. *Laboratory investigation; a journal of technical methods and pathology* 79: 1311-1323, 1999.
132. **Xie Z, and Askari A**. Na(+)/K(+)-ATPase as a signal transducer. *European journal of biochemistry / FEBS* 269: 2434-2439, 2002.
133. **Xie Z, and Cai T**. Na+-K+--ATPase-mediated signal transduction: from protein interaction to cellular function. *Molecular interventions* 3: 157-168, 2003.

134. **Yamaguchi T, Hempson SJ, Reif GA, Hedge AM, and Wallace DP**. Calcium restores a normal proliferation phenotype in human polycystic kidney disease epithelial cells. *J Am Soc Nephrol* 17: 178-187, 2006.
135. **Yamaguchi T, Pelling JC, Ramaswamy NT, Eppler JW, Wallace DP, Shizuko N, Rome LA, Sullivan LP, and Grantham JJ**. cAMP stimulates the in vitro proliferation of renal cyst epithelial cells by activating the extracellular signal-regulated kinase pathway. *Kidney international* 57: 1460-1471, 2000.
136. **Yamaguchi T, Wallace DP, Magenheimer BS, Hempson SJ, Grantham JJ, and Calvet JP**. Calcium restriction allows cAMP activation of the B-Raf/ERK pathway, switching cells to a cAMP-dependent growth-stimulated phenotype. *The Journal of biological chemistry* 279: 40419-40430, 2004.
137. **Yan Y, Haller S, Shapiro A, Malhotra N, Tian J, Xie Z, Malhotra D, Shapiro JI, and Liu J**. Ouabain-stimulated trafficking regulation of the Na/K-ATPase and NHE3 in renal proximal tubule cells. *Molecular and cellular biochemistry* 367: 175-183, 2012.
138. **Yang B, Sonawane ND, Zhao D, Somlo S, and Verkman AS**. Small-molecule CFTR inhibitors slow cyst growth in polycystic kidney disease. *J Am Soc Nephrol* 19: 1300-1310, 2008.
139. **Ye M, and Grantham JJ**. The secretion of fluid by renal cysts from patients with autosomal dominant polycystic kidney disease. *The New England journal of medicine* 329: 310-313, 1993.
140. **Ye Q, Lai F, Banerjee M, Duan Q, Li Z, Si S, and Xie Z**. Expression of mutant alpha1 Na/K-ATPase defective in conformational transition attenuates Src-mediated signal transduction. *The Journal of biological chemistry* 288: 5803-5814, 2013.
141. **Yingst DR**. Modulation of the Na,K-ATPase by Ca and intracellular proteins. *Annual review of physiology* 50: 291-303, 1988.
142. **Yoder BK, Hou X, and Guay-Woodford LM**. The polycystic kidney disease proteins, polycystin-1, polycystin-2, polaris, and cystin, are co-localized in renal cilia. *J Am Soc Nephrol* 13: 2508-2516, 2002.
143. **Zatti A CV, Rajendran V, Kimura T, Pagel P, Caplan MJ**. The C-terminal tail of the polycystin-1 protein interacts with the Na,K-ATPase alpha-subunit. *Mol Biol Cell* 16: 5087-5093, 2005.
144. **Zhang D, Ciciriello F, Anjos SM, Carissimo A, Liao J, Carlile GW, Balghi H, Robert R, Luini A, Hanrahan JW, and Thomas DY**. Ouabain Mimics Low Temperature Rescue of F508del-CFTR in Cystic Fibrosis Epithelial Cells. *Frontiers in pharmacology* 3: 176, 2012.