Contributions of Ouabain to the Autosomal Dominant Polycystic Kidney Disease Phenyotype

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Autosomal Dominant Polycystic Kidney Disease Phenyotype

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ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD) is a genetic disorder, caused by mutations in either of the *PKD1* or *PKD2* genes, which leads to the development and growth of multiple fluid-filled cysts in the kidney. The cysts are present at birth and grow throughout the lifetime of the afflicted individual, eliciting pain and eventually leading to end-stage renal disease (ESRD). The *PKD1* and *PKD2* genes and their corresponding encoded proteins, polycystin-1 (PC1) and polycystin-2 (PC2), are necessary for proper tubulogenesis, and for the repair processes that maintain the architecture of the tubules after injury in the adult kidneys. Mutations in PC1 and PC2 results in phenotypic changes in renal epithelial cells, which consist, among others, in a more dedifferentiated state of the cells, increased proliferation, increased fluid secretion, increased apoptosis, epithelial-to-mesenchymal (EMT)-like features, abnormal activity of various cell signaling pathways, and alterations in intracellular calcium.

An enzyme essential for the proper function of the kidney is the Na,K-ATPase (NKA). NKA uses the free energy from ATP to catalyze the movement of Na^+ in exchange for K^+ across the cell plasma membrane. Its function in the kidney is key for solute and water reabsorption and formation of urine. In addition, NKA is the receptor and signal transducer for the hormone ouabain, which regulates the sodium transport of normal renal epithelial cells. In ADPKD kidneys, the binding of ouabain to NKA has an important role in exasperating cystic progression. Specifically, ouabain stimulates both the proliferation and the fluid secretion of ADPKD cells, as well as cystogenesis in embryonic kidneys from a mouse model of ADPKD. This thesis was undertaken to determine how ouabain contributes to the overall phenotype of ADPKD cells. In particular, emphasis was placed on the effect of ouabain on the cystic phenotypes of apoptosis, EMT and intracellular calcium.

Ouabain significantly increased the apoptotic rate in renal cystic epithelial cells obtained from the kidneys of patients with ADPKD (ADPKD cells). Ouabain-mediated apoptosis occurred via the intrinsic pathway, and was reliant on cytochrome c release from the mitochondria and on an altered ratio of Bcl-2 associated X protein (BAX) to B-cell lymphoma 2 (BCL-2) protein. These results agree with findings from other authors that show that apoptosis of epithelial cells is a characteristic of ADPKD cells. While ouabain can increase apoptosis in ADPKD cells, it also increases proliferation, and overall proliferation remained greater than overall apoptosis induced by ouabain. Therefore, ouabain creates a misbalance towards cell growth, which helps the development of ADPKD cysts.

Additionally, epithelial to mesenchymal (EMT)-like signaling was altered by ouabain. A hallmark of EMT is the decrease in expression of E-cadherin and the increase in N-cadherin. Ouabain was able to induce this affect in ADPKD cells, without altering cadherin levels in NHK cells. Additionally, ouabain increased tumor growth factor-β (TGFβ) expression and SMAD3 phosphorylation, as well as increased expression of downstream targets, such as collagen-I and Snail. However, ouabain was not able to induce a complete switch to a mesenchymal phenotype, as evidenced by unchanged levels in the mesenchymal protein vimentin, the presence of β-catenin in the plasma membrane, and the unchanged invasive properties. Interestingly, despite the decreased cell-cell adhesion, ouabain increased transepithelial electrical resistance, but did not affect the permeability of monolayers to neutral dextran, suggesting that the tightness of junctions and the permeability of the paracellular transport pathway are preserved in the cells. In contrast, epithelial cells from normal human kidneys (NHK cells) did not display any of the mentioned effects of ouabain. Together, these results reveal that while ouabain contributes to the development

of the dedifferentiated phenotype of ADPKD cells, but does not lead to a complete EMT, which stimulates cyst formation and growth.

Finally, intracellular calcium concentration ($[Ca^{2+}]_i$) is constitutively lower in ADPKD cells relative to NHK cells. NHK cells were found to respond to ouabain with an increase in $[Ca^{2+}]_i$, while ADPKD cells did not. Further, ADPKD cells had lower concentrations of ER calcium than NHK, but neither ER nor mitochondrial calcium levels appear to be altered by ouabain. The ouabain-induced increase in $[Ca^{2+}]_i$ in NHK cells occurred through L-type calcium channels (LTCC). ADPKD cells did not respond to ouabain in the same manner, and this was found to be due to lower expression of full-length LTCC at the plasma membrane. Indeed, increased calpain activity was observed in ADPKD cells relative to NHK, and this correlated inversely with expression of the endogenous calpain inhibitor, calpastatin. The expression of c-Myc is elevated in ADPKD cells, and this negatively regulates calpastatin expression. Indeed, inhibition of the transcriptional activity of c-Myc resulted in increased calpastatin protein, decreased calpain activity, and decreased cleavage of LTCC yielding increased levels of basal intracellular calcium in ADPKD cells. Although we did not find that ouabain was able to further exasperate the calcium phenotype, we expect longer treatment with ouabain may have a more pronounced effect.

In conclusion, both apoptosis and EMT are enhanced by ouabain in ADPKD cells and unaffected in NHK cells. Conversely, ADPKD cells do not appear to alter calcium in response to ouabain in the time points assayed. However, the knowledge that calpains are more active in ADPKD causing mid-channel cleavage of LTCC is entirely novel and potentially a benefit to the field.

To my marvelous parents, Doug and Donita Johnson,

I dedicate this thesis and the following haiku:

A thesis you see

Instead of a grandbaby

No need to thank me.

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CHAPTER 1: INTRODUCTION

ADPKD

Overview of the disease

Autosomal dominant polycystic kidney disease (ADPKD) is a genetic disorder characterized by the formation of multiple fluid-filled cysts in both kidneys, which continually expand throughout the lifetime of the affected individual. Renal cyst enlargement occurs as a consequence of two major mechanisms, increased cell proliferation of the cystic epithelium, and aberrant transepithelial fluid secretion into the cysts. Expansion of the cysts ultimately leads to mechanical compression and destruction of the renal parenchyma [1-3], resulting in renal insufficiency and eventually end-stage renal disease (ESRD). ADPKD is responsible for roughly 10% of all cases of ESRD [4, 5] and is the fourth leading cause of ESRD requiring dialysis and kidney transplantation therapy in the United States [6, 7]. Even before the patients exhibit significant loss of kidney function, they have symptoms that cause detrimental effects on patient quality of life. Abdominal or flank pain and hematuria are the most common presentations of ADPKD. Pain is attributed to the cyst-mediated compression of the internal organs, while hematuria is due to cyst rupture into the urinary tract [8, 9].

ADPKD is caused by mutations in the *PKD1* and *PKD2* genes that encode for polycystin-1 (PC1) and polycystin-2 (PC2) respectively. While mutations in PC1 account for 80-85% of ADPKD cases, alteration in PC2 accounts for the remaining 15-20% [10]. Recently, mutations in the glucosidase-II alpha subunit, GANAB, have been shown to inhibit the proper localization of PC1 and thus may also give rise to ADPKD [11]. Patients with mutations in PC1 or PC2 have identical renal manifestations [12], with the exception of varying rates of ESRD progression [13].

Specifically, patients with defects in *PKD1* on average progress to ESRD by age 57, while the kidney function of *PKD2* patients persists longer, with 69 years as the average age for the onset of ESRD [13]. PC1 and PC2 interact with each other to form a macromolecular receptor/signaling complex, the function of which is still not fully elucidated. However, there is evidence that PC1/PC2 are involved in the regulation of different pathways in renal epithelial cells that control cell proliferation, polarity, and the normal structure of the renal tubules [14-17].

To date, genetic screens of patients with ADPKD have identified as many as 2323 *PKD1* and 278 *PKD2* unique germline mutations (2016; pkdb.mayo.edu). In the *PKD1* gene, mutations are found in all areas of the gene, from exons 1 to 46, with no clear hotspot identified [18]. However, mutations are twice as frequent in the C-terminal half of the protein [18]. In the *PKD2* gene, mutations have been detected in most of the 15 exons [19], with truncation mutations accounting for 65% of *PKD1* and 83% of *PKD2* mutations [20, 21]. ADPKD is the most common monogenetic disease of the kidney, affecting 1:400-1:1000 births worldwide [22, 23]. This high incidence, coupled with the many mutations identified, illustrates the sensitivity of PC1 and PC2 to any hindrance in their function, and also foreshadows the polymodal nature of these proteins.

ADPKD is diagnosed on the basis of ultrasound and prior family history; although 6-8% of ADPKD patients are *de-novo* mutations and their diagnosis is a random event during clinical examination [24]. Despite the genetic basis of the disease, genetic testing is not performed as part of the standard diagnosis due to the unique technical challenges presented in confirming *PKD1* mutations. [25] The search for mutations in *PKD1* transcript is complicated by the surrounding genomic region. Specifically, 50 kb of genomic DNA encoding the first 31 exons of *PKD1* are duplicated at least three times on chromosome 16 (16p13.1) and this area also encodes 3 genes that share substantial homology to the *PKD1* exons [26, 27]. Currently, there is no specific

treatment for ADPKD approved in the United States, although several guidelines for disease management exist (reviewed in [28, 29]). At present, finding therapeutic approaches to treat ADPKD is necessary to relieve the physical burden of the patients that suffer from this disease, as well as to decrease the health care costs associated with the palliative measures used to prolong the life of the patients.

The genetic basis of ADPKD and its correlation with disease development and progression

ADPKD progression is slow, occurring over several decades. Despite thousands of microcysts already present in the kidneys of ADPKD patients at birth [30, 31], and their relentless growth during the lifetime of the affected individual, overall renal function remains normal due to compensatory hyperfiltration by the nephrons that continue to be functional [32]. However, during the later stages of the disease, the destruction of adjacent renal parenchyma, results in the initiation of fibrotic and inflammatory processes which contribute to the progressive and rapid deterioration in renal function [33]. The growth rate of individual cysts is extremely variable and can range between 2.2 and 71.1% per year [30]. One of the major factors which predicts accelerated ADPKD progression is the type of polycystin mutation [28]. In general, *PKD2* patients have later onset, longer renal survival, and present fewer complications compared to *PKD1* patients [12]. The next best prognosis goes to *PKD1* non-truncating mutations, while *PKD1* truncations present the worst phenotype [28]. Within the *PKD1*-truncation mutations, it appears that the severity of the *PKD1* mutation depends on the length of the truncated *PKD1* protein, with truncations in the N-terminal portion leading to ESRD more quickly [34, 35]. Other subtleties in genetic expression may also impact disease progression, such as the expression levels of PC1 and PC2, penetrance of

pathogenic alleles, and the stage of kidney development affected by the particular *PKD1* mutation [36-40].

Interestingly, microdissection studies on ADPKD kidneys have shown that, despite the presence of polycystin germline mutations in every kidney cell, just 1% of nephrons form cysts [41]. This phenomenon has been explained by a "second hit" theory [41]. This theory states that in addition to the germline mutation, a somatic mutation must occur in the second normal allele for cyst initiation. In support of this theory, cystic epithelial cells show a loss of heterozygosity in the *PKD1* gene in a subset of cysts [41, 42]. Environmental factors could influence the rate of the second hit, which may account for the variability in cystogenesis and renal disease progression [43]. Indeed, the genetic defect alone cannot account for the observed high intrafamilial variability of the disease. The heritability of ADPKD severity has been estimated at just 42% within family members whose kidney function has not yet declined to ESRD [44]. Comparison between the age of ESRD onset in siblings to that of monozygotic twins [45] has shown that siblings underwent ESRD within 6.9±6 years of each other, while in monozygotic twins, the variability to kidney failure was shortened to 2.1±1.9 years difference [45]. Thus, while the progression of ADPKD to ESRD is clearly affected by the presence of modifier genes, a difference in ESRD onset of up to 4 years is observed between genetically identical monozygotic twins, and this difference can be attributed to environmental factors [45].

Other major factors which predict accelerated progression in ADPKD are the patient's age at diagnosis, total kidney volume, kidney function, and gender [28]. The first three parameters are indicative of a worse disease phenotype. Younger age at diagnosis, early renal enlargement, and lower glomerular filtration rate (GFR) are bound to be correlative not causative factors, but are useful to clinicians in disease management and counseling. With respect to gender, males and

females have the same incidence of ESRD [10], but males more rapidly progress to ESRD [1, 3, 46]. However some studies have found that the gender effect was not present in *PKD1* families [34, 47, 48] and might be restricted to families where *PKD2* is affected [49].

ADPKD as a ciliopathy and defective wound healing process

Two conceptual ideas of APDKD pathophysiology co-exist, and are not mutually exclusive. The first is that ADPKD is a ciliopathy, or a disease caused by defects in the structure or function of cilia [50]. This view was derived by the discovery that the phenotype of the Oak Ridge Polycystic Kidney mouse, a model of polycystic kidney disease, was caused by a mutation in IFT88, a protein required for the assembly of primary cilia and intraflagellar transport [51]. Subsequently, both polycystins, as well as fibrocystin, the cause of autosomal recessive polycystic kidney disease (ARPKD), were found to localize to the primary cilium [52]. In addition, other diseases that cause renal cysts are induced by mutations in proteins which normally localize to cilia; these diseases include nephronophthisis, Bardet-Biedl syndrome, orofaciodigital syndrome and others [53, 54].

Like most cells of the body, the majority of renal tubular epithelial cells have a primary apical cilium [55], which functions as a receptor that senses urine fluid flow. When mouse embryonic kidney cells are subjected to fluid flow, at a rate comparable to the physiological urine fluid flow rate, a calcium influx into the cells is observed [56]. In contrast, *PKD1*-null cells, or cells with truncated forms of *PKD1*, fail to respond to fluid flow with calcium influx [56, 57]. Also, renal cells lacking a primary cilium, but expressing both polycystins, also lack this response to fluid flow [58]. As will be discussed in more detail later, the polycystin proteins create a

functional, nonselective cation channel permeable to Ca^{2+}, Na^{+}, and K^{+} ions [14]. PC2 contains the pore-forming domains of this channel, while PC1 is thought to regulate PC2 channel activity [56]. On the primary cilium, the large extracellular N-terminal domain of PC1 is hypothesized to function as a sensing antenna for mechanical [59], chemical [60], or peptide stimuli [61]. Therefore, stimulation of the cilium may induce a conformational change in PC1, which activates its binding partner PC2 to mediate calcium entry into the cell [56]. However, this remains controversial due to recent studies by Delling, et al which suggest that the ciliary PC1/2 complex is not responsible for fluid-flow mediated calcium influx [62].

The second view of ADPKD is focused on the hypothesis that upon lack of fluid flow, normal renal cells may use the primary cilia to signal the presence of injury. Several renal injuries, such as ureteral obstruction and ischemic injury halt fluid flow [63, 64]. Therefore, it has been theorized that PC1/2 signals "all is well" in response to fluid flow [63]. However, in ADPKD cells, the mutant PC1 or PC2 fail to signal this response even under normal fluid flow, leading to the reactivation of developmental signaling pathways and cell proliferation [65, 66]. Indeed, several observations suggest that polycystins are instrumental in normal renal repair. PC1/2 levels have been observed to be elevated after ischemic injury [67-69]. Also, kidneys heterozygous for *PKD1* or *-2* mutations cannot repair themselves as effectively as kidneys from wildtype mice, and instead tubule dilation and formation of microcysts occur [70, 71]. These observations have led to the idea of the "third hit" hypothesis [72]. This theory postulates that, as an individual ages, the kidneys are more likely to suffer transient obstructive or ischemic injuries that stimulate tissue repair signaling pathways, which reactivate development and epithelial cell proliferation of the renal tubular epithelial cells [65, 66]. Thus, in renal injury, the kidney may rely on the activities of polycystins for its repair. The third hit theory is supported by observations of increased cystic

expansion after injury induction in adult polycystic mouse models with slowly progressive cystic disease [40, 72-74]. Ischemic injury also triggers cyst formation in kidneys lacking cilia [75-77], emphasizing the importance of proper ciliary signaling in the wound healing processes.

Additional support for the third hit theory is the observation of tissue repair processes occurring in the interstitium surrounding the cystic cells [78-81]. Thus, the distortion of tubules and vessels sets up an inflammatory response that becomes chronic and leads to fibrosis as well as dedifferentiation in kidneys from ADPKD patients [82, 83]. In particular, ADPKD kidneys are characterized by severely thickened basement membranes and excessive ECM deposition [78]. Similarly, accumulation of ECM is observed in ischemic acute renal failure and after ureteral obstruction [84-86]. Additionally, infiltrating immune cells are observed after renal ischemic-reperfusion injury, ureteral obstruction, and also in human ADPKD and polycystic models [79, 80]. There is some experimental evidence showing the presence of epithelial to mesenchymal transition (EMT) in ADPKD, which contributes to the appearance of fibroblasts [81]. For example, gene analysis of normal and ADPKD kidneys, has shown the upregulation of smooth muscle-related genes, indicative of cell dedifferentiation and EMT [87].

As will be shown later, the experiments in this thesis support the third hit theory through the abilities of the hormone ouabain to act directly on renal epithelium to enhance the dedifferentiated phenotype of ADPKD cystic cells. However, the non-renal complications of ADPKD will be discussed in the following section in order to give a complete physiological picture of the disease.

Non-renal complications of ADPKD

ADPKD is a systemic disease with a large morbidity burden on the patient. Along with renal manifestations, *PKD1* and *PKD2* patients have identical extrarenal manifestations [12]. In addition to the kidney, cysts can also arise in the liver, spleen, and pancreas. Liver cysts are the most common extrarenal cysts, occurring in about 80% of patients [88]. Women tend to have a higher prevalence of liver cysts, and the number and size of the liver cysts appears to be related to multiple pregnancies or prolonged exogenous estrogen exposure [89, 90]. Liver cysts cause chronic pain, but the function of the liver usually remains normal [91, 92]. Pancreatic cysts have been described in approximately 10% of ADPKD patients [22, 93] and splenic cysts in fewer than 5% [94].

Despite the pro-proliferative phenotype of cystic cells with mutations in polycystins, the incidence of renal cell carcinoma is not increased in ADPKD [95]. However, multiple cardiovascular, musculoskeletal, and gastrointestinal abnormalities are associated with ADPKD (reviewed in [96]). For instance, ADPKD patients have increased incidence of cardiac valve abnormalities, aortic dissection, intracranial aneurysms, and abdominal wall hernias (reviewed in [97, 98]). Hypertension is found in 49-75% of patients, even before the patients show a noticeable loss in renal function [23, 99]. Over 90% of ADPKD patients develop high blood pressure by the time they reach ESRD [100]. One theory attributes this rise in blood pressure to cyst expansion. Compression of the intrarenal vasculature by cysts could cause renin release and activate the renin-angiotensin aldosterone system (RAAS) pathway, leading to increased blood pressure [89, 101]. Coincidentally, the RAAS system can also be activated by renal injury [102], and this mechanism has been found to be involved in the hypertension of ADPKD patients [103-107]. In *PKD1*-null mice, two molecules involved in RAAS activation, pro-renin and angiotensinogen, are increased

[108]. Additionally, elevated levels of angiotensin-II, the effector molecule of RAAS, is increased in cyst fluid, and cyst-derived cells *in vitro* express several components of RAAS [109].

Hypertension in ADPKD is associated with development of left ventricular hypertrophy, increased cardiovascular mortality and morbidity, as well as progression of renal destruction [3, 110]. Importantly, although ESRD is used to measure ADPKD progression, cardiovascular disease is responsible for nearly 80% of deaths in ADPKD [111, 112].

POLYCYSTINS

Expression and Structure

ADPKD was first linked to the *PKD1* gene, on chromosome 16 (16p13.3), in 1985 [113]. A second locus, *PKD2*, on chromosome 4 (4q21-23) was identified in 1993 [114]. A year later, the *PKD1* gene was first cloned and partially characterized [26, 115], while cloning of *PKD2* was achieved by 1996 [116]. It was soon found that PC1 and PC2 interact with one another [14-17] via their C-terminal cytoplasmic tails [15, 16, 117]. It has been proposed that the polycystin complex contains one PC1 and three PC2 subunits [118, 119]. Reinforcing the fact that mutations in either polycystin can cause ADPKD, the interaction between polycystins reciprocally influences the functional properties of each. For instance, the interaction of PC1 with PC2 is important for modulating the ion channel abilities of PC2 [14, 120], while conversely, the interaction decreases the ability of PC1 to activate G proteins [121].

Polycystins are expressed in epithelial cells of the developing and mature renal tubules, as a well as in other somatic tissues including heart, liver, brain, bone, as well as the thyroid and adrenal glands [67, 122-124]. PC1 expression is temporally regulated, with high levels observed

in fetal renal tissue and low, but still detectable, levels present in adult tissue [125]. This early upregulation of PC1 is of significance when considering that cystogenesis occurs *in utero*, during tissue development [30, 31]. Studies of cystic growth rate have concluded that cysts grow at vigorous rates *in utero* and at slower rates thereafter [30, 126]. Indeed, *in vivo* experiments have shown the importance of the developmental expression of polycystin on the growth of cysts. Specifically, temporally controlled inactivation of PC1 or PC2 expression in murine kidneys has revealed that the loss of these proteins during renal development causes a far more severe cystic phenotype than when the loss of PC1 or PC2 occurs in the mature kidney [72, 127, 128].

Further evidence for the importance of *PKD1* expression during nephrogenesis to prevent cysts comes from a mouse *PKD1*-knockout model. Murine PC1 is not detectable in the early stages of nephrogenesis but peaks at E15.5 in the collecting ducts, papillary ducts and renal pelvis [37]. *PKD1* knockout mice develop normally until the induction of cystic dilations at day E15.5 [37]. This suggests a role of polycystin in tubular elongation and maintenance of tubular architecture, rather than in nephron formation [37]. The *PKD2* knockout mouse showed a similar pattern of normal kidney development until E15.5, at which point cysts began to form [129].

In humans, PC1 and PC2 are highly expressed in proximal tubules from 10-24 weeks of gestation, then the expression drops precipitously between gestational week 28 and 38 [125, 130]. In contrast, expression of both polycystin proteins is found in the cortical and medullary collecting ducts by 14 weeks gestation, and this expression is increased and maintained high until term (40 weeks) [130]. Adult human kidneys weakly express polycystins in proximal tubules, while expression of both PC1 and PC2 continues in medullary collecting ducts [130]. Interestingly, although ADPKD cysts derive from all segments of the nephron, a significant portion of the cystic cells are stained by markers that are specific for a collecting duct origin [131, 132]. Moreover,

inactivation of *PKD1* leads to the formation of the largest cysts predominantly in collecting ducts [133]. Therefore, cysts primarily form in the segments in which the strongest expression of polycystins is observed.

Paradoxically, both reducing the expression of PC1, as well as inducing its overexpression, leads to renal cyst growth in transgenic mice [40, 134]. Additionally, PC2 is expressed at higher levels in cyst-lining epithelium of ADPKD [135-137], and cysts of patients with ADPKD have continued expression of PC1 in many instances [122, 138-140]. Therefore, it has been suggested that polycystin proteins need to be expressed at a certain ratio to maintain their stoichiometry and prevent cystogenesis [141, 142].

The polycystins play several roles in both development and in response to injury. Both of those conditions involve certain processes, like increased proliferation, which are important in ADPKD cystic growth. The sections that follow will discuss the structure of the polycystins, and then emphasize the roles of the polycystins in calcium regulation, EMT, and apoptosis - processes which are central to the experiments of this thesis.

PC1 and PC2, also known as TRPP1 and TRPP2, respectively, are members of the transient receptor potential (TRP) superfamily of cation channels, which includes more than 20 members (reviewed in [143, 144]). The activation of TRP channel members is often polymodal, allowing them to respond to multiple forms of stimuli (reviewed in [145]). PC1 shows significant sequence homology with TRP channels [146], but it cannot by itself form an ion channel, as PC1 overexpression alone does not yield any measurable channel activity [147]. Therefore, despite being a TRP channel family member, PC1 is considered to be an integral membrane receptor and not a channel. It bears 11 transmembrane domains, a large N-terminal extracellular region, and a short cytoplasmic C-terminus ([4] and Figure 1.1).

The extracellular N-terminus of PC1 is large, about 3000 amino acids long [148, 149], and is made up of domains thought to play a role in protein-protein or protein-matrix interactions [150-153]. The extracellular domains of PC1 may also be involved in sensing fluid flow and pressure in the kidney (reviewed in [154]). As Figure 1.1 shows, the domains present in the PC1 N-terminus include: 2 complete leucine rich repeat (LRRs) motifs flanked by cysteine-rich sequences, a cell wall stress-responsive component (WSC) domain, a C-type lectin domain, a low density lipoprotein (LDL-A) region, a receptor of egg jelly (REJ) domain [155], and 15 PKD repeat motifs, which are Ig-like [149]. Atomic force microscopy showed that the PKD domains on PC1 exhibit remarkable mechanical strength further supporting the proposed mechanosensory function of PC1 on cilia [147].

PC1 also contains 11 transmembrane domains (~1000 amino acids) [12, 156, 157]. The first cytoplasmic loop after TM1 contains a lipoxygenase domain (also called a PLAT or LH2 domain), which adopts a β-sandwich fold [158]. PLAT domains are usually involved in protein-protein or protein-lipid interactions [159]. Additionally, between the domains TM5 and TM6 is a binding site for the adapter protein, Homer [160].

The cytoplasmic C-terminal tail (CTT) of PC1 is small, yet has garnered much attention as it appears to have many and varied roles in both signaling and transcription. In support of its importance are experiments showing that a naturally occurring truncation mutation which removes the PC1 C-terminal segment is sufficient to cause ADPKD [161]. The domains present in CTT include a G protein binding domain [162], a coiled-coil domain, which is necessary for its interaction with PC2 [67, 163], a PEST domain which may facilitate its ubiquitin mediated degradation [139], and a putative nuclear localization sequence, by which it affects transcription [164]. The functions of the cleaved CTT will be discussed in detail below.

PC1 undergoes cleavage in both its N- and C-terminal domains. The N-terminal cleavage occurs at the GPS cleavage site (T3049), located immediately before the first transmembrane domain [165], and shown in Figure 1.1. This cis-autoproteolysis is caused by a self-catalyzed protein rearrangement and requires the presence of the adjacent REJ domain to occur [165]. GPS cleavage occurs early in the secretory pathway, and results in the N-terminus remaining noncovalently bound to the C-terminal fragment [166]. Mutations that prevent GPS cleavage cause cystic disease in mice and humans [167]. Additionally, expression of a mutant form of PC1 which cannot undergo GPS cleavage does not rescue PC1-null cells *in vitro* or transgenic mice [165, 168, 169], suggesting that GPS cleavage is necessary for PC1 function. However, not all PC1 in a cell are cleaved, yielding a heterogeneous population [166, 168]. The presence of PC2 enhances the GPS cleavage of PC1, which agrees with the proposed role of PC2 as an essential chaperone for PC1 maturation and plasma membrane [170] and ciliary localization [171]. However, the role of PC2-enhanced GPS cleavage in the trafficking of the polycystin complex is not fully agreed upon as some studies have suggested PC2 traffics to the cilia independently of PC1 [172, 173], others claim that PC2 ciliary localization requires PC1 [171] and some suggest that PC1 localization to the cilia does not rely on the GPS cleavage of PC1 or the RVxP sequence found on PC2 [174].

The C-terminal cleavage of PC1 appears to have several physiological roles. Three known cleavage sites, denoted by black arrows in Figure 1.1, have been found in the C-terminal portion of PC1 that release the CTT. Misregulation of PC1 cleavage appears to result in aberrant signaling which may in turn lead to the abnormal cellular growth behaviors that contribute to ADPKD pathogenesis (reviewed in [170]). One CTT fragment (100 kDa), contains the last 6 transmembrane domains and, when cleaved, prevents store-operated calcium entry [175]. This fragment has also been found to directly associate with inositol triphosphate receptors (IP_3R) in the endoplasmic

reticulum (ER) and inhibit calcium release from the ER in response to IP_3 stimulation [176]. Another 15 kDa CTT fragment has a clearly cystogenic action by its interaction with STAT6 and the STAT6-coactivator, P100. This interaction begins at the basal bodies of cilia. Upon flow cessation the 15 kDa CTT fragment of PC1 is cleaved off which promotes the nuclear translocation of itself and STAT6 [139]. Additionally, the presence of this 15 kDa fragment has been found to be increased in cells lining ADPKD cysts, exhibiting a high nuclear localization [139]. Indeed, overexpression of this 15 kDa CTT in zebrafish promoted renal cyst formation [139]. In contrast, cleavage of an anti-cystogenic 35 kDa fragment occurs under the opposite condition than that of the pro-cystogenic 15 kDa fragment. In response to mechanical stimulation by fluid flow, gamma-secretase cleaves this 35 kDa CTT [177], and it is observed to accumulate in the nucleus [125]. Indeed, gamma-secretase inhibition during 3D culture of wild-type renal epithelial cells induced cyst formation, similar to what occurs in *PKD1*$^{-/-}$ cells [177]. This 35 kDa CTT fragment is able to decrease aberrant proliferation as well as aberrant apoptosis in ADPKD cells, by binding to and inhibiting the transcription factors TCF and CHOP, respectively [177]. Production of this 35 kDa CTT fragment is also stimulated by the presence of functional PC2 [178], although increased cleavage of all known fragments occurs in response to PC2 expression [175].

Finally, an engineered C-terminal tail (CTT) containing the last 193 amino acids (~21 kDa) has been used as a dominant negative against full-length PC1 [179]. Overexpression of this CTT was found to increase net fluid secretion and proliferation in response to cAMP, as is what is observed in ADPKD cells [180]. When this construct is co-expressed with α1-NKA in an insect expression system, the cells acquire a phenotype of increased proliferation in response to ouabain [181], which will be discussed in further detail later on.

Figure 1.1

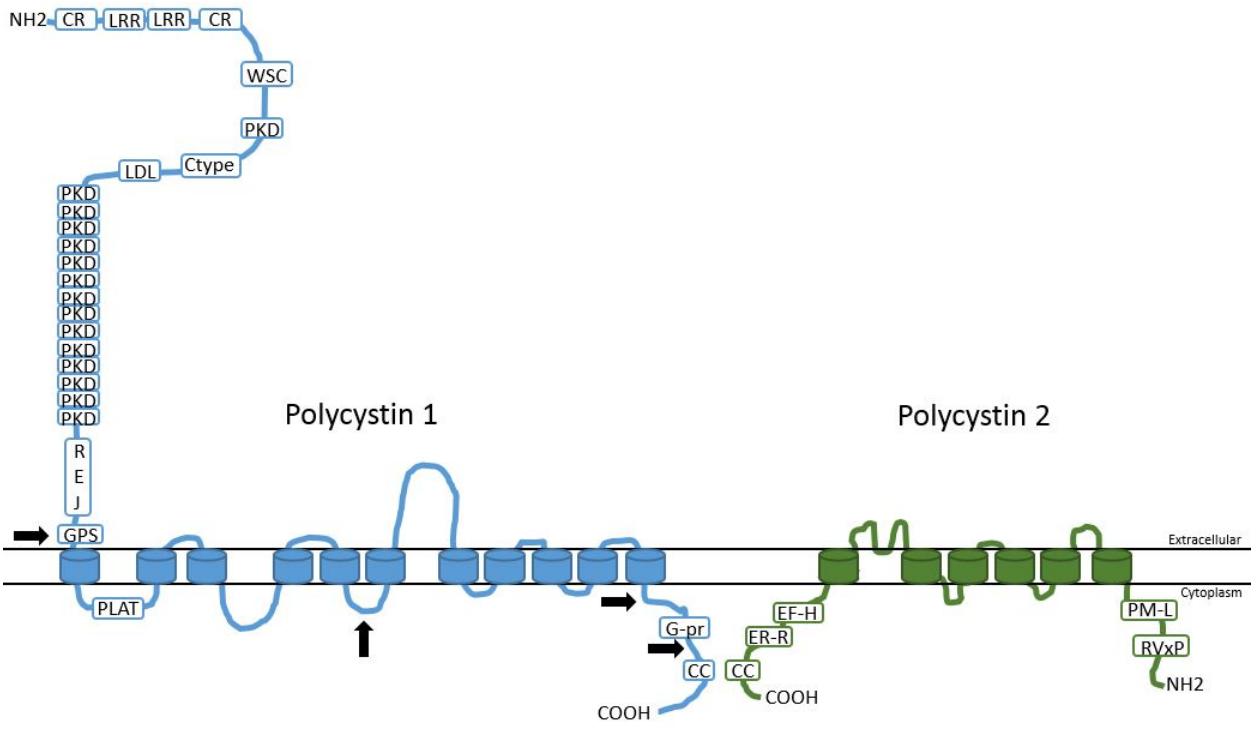

Figure 1.1. The domains of the polycystin proteins. CR, cysteine-rich region; LRR, leucine-rich repeat; WSC, wall-stress component domain; Ctype, C-type lectin domain; LDL, low-density lipoprotein domain; REJ, receptor egg jelly domain; GPS, G-proteolytic site; PLAT, polycystin-lipoxygenase-alpha-toxin domain; G-pr, G-protein binding domain; CC, coiled-coil domain; ER-R, ER-retention signal; EF-H, EF-hand domain; PM-L, phosphorylation site which regulates PM localization of PC2; RVxP, may aid PC2 trafficking to the cilia.

The structure of PC2 is simple in comparison to PC1, as shown in Figure 1.1. PC2 is an integral transmembrane protein with 6 transmembrane regions, beginning and ending with intracellular N- and C-termini [116]. Both the N- and C-terminals of PC2 contain dimerization domains, which according to one model, leads to the formation of PC2 homotetramers, although it is also predicted that PC2 forms heterotetramers with other TRP proteins as well [182]. In fact, PC2 has been observed to interact with several TRP family members, including TRPC1, TRPV4, and PC1 [146, 147]. The N-terminal segment of PC2 is also important for its location within the

cell, as it contains a RVxP amino acid motif which allows PC2 to traffic to the cilia independently of PC1 [173]. This N-terminal portion of PC2 also contains a phosphorylation site (Ser-76) which directs PC2 localization from the plasma membrane to the endoplasmic reticulum [183].

The transmembrane region of PC2 creates a nonselective cation channel, permeable to Ca^{2+}, Na^{+}, and K^{+} ions [14]. The conducting pore of PC2 is likely formed by the loop between the fifth and sixth transmembrane domains with some involvement of the third transmembrane domain [184, 185]. A missense mutation that perturbs this putative conducting pore is causative of ADPKD [185].

Immediately distal to the last transmembrane domain of PC2 is a functionally complex region that includes an EF-hand domain containing one calcium binding site [186, 187], an ER retention domain required for maintaining PC2 in the ER and Golgi [188], and a coiled-coil domain, which is necessary for the interaction with PC1 [16]. The coiled-coil domain of PC2 is also important for interaction with other cellular calcium channels [16, 189-192] as well as other interacting protein partners [193-195]. The deletion of the coiled-coil domain renders the channel properties of PC2 inactive [196].

The Polycystin Cation Channel

Despite the permeability of PC2 to Ca^{2+}, Na^{+}, and K^{+} ions [14], most studies have solely focused on misregulation of calcium levels in response to polycystin alterations. Cystic epithelial cells have constitutively lower intracellular calcium than cells derived from normal tubular epithelia [197-199]. The decreased intracellular calcium ($[Ca^{2+}]_i$) of cystic epithelial cells, alters the way cells respond to stimuli. For instance, ADPKD cells activate B-RAF in response to cAMP,

while normal collecting duct cells do not, and this response can be inhibited by pharmacologically increasing $[Ca^{2+}]_i$ in ADPKD cells [197, 198]. Other manipulations have underlined the importance of alterations in $[Ca^{2+}]_i$ to cyst growth. For example, the size of cysts could be decreased by the pharmacological increase of $[Ca^{2+}]_i$ in metanephric organ culture [200]. Conversely, the knockdown of PC2 in 3D microcyst cultures, presumably decreasing $[Ca^{2+}]_i$, supports cyst growth [199].

The subcellular localization of the PC1/2 complex is still contentious. PC2 is widely expressed in either the endoplasmic reticulum, where perhaps 99% of the protein resides [185, 188] or in the primary cilia, a compartment which PC2 reaches through a highly regulated trafficking mechanism [172, 201]. In some studies a small percentage of PC2 can be isolated with the plasma membrane fraction of epithelial cells [17, 183]. The ability of the PC1/2 complex to bring calcium into the cell is maintained while localized on the plasma membrane [14], or on the cilia [56]. As mentioned previously, in the cilium, PC2 forms a complex with the mechanosensitive PC1, and is theorized to convert shear forces on the cilium membrane into ciliary calcium influxes. These influxes could further induce the release of calcium from intracellular stores through the calcium-induced calcium response [56, 59]. In support of this theory, loss of ciliary polycystins leads to defective calcium transients in response to several stimuli, such as fluid flow, angiotensin, and vasopressin [169]. However, recent studies have suggested that the ciliary PC1/2 complex is not responsible for fluid-flow mediated calcium influx [62, 202]. Despite these new observations, ciliary localization of PC1/2 must still play some role in cystic progression, however, as ablation of cilia in PC1- or PC2-deficient cells reduces cyst growth [203]. Perhaps in future studies, ciliary PC1/2 will be found to mediate cyst growth via influx of Na^+ or K^+, or via mechanically mediated PC1-CTT cleavage.

Whether the PC1/2 complex does or does not bring in calcium into the cell in response to fluid flow, the bulk of PC2 appears to be present in the endoplasmic reticulum to mediate release of calcium from intracellular stores [185] in response to local increases in intracellular calcium [204]. Additionally, PC2 has been suggested to act as an ER calcium leak channel [185]; such leak channels determine the steady-state levels of ER luminal calcium concentration (reviewed in [205]). PC2 can also indirectly regulate cellular calcium by interacting with two ER calcium channels. In cardiomyocytes, PC2 inhibits ryanodine receptors (RyR) by binding and decreasing RyR conductance [190]. PC2 is also able to bind inositol triphosphate receptors (IP_3R) and modify IP_3-induced calcium influx [189, 206]. Additionally, PC1 on the plasma membrane may still interact with PC2 on the ER [10] and indeed, PC1 may also help position the ER to facilitate an interaction between PC2 localized at the ER and molecules at the cell surface [185, 207, 208]. In general, overexpression of PC2 results in decreased ER calcium concentration, while the opposite occurs with PC1 overexpression [209-212].

The open probability of the PC2 channel is regulated by $[Ca^{2+}]_i$ in a bell-shaped curve, with highest PC2 activity at 0.1 uM $[Ca^{2+}]_i$ and comparably less activity at both 0.001 uM and 10 uM $[Ca^{2+}]_i$ [185]. The calcium-dependent activity of the PC2 channel can also be affected by direct interactions at the C-terminal tail of PC2 with a number of protein partners, including PC1 [16, 17], actin binding proteins [213], IP_3R [189, 192], and RyR [190]. The deletion of the coiled-coil renders PC2 inactive and not responsive to alterations in calcium levels [196]. Additionally, PC2 activity can be altered by phosphorylation by casein kinase II [214] or by high levels of PI(4,5)P2 in the membrane [215]. The importance of the ionic function of the PC1/2 complex is undeniable, however, the polycystin complex appears to have additional roles important during both

development and wound-healing processes which involve cell structure, polarity, migration, and EMT-related signaling.

The Polycystin Structural Complex

The PC1/2 complex interacts with many cytoskeletal structural components. In embryonic tubular epithelial cells, PC1 has a basolateral distribution [130, 135, 150, 216-220], where it has been localized to adherens junctions [217]. At these junctions, PC1 associates with E-cadherin as well as α-, β-, and γ-catenins [217, 221]. In renal epithelial cells from ADPKD patients, both PC1 and E-cadherin are depleted from the plasma membrane, while N-cadherin is upregulated in an attempt to maintain the structure of adherens junctions [222]. PC1 also localizes to desmosomes [223, 224], and a severe mispolarization of desmosomal elements has been observed in cells from ADPKD cysts [225, 226]. The carboxy-terminus of PC1 binds vimentin, cytokeratins and desmin [224]. Additionally PC1 binds to extracellular-structural components. For instance, the LRR domain of PC1 binds to collagen, fibronectin, and laminin [227]. Indeed, the PKD domains of PC1 itself are proposed to from homophilic interactions with other PC1 molecules on neighboring cells [151]. Therefore, not surprisingly, the intercellular adhesions between diseased cells are significantly more fragile and more sensitive to shear stress than normal renal cells [226].

The other polycystin, PC2, also co-localizes to cell-cell contacts [228], and interacts with tropomyosin-I, a protein involved in the stabilization of the actin cytoskeleton [229]. However, the interaction of PC2 with the cytoskeleton is likely to be important for its own stabilization and activity [230]. Thus PC2 needs to bind to actin [231], α-actinin [213, 232] and microtubules [231, 233] for activity, while binding to filamin-A inhibits PC2 activity [142, 195, 213].

The interaction of PC1/2 with cytoskeletal and transcriptional pathways is important for the facilitation of tubulogenesis during development [234, 235]. While normal tubular epithelial cells assemble into a tubule-like structure when suspended in a collagen-matrigel mixture, cells lacking either PC1 or PC2 form cyst-like structures [236, 237]. The PC1 C-terminus is important for this role of the PC1/2 complex, as overexpression of it in murine inner medullary collecting duct (mIMCD3) cells can induce tubulogenesis, while expression of a truncation mutation lacking the coiled-coil domain of PC1 abrogated this affect [234].

Three overarching mechanisms may account for PC1/2-mediated tubulogenesis. First, the functional PC1/2 complex has a role in cell migration. Overexpression of full length PC1 in MDCK cells results in a strong migratory effect when the cells are challenged by wound healing [238]. This ability to induce migration was found to occur through G protein-mediated activation of the PI3K/AKT pathway [238]. The other polycystin, PC2, has been observed to localize to the lamellipodia of migrating cells where it interacts with Hax-1 [239], a protein which promotes migration by interacting with cortactin and Rac, two ARP2/3 complex regulators [240, 241]. Additionally, PC2 also associates with CD2AP, an adapter protein that regulates the assembly of focal adhesion complexes [242], however the functional consequences of these interactions with PC2 have not been fully investigated.

Second, the PC1/2 complex may affect tubulogenesis through its role in the proper polarization of renal tubular cells. During tubulogenesis, cells undergo convergent extension, in which the tissue of an embryo is restructured along one axis and elongated along a perpendicular axis [243]. PC1 and its interaction with the polarity protein Par3 are necessary for convergent extension [243]. This interaction between PC1 and Par3 may also be important for the establishment of front-rear polarity during cell migration [243], which is defective in PC1-null

cells [244-246]. PC1 can affect cell polarity in yet another way, through the regulation of microtubule stability and dynamics. This effect is important for the regulation of the turnover rates of focal adhesion and ultimately impacts cell migratory rates and adhesive properties [247]. This dynamic regulation of microtubules is essential in PC1-mediated cell-orientation during migration [247]. In addition, polarized trafficking, which is necessary for proper apical-basal polarity, is also abnormal in ADPKD. Abnormalities in the exocyst complex, which is a docking factor for vesicles and is responsible for polarized trafficking, have been reported in ADPKD [248].

Finally, the polycystins mediate transcriptional pathways important in both development and wound-induced epithelial-to-mesenchymal transition (EMT). Overexpression of full-length PC1 activates STAT1 and STAT3 via increased phosphorylation of JAK2 [249]. The effect requires the presence of the coiled-coil domain in PC1 [249]. Further studies corroborated that full length PC1 can bind JAK2, which leads to the phosphorylation and activation of STAT3 [138]. Indeed, tyrosine phosphorylation of STAT3 is abundant in cystic cells [138, 250]. Additionally, cleaved PC1 CTT can co-activate STAT3 in a JAK-independent manner, by direct interaction with STAT3 [138]. This 30 kDa fragment of PC1 CTT was further found to enhance STAT3 activation, and even allow STAT3 to be insensitive to its endogenous inhibitor, SOCS3, via a PC1-CTT interaction with SRC [251]. The WNT pathway is also important in ADPKD. Several WNTs have recently been found to be ligands for PC1 and to activate PC2-mediated calcium influx when bound [61]. Indeed, the persistent activation of β-catenin-mediated transcription causes renal cyst formation in transgenic mouse models [252, 253]. Increased stabilization of β-catenin results in cystogenesis in both *PKD1* and *PKD2* mutants. Thus, in PC2-null cells, β-catenin levels are increased [254], and WNT/β-catenin-mediated pathways are upregulated in *PKD1* mutants, as determined by gene expression profiles [255]. Alternatively, MDCK cells engineered to

overexpress full-length PC1 have a more efficient turnover of β-catenin at adherens junctions, and this results in reduced mechanical strength at cell-cell adhesion [238]. The increased turnover of β-catenin was found to be due to increased activity of GSK3β induced by PC1 overexpression [238]. These structural roles of the polycystin complex are expected to play an important role in tubulogenesis as well as in response to renal injury. Another aspect of polycystin expression important in both renal injury and ADPKD is the induction of apoptosis.

The Polycystins and Apoptosis

ADPKD progression occurs relatively slowly throughout the lifetime of the affected individual, with GFR not decreasing below normal levels until around age 40 [33, 256], despite cysts having formed before birth [30, 31]. This slow disease progression is difficult to explain in an ailment characterized by increased cell proliferation. Interestingly, renal cyst formation is coupled to increased rates of apoptosis in models of several cystic diseases, including ADPKD [140, 257-259], ARPKD [258, 260, 261], and nephronophthisis [258]. Furthermore, cystogenesis has been found to be attenuated when apoptosis is inhibited pharmacologically [262]. It has been proposed that in the ADPKD cyst epithelium, the balance between cell proliferation and cell death is perturbed, and that loss of some tubular cystic cells by apoptosis may even stimulate proliferation in the surrounding cells [257]. Thus, the aberrant increase in programmed cell death of the cystic epithelium could be a mechanism that influences progression of ADPKD [67, 263].

Direct manipulations of PC1/2 protein expression has found that sensitivity to apoptosis is altered by polycystins. PC1-null arterial myocytes that are subjected to mechanical stress undergo more cell death than cells expressing even just one allele of *PKD1* [264]. Lack of mechanical

stimulation inhibits cleavage of the anti-cystogenic 35 kDa CTT of PC1, which increases CHOP-mediated cell death [177]. Conversely, apoptosis is prevented in MDCK cells when full length PC1 is expressed, via activation of the PI3K/AKT pathway [235, 238]. PC2 also has been found to play a role in apoptosis. Functional PC2, via its purported role as an ER calcium release channel, can decrease cell sensitivity to apoptotic stimuli by lowering ER calcium [211]. Thus, several pathways exist by which dysfunctional PC1/2 proteins can lead to apoptosis, a cell death program which plays a definite, yet not clearly understood role in ADPKD progression.

In summary, ADPKD cysts arise *in utero* during the particular developmental phase in which PC1/2 expression occurs for proper tubule formation. The pathophysiology of ADPKD renal cysts involves a complex cellular phenotype that includes increased proliferation, increased apoptosis, increased fluid secretion, altered apicobasal polarity, extracellular matrix abnormalities, changed calcium management and alterations to cilia or centrosomal fidelity [265, 266]. Cells lacking proper expression of PC1/2 protein products contain abnormal intracellular calcium concentrations, are mispolarized in several respects, and have altered sensitivity to apoptosis. These characteristics of PC1/2 mutants are instrumental in both cystogenesis and in the continued growth of the cysts. Intriguingly, cysts grow at varying rates. While genetic background does play a role in this variation, it cannot account for the discrepancy between ESRD onset entirely.

THE NA,K-ATPASE

Most animal cells maintain high intracellular concentrations of K^+ and low cytoplasmic levels of Na^+ compared with the extracellular milieu. This observation puzzled researchers, who

started looking for the mechanisms responsible for the transmembrane ion differences of cells (reviewed in [267]). In 1957, Skou discovered that the Na,K-ATPase (NKA) is the plasma membrane enzyme responsible for the countertransport of Na^+ for K^+ across the cell plasma membrane [268]. NKA uses the energy of the hydrolysis of ATP to transport 3 Na^+ out in exchange for 2 K^+ that are brought into the cell [269]. The ionic gradients generated by the NKA contribute to creating the resting membrane potential of most cells and allows the generation of an action potential in excitable cells of the nervous system and muscles [270, 271]. In the kidney, NKA plays an important role in the reabsorption of sodium, water and various vital solutes (glucose, amino acids), which are linked to sodium transport [272]. NKA ensures basic cellular homeostasis in all cells, but it also contributes to the specialized roles of tissues. In heart and skeletal muscle cells, the activity of NKA, tightly coupled to the sodium-calcium exchanger, contributes to the control of intracellular calcium and contractility of the cells [273, 274]. In nerve and glial cells, NKA aids in the restoration of ion gradients after an action potential is triggered, to reestablish the basal transmembrane ion gradients of cells [270, 271]. Other roles of NKA derive from its functional association with other channels, including ATP-sensitive K^+ channels [275, 276], glutamate transporters [277] and BK_{Ca} channels [278].

Structure

The NKA is a protein complex composed of an α, β, and γ subunit ([279-281], and Figure 1.2) in a 1:1:1 stoichiometric ratio [282]. While the α and β subunits are essential for ATPase activity [283, 284], the γ subunit is an accessory protein which regulates NKA activity, but is not essential for NKA function [285]. The α and β subunits are synthesized and assembled

independently in the ER, from which they are trafficked to the cell surface. Only assembled α-β subunits are exported out of the ER [286]. In the absence of the β subunit, α-NKA is rapidly degraded [287].

Figure 1.2

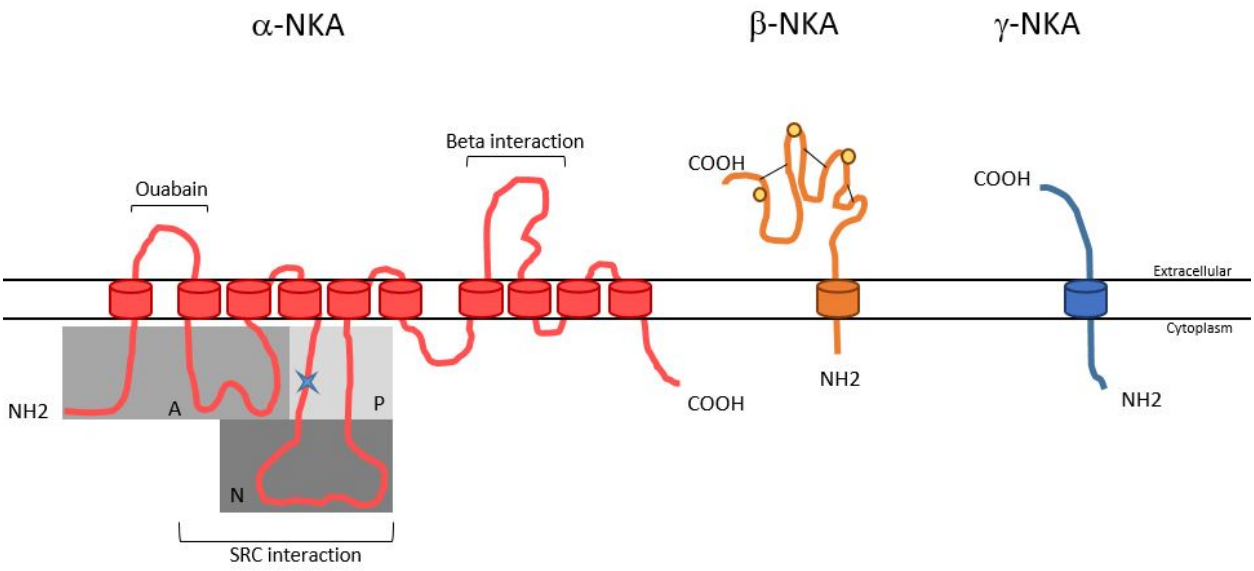

Figure 1.2. The domains of the Na,K-ATPase. On the α subunit: A, actuator domain; N. nucleotide binding domain; P. phosphorylation domain. Blue star denotes the Asp371 to which the terminal phosphate of ATP is attached during the catalytic cycle. On the β subunit: Yellow circles denote N-glycosylation sites and thin black lines denote the relative location of disulfide bridges. The γ subunit is drawn to represent FXYD2.

α-NKA is considered the catalytic subunit of the complex. It has a molecular mass of 110 kDa and contains the binding sites for Na^+ and K^+ and ATP. As shown in Figure 1.2, it is composed of ten transmembrane helices as well as three cytoplasmic domains: the nucleotide binding domain (N), the phosphorylation domain (P), and an actuator domain (A) [288], which is involved in the transmission of conformational changes required for the movement of the ions [289]. The N and P domains are formed by the loop between TM4 and TM5 while the A domain is formed by the

N-terminal tail and the TM2-TM3 loop [289]. Another important structural feature of the α-NKA include the extracellular loop between TM7 and TM8, which provides contact sites between the α and β subunits [290]. Additionally, a caveolin binding domain, present in the α-NKA N-terminus, allows the localization of NKA to caveolae via association with caveolin-1 [291]. Four isoforms of α-NKA exist in mammalian cells (α1, α2, α3 and α4), which share an overall identity of ~80% [292]. α1 is ubiquitously expressed and is the only α subunit present in the kidney [293-295]. The α2 isoform is expressed in skeletal muscle, smooth muscle, heart, brain, lung, and adipocytes [294, 296, 297]. The α3 isoform is expressed in neurons, ovaries, and adult human heart [279, 281, 297]. Expression of α4 is limited to male germ cells of the testis and sperm [298-305].

The β-NKA subunit, is a 36 kDa type II membrane protein. It has a small N-terminal cytoplasmic domain, one transmembrane helix, and a large, highly glycosylated, extracellular domain [281]. β-NKA enhances the rate of synthesis, half-life, trafficking, and membrane stabilization of α-NKA [287, 306-308]. The presence of the β-NKA is required for the maturation and insertion of the NKA complex into the plasma membrane [294, 309, 310]. Three disulfide bridges are also present in the β-NKA extracellular domain, which are important for the targeting of the NKA complex to the plasma membrane [283, 311]. The disulfide bridges also have some role in α-NKA catalytic activity, as their disruption decreases the binding and occlusion of K^+ by the NKA [310]. Finally, the glycosylation of the extracellular domain of β-NKA has little effect on NKA catalytic activity [312] or on its delivery to the plasma membrane [313], but glycosylation of β-NKA does affect the structural role of NKA (see the section "Ouabain as an effector of EMT"). Three isoforms of β-NKA exist (β1, β2 and β3), which share 39-48% sequence homology [314, 315]. All three β isoforms can associate with all four α isoforms [316-319]. Similar to α-

NKA, the β subunits are differentially expressed by tissues. β1 is expressed ubiquitously, β2 is observed in the heart, skeletal muscles, and glial tissue, and β3 is found in testes, retina, liver, and lung (reviewed in [320]). The cation affinity of each α-NKA is affected by its association with different β subunits [316-318, 321]. This allows for the fine-tuned control of NKA in each specific tissue. In the normal kidney, as well as in ADPKD cysts, the only isoforms expressed at significant levels are α1 and β1 [322].

The γ subunit (or FXYD2) is an accessory protein that is not required for NKA catalytic activity [323]. This subunit was at first found in only renal tissue [324]. Since then, at least 6 other isoforms have been identified and grouped together into a family of proteins, characterized by a distinct amino acid motif, "FXYD" (reviewed in [325]). These are all small (7.5 - 19 kDa) single-span membrane proteins [285, 326]. Members of the FXYD family are also expressed in a tissue specific manner [285, 327, 328], and they are more abundant in tissues that are either involved in fluid transport or are electrically excitable [326]. Specifically, FXYD1 is present in heart and skeletal muscle [329, 330], FXYD3 has been detected in uterus, stomach, colon and skin [331, 332]. FXYD5 is not well characterized but is upregulated in several cancer tissues [333], while FXYD6 and FXYD7 have roles in the brain (reviewed in [334, 335]). In the kidney, FXYD2 and FXYD4 are present [336, 337]. FXYD4 induces a 2-3 fold increase in the affinity of the NKA to Na^+ [338, 339]. It has therefore been suggested that in conditions of systemic Na^+ depletion, the presence of a FXYD4-NKA complex will favor efficient Na^+ reabsorption [338]. Indeed FXYD4 expression is increased by a low salt diet [337]. The expression status of FXYD4 in ADPKD is unknown, however FXYD2 expression is known to be increased in ADPKD cells [322]. FXYD2 increases the affinity of α-NKA for ATP [340, 341] and expression of FXYD2 is upregulated in the kidney by increased hypertonicity, or high salt [342, 343]. Interestingly, collecting duct cells

subjected to high hypertonicity, a condition which induces FYXD2 expression, became more ouabain-sensitive [342], and elevated ouabain-sensitivity is also observed in ADPKD cells [322].

The NKA Catalytic Cycle

The NKA is a member of the P-type ATPase family. This distinction refers to the intermediate formed during ATP hydrolysis in which the terminal phosphate of ATP is attached to α-NKA via an aspartate residue (Asp371) [344, 345]. The active transport of Na^+ and K^+ ions by NKA occurs at a fixed stoichiometry (3:2, respectively) and this transport is accomplished through a continuous cycling of the enzyme through different conformations [346, 347]. Two main NKA conformations exist: the sodium-bound E1 state and the potassium-bound E2 state [281]. As illustrated in Figure 1.3, the entire catalytic cycle is described as follows: The E1 form has cation binding sites that face the cell cytoplasm and shows high affinity for Na^+. Intracellular Na^+ ions bind to α-NKA and catalyze the phosphorylation of NKA by a

Figure 1.3.

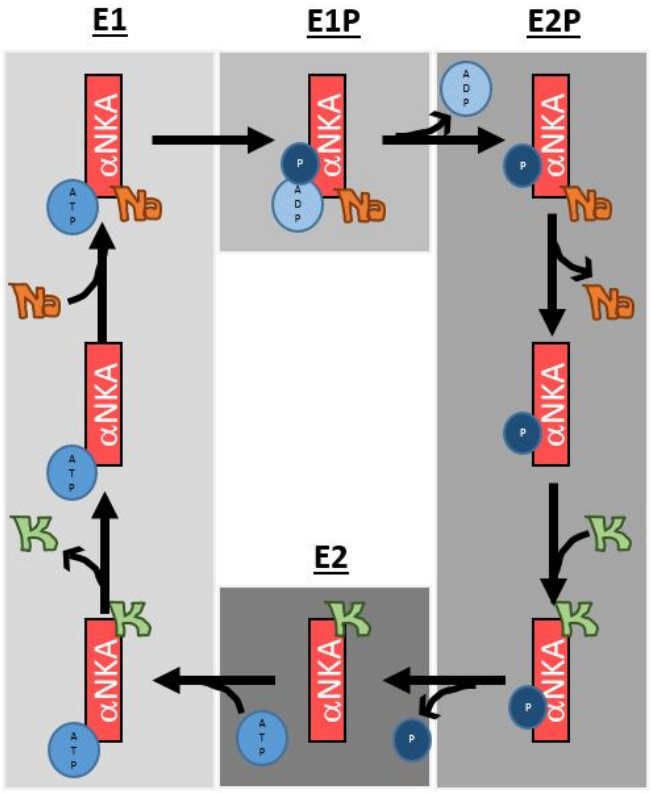

Figure 1.3. The catalytic cycle of the Na,K-ATPase. Shaded areas denote each particular conformation of the α-NKA. Ouabain binds when the enzyme is in E2P and SRC binds when in the E1 conformation. ATP and Na^+ binds to the intracellular portion of NKA while K^+ binds to the extracellular portion.

previously bound ATP molecule. The Na^+-catalyzed phosphorylation produces a conformation called E1P. Then, ADP leaves the enzyme, and the 3 Na^+ ions bound to the enzyme become occluded within α-NKA [348]. Another conformational transition occurs as the enzyme moves into the E2 conformation, losing affinity and releasing Na^+ ions to the extracellular side. This conformation also has a high affinity for K^+ ions, which become bound from the extracellular surface. The β subunit helps α-NKA to trap K^+ in a pocket [349]. The binding of 2 K^+ ions results in the dephosphorylation of NKA, and P_i is released at the cytoplasmic surface. At this point, the 2 K^+ ions are occluded. Then ATP binds to the ATP regulatory site in α-NKA, causing α-NKA to return from the E2 to the E1 form, driven by the difference in binding energy for ATP. The E2 conformation has a low affinity to K^+ ions, and therefore the K^+ ions are released into the cytoplasm. The NKA enzyme is then ready to initiate a new catalytic cycle [281].

Subcellular localization of NKA

In epithelial cells, the NKA has two different but integrated roles. The first, as previously discussed, is the translocation of ions across the plasma membrane [268]. The second role involves the movement of ions and solutes across the entire epithelium [350]. The NKA resides exclusively in the basolateral surface of most epithelial cells [270, 351, 352]. It is this polarized localization of NKA, and that of other co- and countertransporters, which drives the net transport of solutes across the whole epithelium [353, 354]. The targeting of NKA to the basolateral membrane may simply be attained due to the lack of a specific signal on NKA typically needed to join membrane rafts headed to the apical membrane during sorting in the Golgi [355]. Mislocalization of 25% of NKA has been described in some ADPKD models [356-358], but not consistently [359]. In the

ADPKD cells used in this lab, NKA is commonly found at its proper place in the basolateral membrane [322]. Thus, in the normal kidney, the exclusive localization of NKA in the basolateral membrane is the driving force for the Na^+ reabsorption essential to maintain extracellular volume and blood pressure [272]. Meanwhile, in the ADPKD cystic epithelium, the basolateral localization of NKA helps drive fluid secretion into the cyst lumen [360, 361], promoting cyst growth.

Mechanisms of NKA regulation

The activity of the NKA is essential for the function of each cell. However, both the expression and activity of NKA can be regulated at multiple levels to fine tune responses to stimuli or to cater to a cell's needs. Numerous mechanisms are involved in this regulation of NKA to adapt to changing physiological demands. These mechanisms can be broadly placed into 4 groups. 1) Any change in intracellular sodium or ATP affects the NKA activity, as both are limiting factors for the catalytic cycle of NKA (reviewed in [362]). 2) Peptide hormones or neurotransmitters cause the phosphorylation of NKA by protein kinases, such as PKA or PKC [363], which modulates NKA cell surface expression [324]. 3) NKA can also be regulated by glutathionylation and palmitoylation of cysteine residues [364, 365]. Thus, oxidant stress can cause the modification of NKA thiols, which leads to NKA inhibition [366, 367]. 4) Steroid hormones, like aldosterone, can affect α-NKA and β-NKA gene transcription, leading to an increased number of NKA pump units [295]. Indeed, several hormones are involved in the regulation of the NKA [295, 368, 369], the most important of which, for this thesis at least, is the hormone ouabain.

OUABAIN

Ouabain is a steroid derivative [284] which has a high specificity for binding to the NKA [370]. Ouabain binds directly to the extracellular regions of the α-NKA subunit, between the transmembrane helices TM1 and TM2 [370], an interaction which does not involve β-NKA [371]. In particular, ouabain selectively binds to E2P conformation of α-NKA [372, 373], and inhibits the pumping abilities of NKA [374-376]. Depending on the species, the affinity of ouabain for α-NKA varies among the α-NKA isoforms. In rodents, α1 has a very low affinity for ouabain (with an inhibition constant (K_i) of ~10^{-5} M), while the other isoforms are progressively more sensitive (K_i of ~10^{-7} and 10^{-8} M for α2 and α3, respectively), while the α4 isoform has the highest ouabain affinity ((K_i of ~10^{-9} M). In contrast, in humans, α-NKA isoform affinities for ouabain are more similar, mainly due to α1 being much more sensitive to ouabain than its rodent homolog [377]. The structural basis for the isoform specific sensitivity to ouabain has been found to reside, at least in part, in the first extracellular domain of the α-NKA. Specifically, ouabain binding depends on two positively charged amino acids located at the boundary of TM1 and TM2 with the extracellular loop that connects them, whereas the other α-NKA remain highly sensitive [378, 379].

Ouabain Synthesis and Regulation

Ouabain is synthesized and released from the adrenal glands, similar to other steroid hormones [380]. This conclusion was reached by several observations. First, cultured bovine adrenal cells were found to secrete ouabain [380]. High concentrations of ouabain were reported in the adrenals of several species [380, 381]. Second, adrenalectomy can decrease levels of ouabain in blood [380, 382]. Interestingly, adrenal cortex tumors have been observed to overproduce and

secrete ouabain [383], and patients with these tumors have abnormally high ouabain plasma levels. Ouabain levels were normalized in those patients after removal of the adenomas [383-385]. Third, the administration of anti-ouabain antibodies to rats produces adrenal cortex enlargement, further implicating the adrenal gland as a source of ouabain [386]. In addition to production by the adrenal glands, ouabain can also be produced by other tissues, such as the hypothalamus [387, 388].

The synthesis of ouabain is believed to follow a pathway similar to that of aldosterone. This is supported by the observed increase in secretion of ouabain by administration of the biosynthetic precursors of aldosterone: hydroxycholesterol, pregnenolone, and progesterone [389, 390]. Inhibition of 11 β-hydroxylase, the pen-ultimate enzyme in the aldosterone synthesis pathway, prevented the secretion of both aldosterone and ouabain [391], further implicating a similar synthesis mechanism. In addition, the molecular mechanisms which induce ouabain synthesis appear to be related to molecules involved in blood pressure regulation (illustrated in Fig 1.4). Adrenocorticotropic hormone (ACTH), which stimulates production of steroid hormones in adrenal cortex cells, also increases ouabain production [392, 393]. ACTH administration also increases blood pressure, and this event is abrogated in mice expressing a ouabain-resistant form of the α2-NKA isoform [394], and exacerbated in mice expressing ouabain-sensitive α1-NKA isoform [395]. Ouabain synthesis is also increased by angiotensin II, an effector of the RAAS pathway which causes vasoconstriction and Na^+ reabsorption [385, 388, 396, 397]. Other molecules which can increase ouabain expression include vasopressin, phenylephrine, and epinephrine [385, 392], which all cause vasoconstriction and increased blood pressure. In general, ouabain concentrations detected in serum can range between 0.1 - 20 nM [380, 398-405].

Figure 1.4

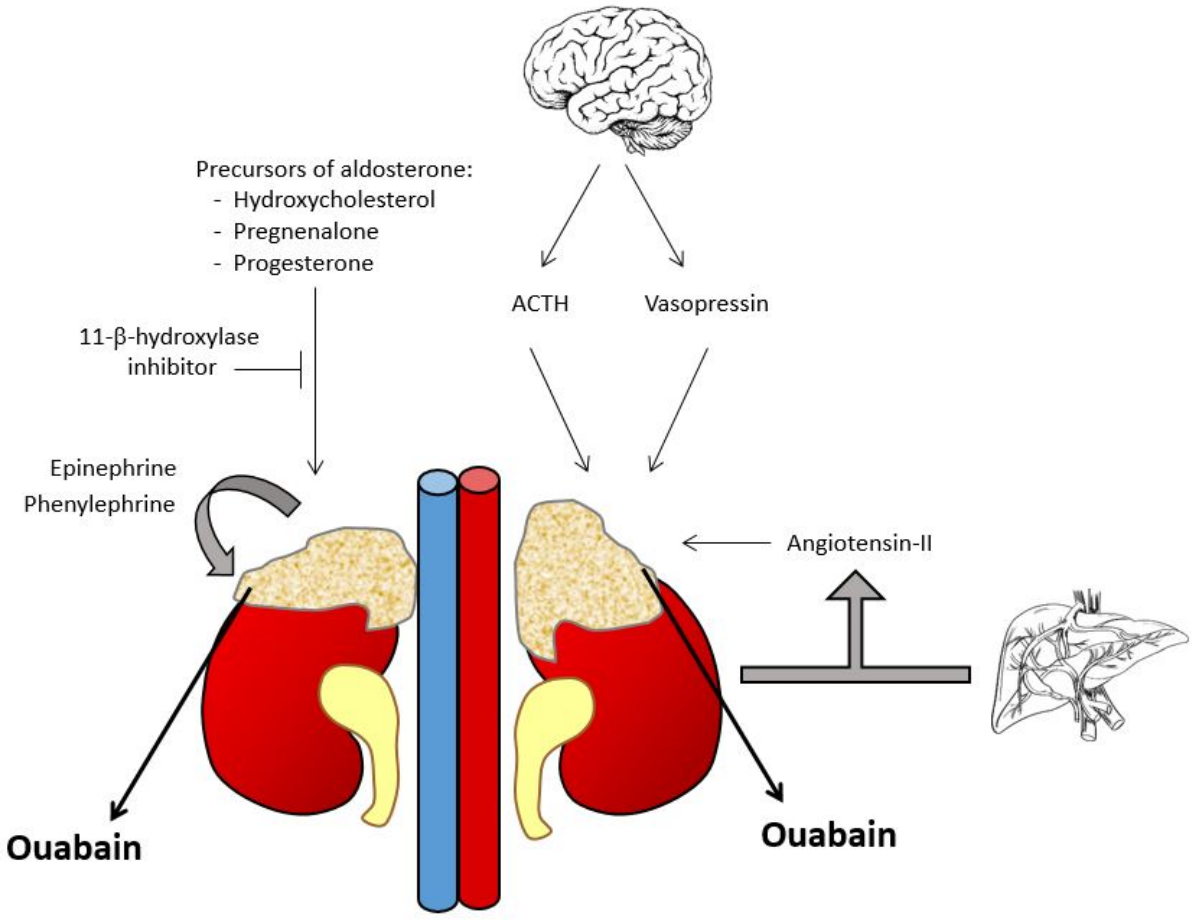

Figure 1.4. Known stimuli of ouabain synthesis by the adrenal glands. Precursors of aldosterone synthesis increase ouabain concentrations; this effect can be inhibited by 11-β-hydroxylase inhibition. Epinephrine, produced by the adrenal glands, and its synthetic mimetic, phenylephrine, can increase plasma ouabain. ACTH and Vasopressin, secreted from the brain, increase ouabain concentrations. Angiotensin-II which is a joint product of enzymes in the liver and kidney, also increases plasma ouabain.

Elevated ouabain concentrations are particularly prominent in states of volume expansion. Four physiological conditions have so far been observed to result in increased levels of ouabain. 1) Salt-loading increases plasma ouabain concentrations in both humans and rats [406-408]. Excessive salt intake is well known to cause volume expansion [287, 409]. 2) High circulating levels of ouabain also occur in pregnancy [410], and pregnancy is associated with plasma volume expansion as a result of renal Na^+ and fluid retention [411]. 3) Exercise also induces blood volume

expansion, increasing volume 10-12% within 24 h of exercise [412]. Elevated plasma ouabain after exercise has been recorded in both humans and dogs [413]. Additionally, physical exercise is associated with increased epinephrine and norepinephrine [414], which augments the synthesis of ouabain, as mentioned previously. 4) Importantly, renal stress increases ouabain levels. Following nephrectomy, serum levels of ouabain are substantially elevated [415]. Nephrectomy can cause elevations in blood pressure, as well as diastolic dysfunction, ventricular hypertrophy, and cardiac fibrosis [405, 416, 417]. These symptoms can also be observed in ESRD, another condition in which patient serum levels of ouabain are substantially elevated [418, 419]. In fact, plasma ouabain levels have been suggested as a biomarker to predict acute kidney injury after cardiac surgery [420, 421]. Additionally, as progressive congestive heart failure is often associated with a rapid fall in renal functions (reviewed in [422]), it is unsurprising that heart failure patients were observed to have nearly a 4-fold increase in mean plasma ouabain concentrations compared to healthy controls [423].

Ouabain targets the heart and vasculature

The ionic gradients generated by NKA are important to maintain membrane potential excitability in cells like myocytes [272]. Ouabain, as well as other structurally similar molecules capable of inhibiting NKA, are called cardiac glycosides due to their ability to increase heart contractility and cardiac output. For this reason ouabain is considered a cardiotonic steroid. Myogenic tone is controlled by NKA activity via the functional relationship that exists between NKA, and the sodium-calcium exchanger (NCX) [273, 274]. The NCX is the major extrusion mechanism for the calcium in cardiac cells. The removal of calcium is necessary for muscle relaxation between beats and for cellular calcium balance [424]. Ouabain, by inhibiting NKA,

causes intracellular Na^+ levels to rise, which reduces the gradient necessary for NCX to pump calcium out of the cell. The NCX and NKA exist together in a complex with the cytoskeletal protein ankyrin-B [425], and thus local, subcellular increases in Na^+ are enough to mediate this process.

The increased intracellular calcium caused by ouabain and other cardiotonic steroids enhances the ability of myocytes to contract. Since the 18th century, cardiac glycosides have been prescribed for congestive heart failure, in the form of herbal remedies, such as foxglove [426]. Later, cardiac glycosides were isolated from these natural sources and used medically until they were set aside for safer alternatives, although in some countries they are still being used [426, 427]. The main isoforms of the NKA in cardiac and vascular smooth muscle cells are α1 and α2 [428-430]. The α2 subunit has been suggested to play a specific subcellular role in the cardiotonic effect, while the α1 subunit may play more of a housekeeping role in regulating bulk cytoplasmic Na^+ [394]. In support for the functional linkage of α2-NKA to NCX, mice heterozygous for the α2 isoform have a hyper-contractile phenotype as a result of increased calcium transients, while α1 heterozygotes were hypocontractile [431].

The cells of the vasculature are also a target of ouabain effects, via the same mechanisms described for the heart. Contraction of the vasculature by ouabain results in increased peripheral vascular resistance and higher blood pressure, and thus the infusion of ouabain is observed to increase blood pressure [432, 433]. Other situations have also revealed the importance of the activity of NKA in the vasculature in regulating blood pressure. For instance, NKA overexpression in mouse vascular smooth muscle, caused the opposite effect of ouabain administration, a decrease in blood pressure [434]. In cardiac hypertrophy, which is an adaptive response to high blood pressure, NKA active ty was observed to be reduced [435-438]. Finally, chronic treatment with

ouabain induces the upregulation of the arterial expression of the NCX1, SERCA2, and TRPC6 and so may contribute to arterial remodeling and thus lead to established hypertension [439-441].

Ouabain targets the kidney

One of the most essential functions of the kidney is the maintenance of fluid and salt balance. The mechanisms in place for this balance require the function of the NKA. For instance, under systemic excess of K^+, basolateral NKA activity is required to provide the necessary gradient to move K^+ across the renal epithelium in order for its secretion [442]. Under low Na^+, the kidney maintains Na^+ blood plasma levels by increasing Na^+ reabsorption in the renal tubules. This depends on the activity of NKA, which moves Na^+ across the basolateral side of the tubular cells into the interstitium, generating a Na^+ gradient which allows the apical transport of Na^+ via Na^+-dependent channels.

Ouabain is especially important for body fluid homeostasis in response to volume expansion. Low volume and low sodium is mainly handled by RAAS activation, resulting in increased volume retention and increased Na^+ reabsorption by the kidney [102]. While the activation of RAAS accounts for the physiological and pathophysiological responses to volume depletion, it cannot fully explain the responses to acute or chronic blood volume expansion [443, 444]. Originally, two factors, aldosterone and glomerular filtration rate (GFR) were considered instrumental in natriuresis, the excretion of Na^+ in urine, which is a mechanism to decrease blood pressure. However, saline infusion-induced natriuresis was found to be maintained even under conditions that held these factors static, and thus unable to cause the observed natriuresis [445]. This led to the postulation of the "Third Factor Theory," which suggests that volume expansion is

associated with an increase in a circulating factor that is natriuretic [445]. Subsequently it was found that a factor in plasma from volume-expanded animals could inhibit the renal NKA [446-449]. Studies from many researchers point to either ouabain, or other cardiac glycosides, as being this third factor. For instance, the presence of a plasma factor that could inhibit NKA correlated positively with blood pressure in patients with essential hypertension [402]. Additionally, plasma volume expansion is associated with elevated levels of cardiac glycoside-like immunoreactive material in dogs [450], and anti-cardiac glycoside antibody lowers blood pressure in rats with DOCA-induced hypertension [451]. However, a *bona fide* natriuretic factor would be produced in response to elevated blood pressure in order to reduce blood pressure by increasing natriuresis. While ouabain is increased in response to blood pressure, its ability to decrease blood pressure by natriuresis has not been definitively proven. While studies in both normotensive [386] and hypertensive [452] animals showed reduced natriuresis in response to administration of anti-ouabain antibodies, administration of ouabain itself does not consistently increase natriuresis [453]. Thus, the exact reason by which ouabain is increased during volume expansion still requires further investigation. In any case, it is clear that excessive ouabain can lead to high blood pressure conditions, such as pre-eclampsia [454] and essential hypertension [402, 451]. As cystic expansion in ADPKD is accelerated by high blood pressure [455], it is tempting to speculate that ouabain may also play a role in the generation of ADPKD related hypertension, and thus cystic progression. The experiments of this thesis have been focused exclusively on the direct actions of ouabain on renal collecting duct cells, not its actions on blood pressure. However, the systemic effects of ouabain add an interesting layer to the theory that ouabain is able to promote cystic growth.

The Na,K-ATPase as a signal transducer

Not only does ouabain inhibit the catalytic function of NKA to elicit many of its effects, but it also induces the activation of signaling pathways via conformational changes in the NKA. This role of ouabain was first discovered in 2002 by Xie and Askari and it depends on the ability of NKA to serve as a receptor and signal transducer for ouabain effects [456]. The ouabain-induced and NKA-mediated signaling pathway begins with the activation of the kinase SRC. In its E1 conformation, NKA binds to SRC [457], which sequesters SRC from the cytosol and inhibits its activity [458]. Ouabain selectively binds to the E2P conformation of α-NKA and blocks it from further progressing in the catalytic cycle [372, 373]. In the E2P conformation, NKA releases SRC, which becomes active [457, 458]. As proof of the validity of this model is the enhancement of SRC activity obtained by shifting the NKA equilibrium towards E2, with low extracellular K^+ concentrations [457]. Not all NKA expressed in the cell is able to signal ouabain effects; only a NKA subpopulation residing in caveolae forms part of the ouabain activated cascade of cellular events [459]. Once SRC has been activated, several other downstream pathways are activated by ouabain, including the transactivation of the epidermal growth factor (EGFR), the RAS-RAF-ERK pathway, and the PI3K-AKT pathway [460-472]. NKA may exhibit different signaling pathways (shown in Fig. 1.5) depending on the nature of the existing neighboring proteins and those that are recruited upon stimulation in a cell-specific, locus-specific, and stimulus-specific manner [473]. Therefore, ouabain has been observed to elicit diverse cellular functions such as proliferation, adhesion, motility, calcium alterations, and metabolic pathway modifications important in cardiac hypertrophy, ischemia, as well as the development and postnatal maturation of kidneys [322, 403, 460, 471, 474-486].

The signaling abilities of NKA also have a role in the previously described tissues targeted by ouabain. For instance, in the heart, ouabain can activate signaling pathways that lead to cardiac myocyte hypertrophy, specifically by induction of the PI3K/AKT pathway [472]. Ouabain aids the differentiation of cardiomyocytes via ERK activation [487]. Finally, ouabain treatment before ischemic injury is cardio-protective via a pathway that relies on SRC activation [483, 484], and/or PI3K and PKCε [485, 486].

Additionally, the signaling abilities of ouabain can also have a role in the salt handling in the renal proximal tubule. This was clearly shown in experiments using Dahl salt-sensitive and salt-resistant rats, animals which respond to salt-loading with hypertension and without hypertension, respectively. The salt-sensitive animals did not respond to ouabain with SRC pathway activation. However, in salt-resistant animals ouabain induced the SRC/MEK/ERK signaling cascade, which culminated in the decreased plasma membrane localization of the apical sodium transporter, NHE3, leading to an increase in sodium excretion [488, 489]. Thus, ouabain-mediated signaling in proximal tubule cells promoted natriuresis, while conversely, the impairment of NKA signaling may contribute to the salt-induced hypertension of the salt-sensitive rats [490].

Importantly, NKA signaling is activated by physiological levels of ouabain in ADPKD cells but not in NHK cells. Nguyen and colleagues showed that ouabain enhanced ADPKD cell proliferation, but not that of NHK cells [322]. They further showed that this increased proliferation was due to increased phosphorylation of ERK in response to ouabain, which did not occur in NHK. Interestingly the α1-NKA of ADPKD cells is more sensitive to ouabain. Thus, ADPKD cells have approximate 25% of their total NKA activity inhibited by ouabain with a IC_{50} value of ~1 nM, while the remaining 75% has the typical lower affinity of the α1-NKA of normal cells (IC_{50} of 0.1 μM) [322]. This increase in ouabain affinity for a subpopulation of NKA in ADPKD cells is not

due to the expression of different α isoforms in the cells, but appears to rather depend on different protein/protein associations [322]. Subsequently, SRC and B-RAF activity were also found to be increased by ouabain in ADPKD cells, confirming the pathway induced by ouabain in ADPKD cells is the NKA-SRC-RAS-BRAF-MEK-ERK cascade [491]. This signaling pathway required the presence of caveolae, considered to be the prime location for NKA-mediated signaling [492], and ultimately caused increased proliferation by the downregulation of p21 and p27, two cell cycle inhibitors [491].

Another characteristic of ADPKD cystic cells is their penchant to increase apical fluid secretion in response to cAMP [493-496], while NHK cells do not significantly increase secretion in response to the same stimulus [493, 497]. Ouabain was not able to increase fluid secretion on its own, but significantly enhanced cAMP-stimulated fluid secretion in ADPKD cells [360]. In contrast, ouabain did not have a significant effect in fluid secretion in normal kidney cells. Interestingly, the ability of ouabain to enhance cAMP-mediated cyst area expansion was subjective to the expression of *PKD1*, as the ability of ouabain to increase cyst size was greatest in a $PKD1^{-/-}$ kidney, compared to $PKD1^{+/-}$ and $PKD1^{+/+}$ kidneys, during metanephric organ culture [360]. These effects of ouabain on fluid secretion and on the total surface area of microcysts could be abrogated by the pharmacological inhibition of components of the ouabain-mediated signaling cascade [360]. Subsequent studies found that ouabain could not induce cyst formation in a metanephric organ culture without the presence of the chloride channel CFTR [361]. Fluid secretion in response to cAMP is mediated by chloride transport in ADPKD cells [493], and ouabain increased CFTR membrane expression, and also elevated the expression of the positive CFTR-regulator, PDZK1 [361].

Figure 1.5

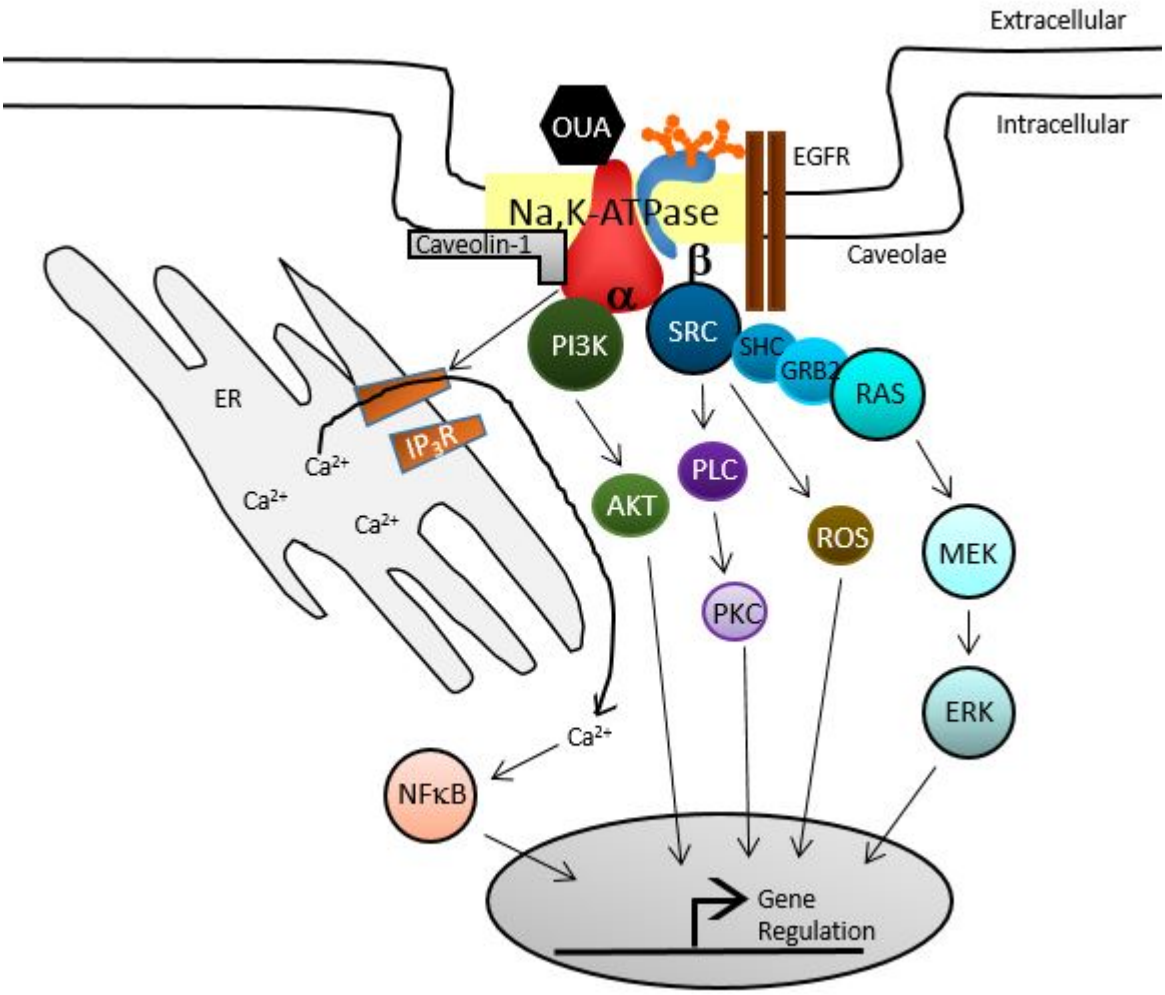

Figure 1.5. Ouabain-mediated signaling pathways. Ouabain-bound NKA by acting through a subpopulation of Na,K-ATPase in caveolae, ouabain activates the Na,K-ATPase signaling pathway, which involves a series of intracellular intermediates that ultimately modulate gene expression and cause a variety of cell type-specific effects.

The compelling data outlined in the previous paragraphs, shows ouabain as a novel factor able to enhance the ADPKD phenotype and perhaps speed the progression of the disease. The data also suggests that ouabain might enhance other characteristic features of the cystic epithelium. In this thesis, focus has been placed on three cellular processes that are particularly related to the

developmental and wound response phenotype of ADPKD cells, including apoptosis, EMT, and altered intracellular calcium of the cells. A brief overview of ouabain-mediated alterations regarding these three processes is given below, followed by a section outlining the multiple points at which the actions of polycystins and that of ouabain-bound NKA might intersect.

Ouabain as an effector of apoptosis

Ouabain has been shown to influence programmed cell death in a cell-type specific manner. For instance, ouabain has pro-apoptotic effects in normal neuronal cells, as well as in neuro- and glioblastoma cells, hepatic cells, blood peripheral lymphocytes, lymphoma cells, and prostate cancer cells [498-506]. In contrast, ouabain protects endothelial cells, cerebellar granule cells, renal proximal tubule cells, and COS-7 cells against apoptosis [476, 507-510]. Ouabain also has a dual pro- and anti-apoptotic effect in smooth muscle, umbilical vein endothelial cells, and fibroblast [511-513].

One would hypothesize that the inhibition of enough NKA pumps by ouabain would lead to the dissipation of the Na^+ and K^+ ion gradients that are essential for the cell and thus cause cell death. However, even when a sharp elevation in the cell $[Na^+]/[K^+]$ ratio is caused by ouabain, a decrease in cell survival does not occur in all cell types (reviewed in [514]). In some cases, ouabain-mediated cell death can be blunted or fully abrogated by inhibition of ouabain-induced signaling pathways, [515, 516], suggesting that ouabain-mediated cell death isn't solely reliant on ionic alterations. The observed cell death mediated by ouabain can occur by conventional mechanisms, such as apoptosis [503, 517-522] anoikis [523], autophagy [524, 525], and necrosis

[508, 526]. In some cases, researchers have observed hybrid forms of cell death, including features of both necrosis and apoptosis, in response to ouabain [499, 515, 527, 528].

Interestingly, the NKA has been suggested to act as a sensor protein capable of facilitating a rapid response to apoptotic stimuli [362, 529, 530], which may account for the cell type-specific induction towards, or protection from, cell death. The strongest evidence for NKA as an apoptotic-sensor comes from Lauf and co-workers, who found canonical BCL-2 protein motifs in the N-terminal domain of α-NKA subunits and who subsequently co-immunoprecipitated the BCL-2 proteins, BCL-XL and BAK, with the α-NKA enzyme [530, 531]. Indeed, cells overexpressing BCL-2 are less susceptible to ouabain-mediated cell death [523, 532], and conversely, protection from radiation-induced cell death due to BCL-2 overexpression is abrogated by ouabain [533]. It has also been suggested that the pro-apoptotic effects of ouabain may have some function on tissue repair [519].

Ouabain as an effector of EMT

Along with its pumping and signaling functions, the NKA also has a structural role. Thus, the extracellular domain of β-NKA subunits form bridges between adjacent cells [286, 308, 534-537]. It is estimated that half of β-NKA subunits interact with other β subunits in adjacent cells (MDCK) [537]. β1-NKA has 3 N-glycosylation sites, all occupied by N-glycans [538]. Prevention of N-glycosylation has little effect on α-β assembly [312], trafficking to the plasma membrane [311, 539, 540] or NKA activity [311, 312, 541]. However, N-glycans on β-NKA are important for the initiation, maintenance and regulation of epithelial junctions, as shown by Vagin and colleagues [539, 542].

Additionally, NKA co-distributes with the adherens junctional proteins, E-cadherin, β-catenin, α-catenin, and occludin [543]. As ouabain-bound NKA leads to the internalization of the NKA complex, ouabain promotes turnover of these β-bridges, which alters cell adhesion and permeability. Ouabain was found to decrease the adhesion of MDCK cells to substrate, and this depended on activation of MAPK (ERK) [461, 462]. In addition, ouabain has been observed to cause the removal of occludin and β-catenin from the plasma membrane of MDCK cells due to the endocytosis of β-NKA [544]. Also, depending on the dose, ouabain modifies the transepithelial electrical resistance (TER), which is dependent on the "tightness" of the adhesions between cells [461, 536, 545, 546].

Interestingly, in some cases, ouabain-dependent effects on cell adhesion are similar to those elicited by either low extracellular K^+ concentration or the presence of the Na^+ ionophore, gramicidin [461, 547, 548]. This demonstrates that the maintenance of low intracellular Na^+ by the NKA is crucial for the integrity of intercellular junctions (reviewed in [542]). Additionally, the polarization of cells requires the formation of tight junctions, and thus proper NKA localization to the basolateral membrane relies on these tight junctions [549]. However, the β-NKA bridges themselves are critical for maintaining the integrity of tight junctions and apical-basolateral polarity, and therefore β-NKA is also important in preventing the translocation of proteins from apical to basolateral membranes [286, 308, 534, 536, 537, 547, 548, 550, 551].

Finally, the binding of ouabain to NKA affects several EMT-related signaling pathways. The nuclear translocation of β-catenin from the plasma membrane is induced by ouabain in MDCK cells [461]. Also ouabain stimulates the expression of TGFβ1 in cardiac myocytes [552]. Finally,

collagen production increases due to EMT [553] and, through SRC activation, ouabain induces collagen-I synthesis in cardiac fibroblasts [417] and in dermal fibroblasts [554].

Ouabain as a regulator of intracellular calcium

Ouabain has a role in calcium signaling. In response to ouabain binding to α-NKA, activation of a calcium channel is often observed; however, the specific channel involved appears to be cell type dependent, as illustrated in Figure 1.6. As mentioned previously, in myocytes of the vasculature and heart, ouabain inhibition of NKA leads to increase in calcium via the NCX [374-376, 555, 556]. This mechanism is responsible for the cardiotonic effect of ouabain and other cardioglycoside steroids, as well as for the changes in blood pressure, secondary to contraction of the vascular smooth muscle cells (reviewed in [555, 557]). Alternatively, in renal proximal tubule (PT) cells, ouabain-bound NKA causes slow calcium oscillations that have a periodicity of 4-5 minutes [403, 477, 558-560]. In these cells, the mechanism responsible does not depend on NCX, but rather, is due to a direct interaction between α-NKA on the plasma membrane and IP_3Rs on the ER membrane [558-560].

The binding of ouabain induces a conformational change in NKA which leads to release of calcium from intracellular stores, without affecting the pumping activity of NKA [558-560]. Finally, in cardiomyocytes and in some neurons, ouabain-mediated calcium alterations rely at least partially on the activity of L-type calcium channels found on the plasma membrane, which bring in calcium in response to membrane depolarization [561]. This depolarization is a result of increased intracellular sodium caused by ouabain-mediated NKA inhibition [562-567].

Figure 1.6

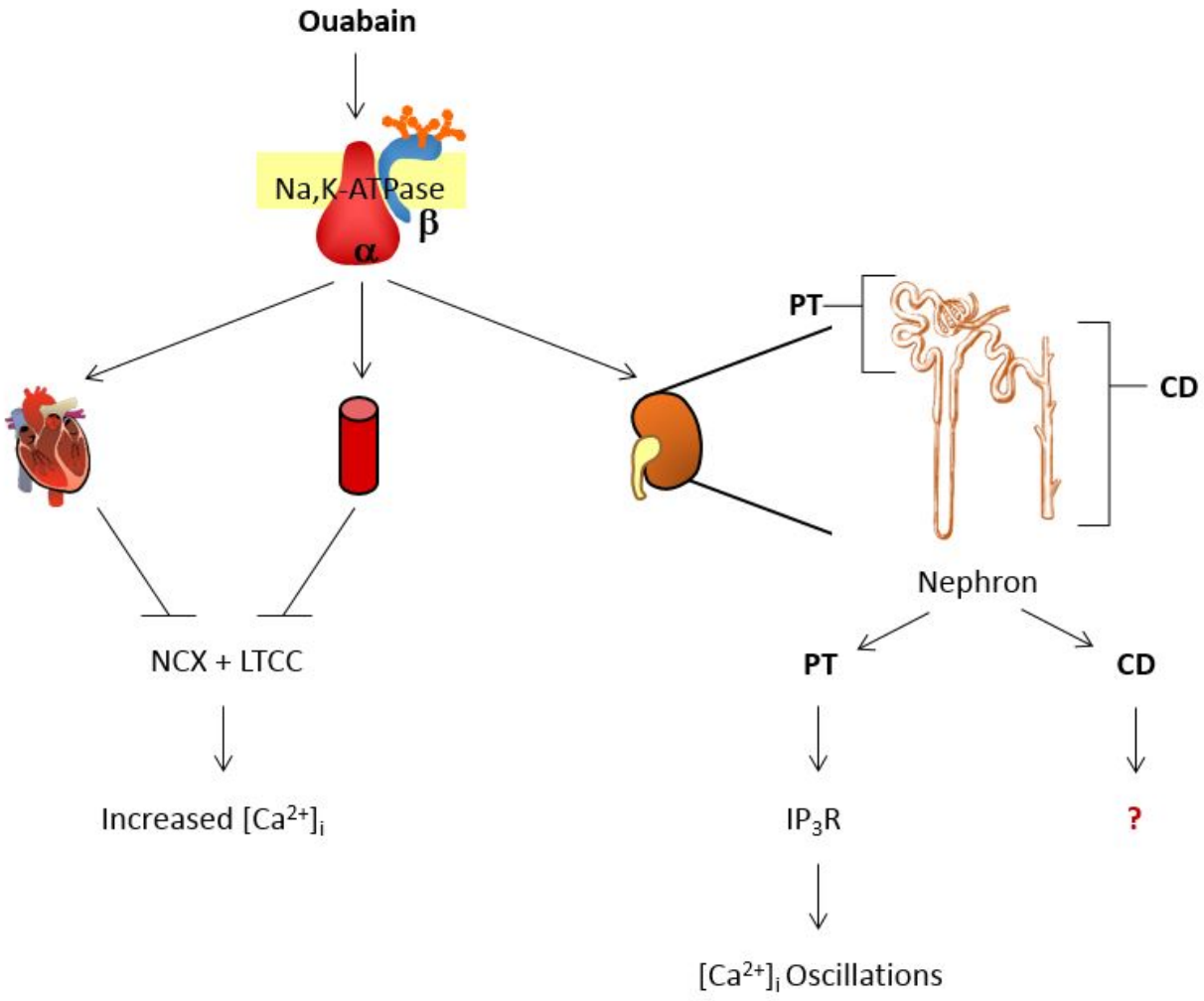

Figure 1.6. Ouabain targeted tissues and calcium alterations. Ouabain-bound NKA decreases the activity of NCX in cardiomyocytes and in smooth muscle cells of the vasculature, resulting in increased intracellular calcium. This effect relies partially on LTCC activity. In proximal tubule (PT) cells of the kidney, ouabain causes the activation of IP_3R channels, yielding calcium oscillations. Before this thesis, the effect of ouabain on calcium in collecting duct (CD) cells was not elucidated.

Summary and areas of investigation

Due to their role in the renal filtration system, renal epithelial cells are constantly working to secrete and reabsorb as necessary. It is likely that renal cells will need to cope with wounding

stresses which could be caused by transport of toxic components, or by free radical generation [568]. ADPKD has many features in common with a kidney that is responding to ischemic damage or ureteral obstruction (reviewed in [63]). For instance, renal epithelial cells partially dedifferentiate and proliferate in response to ischemia, ureteral obstruction, and this also occurs in ADPKD [64, 78, 569]. Additionally, an increased rate of apoptosis is also observed in these cases [64, 78, 569]. Specific calcium signals are known to occur in development and in wound healing [570-573] and abnormal calcium handling also occurs in ADPKD [197-199, 209-212]. Therefore, the central hypothesis of this work is that ouabain enhances the phenotypes which yield the observed dedifferentiated cystic cells of ADPKD [226, 574, 575].

The actions of ouabain may intersect with those of the polycystins at several points. From a purely theoretical point of view, ouabain may act systemically to enhance cystic progression. Polycystins are highly expressed *in utero* during renal tubule development. Ouabain concentrations have also been shown to be increased during pregnancy [410]. In fact, ouabain may aid in the development of the renal system by promoting cell survival during stress [403, 482]. In adults, mutated polycystins eventually lead to increased blood pressure, a condition in which enhanced ouabain levels are detected in plasma. Additionally, 80% of ADPKD patients die from cardiovascular complications [111, 112]. Left ventricle (LV) mass is increased in ADPKD patients [107, 576-578], and abnormal LV are associated with impairment of LV function and impose significant risk for cardiovascular disease morbidity and mortality [579]. Ouabain plasma levels positively correlate with LV mass in hypertensive subjects [580, 581], and are elevated in patients with impaired LV performance [423]. Indeed, sustained infusion of ouabain is sufficient to induce LV hypertrophy in normotensive rats [432], together suggesting that ouabain may significantly decrease the survival of ADPKD patients. The average plasma concentration of ouabain between

ADPKD patients and healthy controls has not been determined. However, cysts undergo rapid growth *in utero* and after GFR decline, and under both conditions high concentrations of ouabain can be expected. However, it is also important to bear in mind that ADPKD cells are more sensitive to ouabain-mediated signaling [322], so increased levels of ouabain may not be necessary.

Systemic conditions then, may or may not provide an intersection between the activities of polycystins and NKA. However, at a cellular level, PC1 and NKA do directly interact. α-NKA can co-immunoprecipitate with full-length PC1 in MDCK cells [582]. This interaction was shown to depend on PC1 CTT (specifically, the last 193 amino acids of PC1) and the intracellular domains of α-NKA (A, P and N domains) [582]. Further, overexpression of PC1 significantly increased NKA activity, without affecting the apparent affinity of Na^+ for NKA [582]. Work from this lab has further shown, using an insect cell expression system, that co-expression of membrane-targeted PC1 CTT with α-NKA increased the sensitivity of α-NKA to ouabain [181] similar to what is seen in ADPKD cells [322]. Additionally, co-expression of the dominant negative PC1-CTT with α-NKA phenotypically altered the cells to increase proliferation in response to ouabain [181], which is once again similar to what is observed in ADPKD cells [322], and was in fact found to use the same SRC/MEK/ERK signaling pathway to accomplish the ouabain-mediated proliferation [322, 491].

In addition to this physical interaction, ouabain has the potential to affect multiple processes which are dysregulated by the mutated polycystin complex. First, dysregulation of polycystins sensitizes cells to apoptosis [211, 235, 264, 583]. Ouabain also affects apoptosis in a concentration and cell-type dependent manner, potentially by an integration of life-death signals [362, 529, 530]. Therefore, in Chapter 2, the ability of ouabain to induce apoptosis in ADPKD was determined. Secondly, in addition to their roles in maintaining ionic concentrations, both NKA

and PC1 have structural roles, accomplished through homophilic interactions with themselves and through interactions with cytoskeletal proteins. Further, both may affect the dedifferentiation processes essential for proper wound-healing through activation of EMT-related signaling. For instance, TGFβ is upregulated by both ouabain treatment and in cystic epithelium [552, 584-586]. Also, the basement membrane of ADPKD cells is abnormally thick [78], and ouabain increases collagen deposition [417, 554]. Therefore, in Chapter 3, the junctional properties of ADPKD cells in response to ouabain have been assayed and it was also determined if EMT-related pathways are altered. Thirdly, dysregulated calcium concentrations are a driving force of ADPKD progression. Mutations in the polycystin proteins are intimately tied to this dysregulated calcium [197-199, 209-212]. As ouabain is known to induce alterations in intracellular calcium in several cell types [374-376, 403, 477, 556, 558-560, 562-567], in Chapter 4, therefore, the ability of ouabain to alter calcium concentrations in NHK and ADPKD cells was determined.

Cystic cells are characterized by a complex cellular phenotype that includes changes in proliferation, apoptosis, fluid secretion, apicobasal polarity, directional cell migration, matrix abnormalities, calcium management, and cilia or centrosomal fidelity [265, 266]. These processes are also involved in development and in response to injury. The effects of ouabain on proliferation and fluid secretion have previously been elucidated. In both cases, ouabain enhanced the cystic phenotype without altering the normal function of NHK cells, suggesting that any method of decreasing ouabain may be beneficial for slowing the progression of ADPKD. Thus, outlined in the following chapters is the effect of ouabain on three other processes dysregulated in ADPKD cystic epithelium, apoptosis, EMT, and calcium concentrations, with the hope of increasing the understanding of how ouabain promotes cystic progression.

OVERALL HYPOTHESIS

In this thesis, the hypothesis addressed was that ouabain enhances the ADPKD phenotype by inducing apoptosis, stimulating epithelial-to-mesenchymal transition (EMT) and affecting calcium signaling. Renal epithelial cells obtained from normal and diseased kidneys, affected with ADPKD, were used. To address this hypothesis, the following specific aims were utilized:

1. Understand the effect and mechanism of action by which ouabain mediates apoptosis in ADPKD cells. In ADPKD, it has been proposed that there is a misbalance between cell proliferation and programmed cell death. Previous reports have shown that ouabain induces cell growth; however, the role of ouabain in ADPKD cell apoptosis is unknown. The working hypothesis was that ouabain stimulates ADPKD cell apoptosis through BCL-2, BAX and cytochrome c release.

2. Determine the role and mechanism of action of ouabain in inducing EMT changes in ADPKD. ADPKD cells have been shown to have a dedifferentiated phenotype, losing properties typical of the mature renal epithelium, such as cell apical-to-basal polarity, as well as planar polarity, and the development of a mesenchymal-like phenotype. The working hypothesis was that ouabain, via the TGFβ pathway, induces EMT-related changes that cause upregulation of mesenchymal markers, relaxation of cell adhesion properties, and increases motility and fibrosis in APDKD cells.

3. Investigate the effect of ouabain on calcium signaling in NHK and ADPKD cells. Intracellular calcium concentration is decreased in ADPKD cells relative to NHK cells. In addition, the low calcium of ADPKD cells has been shown to alter signaling pathways that lead to cell proliferation and fluid secretion by the cells, which maintains the ADPKD phenotype. The working hypothesis was that ouabain regulates the intracellular calcium concentration in NHK and ADPKD cells differently, to favor the cystic phenotype in ADPKD cells.

CHAPTER 2: OUABAIN ENHANCES APOPTOSIS VIA THE INTRINSIC PATHWAY

ABSTRACT

Progression of autosomal dominant polycystic kidney disease (ADPKD) is highly influenced by factors circulating in blood. We have shown that the hormone ouabain enhances several characteristics of the ADPKD cystic phenotype, including increase in cell proliferation, fluid secretion and the capacity of renal epithelial cells obtained from kidney cysts of patients with ADPKD (ADPKD cells) to form cysts. In this work, we found that physiological levels of ouabain (3 nM) also promote programmed cell death in ADPKD cells. This was determined by Alexa Fluor 488 labeled-Annexin-V staining and TUNEL assays, both biochemical markers of apoptosis. Ouabain-induced apoptosis also takes place when ADPKD cell growth is blocked; suggesting that the effect is not secondary to the stimulatory actions of ouabain on cell proliferation. Ouabain alters the expression of BCL family of proteins, reducing BCL-2 and increasing BAX expression levels, anti- and pro-apoptotic mediators respectively. In addition, ouabain caused the release of cytochrome c from mitochondria. Moreover, ouabain activates caspase-3, a key "executioner" caspase of the cell apoptotic pathway, but did not regulate caspase-8. This suggests that ouabain triggers ADPKD cell apoptosis by stimulating the intrinsic, but not the extrinsic pathway of programmed cell death. The apoptotic effects of ouabain are specific to ADPKD cells and do not occur in normal human kidney cells (NHK cells). Taken together with our previous observations, these results show that ouabain causes an imbalance in cell growth/death, to favor growth of the cystic cells. This event, characteristic of ADPKD, further suggests the importance of ouabain as a circulating factor that influences ADPKD progression.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is the most common genetic disorder of the kidney, characterized by the formation and progressive enlargement of numerous fluid filled cysts, which severely distort renal morphology and function, reviewed in [587, 588]. ADPKD cysts are formed *in utero* and continue progressing after birth at a relatively slow, but relentless rate throughout the life of the affected individual [30]. Patients with ADPKD eventually develop renal insufficiency and end-stage renal disease (ESRD), requiring dialysis or kidney replacement therapy [589-591].

ADPKD is caused by mutations in the genes that encode for polycystin-1 and polycystin-2 (*PKD1* and *PKD2* respectively); however, progression of the disease is highly influenced by factors circulating in the bloodstream [25, 592, 593]. We have shown that the hormone ouabain, in concentrations similar to those present in plasma, stimulate the proliferation of renal epithelial cells obtained from kidney cysts of patients with ADPKD (ADPKD cells), the growth of microcysts generated by ADPKD cells, and cyst-like tubule dilations in embryonic kidneys from a mouse model of ADPKD [322, 360]. In contrast, ouabain does not significantly influence cell proliferation and cyst formation in normal kidney cells (NHK cells) and metanephric organs from wild type mice [475].

The slow progression of ADPKD is difficult to explain in a condition that is primarily characterized by continuous cell proliferation. Cell growth is maintained by a balance between cell proliferation and apoptosis, a process of programmed cell death [594, 595]. Interestingly, an imbalance between increased rates of cell apoptosis have been reported in kidneys from animal models of ADPKD and in humans carrying the disease, a phenomenon that may contribute to the uncontrolled, but slow progression of the disease [67, 140, 257, 263, 596-599].

Apoptosis is an essential process during normal tissue development and aging and is also found in several pathological situations [600-603]. Apoptosis involves an intricate cascade of molecular events, with the B-cell lymphoma 2 (BCL-2) protein family and a series of cysteine proteases, the caspases, being essential mediators of the process. The BCL-2 family include several members that are pro-survival and pro-apoptotic factors, such as BCL-2 and BAX respectively. The proteolytic caspases include the "initiator" caspases-8, -9 and -10, and the "executioner" caspases-3 and -7 [594, 600, 604]. Two main caspase-mediated pathways control programmed cell death. The extrinsic pathway, a ligand triggered and transmembrane receptor mediated cascade [605], and the intrinsic pathway, which comprises mitochondrial changes and the release of cytochrome c from the mitochondrial intermembrane space to the cell cytosol [606]. Both intrinsic and extrinsic pathways converge to stimulate the activity of caspases-3 and -7, which are responsible for the events that are characteristic of apoptosis, including DNA fragmentation, protein cross-linking and degradation, and cell disintegration into apoptotic bodies [607].

While apoptosis has been described as a feature of ADPKD, the factors and mechanisms that influence programmed cell death in ADPKD cells are poorly understood. Ouabain has been shown to influence programmed cell death in a cell type specific manner. For instance, ouabain has pro-apoptotic effects in normal neuronal cells, neuro- and glioblastoma cells, hepatic cells, blood peripheral lymphocytes, lymphoma cells, and prostate cancer cells [498-506]. In contrast, ouabain protects endothelial cells, cerebellar granule cells, renal proximal tubule, and COS-7 cells against apoptosis [403, 476, 507-510]; and has a dual pro- and anti-apoptotic effect in smooth muscle, umbilical vein endothelial cells, and fibroblast [511-513]. At present, the role of ouabain in ADPKD apoptosis is unknown. In this work, we show that ouabain enhances apoptosis in ADPKD, but not in NHK cells, by activating the intrinsic pathways of programmed cell death.

MATERIALS AND METHODS

Cell culture:

Primary cell cultures of ADPKD cells, derived from surface cysts of ADPKD kidneys and NHK cells were generated by the PKD Biomaterial Core at University of Kansas Medical Center (KUMC). A protocol for the use of discarded human kidney tissues was approved by the Institutional Review Board at KUMC. Primary cultures were prepared as described [608]. Cells were seeded and grown in DME/F12 supplemented with 1% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin G and 0.1 mg/ml streptomycin, 5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL sodium selenite (ITS). Twenty-four hours before cells were subjected to various experimental manipulations, serum was reduced to 0.002% and ITS removed. As previously shown, these cells are epithelial in nature and they stain positive for specific lectin markers for the collecting duct and distal nephron, indicating that they are derived from the distal nephron [198].

Annexin-V and propidium iodide staining:

Alexa Fluor 488 labeled-Annexin-V and propidium iodide (PI) staining were utilized as biomarkers for the detection of apoptosis and necrosis in ADPKD and NHK cells by flow cytometric analysis, following the manufacturer's protocols (Invitrogen, Carlsbad, CA, USA). Briefly, cells were trypsinized, washed in PBS, and resuspended in binding buffer (50mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4). After addition of 5µL Annexin-V and 1µL PI, cells were incubated at 37 °C and protected from light for 15 minutes. Samples were diluted with binding buffer and were analyzed using a LSRII flow cytometer (Beckton Dickinson, Franklin Lakes, NJ). Alexa Fluor 488 labeled-Annexin-V, which detects changes in distribution of phosphatidylserine

and phosphatidylethanolamine at the cell plasma membrane; and propidium iodide (PI) staining, which reveals loss of cell plasma membrane integrity, allowed distinguishing cells undergoing early and late stages of apoptosis, and necrosis [609].

Measurement of fragmented DNA by TUNEL assay:

Cells cultured on glass coverslips were treated with 3nM ouabain for 24h and analyzed for apoptosis using the DeadEnd fluorometric TUNEL system (Promega, Madison, WI). This method determines nuclear fragmentation, an important biochemical marker for cell apoptosis, by using terminal deoxynucleotidyl transferase to transfer fluorescein (FITC)-12-dUTP to the free 3'-OH of cleaved DNA. Cells were counter-stained with DAPI to label the nuclei. The percentage of cells undergoing apoptosis was determined using fluorescence microscopy. Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoro-methylketone (z-VAD-fmk), at a concentration of 20 μM and camptothecin, at 5 uM (Promega Corporation, Madison, WI), were applied as an inhibitor and inducer of apoptosis, respectively.

Immunoblot analysis:

Cells treated with and without 3nM ouabain for 24 h were washed once with ice-cold phosphate buffered saline (PBS) and lysed in a solution containing 10 mM Tris-Cl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, and 0.1% NP-40. Samples were centrifuged at 10,000 x g for 10 min. Protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Fifty μg of the cleared lysates were subjected to SDS-PAGE (15% gel) and blotted on to nitrocellulose membranes. Immunoblots were probed with different primary antibodies that recognize PARP-1,

BCL-2, BAX, caspase-3 or caspase-8 (Cell Signaling Technology, Boston, MA). Species-specific secondary antibodies conjugated to horse-radish peroxidase and enhanced chemiluminescence was used for protein detection (Santa Cruz Biotechnology, Dallas, TX). Protein expression levels were determined by densitometry and were expressed as a ratio of the corresponding untreated controls.

Cytochrome c analysis:

Cytochrome c release from mitochondria was studied in NHK and ADPKD cells after treatment with or without 3nM ouabain for 24 h by immunoblot and immunocytochemistry. For the immunoblot analysis, cells were harvested and cytosolic or mitochondrial fractions were prepared using the Cell Fractionation Kit ab109719, according to the manufacturer's instructions (AbCam, Cambridge, UK). Samples were subjected to SDS/PAGE and proteins transferred to nitrocellulose membranes. Cytochrome c was determined using a monoclonal antibody from BD Biosciences (San Diego, CA) and horse-radish peroxidase conjugated secondary antibodies; and its levels were estimated by densitometric analysis of the obtained bands. For the immunocytochemical analysis, cells were plated on coverslips and treated with or without ouabain for 23.5 h. Then, 100nM MitoTracker Red CMXRos (Thermo Fisher Scientific, Waltham, MA) was added to the cells and they were incubated for an additional 30 minutes at 37 °C, protected from light. Cells were fixed with 3.7% paraformaldehyde in serum-free media for 15 min at 37 °C. Samples were washed in PBS and permeabilized with acetone for 5 minutes. Anti-cytochrome c antibody (BD Biosciences, San Diego, CA) (1:75) was applied to the cells overnight at 4 °C. Coverslips were washed once with PBS, and then slides were incubated with secondary Alexa 488-conjugated antibodies for 1 hour at room temperature. Samples were washed in PBS and mounted onto microscope slides with DAPI Slowfade Gold solution (Thermo Fisher Scientific,

Grand Island, NY). Slides were viewed using an Eclipse 80i Upright microscope (Nikon Instruments, Inc., Melville, NY). Analysis of cytochrome c release from the mitochondria was determined from the obtained images, by quantifying the number of pixels in the cell cytosol divided by the number of pixels in mitochondria. Values were expressed as the ratio of cytosolic/mitochondrial cytochrome c levels, as previously described [610]. This allowed characterizing the mitochondrial to cytosolic distribution of cytochrome c.

Caspase-3/7 activity determination:

Caspase-3/7 activity was determined using the Caspase-Glo-3/7 Assay according to manufacturer's instructions (Promega, Madison, WI). Briefly, NHK and ADPKD cells were plated into black-walled, clear-bottomed 96-well plates (Corning Inc., Corning, NY) at a density of 4,000 cells per well and treated with or without ouabain for 24 h. The luminescent caspase-3/7 substrate was added following the manufacturers specifications. The luminescent signal resulting from cleavage of the substrate specific to caspase-3 or -7 is proportional to the amount of caspase activity present. Data were expressed as a percentage of untreated controls.

Data analysis:

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

RESULTS

Ouabain stimulates apoptosis in ADPKD but not in NHK cells

Ouabain has been reported to stimulate apoptosis in a cell type specific manner [611]. We explored whether ouabain affected programmed cell death in NHK and ADPKD cells. For this, we treated NHK and ADPKD cells with 3 nM ouabain, a concentration of this hormone that is within the levels commonly found to be circulating in blood. Twenty-four hours later, we determined cell apoptosis and necrosis using Alexa Fluor 488 labeled-Annexin-V and PI labeling and flow cytometry. Sorting of the cells based on these markers showed that ouabain treatment significantly increased the number of ADPKD cells undergoing apoptosis compared to untreated controls (Fig. 2.1A, top panel). This increase corresponded to cells showing signs of late apoptosis, while the number of ADPKD cells showing early manifestations of apoptosis, or undergoing necrosis did not significantly change with ouabain administration (Fig. 2.1A bottom panel). Different from ADPKD cells, ouabain did not induce programmed cell death (either early or late apoptosis), or necrosis in NHK cells (Fig. 2.1A, top and bottom panels).

To further estimate apoptosis, we determined DNA nuclear fragmentation by TUNEL assay in NHK and ADPKD cells. In agreement with the Alexa Fluor 488 labeled-Annexin-V and PI studies, ouabain increased TUNEL staining in ADPKD cells (Fig. 2.1B). This ouabain-induced increase in DNA fragmentation could be rescued by the pan-caspase inhibitor, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoro-methylketone (z-VAD-fmk), further suggesting that ouabain is inducing apoptosis in ADPKD cells. The topoisomerase I inhibitor, camptothecin, was used as a positive control for apoptosis (Fig. 2.1B). Altogether, these experiments show that ouabain stimulates apoptosis in ADPKD, but not in NHK cells.

Figure 2.1

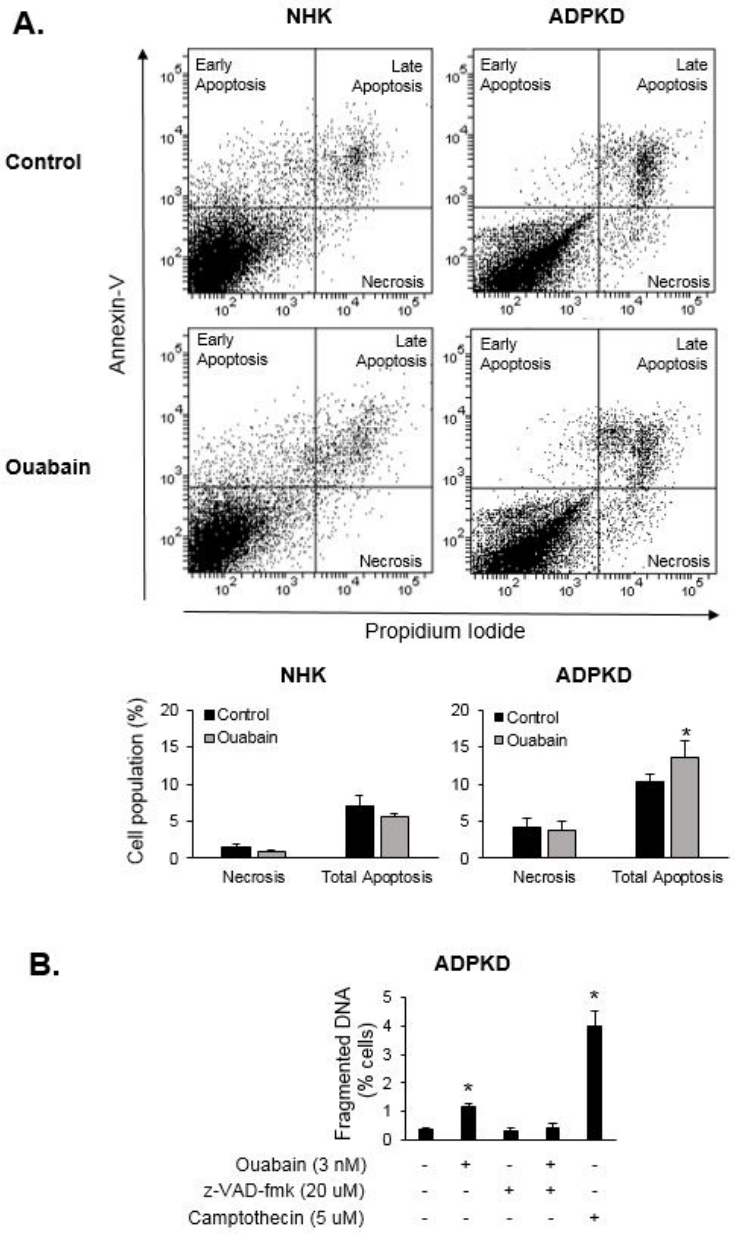

Figure 2.1. Ouabain induces apoptosis in ADPKD, but not in NHK cells. A) Alexa Fluor 488 labeled-Annexin V and PI staining. After treatment with ouabain for 24h, cells were labeled and sorted using flow cytometry. The top panel shows representative plots for the sorted NHK and ADPKD cells, without and with addition of ouabain. Different cell populations undergoing necrosis, early and late apoptosis, identified by differential annexin-V/PI labeling, were quantified and expressed as percent of total cells (bottom panel). B) DNA fragmentation assays. Cells seeded onto glass coverslips were incubated with 3nM ouabain and the indicated experimental conditions for 24 h, fixed and analyzed for apoptosis using the Dead-End d Fluor Metric TUNEL System. At least 10 random fields were analyzed from each of three different ADPKD kidneys. In all graphs, bars represent the mean $^{\pm}$ SEM of three different experiments. The asterisks indicate values that are statistically different compared to the corresponding untreated control, with $P < 0.05$.

Ouabain modulates expression of BCL-2 protein family members in ADPKD cells

Whether a cell undergoes apoptosis is in part determined by the ratio of pro- to anti-apoptotic members of the BCL-2 protein family [594]. Within the BCL-2 members, BAX and BAK function as pro-apoptotic agents, while BCL-2 behaves as an anti-apoptotic mediator [604]. To further characterize the mechanisms by which ouabain induces cell death in normal and cystic renal cells, we examined the role of these apoptotic regulators in NHK and ADPKD cells. Ouabain treatment for 24 h did not alter the expression levels of either BAX or BCL-2 proteins in NHK cells (Fig. 2.2A). However, in ADPKD cells, ouabain caused a significant decrease in the anti-apoptotic BCL-2 protein, with a concomitant increase in the pro-apoptotic BAX protein levels (Fig. 2.2B). This change towards a pro-apoptotic protein ratio supports the role of ouabain as an

Figure 2.2

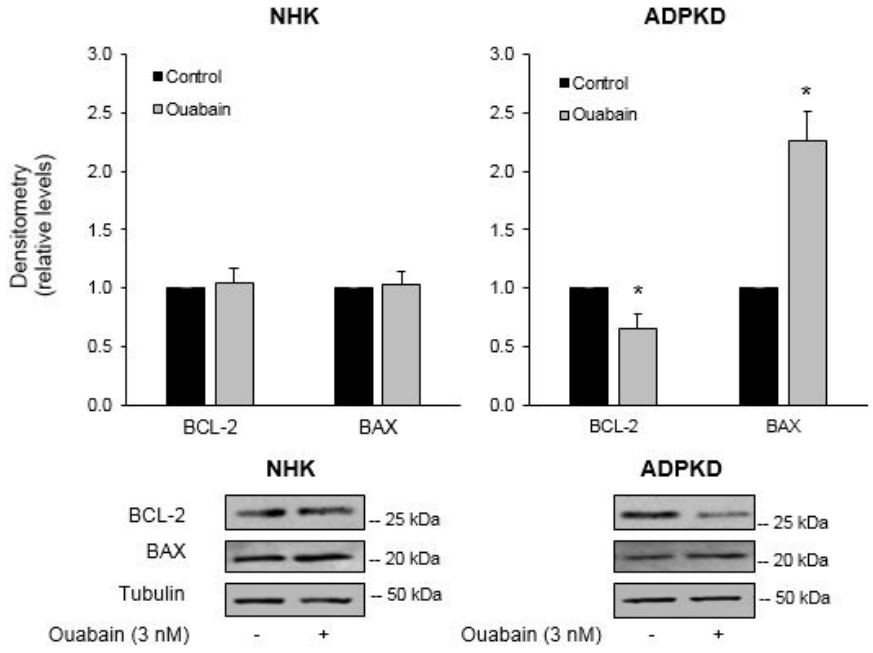

Figure 2.2. Ouabain modifies the expression of BCL-2 and BAX protein levels in ADPKD, but not NHK cells. NHK and ADPKD cells were treated in the absence and presence of 3 nM ouabain and 24 h later, expression levels of BCL-2 and BAX were determined by immunoblot. Tubulin was used as a loading control. Top panels show the densitometric analysis of the protein bands, while bottom panels show representative blots. Bars are the mean $\pm$ SEM of three experiments. Asterisks indicate statistically different values, with $P < 0.05$.

inducer of apoptosis in ADPKD cells, and suggests that its effects are mediated via the intrinsic pathway of programmed cell death.

Ouabain enhances cytochrome-c release from ADPKD cell mitochondria

A pro-apoptotic change in BCL-2/BAX protein ratio is commonly followed by the release of cytochrome c from mitochondria, another event in the activation cascade of programmed cell death [612]. Therefore, we determined the release of cytochrome c from mitochondria to the cytosol in cell fractions from NHK and ADPKD cells by immunoblotting. As shown in Fig. 2.3A, cytochrome c levels were much higher in mitochondrial than cytosolic fractions of both NHK and ADPKD cells. Importantly, cytochrome c was significantly augmented by ouabain in cytosolic fractions from ADPKD, but it was slightly decreased in NHK cells (Fig. 2.3A). This suggest that the change in BCL-2/BAX ratio in ADPKD cells did result in release of cytochrome c from the cell mitochondria.

In addition, changes in cytochrome c localization were studied by immunocytochemistry. For this, cells were labeled with MitoTracker Red, a dye which allows the visualization of mitochondria, and an anti-cytochrome c. Then, the ratio of cytoplasmic to mitochondrial localization of cytochrome c was determined by quantification of pixel density as described [610]. As shown in Fig. 2.3B, the ratio of cytoplasmic/mitochondrial cytochrome c did not change with ouabain treatment in NHK cells. In contrast, this ratio was significantly increased by ouabain in ADPKD cells (Fig. 2.3B). Altogether, these results show that ouabain promotes the release of cytochrome c from the mitochondria of ADPKD cells and are in agreement with the notion that ouabain induces apoptosis in these cells.

Ouabain activates caspase-3/7 in ADPKD cells

Proteases of the caspase family play an essential role in the cleavage of specific substrates that mediate cell apoptosis [613, 614]. To assess the involvement of caspases in ouabain-induced apoptosis of ADPKD cells, we treated NHK and ADPKD cells in the presence and absence of 3nM ouabain for 24h and determined activation of the "executioner" caspase-3 via its cleavage status. Caspase-3 cleavage was determined by immunoblot. As shown in Figure 2.4A, caspase-3 cleavage was not modified by ouabain in NHK cells (left panel). In contrast, in ADPKD cells, ouabain significantly increased the cleavage of pro-caspase-3 into its

Figure 2.3

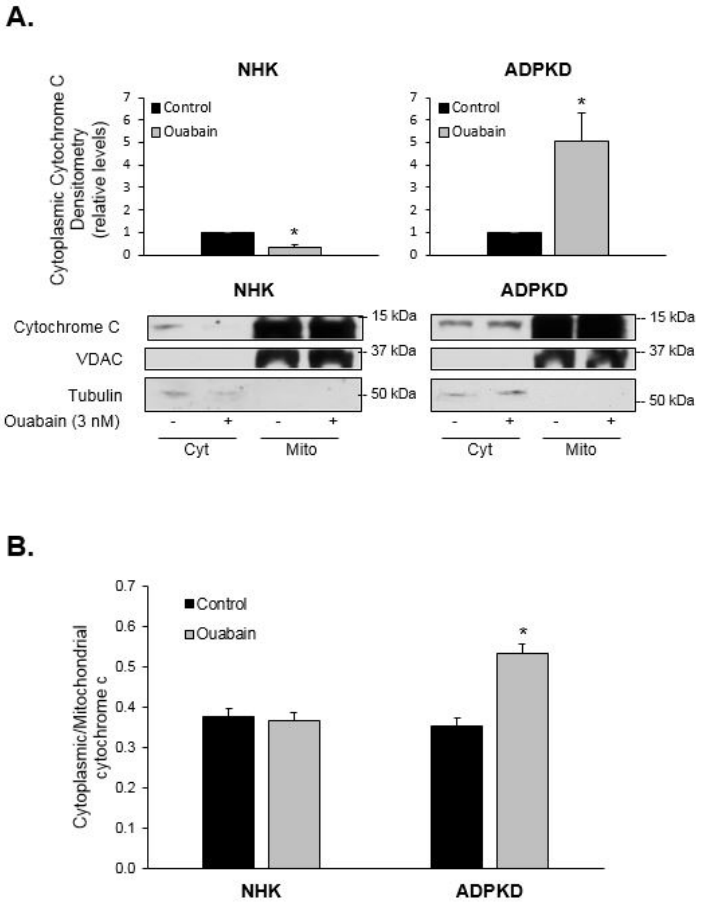

Figure 2.3. Ouabain stimulates cytochrome c release from mitochondria in ADPKD, but not NHK cells. A) Immunoblot analysis. After treatment with or without 3 nM ouabain for 24 h, cells were processed to obtain cell cytoplasmic and mitochondrial fractions, and samples were subjected to immunoblot analysis to determine cytochrome c levels. Tubulin was used as a loading control, while VDAC was used as a mitochondrial marker. Relative densitometric levels for cytochrome c are shown in the upper panels and they represent data compiled from three different experiments. The bottom panels show representative immunoblots. B) Immunocytochemical analysis. After treatment with 3 nM ouabain for 24 h, NHK and ADPKD cells were labeled for cytochrome c and MitoTracker, to visualize mitochondria. Cytochrome c release was quantified and expressed as the cytosol to mitochondrial ratio. Bars represent the compiled data from 3 different experiments. In A and B, bars represent the mean $\pm$ SEM. Asterisks indicate statistically different values, with $P < 0.05$ versus untreated control.

active large (p17) and small (p12) fragments (right panel).

In addition, we directly measured the levels of caspase-3 activity, in ADPKD cells treated with and without 3nM ouabain. Once activated, caspase-3 cleaves the same peptide sequences as caspase-7 and so their activities cannot be distinguished with the assay that we used. In any case, both caspase-3 and -7 are "executioner" caspases, involved in downstream cleavage of substrates that mediate many of the typical biochemical and morphological events of apoptosis [613, 614]. As shown in Figure 2.4B, ouabain significantly stimulated caspase-3/7 activity of ADPKD cells.

Ouabain dependent caspase-3 activation by ouabain was also estimated by immunoblot analysis of poly(ADP-ribose) polymerase-1 (PARP-1), a known target of caspase-3 action [615]. Consistent with activation of caspase-3, ouabain increased the cleavage of PARP-1 in ADPKD cells, but not in NHK cells (Fig. 2.4C).

Ouabain does not activate the extrinsic pathway of ADPKD cell apoptosis

The factors involved in triggering cell apoptosis can act through two main mechanisms, the intrinsic and extrinsic pathways [594]. Our findings that ouabain stimulates the release of cytochrome c from mitochondria suggest a role for the intrinsic pathway in the mechanisms leading to ouabain-mediated apoptosis in ADPKD cells. To determine the involvement of the extrinsic pathway in ouabain-induced ADPKD apoptosis, we measured the activation of caspase-8. This protease is involved in the extrinsic pathway of programmed cell death and its cleavage is a marker for its activation [616]. We treated NHK and ADPKD cells in the presence and absence of 3 nM ouabain for 24 h and determined the total and cleaved forms of caspase-8 by immunoblot. Compared to untreated controls, ouabain unexpectedly, decreased caspase-8 cleavage in NHK

Figure 2.4

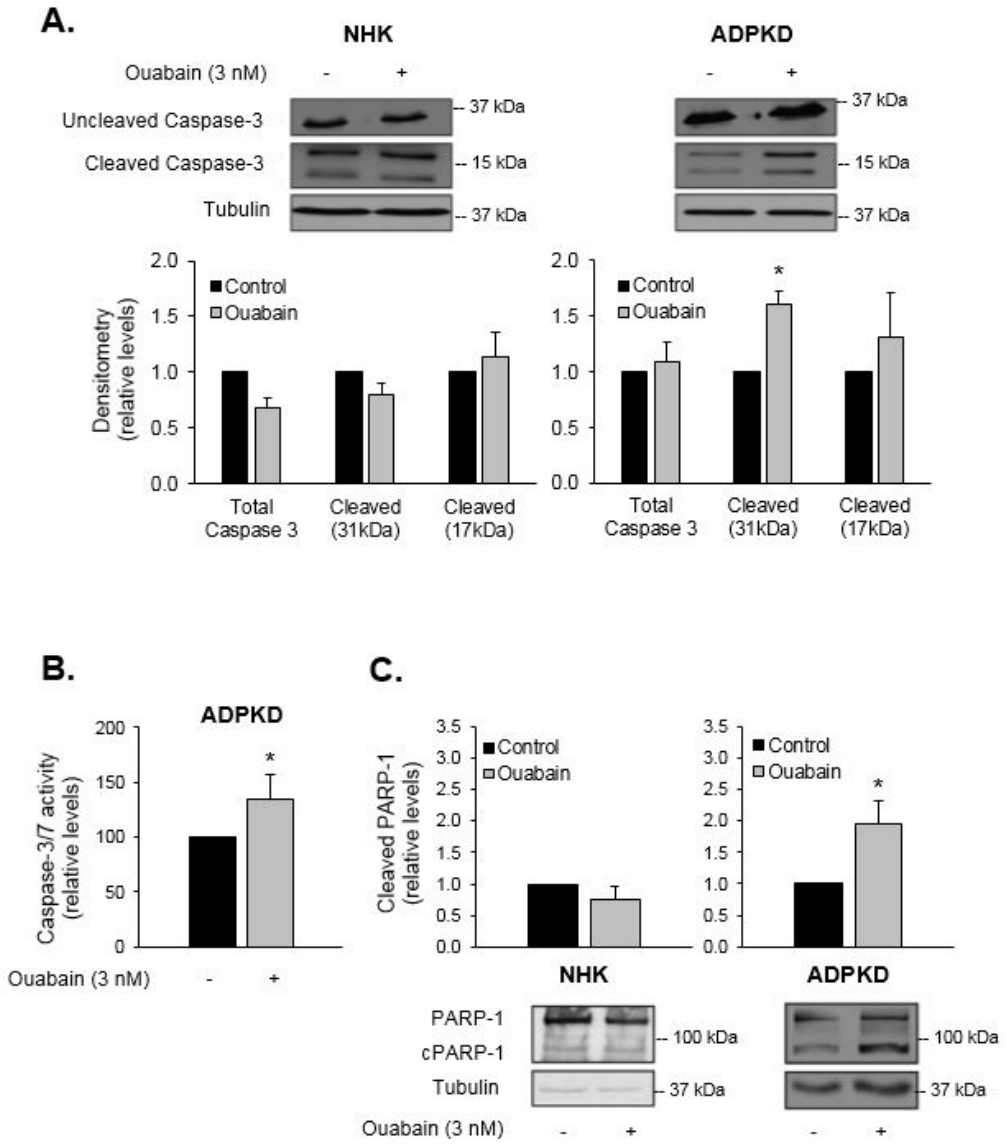

Figure 2.4. Ouabain stimulates caspase-3 cleavage and activity in ADPKD, but not NHK cells. A) Caspase-3 cleavage. After treatment with 3 nM ouabain for 24 h, the total (35 kDa) and cleaved products (17 kDa and 12 kDa) of caspase-3 were determined in NHK and ADPKD cells by immunoblot and densitometric analysis. Upper panels show representative blots and bottom panels the densitometric analysis from three different experiments. Bars are the mean $^{\pm}$ SEM of three experiments. B) Relative caspase-3 and -7 activity levels. ADPKD cells were treated in the absence and presence of 3 nM ouabain and caspase activity was measured using the Caspase-3/7 Glo Assay. Data are the mean $^{\pm}$ SEM of sextuplicate experiments. C) PARP-1 cleavage. After ouabain treatment for 24h, NHK and ADPKD cell lisates were subjected to immunoblot to determine fragmentation of the caspase-3 substrate, PARP-1. The top panels show the densitometric analysis of the total and cleaved (89 kDa) PARP-1 bands. Cleaved PARP-1 is expressed relative to the corresponding untreated controls. The bottom panels show representative immunoblots. Bars represent the mean $^{\pm}$ SEM of 5 different experiments. Asterisks show statistically different values, compared to untreated controls and with $P < 0.05$.

cells (Fig. 2.5A), but it did not affect caspase-8 cleavage in ADPKD cells (Fig. 2.5B). These results suggest that the apoptotic effects of ouabain in ADPKD cells are not mediated by caspase-8 and the extrinsic pathway of programmed cell death. In addition, the reduction in caspase-8 in NHK cells suggests a protective effect of ouabain toward apoptosis in normal cells.

The effect of ouabain on ADPKD apoptosis is independent from cell proliferation

We have previously shown that ouabain enhances the growth of ADPKD cells [322]. The ouabain-induced activation of apoptosis that we observe in ADPKD cells could just be secondary to the hyperproliferative effects of the hormone in these cells. To investigate this possibility, we inhibited cell proliferation in NHK and ADPKD cells with thymidine, and treated the cells with 3nM ouabain for 24 h. Finally, we determined apoptosis levels by cell sorting, after labeling the cells Alexa Fluor 488 labeled-Annexin-V. As shown in Fig. 2.6A, ouabain had no effect on NHK cell apoptosis, either in the presence or absence of thymidine. In ADPKD cells, ouabain-induced programmed cell death was not abrogated by the inhibition of cell proliferation with

Figure 2.5

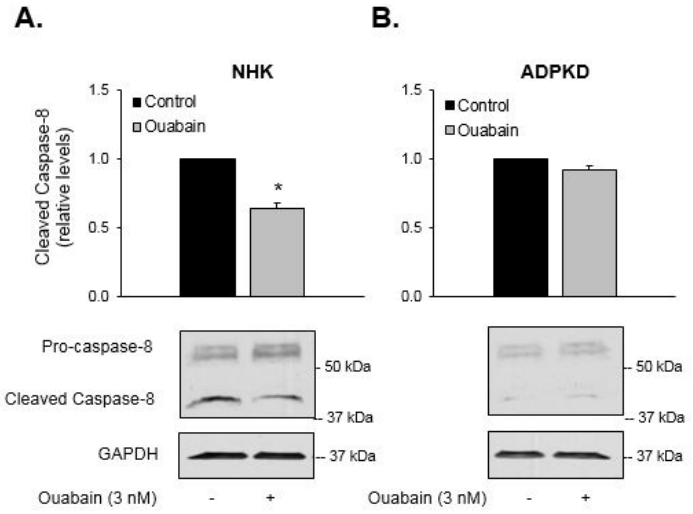

Figure 2.5. Ouabain does not affect the extrinsic pathway for apoptosis in ADPKD cells. NHK (A) and ADPKD (B) cells were treated with 3 nM ouabain for 24 h and the total (57 kDa) and cleaved (43 kDa) forms of caspase-8 were determined by immunoblot analysis and quantified by densitometry. The top panels show the densitometric analysis of the bands from 4 different experiments. The bottom panels show representative blots. Values are the mean $\pm$ SEM. Asterisks indicate statistically different values, with $P < 0.05$ versus untreated control.

thymidine (Fig. 2.6B). This shows that the effects of ouabain on ADPKD cell apoptosis directly target programmed cell death and they are not an indirect consequence of the exacerbated growth that the hormone causes in the cells.

Figure 2.6

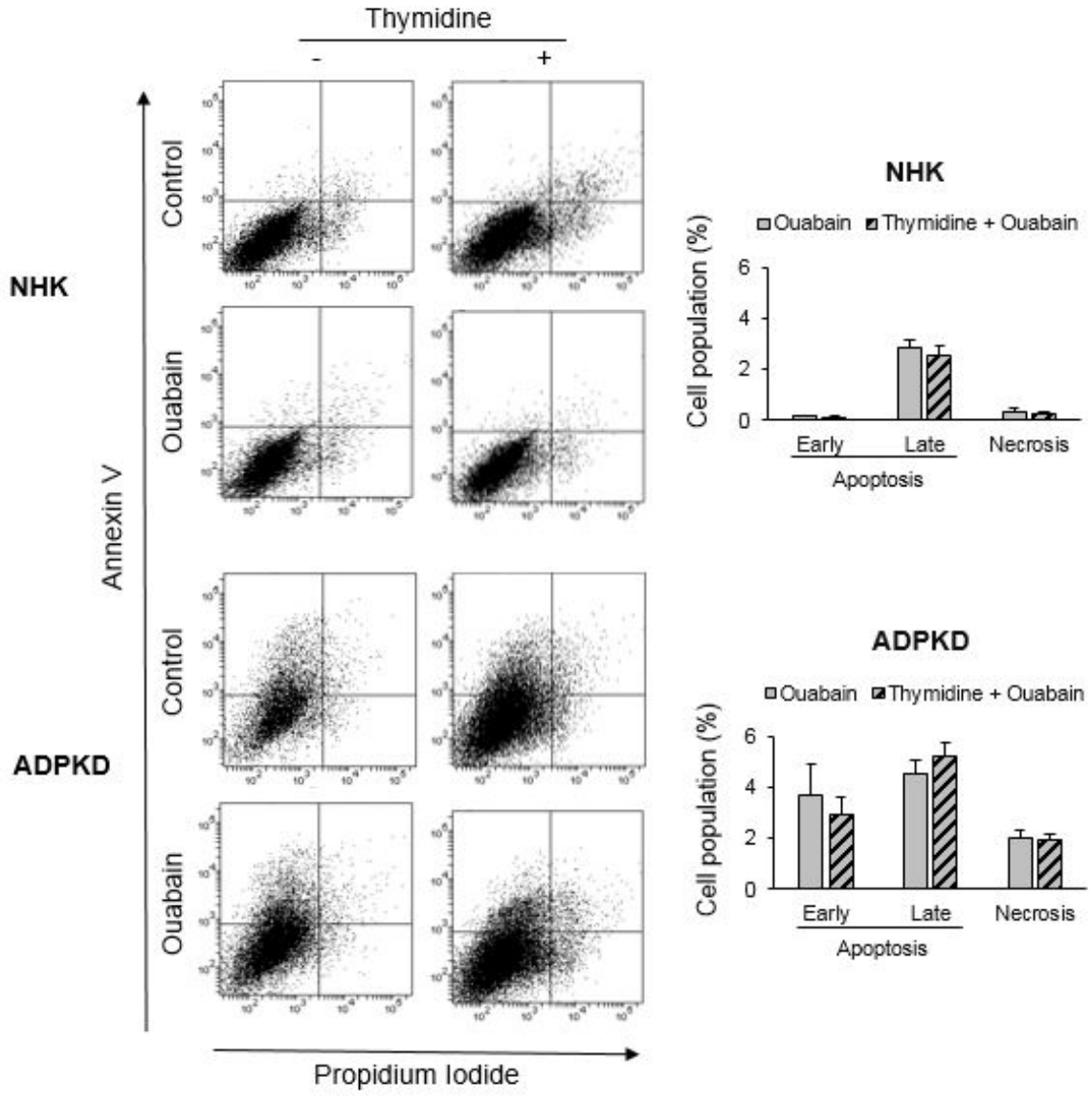

Figure 2.6. Ouabain induces ADPKD cell apoptosis independent from its proliferative effects. NHK (A) and ADPKD (B) cells were treated with or without 3 nM ouabain, in the presence and absence of 2.5 M thymidine to arrest cell growth. Alexa fluor-labeled-annexin-V and PI staining and cell sorting were utilized to detect apoptosis/necrosis. Bars represent the mean $\pm$ SEM of 3 different experiments. Inhibition of proliferation with thymidine did not significantly effect ouabain-induced apoptosis.

DISCUSSION

In this work we have shown that ouabain stimulates apoptosis in human ADPKD cells. Thus, ouabain causes changes in plasma membrane phospholipids, induces DNA fragmentation, alters the balance of BCL-2 protein expression, favors release of cytochrome c from mitochondria, and activates caspase-3/7; all of which are typical events of programmed cell death. Ouabain-induced ADPKD apoptosis occurs even when cell proliferation in the cells is blocked. This suggests that the enhancement of ADPKD cell apoptosis by ouabain is due to a direct action of ouabain on programmed cell death, and not a secondary consequence of increased cell growth, which is another effect induced by ouabain in these cells [475]. The role of ouabain on apoptosis has been shown to be cell type dependent [611]. Similar to ADPKD cells, various cancer cell types either undergo apoptosis when treated with ouabain, or are sensitized for apoptosis triggered by other compounds [500, 504, 515, 519, 527, 617-620]. Therefore, ADPKD are not the only diseased cells that react to ouabain with an apoptotic response.

We show that, different from ADPKD cells, ouabain does not induce apoptosis in NHK cells. In agreement with these observations, ouabain does not trigger programmed cell death in renal proximal tubule cells and explanted embryonic kidneys from normal rats. Moreover, in those studies, ouabain was shown to have a protective effect against apoptosis induced by serum-starvation [403] and Shiga toxin-2 infection [476]. While the goal of our experiments was not to explore the effects of ouabain under stimuli that challenge the cells to undergo apoptosis, we find a decrease in cytochrome c release from mitochondria, a reduction of ouabain-induced caspase-8 cleavage, and a trend for lower caspase-3 cleavage in NHK cells after ouabain treatment. This suggests that ouabain may be shifting NHK cells towards a pro-survival phenotype. In any case, it

appears that, despite the differences in species and renal origin, ouabain acts as a pro-survival agent in normal kidney cells.

Interestingly, increased apoptosis is a feature of early and late stages of human ADPKD [140, 258], and it is linked to cystogenesis in various animal models of polycystic kidney disease [259, 621-623]. ADPKD apoptosis has been shown to take place in the epithelial cells lining the kidney cysts, and in cysts formed by Madin-Darby canine kidney cells (MDCK) grown in collagen matrix, a model of kidney cystic disease [258, 598, 623, 624]. Moreover, polycystin-1, the primary gene altered in ADPKD, has been shown to be a regulator of programmed cell death in renal cells, and its over-expression confers the host cells resistance to apoptosis [235]. Apoptosis has also been detected in non-cystic tissue in ADPKD kidneys, which suggests that programmed cell death of the normal remaining kidney contributes to the progressive deterioration of ADPKD renal function [258, 598, 599]. Therefore, it appears that apoptosis is an essential concomitant event that helps kidney cyst development, and along with cell proliferation contributes to the pathophysiology of ADPKD. Our results show that in human cells, the target for ouabain-induced apoptosis is the cystic ADPKD cells, and not the NHK cells. The apoptotic effect of ouabain in ADPKD cells, along with the aberrant increase that ouabain promotes in ADPKD cell growth [322], places ouabain as a modulator of two essential mechanisms involved in the pathophysiology of ADPKD. It is clear that that the increase in apoptosis caused by ouabain is not sufficient to overcome the ouabain-induced ADPKD cell growth. Therefore, ouabain functions as a factor that creates a dysregulation of ADPKD cell growth/death, in favor of cell proliferation.

Our studies used amounts of ouabain that are within those observed circulating in plasma [625]. This highlights the relevance of ouabain as a factor that contributes to the pathophysiology of ADPKD. At present, the molecular basis for the different responses of ADPKD and NHK cells

to ouabain is unclear; however, we have previously found that, different from NHK cells, ADPKD cells contain a fraction of the total NKA with an abnormal higher affinity for ouabain [322]. Due to their increased ouabain affinity, ADPKD cells may be more susceptible to the endogenous circulating levels of ouabain, to which NHK cells do not respond to the same extent. In addition to their differential sensitivity to ouabain, ADPKD cells also differ from NHK cells in the activity of several intracellular signaling pathways. For example, it is known that the kinase B-RAF and the ERK pathway have an abnormal reactivity to different circulating factors in ADPKD cells [198, 626]. It is therefore possible that, in ADPKD cells, ouabain impinges on pathways which respond in an exacerbated manner on apoptotic effectors to cause programmed cell death. Undoubtedly, more studies are necessary before we can fully understand the molecular mechanisms by which ADPKD cells respond differently to the variety of stimuli that circulate in blood, including ouabain.

As shown from our labeling experiments with PI, ouabain does not cause cell necrosis, which suggests that ouabain effects are non-toxic for the ADPKD cells. This agrees with the notion that, in ADPKD cells, ouabain is not completely inhibiting NKA ion transport, but that at relatively low, physiologic amounts, it activates downstream effectors, as we have shown before [491]. In further support of this, we here found that ouabain activates several mediators in the signaling pathway that leads to apoptosis. Thus, in ADPKD, but not NHK cells, ouabain causes an imbalance in the expression of the BCL-2 and BAX proteins, involved in anti- and pro-apoptotic effects respectively. Ouabain slightly inhibited BCL-2 expression and augmented BAX levels, agreeing with induction of apoptosis in the cells. Interestingly, BCL-2 deficient mice show increased kidney apoptosis and the development of renal cyst disease [627]. The downregulation of BCL-2 and the pro-apoptotic effects of ouabain may represent mechanisms, which together with those on cell

growth and fluid secretion, contribute to the enhancement of the cystic phenotype of ADPKD cells. The decrease in BCL-2 protein concentration in response to ouabain is not unique to ADPKD cells and it has been found in other cell types [502, 517, 524]. Moreover, overexpression of either BCL-2 or BCL-XL has been reported to abrogate the pro-apoptotic effects of ouabain in a lymphoma cell line [533]. Interestingly, the involvement of the BCL-2 family of proteins in ouabain-mediated effects in cells is also supported by the finding that the α–NKA subunit contains BH1- and BH3-like motifs, similar to those involved in the pairing of BCL-2 family proteins among each other [531, 628], and that the BCL-2 family member proteins, BCL-XL and BAK co-immunoprecipitate with NKA in A549 lung cancer cells and in fetal human epithelial lens cells [531]. While it is unknown whether NKA acts as a scaffolding protein to mediate pro-apoptotic effects via its BH1- and BH3-motifs in ADPKD cells, it is clear that BCL family proteins are involved in ouabain-induced apoptotic effects in the renal epithelial cystic cells.

Besides stimulating the expression of BCL-2 protein, ouabain also impacts other important mediators of apoptosis in ADPKD cells, such as the mitochondria, through the release of cytochrome c. Moreover, ouabain activates the executioner caspases 3 and 7. In contrast, ouabain does not activate the cleavage of caspase-8, an essential effector of the extrinsic apoptotic pathway. Therefore, ouabain induces ADPKD cell apoptosis via specific activation of the intrinsic pathway of programmed cell death. Ouabain has been shown to activate the intrinsic apoptotic pathway in other cell types [499, 502, 519]. Importantly, our results concur with studies in the Han:SPRD rat, a rodent model of ADPKD, in which the increase in apoptosis is dependent on activation of the intrinsic pathway of programmed cell death [262, 263]. In this manner, ouabain enhances an apoptotic route, which commonly participates in ADPKD programmed cell death.

In summary, we have further advanced our understanding of the effects of ouabain in ADPKD cells and found that physiological amounts of ouabain stimulate apoptosis in these cells through activation of the intrinsic pathway of programmed cell death. Activation of this characteristic event of ADPKD, together with other enhancing actions of ouabain on the ADPKD phenotype [475], further supports the role of ouabain as a non-genetic factor that can modulate renal cystogenesis and the progression of ADPKD.

Note: This chapter of the thesis was previously published: Venugopal, J. and G. Blanco, *Ouabain Enhances ADPKD Cell Apoptosis via the Intrinsic Pathway.* Front Physiol, 2016. **7**: p. 107.

CHAPTER 3: OUABAIN & THE EPITHELIAL-TO-MESENCHYMAL TRANSITION

ABSTRACT

ADPKD renal epithelial cells are characterized by an undifferentiated phenotype, with changes in cell polarity and misexpression of proteins including markers of epithelial to mesenchymal transition (EMT). We have previously shown that the hormone ouabain has pro-cystogenic effects on human renal epithelial cells from patients with ADPKD (ADPKD cells) and on embryonic kidneys of an ADPKD mouse model. Here, we studied whether ouabain at physiological circulating concentrations (3 nM) could trigger EMT in the cells. Our results show that ouabain decreases the expression of the epithelial marker E-cadherin and increases the expression of the mesenchymal markers N-cadherin, αSMA, and collagen-I. Ouabain also differentially affected the expression of the tight junction protein occludin, which was elevated in response to ouabain. Other adhesion molecules, such as claudin-1, ZO-1, β-catenin and vinculin were not significantly modified. At the cellular level, ouabain stimulated cell migration, but did not influence invasiveness of ADPKD cells. In addition, ouabain reduced cell-cell interaction and the ability of ADPKD cells to form cell aggregates. Moreover, ouabain increased the transepithelial electrical resistance of ADPKD cell monolayers, but did not affect the permeability to neutral dextran, suggesting that the tightness of the tight junctions and permeability of the paracellular transport pathway were preserved in the cells. All of these effects of ouabain were not observed on normal renal epithelial cells (NHK cells). Altogether these results suggest that ouabain promotes changes in ADPKD cells that do not correspond to the full EMT phenotype; however, this partial EMT contributes to the abnormal cystic characteristics of ADPKD cells. These effects further support the role of ouabain as a factor that stimulates ADPKD progression.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is the most common genetic disorder of the kidney, which occurs in approximately 1:400-1:1,000 individuals [22]. ADPKD is caused by mutations in the *PKD1* or *PKD2* genes, which encode for polycystin-1 and -2 respectively (PC1, PC2), which together form a functional nonspecific cation channel [14, 629, 630]. The main pathological manifestation of ADPKD is the development and relentless growth of multiple epithelial-lined cysts in the kidney, which progressively impairs renal function and eventually leads to end stage renal disease, requiring dialysis or transplant therapy [47, 631]. ADPKD cells have been shown to be dedifferentiated and to undergo changes similar to epithelial-to-mesenchymal transition (EMT) as part of their phenotype. Thus, in ADPKD kidneys there is an upregulation of EMT-related genes and increased fibrosis [87, 584, 585, 632-635]. In addition, it has been suggested that in ADPKD, the renal epithelial cells show an abnormal response to injury, signaling a "wounded status," which initiates a futile wound-healing program that exacerbates the progression of the disease [63]. The response to injury in the kidney has been shown to be associated with the conversion into an EMT phenotype [636, 637].

Polycystin-1 has been shown to directly interact with a number of proteins that are involved in the development of the EMT phenotype. For instance, PC1 localizes *in vitro* at cell junctions in epithelial cells [151, 638], and polycystins interact with components of focal adhesions and the extracellular matrix (reviewed by [639]). Importantly, the altered expression of polycystin-1 in MDCK cells has been shown to promote an EMT-like phenotype, which involves cytoskeletal changes, rearrangement of adhesion proteins, and altered migration of the cells, implying that the aberrant expression of polycystin influences downstream pathways that activate EMT changes in the cells [238].

Although ADPKD is a genetic disorder, environmental factors and hormones have been shown to significantly affect the severity of the disease [41, 640]. We have previously shown that the hormone ouabain, a steroid produced in the adrenal glands [388, 641, 642], is one of those effectors. Ouabain has been shown to activate signaling events that lead to changes in metabolism, adhesion, and proliferation in a cell type specific manner [322, 360, 544, 643]. These effects are mediated via the binding of ouabain to its receptor, the plasma membrane Na,K-ATPase (NKA). Ouabain not only causes changes in intracellular ion concentrations by modifying NKA ion transport activity, but ouabain binding to NKA can also stimulate a cascade of intracellular signaling events in target cells. We have previously shown that the NKA of epithelial cells derived from the cysts of ADPKD patients (ADPKD cells) exhibit an altered affinity for ouabain, which is significantly higher than that of normal human kidney epithelial cells (NHK cells) [322]. Ouabain, in physiologically relevant concentrations (3 nM), initiates extracellular regulated kinase (ERK) signaling and increases proliferation of ADPKD cells, while the proliferation of NHK cells is not affected [322, 491]. Moreover, ouabain stimulates the cAMP induced transepithelial secretion of fluid by ADPKD cell monolayers, and enhances microcyst growth in embryonic kidneys from ADPKD mice grown in culture [360, 361]. In contrast, ouabain does not elicit those effects on NHK cells, or in wild type mouse kidney embryos [360, 361]. Increased cell proliferation and fluid secretions are hallmarks in the pathogenesis of ADPKD cyst formation and development. Therefore, ouabain is a factor that circulates in blood, which affects cystogenesis and can potentially influence the progression of ADPKD [322, 360, 491].

Interestingly, signal transduction pathways that are known to promote cell EMT, such as EGFR, SRC, PI3K, ERK1/2, and PKC [360, 644-648], have been shown to be activated by the binding of ouabain to NKA. Additionally, ouabain can alter the protein content of the cell

junctional complexes in MDCK cells, affecting the adhesion of cells to the tissue culture substrate [536, 544, 546, 649]. Interestingly, in mouse kidneys a compound called marinobufagenin, another member of cardiac glycoside steroid family to which ouabain belongs, has been shown to induce fibrosis and other alterations which resemble EMT [398]. It is therefore plausible that ouabain could enhance the ADPKD phenotype, not only by activating cell proliferation and cAMP dependent fluid secretion, but perhaps also by promoting EMT in the ADPKD cells, which would help the progression of cyst growth. In the present study, we examined whether physiological concentrations of ouabain (3 nM) could influence events associated with the EMT process in NHK and ADPKD cells. We show that in ADPKD cells, but not in NHK cells, ouabain alters the expression of some EMT markers and proteins that are specific to the cell junctional complexes, but not all markers are affected. In addition, ouabain increases the motility and adhesion of the cells, but not the paracellular permeability, or the invasive growth of the cells. This suggests that ouabain induces changes in ADPKD cells that do not correspond to a complete EMT phenotype. However, these partial EMT effects of ouabain promote the cystic phenotype of these cells.

MATERIALS AND METHODS

Cell culture:

A protocol for the use of human kidney tissues was approved by the institutional review board at KUMC. The ADPKD and NHK cells derived from discarded human kidney tissues were cultured as described previously [322, 608]. Briefly, cells were seeded and grown in DMEM/F12 supplemented with 1% heat-inactivated FBS, 100 IU/ml penicillin G and 0.1 mg/ml streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite (ITS). The cells were incubated in media with 0.002% serum 24 h prior to ouabain treatment. Cells were cultured on both filter supports (Transwell Costar, Corning, NY) or plastic culture dishes. All the studies were done in triplicate with ADPKD and NHK cells obtained from at least three different patients.

Protein extraction and immunoblots:

ADPKD cells and NHK cells were treated with 3 nM ouabain at 70% confluency for 24 h in low serum (0.002%) media. Untreated cells served as controls. Cells were then washed with ice-cold PBS and lysed with RIPA buffer (1% NP-40, 0.25% sodium deoxycholate, 1 mM NaVO3, 1 mM NaF, 150 mM NaCl, 1 mM EDTA, 50 mM Tris, and protease inhibitor cocktail (Sigma, St. Louis, MO)). The cleared lysates were stored at -80ºC. For the membrane extracts, CNM kit (Biochain Institute, Inc, Hayward, CA) was used as per manufacturer's instructions. Protein concentration was measured using dye binding assay from Bio-Rad (Hercules, CA). Total protein extracts (20 μg) and membrane extracts (10 μg) were electrophoresed on a 10% acrylamide gel and transferred to nitrocellulose membrane. The immunoblots were probed for the levels of occludin, claudin-1, ZO-1, E-cadherin (ThermoFisher Scientific, Waltham, MA), vinculin

(Millipore, Temecula, CA), αSMA (Sigma, St. Louis, MO), N-cadherin, vimentin, pSMAD3 (Cell Signaling, Danvers, MA), TGFβ, fibronectin, and snail (Santa Cruz Biotechnology Inc., Dallas TX) using western blot protocol described previously [322]. After detection by chemiluminescence, the images were scanned and quantified for band intensity using the Gel-Pro software. The protein levels were normalized against tubulin levels and expressed as density units relative to the untreated controls.

Cell aggregation assays:

ADPKD and NHK cells were suspended in 0.002% serum media with or without ouabain (3 nM) at a concentration of 25,000 cells/mL in a 50 mL conical tube (Corning Inc., Corning, NY) with loosened cap at 37˚C, 5% CO^2 for 1 hour. At this point an aliquot of 500 µL was removed from each tube and placed on ice; time point was denoted as "0 Hr." The remaining cells were then incubated in the shaker at 100 rpm at 37˚C to form aggregates. Cells were then collected at 30 and 60 min intervals and placed on ice. Samples were maintained on ice until all the samples were collected and then processed. To each tube containing cells, 500 µL PBS + 0.4% Tween-20 was added to yield a final concentration of 0.2% Tween-20, then DAPI was added to each tube at a concentration of 1x. Cell clusters were counted using flow cytometry (LSRII flow cytometer, Beckton Dickinson, Franklin Laks, NJ).

Cell invasion assays:

Invasion assays were performed using Cultrex 96-well BME cell invasion assay kit, following manufacturer's instructions (R&D Systems, Minneapolis, MN). Briefly, 50,000 serum-

starved cells were plated on to basement membrane extract (BME)-coated transwell membranes followed by an addition of 150 μL cell media either with or without ouabain to the bottom chamber. The cells were allowed to invade for 24 h. and the cells that migrated to the underside of the membrane were dissociated and labeled using Dissociation/Calcein-AM fluorescent solution (BD Biosciences, San Jose, CA) and read at an excitation/emission of 485nm/520nm using a BioTek Synergy HT plate reader (Biotek, Winooski, VT. The number of cells which invaded through the BME was quantified using a standard curve obtained from cells that migrated on uncoated membranes.

Cell migration assays:

ADPKD or NHK cells were plated on 6 well plates and grown until confluency. Cells were then deprived of serum for 24 hours. Using a pipette tip, a scratch was made in the monolayer; ouabain was applied at the time of scratch. Pictures of the well were taken using a Celigo imaging cytometer (Nexcelom, Biosciences LOC, Lawrence MA), at 0, 4, and 8 after the scratch. Images were analyzed using ImageJ. In some experiments, cells were treated with or without ouabain 24 h before the scratch, at which time ouabain was re-applied.

Immunofluorescence analysis:

ADPKD and NHK cells were used. Cells were grown until they achieved confluency on transwell insert plates. Then, they were deprived of serum for 24 h and ouabain at a concentration of 3 nM was applied to the basolateral side of the cells. Cultures were further maintained in culture for 24 h. At these time points, monolayers were washed three times with ice-cold PBS before 20

minutes of methanol at −20 °C to fix and permeabilize the samples. After three more washes with PBS, samples were blocked for 1 h with 0.5% BSA, and incubated for 1 h at 37 °C with a specific primary antibody. Samples were washed 3 times as above. Cells were then incubated with FITC conjugated goat anti-mouse or anti-rabbit antibodies (1:1000) depending on the source of the primary antibody. This was followed by 3 washes with PBS and cells were mounted on glass slides with Slow Fade Gold Antifade reagent with DAPI (ThermoFisher Scientific, Waltham, MA), and viewed on a Nikon Eclipse 80i equipped with digital camera.

Dextran assays:

Cells were grown at a density of 1 x 10^6 cells per well in six-well transwell inserts to form confluent monolayer on transwell filter supports (0.4 uM pore size, from BD Bioschiences, San Jose, CA). Ouabain was added basolaterally at given time points. Control cells had no ouabain in either chamber. Paracellular permeability was assessed by FITC-dextran (4 kDa, Sigma, St. Louis, MO) flux through monolayers treated for 24, 48, and 72 hours, as described [546]. Cells were washed with "P Buffer" [10 mM hepes (pH 7.4), 1 mM sodium pyruvate, 10 mM glucose, 3 mM CaCl2, and 145 mM NaCl]. A freshly prepared solution of 10 μg/mL of FITC-dextran was dissolved in the P buffer. The diluted mixture was added to the upper chamber of the transwell insert to measure the permeability from apical to basolateral (A-BL) direction and to lower chamber to measure the permeability from basolateral to apical direction (BL-A). After 1 h incubation at 37 °C, the basal medium was collected and the fluorescence of the transported FITC-dextran was measured with a synergy HT fluorescence spectrometer (BioTek, Winooski, VT) at 492 nm (excitation) and 520 nm (emission). The FITC was quantified by extrapolating from a standard curve. The unidirectional flux of dextran in the apical to basolateral direction (JDEX in

ng·cm−2·h−1) was calculated by dividing the fluorescence intensity of a given sample of the bottom solution (arbitrary units) by the corresponding value of the upper solution or vice versa. For convenience the JDEX values were normalized with control samples at the 24 h time point and expressed as a relative JDEX value.

Transepithelial Electrical Resistance (TER) measurements:

Transepithelial Electrical Resistance (TER) was performed using transwell permeable supports (0.4μm, 12 well, Corning Inc., Corning, NY). The cells were seeded on the filters in a density of 120,000 cells/cm^2. Cells were grown until they achieved confluency on transwell inserts. Then, they were deprived of serum for 24 h and ouabain was applied at a concentration of 3 nM to the basolateral side of the cells. Cultures were further maintained in culture for 24, 48 and 72 h, then TER was measured by using automated cellZscope (NanoAnalytics, Munster, Germany).

Statistical Analysis:

All the experiments were performed in triplicates with cystic epithelial cells obtained from at least three different ADPKD patients. Statistical significance of the differences between ouabain treated and untreated controls was determined by Student's T-test and Anova. Statistical significance was defined as $P<0.05$.

RESULTS

Ouabain alters the expression of EMT markers in ADPKD cells.

In ADPKD, cells have been shown to undergo changes resembling EMT, which may help their cystic phenotype [574]. Based on our findings that ouabain enhances other aspects of ADPKD cystogenesis, we were interested in studying whether ouabain could induce EMT changes in ADPKD cells. Our first approach to this end consisted in assaying commonly used markers of EMT after 24 h treatment of the cells with or without 3 nM ouabain. Immunoblot analysis of EMT related proteins in NHK cells showed that ouabain had no significant effect on expression levels of E-cadherin, N-cadherin, αSMA, collagen-I, vinculin, or fibronectin. In contrast, ouabain treatment decreased the epithelial marker E-cadherin and increased the mesenchymal markers N-cadherin, αSMA, and collagen-I in ADPKD cells (Fig. 3.1). The expression of other mesenchymal markers, such as vimentin and fibronectin where not changed in ADPKD cells with ouabain treatment (Fig. 3.1). This suggests that ouabain is able to induce protein expression changes that are commonly found in EMT; however, not all mesenchymal markers are upregulated by ouabain in ADPKD cells.

Ouabain modifies cell-cell adhesion properties in ADPKD cells

In order to investigate if ouabain influences cellular events that are associated with EMT in ADPKD cells, we studied the effect of ouabain on the adhesion properties of the cells. ADPKD and NHK cells in suspension were treated with and without 3 nM ouabain and then were allowed to form aggregates under incubation in a shaker. The number of 2n and 4n nuclei, indicative of aggregated cells, was evaluated by flow cytometry. Ouabain reduced the ability of ADPKD cells to form cell aggregates in ADPKD after 30 minutes of incubation and this effect persisted at the

60 minute time point as well (Fig. 3.2B). In contrast, ouabain did not significantly change cell aggregation in NHK cells (Fig. 3.2A). This suggests that ouabain affects the cell to cell attachment properties of ADPKD cells, but not NHK cells.

Figure 3.1

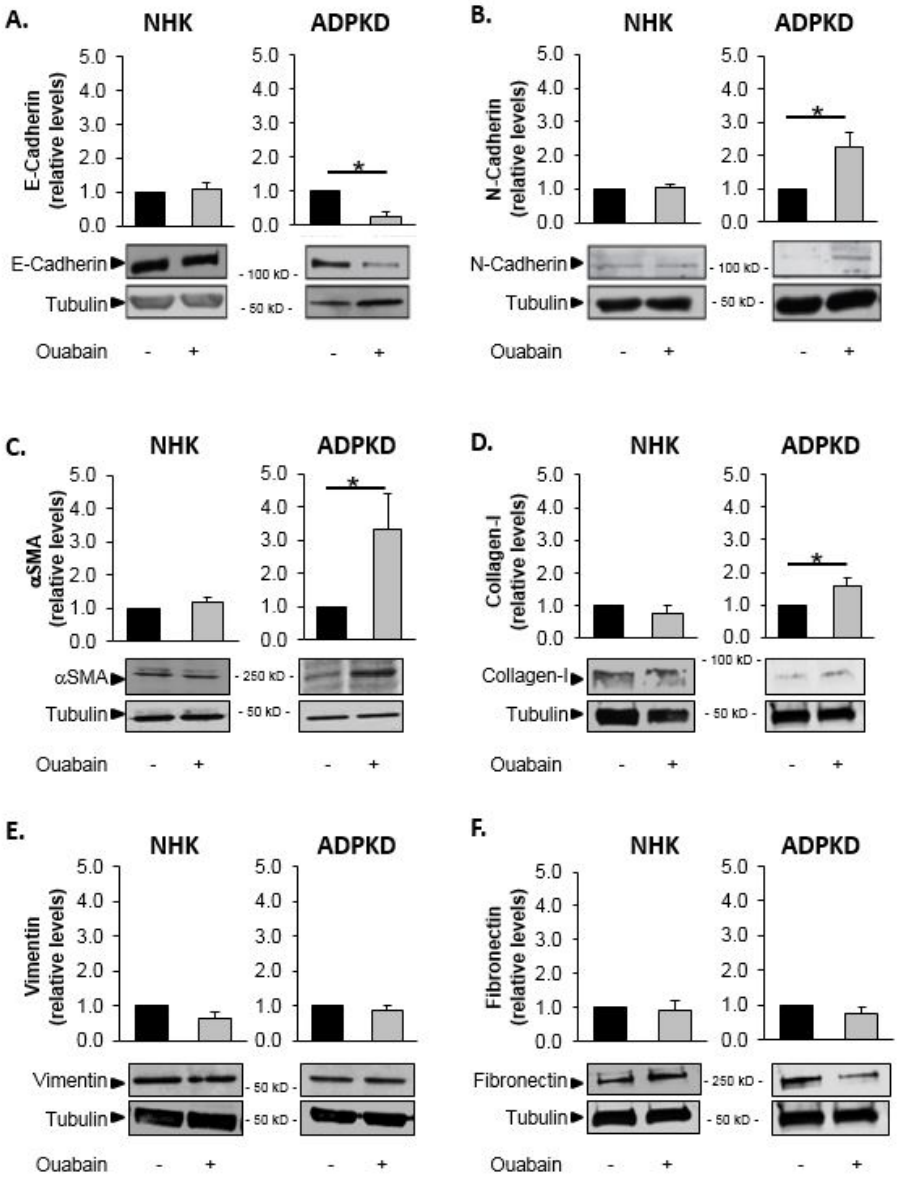

Figure 3.1. Ouabain effects on expression of EMT markers in NHK and ADPKD cells. ADPKD and NHK cells in culture were treated without and with 3 nM ouabain for 24 hours. Cells were harvested, lysed and the proteins of interest identified by immunoblot. A) E-cadherin, B) N-cadherin, c) αSMA, d) collagen-I, E) vimentin, and F) fibronectin. The lower panels show representative blots, while the top panels show the average values ± SEM of the densitometric analysis of 3 different experiments. Statistical significant differences are shown relative to control values, with asterisks indicating $P<0.05$.

Ouabain enhances migration, but not invasion of ADPKD cells

Another functional consequence of EMT is the enhanced ability of the cells to migrate [553, 650]. Therefore, we tested if ouabain was able to alter the migration rate of ADPKD and NHK cells, using wound healing assays. For this, confluent cell monolayers were scratched with a micropipette tip to inflict a wound in the culture and were treated without and with 3 nM ouabain. Repair of the wound was analyzed over time, by taking serial pictures of the culture at 0, 4, and 8 h after the scratch was performed. Ouabain did not affect cell migration in either NHK or ADPKD cells at these short-term treatments (data not shown). However, when cells were pretreated with ouabain for 24 h before wounding, then a significant enhancement of migration occurred 8 h after scratch induction in ADPKD, but not in NHK cells (Fig. 3.3A-C). This suggests that ouabain is able to enhance cell migration, a characteristic of EMT, in ADPKD, but the effect requires relatively prolonged exposure times to ouabain.

Figure 3.2

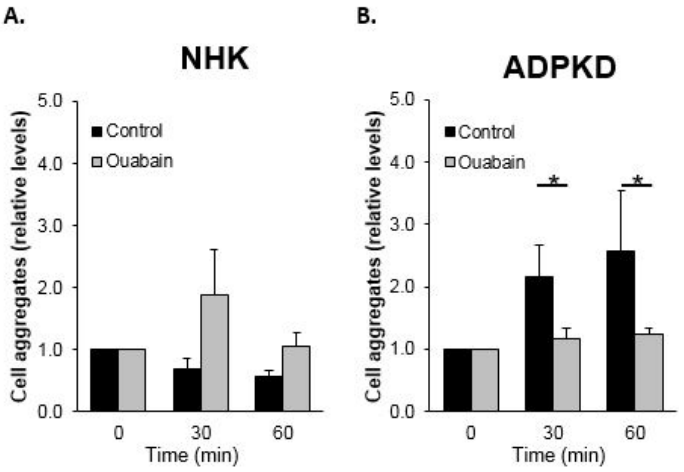

Figure 3.2. Ouabain effect on NHK and ADPKD cell-cell attachment. NHK and ADPKD cells were incubated in suspension on a shaker and were treated with and without 3 nM ouabain. After the indicated times, the number of cell aggregates formed was determined, based on the number of 2n and 4n nuclei, measured by flow cytometry. A) NHK cells, B) ADPKD cells. Bars represent mean densitometry values ± SEM of 3 determinations using cells from different ADPKD kidneys. Asterisks indicate statistical significance, with $P<0.05$.

To determine whether ouabain promotes not only cell migration, but also invasion, we tested the ability of the cells to cross through an extracellular matrix support after the addition of ouabain. Cells were grown on basement membrane extract (BME)-coated filter inserts and were

Figure 3.3

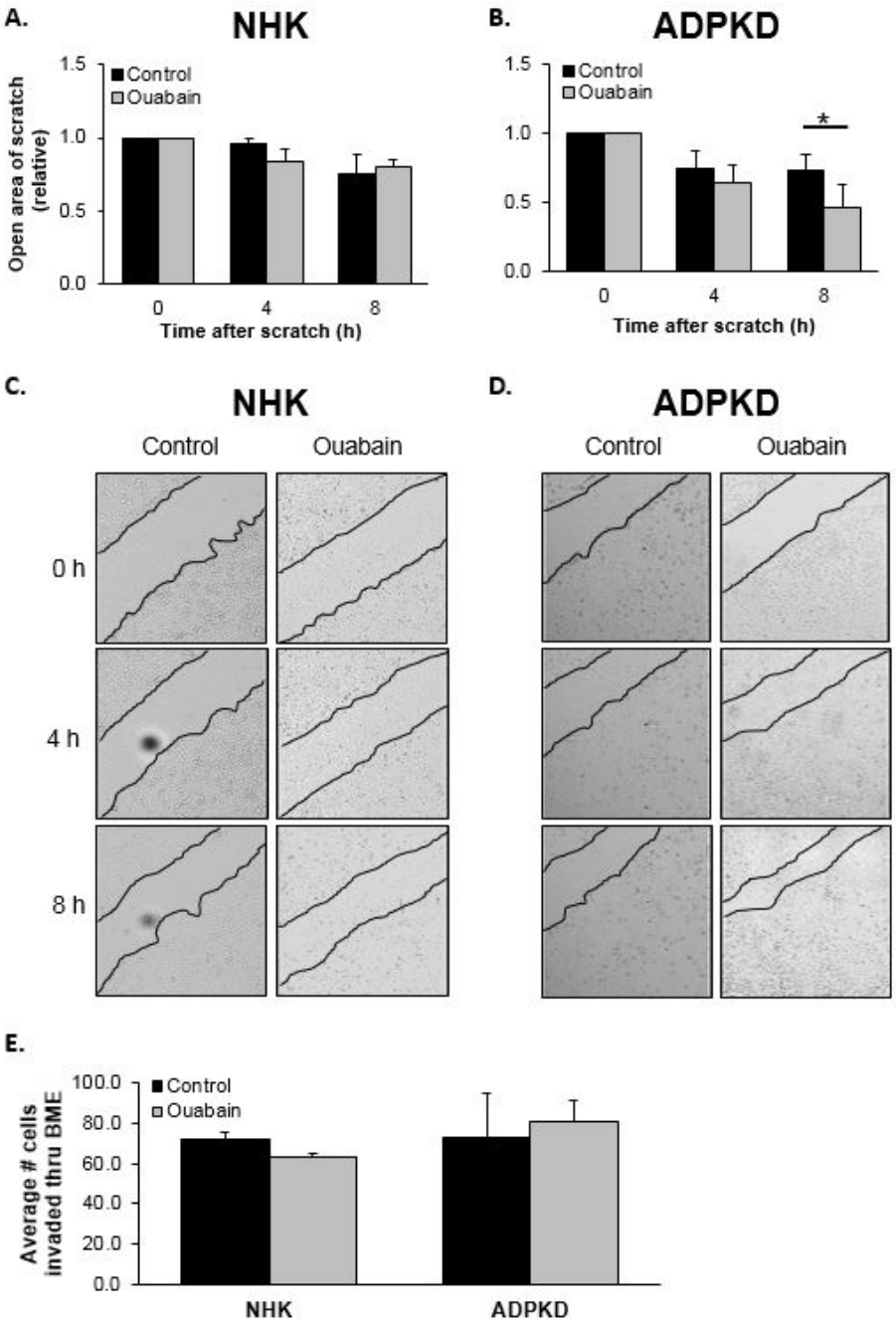

Figure 3.3. Ouabain effects on NHK and ADPKD cell migration and invasive growth. A-D) Cells were pretreated with ouabain for 24 h. Then a scratch was made in the confluent monolayer with a pipette tip and healing of the wound was followed by taking pictures at the indicated times after inflicting the scratch. Representative pictures are shown; black lines delineate edge of the wound. Cells from 4 separate kidneys were assayed, in duplicate. Bars indicate mean ± SEM, expressed relative to control values at time of scratch. Asterisks indicate statistical significance with respect to 0 time, with $P<0.05$. E) 24 h ± ouabain. The number of cells translocated through basement membrane extract (BME). Bars denote mean ± SEM. *$P<0.05$.

treated in the absence and presence of 3 nM ouabain. After 24 h, the number of cells that invaded through to the support was quantified. As shown in Fig. 3.3E, ouabain did not increase invasiveness in either NHK or ADPKD cells. This shows that while ouabain enhances cell migration, it does not change the aggressiveness of cells to grow across their basement membrane support.

Ouabain selectively modulates tight junctions in ADPKD cells.

Tight junctions between epithelial cells maintain the structure of the cell monolayer and form a semipermeable diffusion barrier that restricts the passive diffusion of molecules, according to their charge and size (reviewed in [651]). These are dynamic structures, which consist of multi-protein transmembrane complexes that include claudins and occludins, as well as scaffold proteins, such as the ZO proteins [549]. Since we found ouabain to induce relaxation of cell-cell adhesion and increase cell migration, we hypothesized that ouabain treatment may alter the expression of tight junction

Figure 3.4

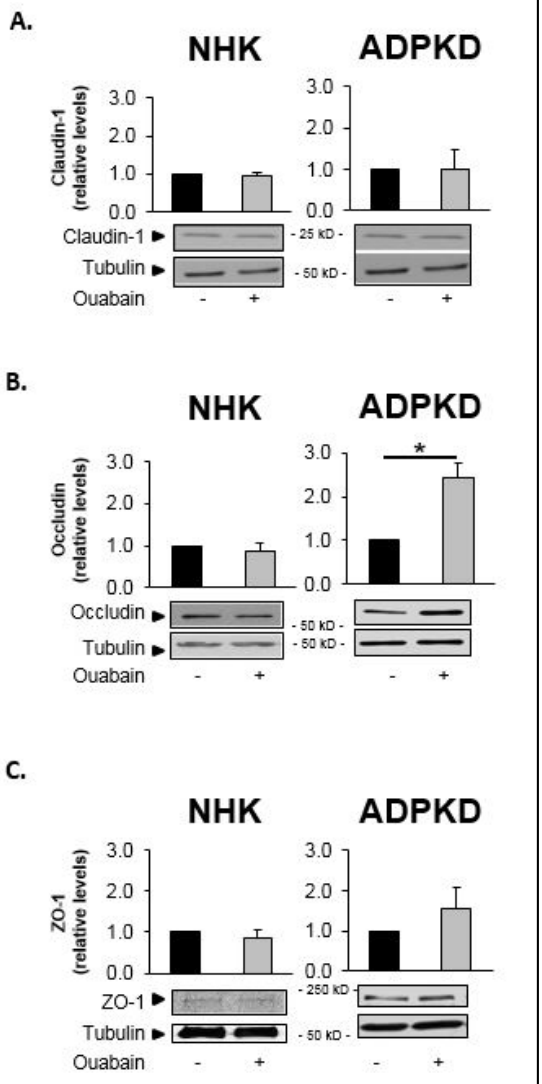

Figure 3.4. Ouabain effect on cell tight junctions in NHK and ADPKD cells. NHK and ADPKD cells were treated without and with ouabain (3 nM) for 24 hours and tight junction protein expression was analyzed by immunoblot and immunofluorescence. Representative pictures of the cultures are shown in the upper panels, while immunoblots of each sample are displayed below. A) Claudin-1, B) Occludin, and C) ZO-1. Bars denote mean densitometry levels ± SEM of 3 determinations using cells from different ADPKD kidneys; relative to control values. Asterisks indicate statistical significance with respect to untreated controls, with $P<0.05$.

proteins in the cells, which will facilitate the capacity of the cells to migrate. In addition, changes in tight junction proteins are observed as part of EMT [553, 650]. To test this, we cultured NHK and ADPKD cells for 24 h with and without 3 nM ouabain, after which protein lysates were prepared and a series of tight junction proteins were analyzed by immunoblot and immunocytochemistry. NHK cells did not respond to ouabain with changes in the tight junction proteins tested (Fig. 3.4A – 4C). In contrast, ouabain differentially affected tight junction protein expression in ADPKD cells. While claudin-1 and ZO-1 were not significantly modified (Fig. 3.4A, and 3.4C), occludin amounts were elevated (Fig. 3.4B). Immunolocalization analysis of these proteins showed that ouabain did not change the plasma membrane localization of tight junction proteins either in NHK and ADPKD cells (Fig. 3.6A). These results show that ouabain is a selective regulator of tight junction protein expression in ADPKD cells, upregulating occludin. While ouabain did not induce subcellular localization changes of the tight junction proteins tested, localization of claudin-1, occludin, and ZO-1 is not altered in ADPKD mouse models, and thus the action of ouabain does not go against the described phenotype of ADPKD epithelium [652].

Figure 3.5

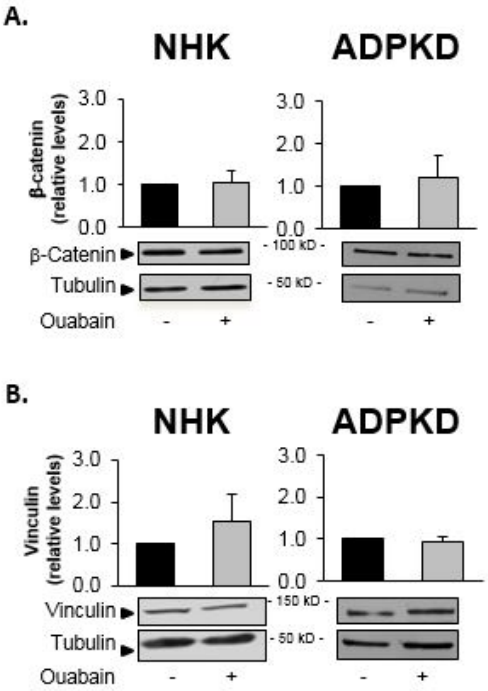

Figure 3.5. Ouabain effect on adherens junction proteins in NHK and ADPKD cells. NHK and ADPKD cells were treated without and with ouabain (3 nM) for 24 hours And the expression of adherens junction proteins was analyzed by immunoblots of each sample are shown A) β-catenin and B) Vinculin. Bars denote mean densitometry ± SEM of 3 determinations using cells from different ADPKD kidneys; relative to control values. Asterisks indicate statistical significance with respect to untreated controls, with $P<0.05$.

Ouabain modulates adherens junctions in ADPKD cells in a selective manner.

Adherens junctions lie more basal to tight junctions and serve to connect the actin cytoskeletons of neighboring epithelial cells. Interestingly, PC1 associates with a multiprotein complex containing E-cadherin, α-, β- and γ-catenins [217, 221]. In ADPKD cells, the polycystin-1/E-cadherin/β-catenin complex is disrupted and both polycystin-1 and E-cadherin have been shown to be depleted from the plasma membrane as a result of the increased phosphorylation of polycystin-1 [153, 222]. Changes in adherens proteins are also typically observed during EMT.

We assayed the effect of ouabain on the expression levels and cellular localization of several adherens junction proteins, including E-cadherin, β-catenin, and vinculin in NHK and ADPKD cells. In NHK cells, ouabain did not have a significant effect on either the expression levels or localization of E-cadherin, β-catenin or vinculin (Fig. 3.1, 3.5A-C, 3.6B). In ADPKD cells, ouabain decreased the expression levels of E-cadherin (Fig. 3.1A). In contrast, the expression and localization of β-catenin and vinculin remained unchanged after ouabain treatment (Fig. 3.1, 3.5A-C, 3.6B). Therefore, similar to what occurred with tight junctions, ouabain also selectively regulated the expression of zonula-adherens proteins, specifically decreasing the levels of E-cadherin.

TER but not paracellular permeability is altered in response to ouabain in ADPKD.

Junctional complexes control the diffusion of ions and hydrophilic solutes across the paracellular route [549]. The ability of ADPKD cells to form cysts suggests that their junctional

Figure 3.6

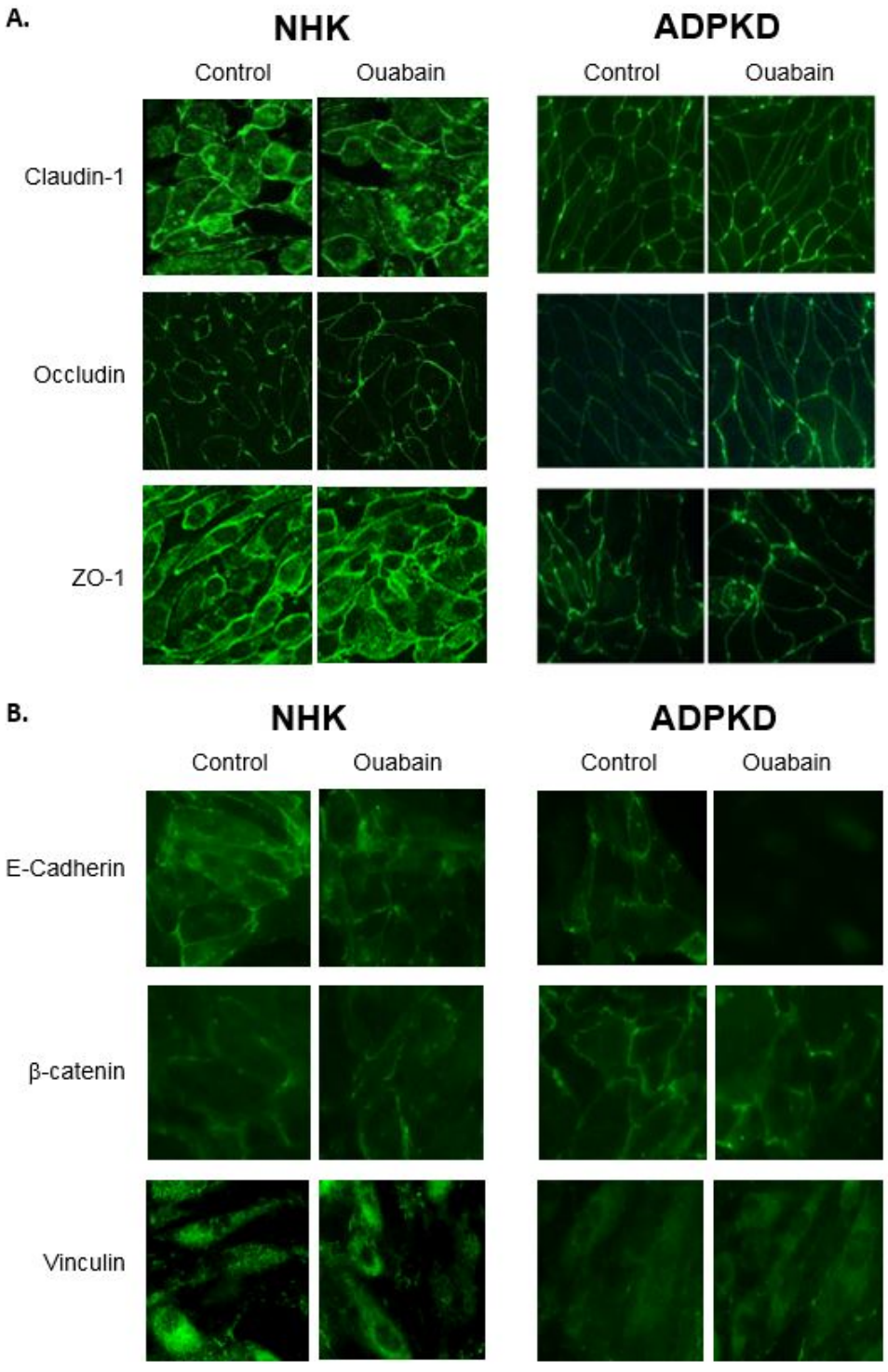

Figure 3.6. Ouabain effect on localization of junction proteins. NHK and ADPKD cells were treated without and with ouabain (3 nM) for 24 hours and the expression of adherens junction proteins was analyzed by immunocytochemistry. Representative pictures of the cultures are shown for A) Tight junction proteins and B) Adherens junction proteins.

complexes continue functioning as a selective barrier to maintain the tightness of the epithelium lining the cysts. However, some reports have shown that ADPKD is accompanied by alterations in the apical junctional complexes and dysregulation of the polarity and growth of the cells [238, 248, 652]. This agrees with the dedifferentiated phenotype described of cystic cells and the EMT-like alterations that ADPKD cells exhibit [226, 574, 575]. We tested the ability of ouabain to influence transepithelial electrical resistance (TER) after treatment without and with 3 nM ouabain for different times. We found that ouabain significantly enhanced TER in ADPKD cells, an effect which was observed at 48 and 72 h treatment with ouabain (Fig. 3.7A). In contrast, no significant change in TER was found in NHK cells (Fig. 3.7A).

The unidirectional flux of dextran (J_{DEX}) from the apical to the basolateral side of the epithelium, or vice versa, is a measure of the restriction offered by tight junctions to the movement of neutral molecules across an epithelium [653]. To further assess the effects of ouabain on paracellular transport of NHK and ADPKD cells, we determined whether ouabain regulated the paracellular transport of solute in the cells. For this, the permeability of neutral dextran molecules (4 kDa in size) was measured in monolayers of NHK and ADPKD cells grown in transwell filters and treated in the absence and presence of 3 nM ouabain for different times. We did not find any significant differences in the transport of 4 kDa dextran between the treatments in either cell type. This occurred for both the apical to basolateral, as well as for the basolateral to apical movement of dextran (Fig. 3.7B and 3.7C). Thus, it appears that ouabain does not cause functional changes in the paracellular pathway involved in moving solutes across the epithelial monolayer of NHK and ADPKD cells.

Figure 3.7

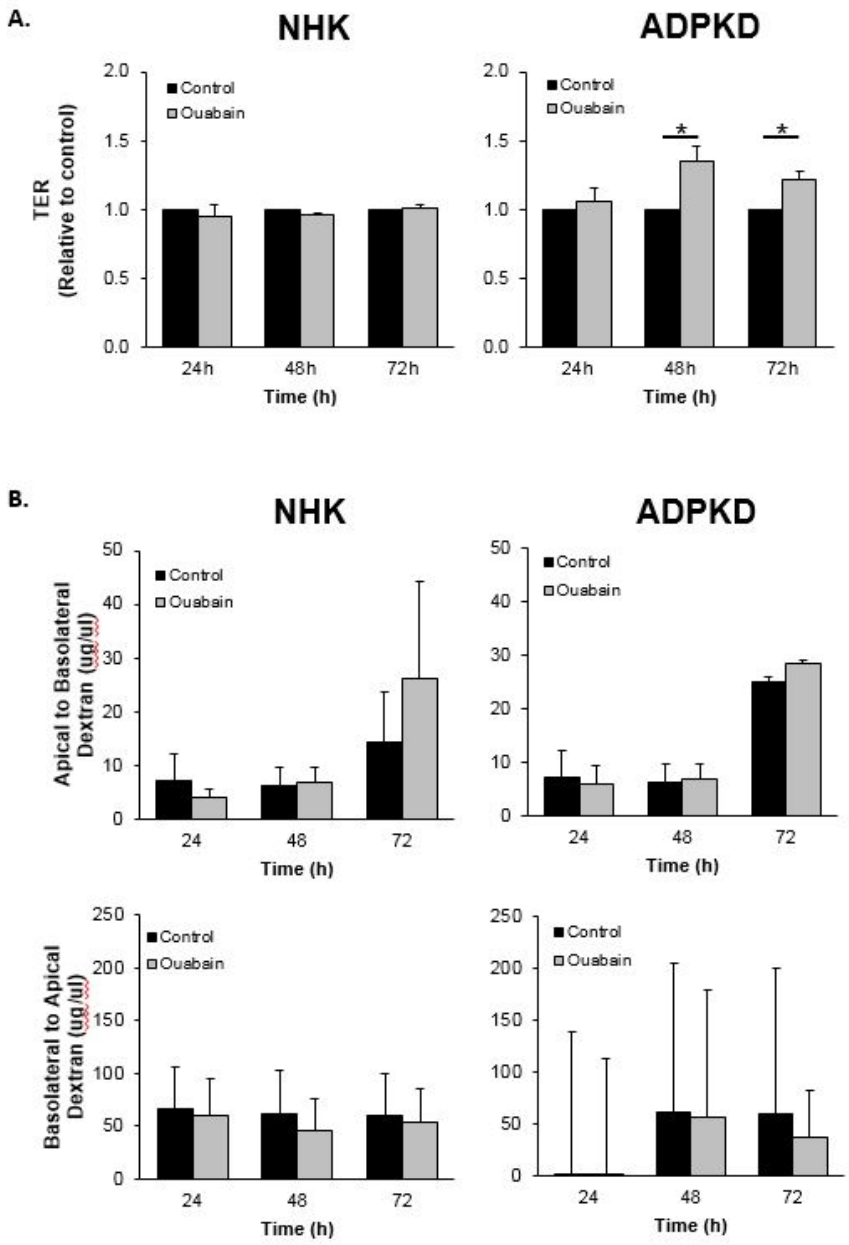

Figure 3.7. Ouabain effect on TER and paracellular movement of dextran in NHK and ADPKD cells. (A) Cells were grown to confluency on transwell inserts and treated with ouabain (3 nM) for the indicated times. TER was measured using an automated cellZscope. Bars denote mean ± SEM of cells obtained from 3 separate kidneys. *P<0.05. (B) Paracellular permeability was assessed by FITC-dextran flux through confluent monolayers treated without and with ouabain (3 nM) for the indicated times. Bars denote mean ± SEM of cells obtained from 4 separate kidneys. Asterisks indicate statistical significance with respect to untreated controls, with P<0.05.

The TGFβ-Smad3 signaling is activated by ouabain in ADPKD cells

TGFβ is a key factor in the development of fibrosis and in EMT promotion in several kidney diseases [585, 650, 654, 655], including ADPKD [584-586]. TGFβ activates TGFβR which then recruits transcription factors Smad2 and Smad3. Together with Smad4, the Smad2/3 complex translocates to the nucleus and binds to regulatory elements on DNA, leading to increased transcription of key genes which promote EMT [654]. We investigated the involvement of the TGFβ signaling cascade in relation to EMT-like alterations that ouabain produces in ADPKD cells. We observed that ouabain increased TGFβ protein levels in ADPKD cells treated with 3 nM ouabain for 24 h, compared to untreated cells (Fig. 3.8A). In addition, we also observed that the expression of Snail, a TGFβ target gene which governs EMT and fibrosis [656, 657], is increased in ADPKD cells in response to ouabain (Fig. 3.8B). These effects on TGFβ and Snail were not found in NHK cells (Fig. 3.8A and 3.8B). When Smad3 signaling was analyzed, we found that ouabain activated the phosphorylation of Smad3 in ADPKD cells within 15 minutes of ouabain application. In contrast, ouabain did not promote Smad3 phosphorylation in NHK cells (Fig. 3.8C). Taken together, these data show that ouabain can activate the TGFβ-Smad pathway, which agrees with the EMT related effects that ouabain elicits in ADPKD cells.

Figure 3.8

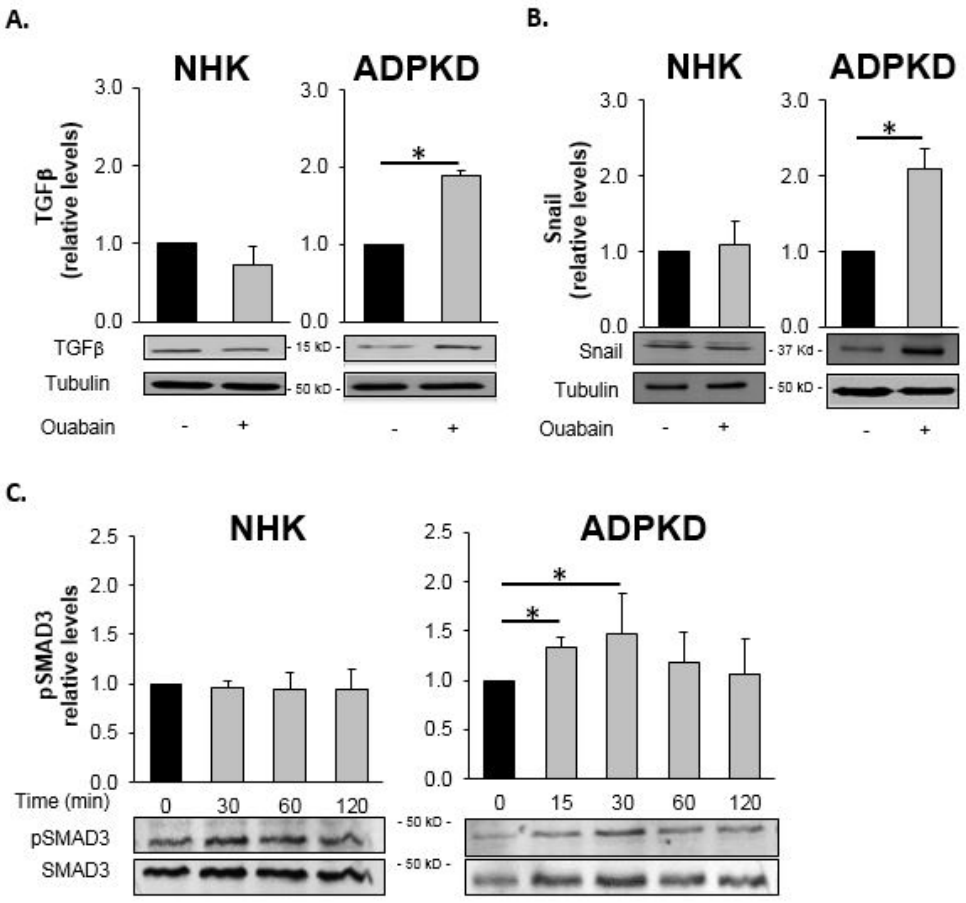

Figure 3.8. Ouabain effect on the TGFβ signaling pathway in NHK and ADPKD cells. (A, B) NHK and ADPKD cells were treated without and with ouabain (3 nM) for 24 hours and the relative levels of TGFβ were determined by immunoblot. (C, D) NHK and ADPKD cells were treated with or without ouabain and total and phosphorylated Smad3 levels were determined at the indicated time points. Bars represent phosphor-Smad3 levels as a fraction of the untreated comtrols and relative to the untreated controls. Bars represent the mean ± SEM of 3 determinations using cells from different ADPKD kidneys. Asterisks indicate statistical significance with respect to untreated controls, with $P<0.05$.

DISCUSSION

Phenotypic changes characteristic of EMT have been described in ADPKD [658] and a number of EMT-related genes have been found to be upregulated in kidney samples from patients with ADPKD [87]. Ouabain and other cardiotonic steroids have been shown to modify cell junctional complexes and induce EMT in various epithelial cells in culture [398, 544, 546]. Previous results from our laboratory have shown that ouabain is a factor that enhances several characteristics of ADPKD cells that are relevant to the progression of the disease. Among those are the exacerbation of ADPKD cell proliferation and the increase in cAMP-mediated fluid secretion [322, 360, 361, 491]. These effects contribute to the dedifferentiated phenotype that renal ADPKD cells are known to present [226, 574, 575]. In this work, we found that, in contrast to NHK cells, ouabain exerts complex effects on ADPKD cells that accentuate the dedifferentiated state of cystic cells, compatible with an EMT phenotype. For instance, ouabain decreased E-cadherin and increased N-cadherin protein levels in ADPKD cells, two major markers of EMT. In addition, other typical markers of a mesenchymal phenotype also increased, including αSMA, and collagen-I. While this supports the idea that ouabain is pushing ADPKD cells towards an EMT phenotype, a complete transition to a mesenchymal phenotype was not observed. Thus, some of the tight and adherens junctional proteins of the cells, such as vimentin and fibronectin appeared not to be affected. The complete transition from an epithelial state to a mesenchymal state is the classic definition of EMT [650]. However, recent studies have recognized the existence of intermediate, "metastable" stages of EMT in epithelial and cancer cells [659-661]. This implies that a range of heterogeneous patterns of mesenchymal change can be observed in cells [659-661]. Our observations on the effects of ouabain on EMT agree with the notion that this compound is causing a partial EMT or a metastable state in the ADPKD cells. Such a state is increasingly

recognized to occur in renal cells under conditions of wounding or stress [634, 662-664]. It is important to note that our experiments were performed on primary cell cultures. It is possible that when placed under *in vitro* conditions, the cells exhibit a degree of dedifferentiation that may not reach that achieved in the native *in vivo* environment. This could result in relatively higher background levels of expression of makers of cell EMT, which may make it more difficult to detect changes in EMT-related gene expression. In any case, our results clearly show a specific response in ADPKD cells in favor of a higher undifferentiated phenotype. Moreover, the increase that we observe in collagen-I suggests that ouabain treated ADPKD cells may be undergoing fibrotic changes. This effect coincides with the deleterious functional consequence that ADPKD has on renal function, which is in great part dependent on the fibrosis that affects and compromises renal function [665]. We also found that ADPKD, but not NHK cells respond to ouabain with selected changes in expression of proteins involved in cell tight junction and adherens junction proteins. Accordingly, we show that ADPKD cells respond to ouabain with a lower ability to form cell aggregates when in suspension cultures. We observed a reduction in E-cadherin, which will reduce adhesion between ADPKD cells. The cellular localization of E-cadherin was not changed by ouabain treatment of the cells. Therefore, it appears that the effect is due to rapid degradation of the protein, rather than its mobilization to intracellular stores. Previous results in our laboratory have shown that ouabain causes an internalization of the NKA to the cytoplasm of ADPKD cells [322]. The β-NKA subunit is known to function as a cell-cell adhesion molecule, which interacts with other β-NKA polypeptides expressed at the plasma membrane of neighboring cells (reviewed in [536] and [542]). Therefore ouabain-induced endocytosis of NKA may be an additional mechanism to explain the decreased cell-cell adhesion observed with ouabain in ADPKD cells.

Interestingly, the expression level of NKA has been proposed to be included as a marker of EMT [666].

Another adhesion protein found to be modulated by ouabain in ADPKD cells is occludin, the protein expression of which increased. This result was unexpected, since occludin commonly decreases in EMT [553]. While this finding does not agree with alterations typical of EMT, it may be related to a compensatory response by the cells to maintain the integral structure of tight and adherens junctions. This may help retain the overall structural integrity of the ADPKD cysts, in spite of the lower E-cadherin amounts. It may also permit the epithelium to continue accumulating fluid within the cyst lumen, despite other ouabain-modified tight junctional complex alterations which might otherwise cause fluid leakage. In support of this, our experiments that show an increase in the TER of the ADPKD cell monolayers. Therefore, ouabain is causing a remodeling of cell junctions that may help the functional needs of the cystic ADPKD epithelium.

Similar to our studies, it has been shown that ouabain at relatively high concentrations (1 uM) induces cell detachment in MDCK cells and this was associated with loss of tight junction complexes [462]. This effect however, was not seen when lower concentrations of ouabain (10 - 50 nM) were used [546]. Lower concentrations of ouabain may be sufficient to elicit an effect on tight junctions in ADPKD cells compared to MDCK cells, as ADPKD cells have an abnormally high affinity to ouabain [322]. In addition, MDCK cells responded to high ouabain doses with changes in a different subset of adhesion proteins than the ones we find modulated by ouabain in ADPKD cells. While this could perhaps be attributed to differences in the cell type used in each of the studies, it appears clear that ouabain plays a role in the regulation of cell adhesion, and that this effect is different in normal and ADPKD diseased cells.

Ouabain decreased the adhesive properties and augmented the mobility of ADPKD cells, further supporting the undifferentiated phenotype of these cells. In contrast, ouabain does not influence the invasive properties of ADPKD cells. As ADPKD cells are not invasive (reviewed in [667]), the phenotype elicited by ouabain does not contradict the non-invasive phenotype of ADPKD, but does promote some migration. In contrast to ADPKD cells, ouabain has no effect on NHK cells.

Our studies assessing dextran permeability also show that ouabain does not change the paracellular transport of neutral solutes in NHK or ADPKD cells. This effect agrees with a maintenance of the function of junctional complexes by ouabain, which is needed to maintain cyst functional and structural integrity [360, 361]. We observed that ouabain induced a time-dependent increase in TER in ADPKD cells. TER is an indicator of the paracellular resistance to ions of the epithelium. Its increase indicates that ouabain favors the ion differences between the cyst lumen and the interstitium, while still allowing the passage of solutes as shown from the constant permeability that the cells exhibit to the passage of dextran. At present, how the changes in TER that ouabain induces in ADPKD cells correlate with the composition of tight junction proteins is unclear. Further studies will be needed to ascertain the structure function relationship of tight junctional complexes in ADPKD cells. However, it is of interest that increases in occludin expression have been correlated with increased TER [545], two conditions which occur upon ouabain treatment in ADPKD. Changes in tight junction proteins in response to ouabain treatment, with an increase in TER values, but not dextran permeability have also been reported by Larre et al [546]. However, similar to our studies, these authors were not able to define how this correlated with the profile of different junctional proteins (reviewed in [651]).

Not only does ouabain induce EMT in ADPKD cells, we also observed activation of the TGFβ/Smad pathway. Thus, we found a ouabain-dependent increase in the expression of TGFβ, the phosphorylation of SMAD3, and the levels of Snail protein. In ADPKD patients, target genes of the TGFβ pathway are upregulated compared to normal controls [574, 584, 585], with SMAD2/3 rather than SMAD 1/5/8 found to be activated [584, 585]. Therefore, our data correlates well with ouabain-mediated enhancement of the EMT phenotype observed in ADPKD. TGFβ is a known driver of EMT, yet the targets of TGFβ signaling depend greatly on the cell type (reviewed in [668]). This may explain why in ADPKD cells we find that not all EMT markers are affected by ouabain.

In conclusion, ADPKD cells, have been shown to exhibit altered apical to basal, as well as planar polarity, increased cell migration, and an undifferentiated cell state [238, 248, 652]. The dedifferentiated phenotype of ADPKD cells was found to be partially enhanced by ouabain. These results further support the role that we previously reported for ouabain as a factor that promotes ADPKD progression.

CHAPTER 4: OUABAIN-MEDIATED ALTERATIONS IN CELLULAR CALCIUM

ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the progressive growth of renal cysts that alter the structure and function of the kidney. Previously, we have shown that the hormone ouabain enhances cell proliferation, fluid secretion, and promotes cell dedifferentiation in cystic kidney epithelial cells from patients with ADPKD (ADPKD cells); but does not have these effects in normal human kidney epithelial cells (NHK cells). Intracellular calcium concentration ($[Ca^{2+}]_i$) plays an important role in the pathophysiology of ADPKD. In this work, we explored the effect of ouabain on $[Ca^{2+}]_i$ levels in NHK and ADPKD cells and the mechanism of action by which ouabain elicits its effects. We found that ouabain increased $[Ca^{2+}]_i$ in NHK cells, but not in ADPKD cells. While ouabain raised Ca^{2+} in the cell cytoplasm, it did not change endoplasmic or mitochondrial Ca^{2+}. Ouabain-induced $[Ca^{2+}]_i$ elevation in NHK cells was blocked by Ca^{2+} removal from the medium, suggesting that plasma membrane ion channels are involved in the response. The Ca^{2+} increase elicited by ouabain in NHK cells was abrogated by the L-type calcium channel (LTCC) inhibitor verapamil. Moreover, the LTCC agonists Bay K8644 and FPL-64176 restored the ouabain-dependent $[Ca^{2+}]_i$ increase in ADPKD cells. LTCC agonists also blocked ouabain-induced proliferation of ADPKD cells. Protein expression levels of full-length LTCC were lower in ADPKD cells than NHK cells. Concomitantly, ADPKD cells contained higher amounts of LTCC cleavage products. We observed that ADPKD cells had higher expression of c-Myc, and that this correlated with decreased expression of the calpain inhibitor calpastatin, as well as increased levels of calpain activity, a protease involved in LTCC cleavage. Our data shows that ouabain stimulates $[Ca^{2+}]_i$ increase in NHK cells by facilitating Ca^{2+} uptake from the extracellular space via LTCC. In contrast, ouabain fails to elevate $[Ca^{2+}]_i$ in ADPKD

cells, due to low LTCC levels, which may be secondary to enhanced LTCC cleavage by the c-Myc dependent increase in calpain. We additionally found that ADPKD cells could be made to respond to ouabain with increased $[Ca^{2+}]_i$ by inhibited Mitogen-activated protein kinase kinase (MEK 1/2), an action which was found to move LTCC channels into the plasma membrane. Therefore due to alterations in LTCC localization and cleavage, ADPKD cells are unable to fully respond to stimuli, like ouabain, with Ca^{2+} flux.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is the most common hereditary alteration of the kidney, afflicting 1 in 400-1000 births worldwide [22, 23, 669]. The disease is primarily characterized by the formation and progressive growth of multiple fluid-filled cysts in the kidney. Additionally, ADPKD can also present with extrarenal manifestations, including cysts in pancreas and liver, aortic and intracranial aneurisms, and cardiac valve prolapse [670]. The renal cysts are believed to form early *in utero* and they slowly expand during the life of the affected individual [30]. ADPKD compromises kidney function, leading to renal insufficiency by the fifth decade of life [13]. ADPKD is responsible for approximately 8-10% of all cases of end stage renal disease (ESRD), irreversibly requiring dialysis or transplant therapy [4, 5].

ADPKD has a multifactorial pathophysiology with the renal cystic cells exhibiting a complex phenotype. Among their characteristics, renal epithelial cells from ADPKD patients show an increased ability to proliferate, a greater capacity for net fluid secretion, altered apical to basal polarity, abnormal directional migration, and changes in the cell matrix (reviewed in [265, 266]). Another distinctive feature of renal epithelial cystic cells from ADPKD patients, as well as various ADPKD animal models is the dysregulation of several intracellular signaling pathways. Interestingly, the intracellular calcium concentration ($[Ca^{2+}]_i$) of ADPKD cells is constitutively lower relative to cells derived from the normal human tubular epithelium [197, 199]. The abnormal low $[Ca^{2+}]_i$ of ADPKD cells alters the manner in which the cells respond to stimuli. Thus, ADPKD cells show excessive activation of B-RAF in response to cAMP, a response that can be reversed by pharmacologically increasing $[Ca^{2+}]_i$ in the cells [197]. Further support for the crucial role of $[Ca^{2+}]_i$ in ADPKD progression is illustrated by the decrease in cyst size upon application of a Ca^{2+} ionophore in metanephric organ culture [200].

ADPKD is caused by mutations in the *PKD1* and *PKD2* genes that encode for polycystin-1 (PC1) and polycystin-2 (PC2) respectively [113, 114]. While mutations in PC1 are responsible of 80-85% of ADPKD cases, the remaining 15-20% are associated with alterations in PC2 [10]. The average onset of ESRD due to ADPKD occurs at approximately the age of 57 for patients with PC1 mutations and age 69 for patients with PC2 mutations [13]. PC1 and PC2 interact with each other to form a macromolecular receptor/signaling complex that appears to have a role in the control of cytoplasmic Ca^{2+} levels. PC2 has homology with the transient receptor potential (TRP) type of ion channels and it functions as a Ca^{2+} non-specific cation channel [14]. PC2 regulates cytoplasmic Ca^{2+} in renal epithelial cells [204, 671] and knockdown of PC2 increases cyst growth in microcyst cultures [199]. PC1 is thought to act as a sensor that regulates the activity of PC2 [15]. The PC1/PC2 complex has been described to have roles both in inducing extracellular Ca^{2+} flux [14, 672], as well as modulating Ca^{2+} release from endoplasmic stores [185]. Thus, overexpression of PC2 results in decreased ER [Ca^{2+}], while expression of PC1 generally increases ER [Ca^{2+}] [176, 209-212]. Although ADPKD is a genetic disorder, the progression of the disease is highly dependent on factors circulating in blood and on different exogenous pharmacological agents (reviewed in [673]). Previous results in our laboratory have shown that ouabain, in concentrations similar to those circulating in blood, increases the proliferation of renal epithelial cystic cells obtained from kidneys of patients with ADPKD (ADPKD cells) [322, 491]. In these cells, ouabain also activates the intrinsic pathway that leads to apoptosis [674] and the cAMP-dependent fluid secretion of ADPKD cell monolayers [360, 361]. Moreover, ouabain stimulates cyst-like tubular dilations in embryonic kidneys from *PKD1* mutant mice [360, 361]. In contrast, ouabain does not cause any of these effects in normal human kidney epithelial cells (NHK cells) or wild type mouse kidneys [322, 360, 361, 491, 674]. Ouabain is a hormone synthesized by the

adrenal glands, which circulates in nanomolar amounts in the bloodstream of mammals [384, 388-390]. The effects of ouabain are mediated by the Na,K-ATPase (NKA) [380, 675, 676]. NKA is a protein complex at the plasma membrane of most animal cells, which had been classically known for its ion transport function, consisting in the ATP driven exchange of cytoplasmic Na^+ for extracellular K^+ across the cell plasma membrane [268, 269]. However, more recently a new role of NKA as a signal molecule of ouabain effects has been established [456]. By binding to NKA, ouabain regulates metabolism, motility and proliferation in a tissue specific manner [322, 403, 460, 471]. The mechanisms by which ouabain exerts its actions are complex, involving several intracellular mediators, including the modulation of intracellular Ca^{2+} concentration. For example, by partially inhibiting the ion transport function of NKA [374-376], ouabain causes a secondary increase in $[Ca^{2+}]_i$ via Na^+ dependent downregulation of function of the sodium/calcium exchanger (NCX) [555, 556]. This mechanism is responsible for the enhanced contraction of myocardial cells and vascular smooth muscle cells, which leads to the well-known regulatory effects of ouabain on cardiac and vascular smooth muscle cells (reviewed in [555, 557]). Alternatively, ouabain controls $[Ca^{2+}]_i$ via activation of downstream effectors, such as the inositol triphosphate receptors (IP_3Rs), which trigger the release of Ca^{2+} from the endoplasmic reticulum [558-560]. This mechanism has been shown to cause slow Ca^{2+} oscillations in renal proximal tubules epithelial cells in response to ouabain [403, 477, 558-560]. In addition, a rise in $[Ca^{2+}]_i$ induced by ouabain has been reported to also take place via changes in activity of L-type calcium channels (LTCC). This mechanism, described in cardiomyocytes and in some neuronal cells, presumably depends on the alterations in plasma membrane potential, which would be secondary to ouabain-mediated NKA inhibition [562-567].

We have previously determined that some of the effects that ouabain exerts on ADPKD cells involves the scaffolding of Na,K-ATPase with epidermal growth factor receptor (EGFR), the activation of the kinase SRC and the downstream stimulation of the extracellular signal-regulated kinase (ERK) pathway [322, 491]. However, the role of Ca^{2+} as a secondary mediator of the effects of ouabain in ADPKD cells has not yet been determined. In this work, we report that, similar to other cell types, NHK cells respond to ouabain with an influx of $[Ca^{2+}]_i$ and this effect is mediated by activation of LTCCs. In contrast, ouabain is ineffective in increasing $[Ca^{2+}]_i$ in ADPKD cells. Upon investigation, we found ADPKD cells have constitutively lower expression of full-length LTCC in ADPKD cells, but increased cleavage of LTCC in the cells.

MATERIALS AND METHODS

Cell culture:

Primary cell cultures derived from surface cysts of ADPKD kidneys (ADPKD cells) were generated by the PKD Biomaterial Core at the University of Kansas Medical Center (KUMC). A protocol for the use of discarded human kidney tissues was approved by the Institutional Review Board at KUMC. Primary cultures were prepared as previously described [608]. Cells were seeded and grown in DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 1U.ml penicillin G and 0.1mg/ml streptomycin, 5 ug/ml insulin, 5 ug/ml transferrin, and 5 ng/ml sodium selenite (ITS). 24 hours before the cells were subject to experimental manipulation, the serum was reduced to 0.002% and ITS was removed. As previously shown, these cells are epithelial in nature and derived from distal nephrons and collecting tubules, as shown by their labeling with lectin markers specific for collecting duct and distal nephron cells, such as Arachis hypogaea (PNA) and Dolichos biflourus agglutinin (DBA) [677].

Chemical reagents:

The Ca^{2+} indicator dyes used in this study were purchased from Molecular Probes (Thermo-Fisher Scientific, Waltham MA). They include Fura-2 AM, Fluoro-5N AM, and Rhod-2, AM. The following compounds were used to elucidate the mechanism of action. From Santa Cruz (Santa Cruz, Carlsbad CA): FPL-64176 (20 nM), Xestospongin-c (5 uM), NAADP (50 nM), 10058-F4 (25 uM). From Sigma (St. Louis, MO): A23187 (1-2 uM), (±)-Bay K8644 (5 uM), 2-APB (5 uM), ORM-10103 (1 uM), Verapamil (2 uM) and Calpain-inhibitor I (N-acetyl-leu-leu-norleucinal) (10 uM). Compounds from other companies include: Thapsigargin (10 uM; Cayman

Chemicals, Ann Arbor, MI), SEA0400 (5 uM; APExBIO, Houston TX), PP2 (10 uM; Calbiochem, Nottinghamshire UK), U0126 (1 uM, Promega, Madison WI), Boc-Leu-Met-CMAC (50 uM; Thermo-Fisher Scientific, Waltham, MA).

Calcium trace:

Cells were seeded at a concentration of 8,000 cells per well in a 96-well black with clear bottom plate (Corning, Kennebunk ME). Media was replaced with serum-free media as described under the cell culture section. To determine cytoplasmic Ca^{2+} levels, 2 uM Fura-2, AM in serum-free media was added to each well and incubated at 37 ºC for 1 h. Then, cells were washed with PBS and the medium was replaced with HBSS and read in a FlexStation3 plate reader, at an excitation (Ex) of 340, 380 nm and an emission (Em) of 510 nm. The same protocol was used for Fluo-5N, except that this compound was used at 4 uM, and at an excitation/emission ratio (Ex/Em) of 488/521 nm; and for Rhod-2 (4 uM, Ex/Em of 552/581 nm). Before the addition of Rhod-2, the dye was reduced to dihydroRhod-2 to facilitate its uptake into the cell mitochondria, using 1 mg/mL of $NaBH_4$ in methanol. For this, freshly prepared $NaBH_4$ solution was added to resuspended Rhod-2 immediately before the addition of the dye to the cells. All Ca^{2+} indicators were resuspended in DMSO before they were added to medium.

Immunoblot analysis:

Cells treated with and without 3 nM ouabain for 24 hours were washed once with ice-cold PBS and lysed with buffer containing 10mM Tris-Cl (pH 7.4), 10mM NaCl, 3mM MgCl2, and 0.1% NP-40. Fifty micrograms of the cleared lysates were analyzed by 8% SDS-PAGE and blotted

onto nitrocellulose membranes. Immunoblots were probed with antibodies at a concentration of 1:1000 or 1:500, depending on the antibody, in TBS-T with 5% w/v BSA (Sigma, St. Louis, MO). The following antibodies were used: p65 NFκB (sc-109), Histone H3 (sc-10809), NCX (sc-32881), pan-αLTCC (sc-98753), c-Myc (sc-764), calpastatin (sc-20779), all from Santa Cruz, (Carlsbad, CA), as well as α-tubulin (#2144) GAPDH (#2118) from Cell Signaling (Danvers, MA). Secondary antibodies, obtained from Santa Cruz (sc-2004, sc-2005), were used at a concentration of 1:2000.

Calpain activity assay:

Cells were plated at a density of 8,000 cells per well on 96-well plates in complete medium. The next day, the medium was replaced with serum-free media. On the third day, cells were pretreated with calpain inhibitor 1 (10 uM), the MEK inhibitor U0125 (1 uM) or ouabain (3 nM) for 30 minutes at 37 ºC before the addition of 50 μM of the calpain substrate, Boc-LM-CMAC (Thermo-Fisher Scientific, Waltham, MA). Cells were incubated for an additional 20 minutes at 37ºC, washed 3x with PBS and placed in a plate reader to quantify activity (Ex/Em of 351/430 nm). Myc inhibitor experiments were nearly the same, with 24h treatment of the c-Myc inhibitor 10058-F4 (25 uM) before Boc-LM-CMAC addition.

Data analysis:

The statistical analysis of samples was determined by one way analysis of variance (ANOVA), followed by Student's T-test and Bonferroni's test. Statistical differences were defined as significant when $P<0.05$.

RESULTS

Ouabain changes cytoplasmic Ca^{2+} in NHK, but not in ADPKD cells

We used the Ca^{2+} indicator Fura-2 to measure $[Ca^{2+}]_i$ in NHK and ADPKD cells. In agreement with previous studies, [197], we observed that ADPKD cells exhibit overall lower $[Ca^{2+}]_i$ than NHK cells (Fig. 4.1A - 4.1C). We treated the cells without and with 3 nM ouabain to determine its effect on $[Ca^{2+}]_i$ in the cells. Ouabain increased $[Ca^{2+}]_i$ in NHK cells (Fig. 4.1A and

Figure 4.1

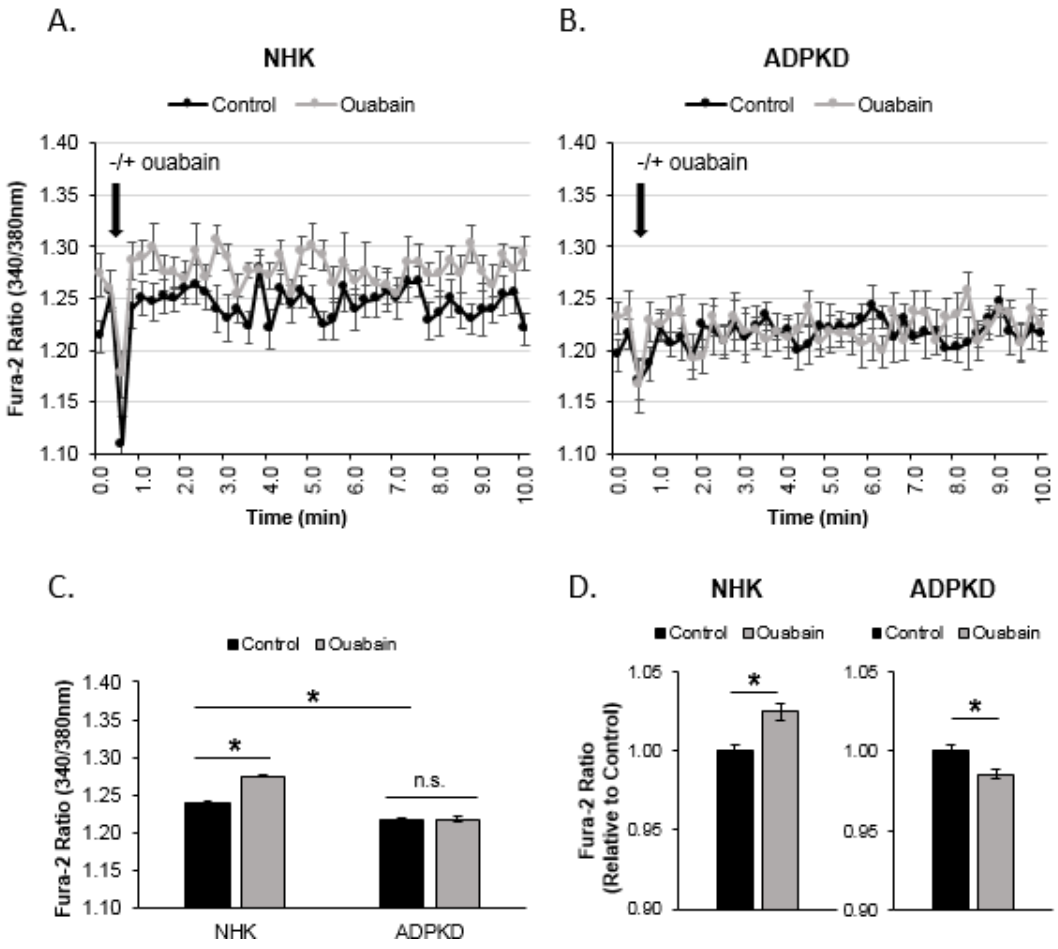

Figure 4.1. Ouabain elicits an intracellular calcium increase in NHK cells. NHK (A) and ADPKD (B) cells in 96-well plates were loaded with Fura-2, and treated with ouabain (3 nM) [arrow]. The trace shown is a composite of the response of cells from 4 separate kidneys, in quadruplicate. (C) Average fura-2 fluorescence of trace between untreated NHK and ADPKD. Bars indicate mean ± SEM of untreated cells. C. Average fura-2 fluorescence in response to 24 h treatment ouabain. Bars indicate mean ± SEM of 4 separate kidneys. *P<0.05.

4.1C), but did not significantly affect $[Ca^{2+}]_i$ in ADPKD cells (Fig. 4.1B and 4.1C). To explore the long term effects of ouabain on $[Ca^{2+}]_i$, we measured $[Ca^{2+}]_i$ after treatment of the cells with ouabain for 24 h. NHK cells, but not ADPKD cells showed a ouabain induced increase in $[Ca^{2+}]_i$ (Fig. 4.1D). Therefore, while ouabain elicits a Ca^{2+} response in NHK cells, it does not affect $[Ca^{2+}]_i$ in ADPKD cells.

Ouabain has no effect on endoplasmic reticulum and mitochondrial Ca^{2+} in both NHK and ADPKD cells

In order to determine if ouabain was able to influence Ca^{2+} levels in the endoplasmic reticulum we used the Ca^{2+} indicator dye Fluo5N which has low affinity for Ca^{2+}, making it suitable for detecting Ca^{2+} levels in the 1 uM-1mM range [678, 679]. Due to typical cytosolic Ca^{2+} concentrations of approximately 100 nM, Fluo5N is not able to detect Ca^{2+} in this cell compartment. In contrast, Fluo5N can detect the higher Ca^{2+} amounts of the endoplasmic reticulum (ER) [680], which has a Ca^{2+} concentration that ranges between 100-800 μM and is generally considered to be the largest Ca^{2+} store of cells [680]. Therefore, we used Fluo5N as an indicator of ER Ca^{2+} in NHK and ADPKD cells. We were also interested in the effect of ouabain on the mitochondria (which is an organelle with a typical Ca^{2+} concentration of ~100 nM Ca^{2+} [681]). Since the free mitochondrial $[Ca^{2+}]$ is similar to that of the cytosol [682], it is not detected by Fluo5N. Therefore, we used a second Ca^{2+} dye, Rhod-2, which preferentially enters mitochondria, to determine Ca^{2+} in this organelle.

We initially validated the ability of Fluo5N to detect ER Ca^{2+} by treating the cells with the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin, which inhibits ER Ca^{2+}

Figure 4.2

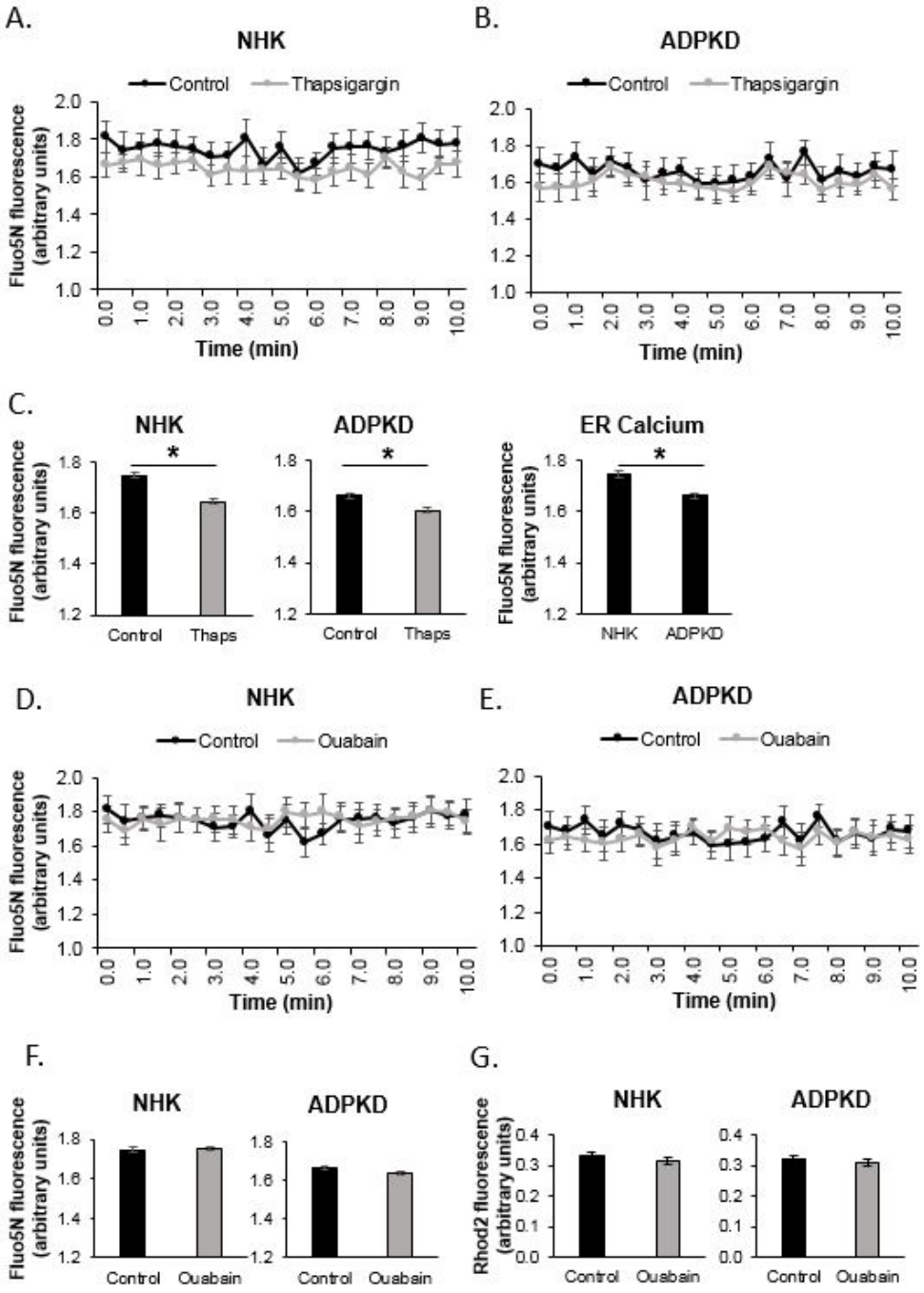

Figure 4.2. Ouabain does not significantly change ER or Mito calcium concentration. NHK (A) and ADPKD (B) cells loaded with Fluo5N show decreased fluorescence when ER stores are depleted using thapsigargin; composite of cells from 3 separate kidneys, in quadruplicate. (C) Average fluorescence of traces. Bars indicate mean ± SEM of trace values. Average ER calcium between untreated NHK and ADPKD cells is also shown. Fluo5N trace showing average ER calcium response with and without ouabain in NHK (D) and ADPKD (E) cells. (F). Average ER calcium response. Values indicate mean ± SEM of trace values. (G) Average mitochondrial calcium response. Bars indicate mean ± SEM of 3 separate kidneys. *P<0.05.

uptake. We observed decreased Fluo5N fluorescence with thapsigargin treatment consistent with our expectation (Fig. 4.2A and 4.2B). Using this model, we tested if ER Ca^{2+} concentrations were different between NHK and ADPKD cells. Indeed, ADPKD cells had significantly lower ER Ca^{2+} relative to that of NHK cells (Fig. 4.2C). This is in agreement with Xu, et al, whose work showed through the use of thapsigargin that the ER Ca^{2+} stores of ADPKD cells are lower than that of NHK cells [169]. Following the addition of ouabain no significant change in the ER Ca^{2+} concentration was observed in either NHK or ADPKD cells, (Fig. 4.2D-F). When mitochondrial Ca^{2+} was determined using Rhod-2, no significant difference in fluorescence was observed between NHK and ADPKD cells. Moreover, ouabain did not modify mitochondrial Ca^{2+} in either cell type (Fig. 4.2G). These results show that ouabain does not affect Ca^{2+} levels in ER or mitochondria in either NHK or ADPKD cells.

The increase in $[Ca^{2+}]_i$ caused by LTCC agonists, but not IP_3R inhibitors rescues the normal Ca^{2+}-response induced by ouabain in ADPKD cells

In a study by Yamaguchi, et al., the proliferative response that ADPKD cells have towards forskolin was inhibited by pharmacologically increasing the $[Ca^{2+}]_i$ in ADPKD cells [197]. This maneuver of Ca^{2+} rescue reverted the response of ADPKD cells to forskolin to mimic that of NHK cells, in which growth is not stimulated by forskolin. Conversely, in the reciprocal experiment, decreasing $[Ca^{2+}]_i$ of NHK cells caused the cells to respond to forskolin in a similar manner as ADPKD cells [683]. Following this strategy, we hypothesized that the response of ADPKD cells to ouabain could be made to resemble that of NHK cells by increasing their $[Ca^{2+}]_i$. We increased the $[Ca^{2+}]_i$ by pretreating ADPKD cells for 1 h with the dihydropyridine L-type calcium channel (LTCC) agonist Bay-K8644, or the benzoyl pyrrole LTCC agonist FPL-64176 [684]. Both

agonists increased $[Ca^{2+}]_i$ in ADPKD cells (Fig. 4.3A and 4.3B) When ouabain was applied to ADPKD cells which had previously received LTCC-agonist pre-treatment, the cells responded to ouabain with increased $[Ca^{2+}]_i$, in a manner similar to that of NHK cells (Fig. 4.3A). To determine whether this rescue of a normal $[Ca^{2+}]_i$ response in ADPKD cells has functional consequences, we co-treated the cells with Bay-K8644 and 3 nM ouabain and measured cell proliferation 24 h later. In contrast to the typical stimulatory effect that ouabain elicits in ADPKD cells [322, 491], ouabain did not increase proliferation of ADPKD cells when co-treated with the $[Ca^{2+}]_i$ enhancer Bay-K8644 (Fig. 4.3C).

As described above, ADPKD cells have lower Ca^{2+} levels in the ER than NHK cells (Fig. 4.2C). Reduced ER Ca^{2+} levels can decrease the sensitivity of Ca^{2+} channels on the ER membrane to stimuli, which could in turn explain the lack of ouabain-induced Ca^{2+} increase in ADPKD cells [685, 686]. Additionally, reduced ER Ca^{2+} could increase Ca^{2+} uptake by SERCA [687, 688], which could mask any ouabain-induced $[Ca^{2+}]_i$ increase in ADPKD cells. To ascertain whether this mechanism is involved in the insensitivity of ADPKD cells to the ouabain induced Ca^{2+} raise, we increased ER Ca^{2+} levels and assessed the effect of ouabain on $[Ca^{2+}]_i$ thereafter. To achieve this, two different IP_3R inhibitors, xestospongin-c or 2-APB, were used. As shown in Figure 4.3D, pretreatment with the IP_3R inhibitors did increase the Ca^{2+} concentration in the ER of ADPKD cells. However, despite the effect of xestospongin-c or 2-APB, ouabain was ineffective in raising the $[Ca^{2+}]_i$ of ADPKD cells (Fig. 4.3E and 4.3F). Overall, our findings show that the failure of ADPKD cells to increase $[Ca^{2+}]_i$ in response to ouabain can be reverted with agents that restore Ca^{2+} in the cell cytoplasm, but not by increasing Ca^{2+} storage in the cell ER.

Figure 4.3

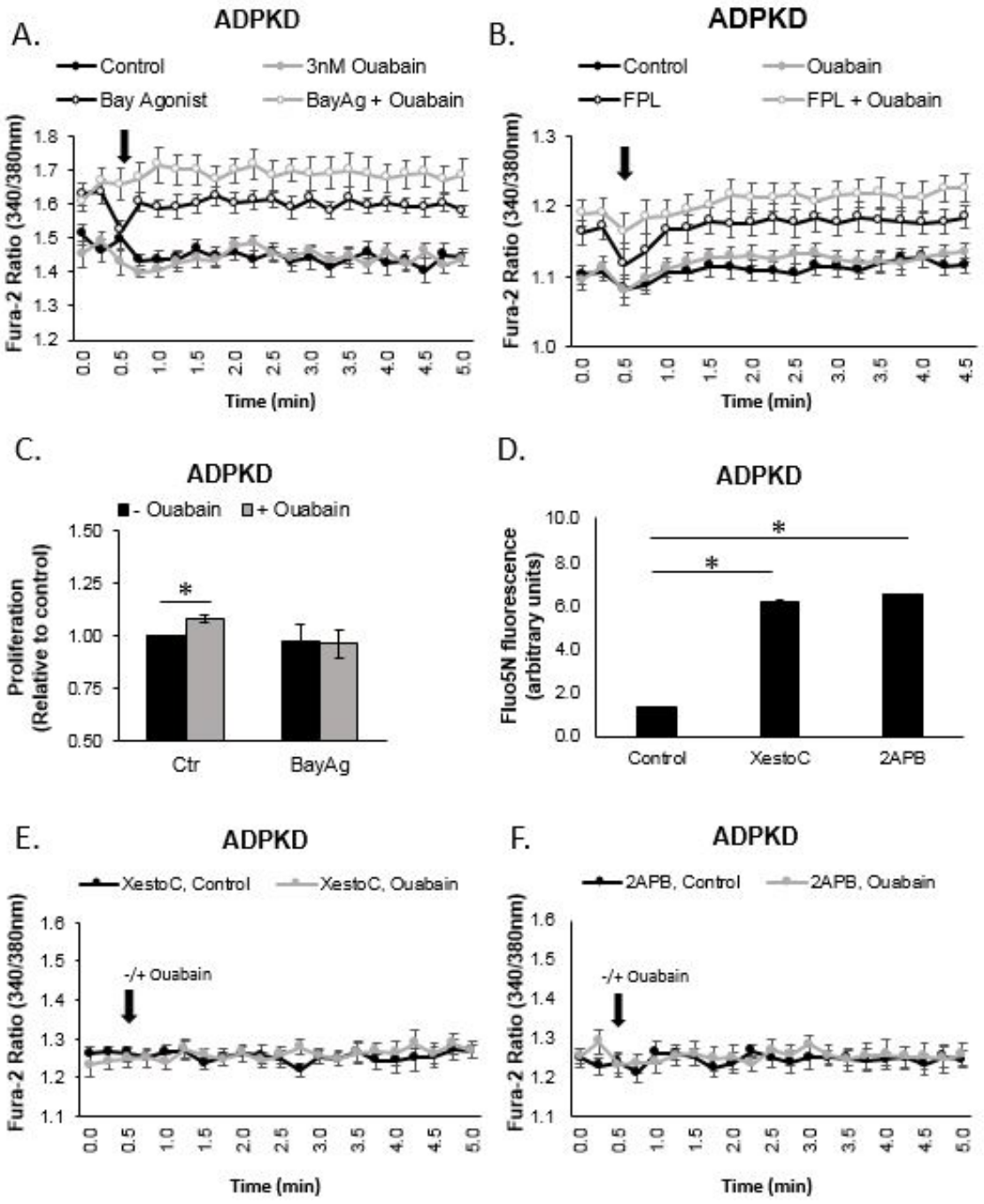

Figure 4.3. Pretreatment of ADPKD cells with LTCC agonists rescues the calcium response. A. ADPKD cells were pretreated with Bay K8644 or FPL-64176 for 1 h before being read. Cytoplasmic calcium is increased in response to the LTCC agonists. Cells pretreated with LTCC agonists respond to ouabain by increasing intracellular calcium. B. Ouabain-mediated proliferation is abrogated by Bay K8644 co-treatment. MTT assay. C. Low ER calcium in ADPKD can be rescued by 1 h pretreatment with IP_3R antagonists. D. 1 h pretreatment with IP_3R antagonists does not lead to an increase in intracellular calcium in response to ouabain. *P<0.05.

IP_3R channels are not involved in the differential ouabain-mediated Ca^{2+} response of NHK and ADPKD cells

Our next goal was to characterize the mechanisms by which ouabain modulates Ca^{2+} in NHK cells and to understand why they are unresponsive to ouabain in ADPKD cells. To achieve this, we first determined if the rise in Ca^{2+} in NHK cells was secondary to Ca^{2+} coming from the extracellular space, or if it originated by its release from the ER. Therefore, we stimulated NHK cells with or without 3 nM ouabain in standard medium, or in medium devoid of Ca^{2+}. As shown in Fig. 4.4A, the absence of Ca^{2+} in the medium prevented ouabain from increasing $[Ca^{2+}]_i$ in NHK cells. This suggests that the rise in $[Ca^{2+}]_i$ in response to ouabain treatment in NHK cells involves the transmembrane transport of Ca^{2+} from the medium into the cells.

However, we wanted to further confirm the $[Ca^{2+}]_i$ response to ouabain did not require ER Ca^{2+} an so we directed our focus to the inositol triphosphate receptors (IP_3Rs). The IP_3Rs are $[Ca^{2+}]_i$ release channels that are predominately localized in the ER membrane and release Ca^{2+} into the cytosol in response to IP_3 (reviewed in [689-692]). Activation of IP_3Rs often leads to formation of complex spatio-temporal Ca^{2+} signals which regulate various cell processes, including proliferation, differentiation, metabolism, secretion and cell death [693]. We found that depletion of ER Ca^{2+} using the SERCA antagonist, thapsigargin, did not inhibit the ouabain-induced Ca^{2+} response in NHK cells (Fig. 4.4B). Additionally, inhibition of IP_3Rs with either 2-APB or xestospongin-c pretreatment did not completely inhibit the Ca^{2+} response in NHK cells (Fig. 4.4C and 4.4D).

Ouabain has been shown to increase $[Ca^{2+}]_i$ in rat renal epithelial proximal tubule cells, via activation of the IP_3Rs [403, 477, 558-560], resulting in the translocation of the p65 subunit of NFκB into the nucleus [403]. We tested the possibility that the p65 subunit of NFκB is involved

in the Ca^{2+} changes observed in NHK cells. For this, NHK and ADPKD cells were treated with or without 3 nM ouabain for 24 h and NFκB levels were subsequently determined in cytosolic and nuclear fractions. We did not observe NFκB translocation in response to ouabain in either NHK or ADPKD cells (Fig. 4.4E). These results show that the ouabain induced $[Ca^{2+}]_i$ response of NHK relies very little on ER Ca^{2+} stores and that the changes in ER Ca^{2+} between NHK and ADPKD cells are not the basis for the different Ca^{2+} response to ouabain displayed by these cell types. In addition, our data show that NHK cells, which are from collecting duct origin, respond to ouabain

Figure 4.4

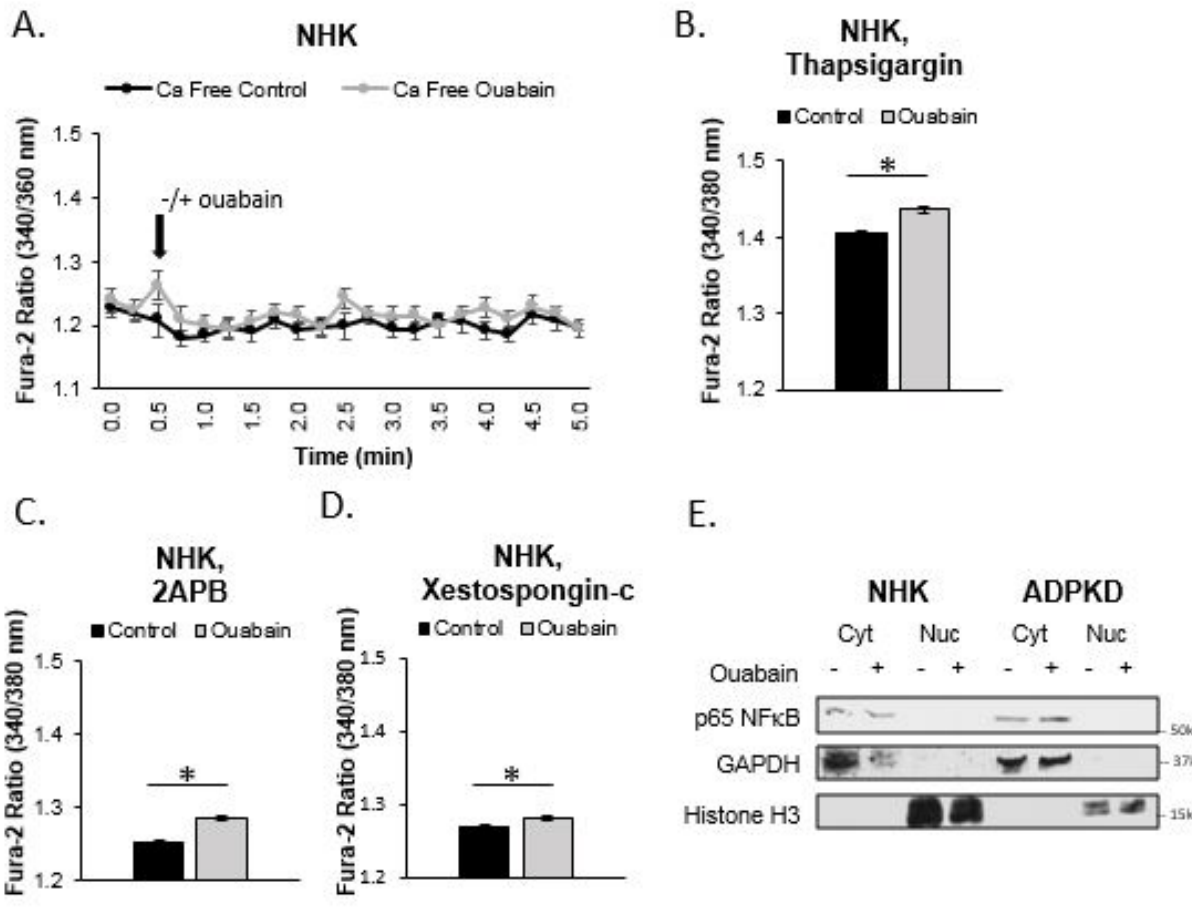

Figure 4.4. The ouabain-mediated response does not rely on ER stores. A. Fura-2 loaded NHK cells treated in Ca^{2+}-free media. B. Depletion of ER Ca^{2+} with 1 h pretreatment with thapsigargin. C. NHK cells pretreated with IP_3R antagonists (1 h). D. 24 h ouabain treatment did not increase p65 NFκB nuclear translocation. All cells from 3 separate kidneys used, in quadruplicate *P<0.05.

with Ca^{2+} increase by using mechanisms that are different from those of renal cells from proximal tubular origin.

NCX function is not involved in the different ouabain-mediated Ca^{2+} response of NHK and ADPKD cells

We next studied whether the sodium-calcium exchanger (NCX) plays a role in the response of NHK cells to ouabain induced $[Ca^{2+}]_i$ increase, and if NCX is involved in the differences between NHK and ADPKD cells. NCX catalyzes the 3:1 counter-transport of Na^+ and Ca^{2+} across the cell plasma membrane, using the energy of the inward Na^+ gradient maintained by the NKA (reviewed in [694]). In fact, this mechanism is responsible for the "cardiotonic effect" that ouabain produces in the heart [425]. NCX expression has been shown in human collecting duct cells [695]. We found that NHK cells respond to the NCX inhibitor ORM10103 with an increase in $[Ca^{2+}]_i$ (Fig. 4.5A). The NCX inhibitor did not increase $[Ca^{2+}]_i$ above the levels already induced by ouabain. Comparison of the effect of ORM10103 in the absence and presence of ouabain shows no differences in $[Ca^{2+}]_i$. In contrast, ADPKD cells did not change their $[Ca^{2+}]_i$ in response to ORM10103 (Fig. 4.5B), either in the absence or presence of ouabain (Fig. 4.5B). We confirmed that NHK and ADPKD cells express similar levels of NCX (Fig. 4.5C). These results show that, different from NHK cells which have a functional NCX transporter, ADPKD cells cannot increase $[Ca^{2+}]_i$ by inhibition of NCX. The NCX is known to be allosterically regulated by cytoplasmic Ca^{2+}. Specificallty, the binding of calcium to intracellular regulatory domains increases NCX activity [696, 697]. Therefore, we hypothesized that increasing $[Ca^{2+}]_i$ with Bay K8644, as performed before (see Fig. 3), could produce a Ca^{2+} response to ORM10103 in ADPKD cells. However, pretreatment with Bay K8644 did not result in increase of $[Ca^{2+}]_i$ by ORM10103 in the

ADPKD cells (Fig. 4.5D). In summary, although pretreatment with Bay K8644 was able to rescue the ouabain-induced Ca^{2+} response in ADPKD cells, it was not able to rescue the lack of ORM10103-mediated calcium increase in these cells. Therefore, altered NCX activity between the cell types cannot explain the difference in the response of NHK and ADPKD cells to ouabain.

Figure 4.5

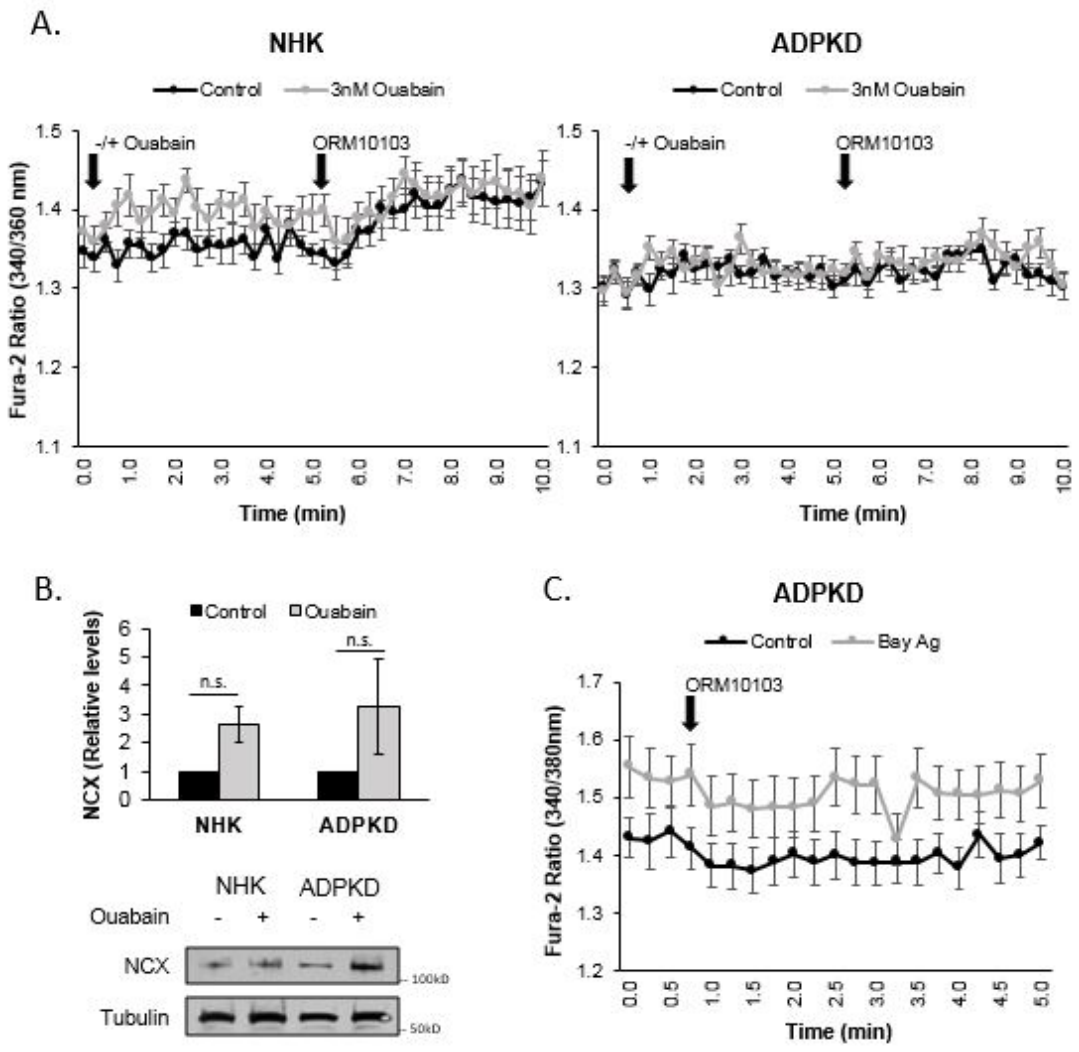

Figure 4.5. ADPKD cells do not respond to NCX inhibition with calcium influx. A. Cells were loaded with Fura-2, and treated ± ouabain (3 nM) or the NCX inhibitor, ORM-10103, where indicated (arrows). B. Western blot analysis of NCX protein expression, 24h ± ouabain (3 nM). C. Pretreatment of ADPKD cells for 1 h with Bay-K8644 did not rescue the response to ORM-10103. All: cells from 3 separate kidneys used, in quadruplicate *P<0.05.

LTCC mediates the $[Ca^{2+}]_i$ response to ouabain in NHK cells

L-type calcium channels (LTCCs) have been shown to become activated after ouabain addition [562-567]. The mechanism appears to depend on inhibition of NKA, which causes membrane depolarization and in turn, activation of LTCC, with the subsequent entry of Ca^{2+}. Ouabain has been found to increase $[Ca^{2+}]_i$ via this mechanism in cardiomyocytes and in some neurons [562-567]. LTCCs are hetero-tetrameric complexes which are composed of the pore-forming α_1 subunit and the auxiliary α_2/δ and β subunits (reviewed in [561, 698]). At least one subtype of α_1-LTCC, CaV1.2, is present in renal collecting duct cells [699]. In Figure 4.3A we show that LTCC agonists can rescue the ouabain dependent Ca^{2+} response in ADPKD cells. Therefore, we performed the reciprocal experiment and assayed whether the Ca^{2+} increase induced by ouabain could be inhibited in NHK cells by the LTCC antagonist verapamil. As shown in Figure 4.6, pretreatment with verapamil blocked the ouabain induced Ca^{2+} rise in NHK cells. This suggests that in NHK cells, Ca^{2+} flux in response to ouabain relies on the function of LTCCs.

Figure 4.6

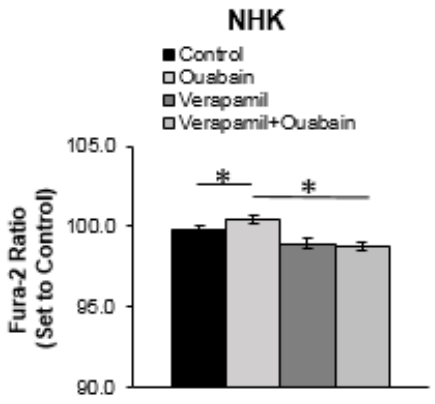

Figure 4.6. L-type calcium channels are what mediate the influx of ouabain in collecting duct cells. The ouabain-mediated Ca^{2+} increase is abrogated in NHK cells co-treated with verapamil. All: cells from 3 separate kidneys used, in quadruplicate *P<0.05.

Different levels of LTCC are responsible for the dissimilar ouabain induced $[Ca^{2+}]_i$ response in NHK and ADPKD cells

We next assayed the expression levels of α_1-LTCC by western blot analysis and found significantly lower expression of the band corresponding to the full-length α_1-LTCC (of 240 kDa) in ADPKD than in NHK cells (Fig. 4.7A). Decreased expression could be due to altered stability of the α_1-LTCC protein. Indeed, Pedrozo, et al. showed in cardiomyocytes that polycystin-1 expression is required to stabilize α_1-LTCC, especially under conditions of stress [700]. Therefore, we assessed the stability of the α_1-LTCC protein in NHK and ADPKD cells by inhibition of protein translation with cycloheximide and followed the expression of the mature protein for different times. This provides an estimation of the half-life of α_1-LTCC protein. As shown in Fig. 4.7B, the half-life of the α_1-LTCC protein was not significantly different between NHK and ADPKD cells. In addition, to determine if both cell types present differences in α_1-LTCC degradation, we assessed α_1-LTCC protein levels over time in the

Figure 4.7

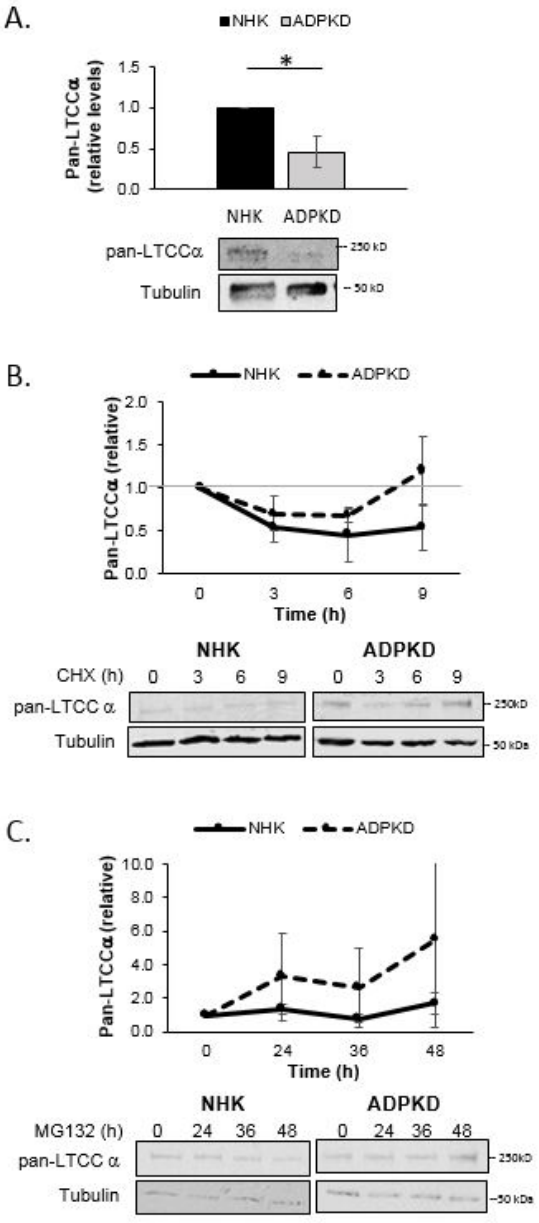

Figure 4.7 A. ADPKD cells have less full-length (240 kDa) LTCC protein than NHK cells. B. Cycloheximide time course reveals that ADPKD cells do not have less stable LTCC protein. C. MG132 time course confirms that ADPKD cells do not have less stable LTCC protein. *P<0.05.

presence of the proteasome inhibitor, MG132. This showed no differences in degradation of α_1-LTCC between ADPKD and NHK cells (Fig. 4.7C). Therefore, while ADPKD cells have an abnormally low level of expression of α_1-LTCC, this is not due to altered stability of this channel.

LTCC undergoes increased cleavage in ADPKD than NHK cells

When performing the immunoblots to detect α_1-LTCC, we observed that in addition to the full length protein, there were lower molecular weight bands identified by the anti-LTCC antibody (Fig. 4.8A). Cleavage is an important mechanism that regulates α_1-LTCCs, and has been well studied in neurons and cardiomyocytes [701-703]. The full length LTCC protein (240 kDa) is fully functional, while the cleaved products (150 kDa and 90 kDa) are known to bind to the full-length form and decrease the open probability of the channel [701-703]. Therefore, we analyzed and compared the expression levels of the lower molecular weight bands of α_1-LTCC in NHK and ADPKD cells. We calculated the cleavage index of α_1-LTCC, which has been previously defined as the α_1-LTCC 150 kDa band / 240 kDa band ratio [701]. We found that a greater cleavage of α_1-LTCC occurs in ADPKD cells compared to NHK cells (Fig. 4.8B)

The α_1-LTCCs are known to be cleaved by the calpain family of proteases [704]. Therefore, we assessed the activity of calpains in NHK and ADPKD cells. Using a fluorescent calpain substrate, Boc-LM-CMAC, we found that, calpains are more active in ADPKD cells than in NHK cells (Fig. 4.8C). Altogether, these results suggests increased cleavage of α_1-LTCCs may be the cause for the decreased capacity of ADPKD cells to increase $[Ca^{2+}]_i$ in response to ouabain.

Figure 4.8

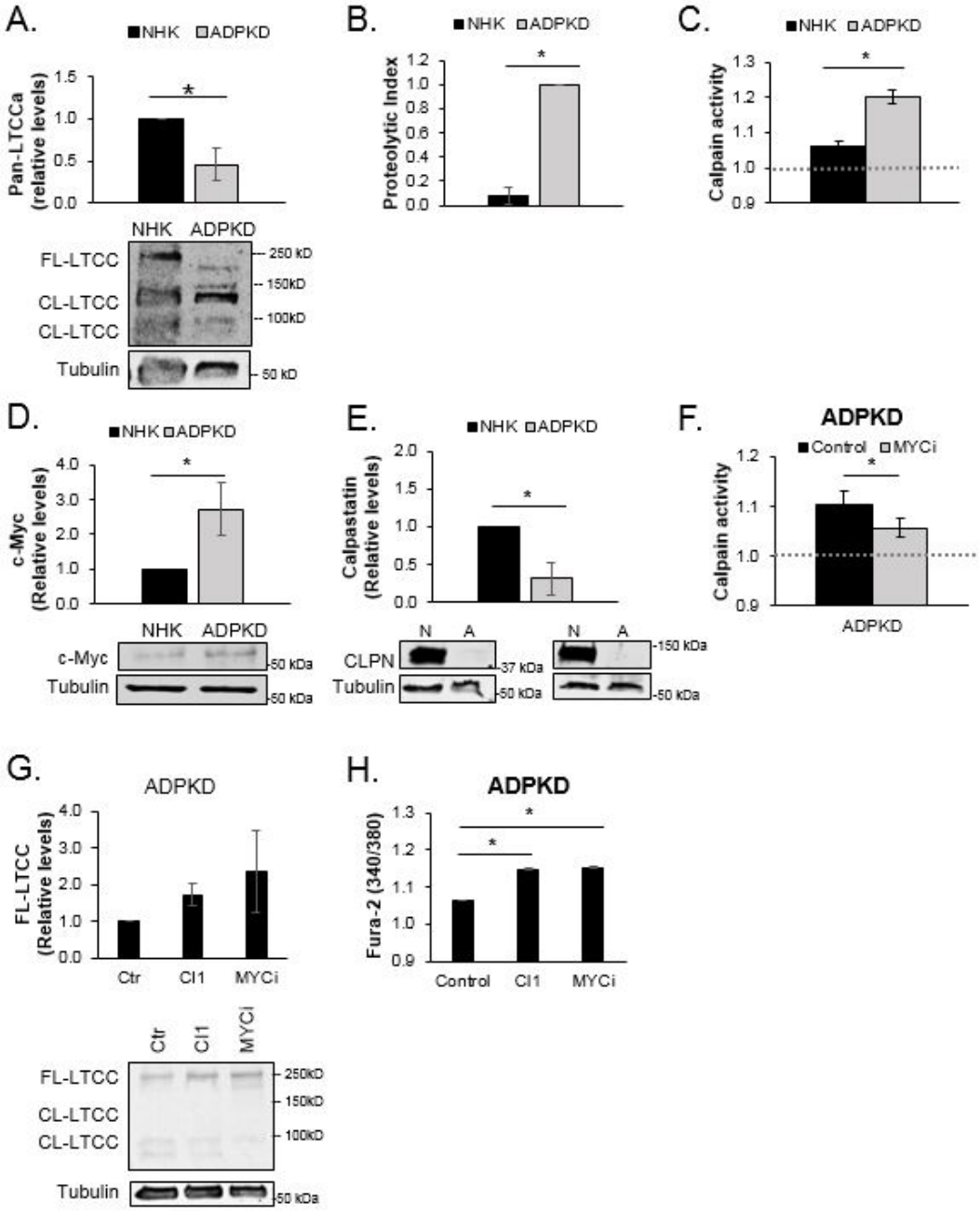

Figure 4.8. L-type calcium channels undergo increased cleavage in ADPKD cells due to increased calpain activity. (A) Lower bands observed in western blot analysis of LTCC protein. (B) Proteolytic index of LTCC (150 kDa band / 250 kDa band) is significantly greater in ADPKD cells. (C) Cleavage of the calpain-specific substrate, Boc-LM-CMAC; calpain inhibitor-1 (CI1) was used as a negative control, value shown in dotted line. (D) Western blot of c-Myc protein. (E) Western blot of calpastatin (CLPN). (F) Inhibition of c-Myc transcriptional activity with 10058-F4, decreased cleavage of Boc-LM-CMAC. (G). Pretreatment with CI1 or 10058-F4 increases increases expression of full length LTCC. (H) CI1 or 10058-F4 pretreatment both increase intracellular calcium in ADPKD cells (average Fura-2 fluorescence). *P<0.05.

Calpain activity is increased by c-Myc overexpression in ADPKD cells

To gain insight into the mechanisms by which calpain activity is increased in ADPKD cells, we explored the expression levels of c-Myc. The c-Myc transcription factor is a regulator of cell growth, proliferation, dedifferentiation and apoptosis [705, 706]. c-Myc is known to be upregulated in human APDKD cells [140, 574, 707], as well as in several ADPKD models, including *PKD1* transgenic animals, SBM mice, CPK mice, and Han:SPRD rats [356, 622, 708-711]. We confirmed that c-Myc is increased in ADPKD cells compared to NHK cells (Fig. 8D).

c-Myc transcriptionally represses an endogenous inhibitor of calpains, the protein calpastatin [712]. Therefore, we determined the expression of calpastatin in NHK and ADPKD cells and indeed found that ADPKD cells showed significantly lower calpastatin levels than NHK cells (Fig. 8E). Calpastatin appeared as a double band in our immunoblots. This may reflect the presence of different isoforms of calpastatin, derived from alternative splicing or proteolysis, as previously reported [713]. We combined both bands into "total calpastatin" when performing the densitometric analysis. To further confirm a relationship between c-Myc and calpain activity, we pretreated ADPKD cells for 24 h with the compound 10058-F4, which inhibits the transcriptional effects of c-Myc, and then measured calpain activity of the cells. We observed that the Myc inhibitor 10058-F4 significantly decreased calpain activity of ADPKD cells (Fig. 4.8F). Concomitant with this effect, inhibition of calpain or c-Myc increased $[Ca^{2+}]_i$ of ADPKD cells (Fig 4.8G). Moreover, inhibition of either calpain or c-Myc decreased the cleavage of α_1-LTCC in ADPKD cells (Fig. 4.8H). Altogether, these experiments show that in ADPKD cells, the abnormally high levels of c-Myc are associated with a reduction in calpastatin expression, which further leads to increased calpain activity and higher cleavage of α_1-LTCC.

Ouabain has been shown to enhance the expression of c-Myc [498, 714], therefore we were interested in testing whether ouabain could enhance α_1-LTCC cleavage. Treatment with ouabain for 24 h had no effect on the amounts of full length α_1-LTCC or the α_1-LTCC cleavage index (Fig. 4.9A and 4.9B). In addition, calpain activity was not changed by 24 h treatment with ouabain (Figure 4.9C).

Figure 4.9

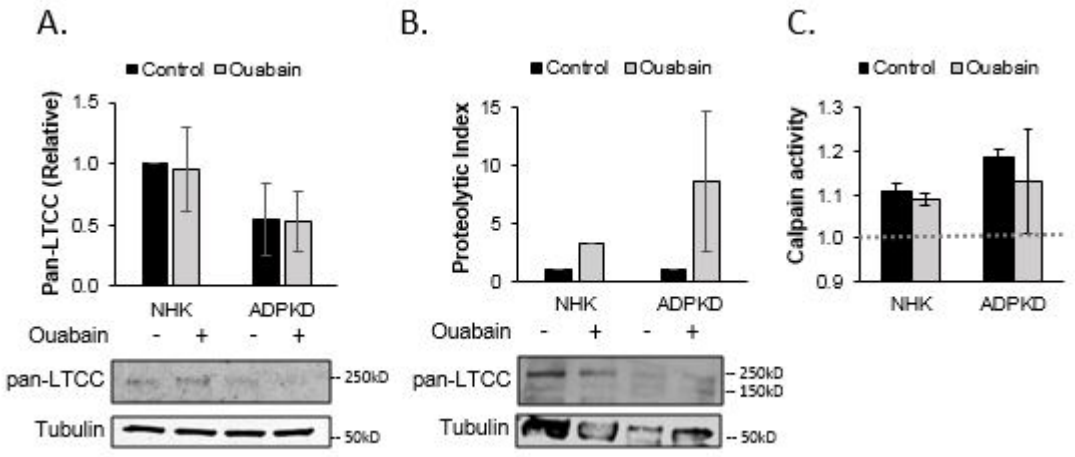

Figure 4.9. Ouabain does not enhance the cleavage of LTCCs. A. Full length LTCC protein concentration is not decreased by 24 h treatment with ouabain. B. The proteolytic index of LTCC is not significantly increased by ouabain. C. Calpain activity is not significantly altered by ouabain. Dotted line represents CI1 value. *P<0.05.

LTCC activity is decreased by aberrant ERK signaling in ADPKD

ADPKD cells are known to have aberrantly active ERK signaling [133, 715-717]. Furthermore, the inhibition of MEK1/2 kinases, which activate ERK, have been shown to increase the membrane localization of α_1-LTCC, and thus increase channel activity [718]. Therefore, we hypothesized that ADPKD cells may have less functional α_1-LTCC due to a decreased presence of α_1-LTCC in the membrane. We observed α_1-LTCC localization by confocal microscopy and found that 30 minutes of pretreatment with the MEK1/2 inhibitor, U0126, increased the

localization of α_1-LTCC to the plasma membrane in ADPKD cells (Fig. 4.10A). To further confirm this mechanism, we pretreated ADPKD cells with U0126, and assayed $[Ca^{2+}]_i$, with and without ouabain treatment. Ouabain was able to increase $[Ca^{2+}]_i$ after pretreatment of the cells with U0126 (Fig. 4.10B). This suggests that decreased insertion of α_1-LTCC into the plasma membrane as a result of MEK1/2 overactivation in ADPKD cells is an additional mechanism that prevents the ouabain induced $[Ca^{2+}]_i$ rise in ADPKD cells.

Figure 4.10

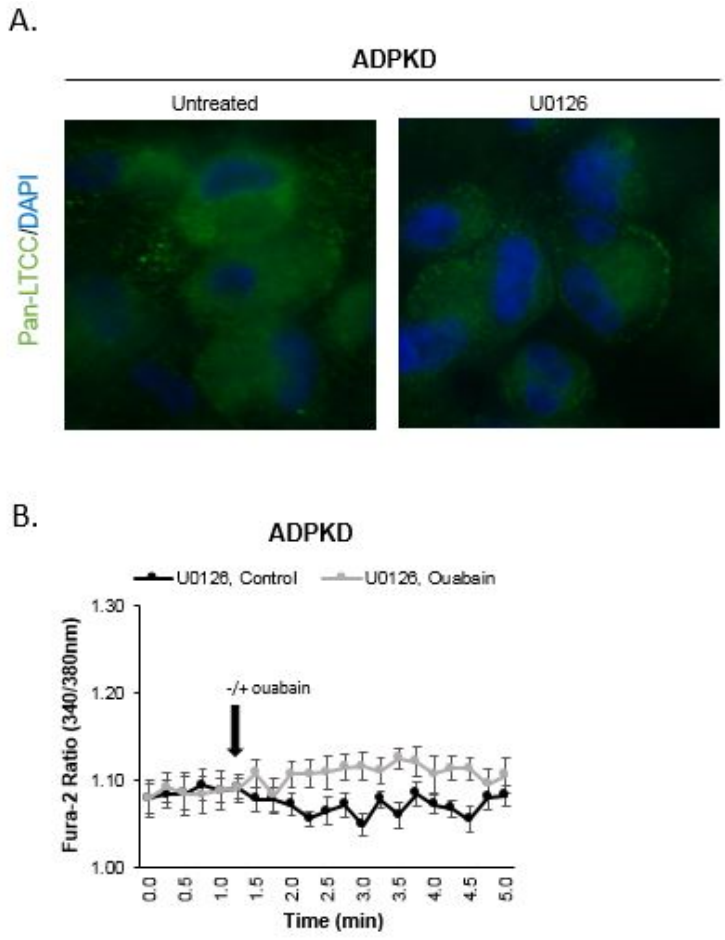

Figure 4.10. MEK activity affects the localization of LTCCs. (A) Representative images of LTCC Immunolocalization after 30 min ± U0126. (B) Fura-2 trace showing U0126 pretreatment rescued the calcium response in ADPKD cells; composite of the response of cells from 4 separate kidneys, in quadruplicate.

DISCUSSION

In this study, we found that ouabain is able to induce $[Ca^{2+}]_i$ increase in NHK cells, but not in ADPKD cells. The $[Ca^{2+}]_i$ rise in NHK cells was rapid, occurring in a short interval after ouabain addition (before one minute). This effect of ouabain is also sustained, since the changes in $[Ca^{2+}]_i$ were found to persist even 24 h after ouabain incubation. Similar to our results, ouabain was shown to increase $[Ca^{2+}]_i$ in different cell types, including normal renal proximal tubule cells. In this previous study, ouabain was shown to produce a Ca^{2+} wave, with an oscillatory pattern that had a periodicity of 1 Ca^{2+} spike every 4-5 minutes [403, 477, 558, 559]. We did not find a rhythmicity for the ouabain induced $[Ca^{2+}]_i$ increase in NHK cells. However, this may reflect differences in the experimental design used in each study. We carried out our assays on cell populations, while other studies performed single cell recordings [403, 477, 558-560]. Assessment of the ouabain-mediated $[Ca^{2+}]_i$ response in aggregate cells may have masked the oscillations of individual cells, producing an asynchronous pattern derived from the combined firing of multiple cells. Some reports show that ouabain-induced Ca^{2+} oscillations have a lag-time of several minutes after ouabain addition [558, 560]. We have performed Ca^{2+} traces for up to 30 minutes after ouabain treatment (data not shown), which failed to show an oscillatory pattern. Alternatively, $[Ca^{2+}]_i$ oscillations in response to ouabain may depend on differences in cell types in each study. This includes differences in species (mouse vs human) and the dissimilar renal origin of the cells (proximal tubule versus collecting duct). In this regard, it is interesting to postulate that the ouabain response uses different pathways in different cells types, pathways which can differentially affect $[Ca^{2+}]_i$ or operate downstream of Ca^{2+}. Despite the possible oscillatory differences, it is clear that normal renal epithelial cells respond to ouabain with an increase in $[Ca^{2+}]_i$ that is not present in ADPKD cells.

Our experiments show that the $[Ca^{2+}]_i$ rise caused by ouabain in NHK cells is prevented by eliminating Ca^{2+} from the medium. In addition, we find that depletion of ER Ca^{2+} with thapsigargin, or inhibition of IP_3R with xestospongin-c or 2-APB did not inhibit the ouabain induced $[Ca^{2+}]_i$ response in NHK cells. Moreover, ouabain did not modify ER or mitochondrial Ca^{2+} in NHK cells. Altogether, this shows that the $[Ca^{2+}]_i$ rise that follows ouabain administration depends on an influx of Ca^{2+} from the medium bathing the cells and is not the result of Ca^{2+} translocation from intracellular stores to the cell cytosol. This observation is different from findings in renal proximal tubular cells. In those experiments, removal of extracellular Ca^{2+} did not inhibit the Ca^{2+} response of proximal tubule cells to ouabain [403, 560]. In addition, treatment with thapsigargin, or inhibition of IP_3R completely abrogated the ouabain induced $[Ca^{2+}]_i$ response in renal proximal tubular cells [558-560]. Furthermore, we did not observe the translocation of p65 NFκB into the nucleus, which had been observed in renal proximal cells. This reinforces the idea that epithelial cells from different nephron segments activate different mechanisms in response to ouabain.

Yamaguchi et al. found NHK cells have constitutively higher $[Ca^{2+}]_i$ than ADPKD cells [197], while Xu, et al. observed ADPKD cells to have lower ER Ca^{2+} than NHK cells [169]. In agreement with those studies, we observed that cytoplasmic and ER Ca^{2+} levels are intrinsically lower in ADPKD than in NHK cells. We extended these observations to mitochondria and found no differences in Ca^{2+} content of this organelle between NHK and ADPKD cells. At present the cause for the lower Ca^{2+} levels of ADPKD cells is not precisely known. PC2 is a non-specific Ca^{2+} channel and it appears to have a role in release of Ca^{2+} from the ER, either on its own [185] or in conjunction with IP_3Rs [176, 212]. Overexpression of PC2 has been shown to decreases ER Ca^{2+} levels [211]. In contrast, expression of PC1 inhibits the Ca^{2+} leak from the ER, increases ER Ca^{2+}

refill [209, 210], and reduces ER Ca^{2+} release in response to stimulation [176, 212]. Thus, the mutations in polycystins leading to ADPKD may be responsible for the low ER and cytoplasmic Ca^{2+} observed in ADPKD cells.

We found that the mechanisms that mediate the differences in the ouabain induced $[Ca^{2+}]_i$ increase between NHK and ADPKD cells, depends on the action of LTCC. Therefore, the LTCC antagonist verapamil blocked the ouabain induced Ca^{2+} rise in NHK cells. In addition, the LTCC agonist Bay K8644 rescues the ouabain dependent Ca^{2+} response in ADPKD cells. A similar activation of LTCC by ouabain has been reported in non-renal cells [562-567]. Thus, we have established LTCCs as a new mechanism by which ouabain increases $[Ca^{2+}]_i$ in kidney cells. In contrast to NHK cells, ouabain is not able to stimulate $[Ca^{2+}]_i$ influx into ADPKD via LTCC. Alterations in LTCC, in particular the LTCC subclass CaV1.2, have been shown to play a role in cyst formation. In zebrafish, CaV1.2 localizes to primary cilia and its knock down results in the development of renal cysts [719]. Additionally, CaV1.2 is altered in *PKD1* or *PKD2* knockout mice [720]. Specifically, a band the size of the CaV1.2 cleavage product increases after knockout of either polycystin [720]. This suggests that the cleavage of α_1-LTCC is directly linked to the proper function of *PKD1* or *PKD2*. Furthermore, the complete knock-down of CaV1.2 in *PKD1*$^{+/-}$ zebrafish and in *PKD1*$^{+/-}$ mice greatly enhanced the cystic phenotype [720]. The importance of functional LTCCs in normal renal epithelial cells is further illustrated by studies in which treatment with verapamil, an LTCC antagonist, greatly enhanced cyst formation in the Cy/+ Han:SPRD rat model of ADPKD [683].

A known regulator of LTCC cleavage is the calpain protease family. Our findings show that ADPKD cells have higher calpain activity than NHK cells. Another difference that we found in ADPKD cells, compared to NHK cells is the decrease in the calpain inhibitor calpastatin. The

lower levels of calpastatin explains the higher calpain activity that we measured in ADPKD cells. Dysregulation of calpain activity and alterations in calpastatin expression have not been reported before in ADPKD cells; therefore, we have encountered a new characteristic that distinguishes these cells from normal renal epithelial cells. We found that pharmacological inhibition of calpain reduces LTCC cleavage in ADPKD cells. The calpain inhibitor that we used (CI1) is known to target both calpain-1 and -2; however, it is possible that other members of the calpain family of proteins are also targeted. The calpain family includes 14 members [721]. While calpain-1 and calpain-2 have been detected in collecting duct cells [722, 723], the presence of other calpains in the kidney is unclear. In general, calpains 1, 2, 5, 7, and 10 are ubiquitously expressed (reviewed in [724]); and The Human Genome Atlas (www.proteinatlas.org) lists a number of calpain family members as expressed in the kidney. Therefore, it is possible that other members of the calpain family are involved in the exacerbated cleavage of LTCC in ADPKD cells. Calpains are hypothesized to function at sub-maximal capacity at normal physiological concentrations of $[Ca^{2+}]_i$ as a safety mechanism to prevent unrestrained cleavage of substrates [725]. The binding of calpastatin to calpains limits the number of calpain molecules susceptible to activation [713]. Therefore, decreased expression of calpastatin expression would allow some calpain to become active even at low $[Ca^{2+}]_i$, as we have witnessed.

ADPKD cells and several ADPKD experimental models are known to express abnormally high amounts of c-Myc [140, 356, 574, 622, 707, 709-711], and c-Myc overexpression alone is enough to cause renal cysts, as observed in SBM mice [356, 622, 708-711]. In this study we confirmed that ADPKD cells express higher levels of c-Myc than NHK cells. While we found c-Myc significantly elevated in ADPKD cells, the increase we observed is not as robust as that reported by others. c-Myc is upregulated by serum growth factors [726-728], and therefore our use

of serum-free media in all experiments may have blunted c-Myc expression in the cells, preventing even higher expression levels of this transcription factor. Importantly, we showed that c-Myc overexpression is linked to downregulation of LTCC activity. Thus, inhibition of c-Myc transcriptional activities increased the expression of calpastatin, decreased calpain activity, and increased the amount of full-length LTCC as well as the $[Ca^{2+}]_i$ in ADPKD cells. These results show that in NHK cells, normal levels of c-Myc and calpastatin maintain the activity of calpain at relatively low levels. This in turn favors the expression of higher levels of LTCC, through which ouabain acts to ultimately cause the increase in $[Ca^{2+}]_i$ observed to occur in the NHK cells (Fig. 4.11). Our results show that ouabain did not alter the expression of c-Myc, calpastatin, or calpain in NHK or ADPKD cells. Therefore, the difference in effect that ouabain has on $[Ca^{2+}]_i$ in NHK and ADPKD cells is due to the dissimilar intrinsic activity of the c-Myc-calpastatin-calpain pathway that influences LTCC activity in the cells.

An additional difference that we observed between the mechanisms that control $[Ca^{2+}]_i$ in NHK and ADPKD cell is their response to NCX inhibition. We determined that NCX is expressed in both cell types. However, while our experiments cannot discard a role of NCX in the $[Ca^{2+}]_i$ increase induced by ouabain in NHK cells, NCX appears not to be functional in ADPKD cells. This idea is based on the lack of effect that ORM10103 has in ADPKD cells. At present, the lack of activity of NCX in ADPKD cells is unclear. It is possible that the low $[Ca^{2+}]_i$ levels in these cells and the continuous translocation of Ca^{2+} to replenish intracellular stores, prevent any possible increase in $[Ca^{2+}]_i$ in the cytoplasm of the cells. Additional experiments will be needed to directly assess the activity of NCX and its role in Ca^{2+} maintenance in NHK and ADPKD cells.

In conclusion, we have discovered molecular mechanisms by which ouabain increases $[Ca^{2+}]_i$ in NHK cells and identified phenotypic changes which prevent ADPKD cells from responding to ouabain with a Ca^{2+} rise. The inability of ouabain to raise $[Ca^{2+}]_i$ in ADPKD cells is relevant to the pathophysiology of ADPKD, since low $[Ca^{2+}]_i$ is known to enhance ADPKD cystogenesis. This effect along with our previous results showing that ouabain stimulates cell proliferation and favors secretion of the cystic epithelium, highlights the importance of ouabain as a factor that enhances progression of ADPKD.

Figure 4.11

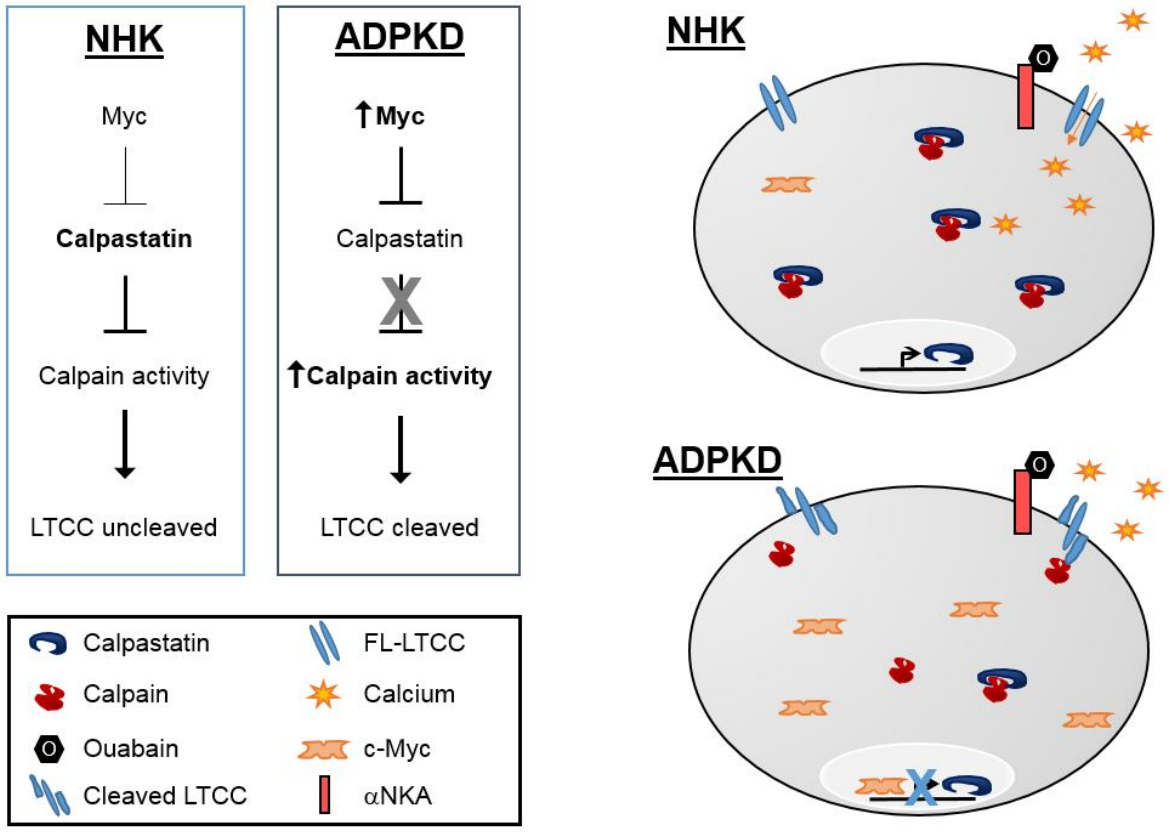

Figure 4.11. In ADPKD cells, high levels of c-Myc transcriptionally inhibit the expression of calpastatin. Without calpastatin to inhibit calpains, increased cleavage of α1-LTCC occurs which contributes to the low cytoplasmic calcium phenotype of cystic cells. In contrast, NHK cells have ample calpastatin to control calpain activity, allowing sufficient levels of full length α1-LTCC to be available to respond to ouabain with calcium influx, which does not occur in ADPKD cells.

OVERALL DISCUSSION and FUTURE DIRECTIONS

The second hit [41] and third hit [72] theories of ADPKD progression suggest that unknown environmental factors play a large role in accelerating cyst growth. Indeed intrafamilial studies have shown that genetics alone cannot explain the variability observed in the progression of ADPKD to ESRD [44, 45]. One non-genetic factor shown to enhance the ADPKD phenotype is ouabain. This hormone acts directly on cystic epithelial cells collected from ADPKD patients to elicit both increased growth and increased fluid secretion though activation of the B-RAF/MEK/ERK signaling cascade [322, 360, 491]. In contrast, ouabain did not elicit these effects in normal human collecting duct cells [322, 360, 491]. The results in this thesis have expanded our knowledge of the effects of ouabain on ADPKD cells, showing that ouabain is a factor that contributes to the abnormal phenotype of ADPKD cells. First, ouabain was found to increase apoptosis in the cells, which is a characteristic of ADPKD. Second, ouabain induced changes compatible with partial EMT of the cells, which corresponds to the dedifferentiated characteristics shown by ADPKD. Finally, ouabain failed to increase $[Ca^{2+}]_i$ in ADPKD cells due to their constitutive low expression of LTCC; and possibly via the ERK-mediated maintenance of LTCC in intracellular compartments.

Specific Aim 1: Define the action of ouabain-mediated apoptosis on ADPKD cells.

ADPKD is paradoxically characterized by both increased proliferation and apoptosis. The working hypothesis for Chapter 2 was that ouabain would be able to stimulate ADPKD cell apoptosis through BCL-2 and BAX alterations, resulting in cytochrome c release from the mitochondria. This was found to be correct; ouabain was able to enhance the ADPKD phenotype

of increased apoptosis, as expected. The balance between proliferation and apoptosis was maintained, however, as ouabain also enhanced ADPKD-mediated proliferation, resulting in net increased proliferation, which would serve to aid cyst growth.

Apoptosis is involved during kidney development, and is theorized to be important for lumenization or other morphogenetic processes [729]. In particular, cavitation, which involves lumen generation by apoptosis, is employed during tubulogenesis (reviewed by [730]). Additionally, apoptosis can also be instrumental in wound responses. Indeed, the enhanced caspase-3/7 activity caused by ouabain in ADPKD cells, may also have non-apoptotic roles. For instance, low levels of caspase activity can affect cytoskeletal rearrangements and cell fate decisions (reviewed in [731]). In fact, after partial hepatectomy, liver regeneration is dependent on caspase-3 and -7 activation [732]. It has been hypothesized that some apoptotic cells die slowly, affording the time necessary to induce expression of mitogenic factors which prepares neighboring cells for the departure of the apoptotic cell [731]). Signals that are transmitted from apoptotic cells include activators of the WNT, TGFβ and Hedgehog families, which are all active during cystic progression [254, 255, 584, 733]. As the data in Chapter 3 shows, ouabain increased TGFβ expression in ADPKD cells, while β-catenin localization and expression was not found to change. The effect of ouabain on the hedgehog pathway in ADPKD has not yet been investigated, but may prove to be an interesting avenue for future study.

ADPKD cells have constitutively altered concentrations of Ca^{2+}, an element which can both inhibit or promote apoptosis, depending on cellular context. Ca^{2+} was first shown to induce apoptosis when in excess [734]. However, low $[Ca^{2+}]_i$ is able to increase both proliferation and apoptosis in vascular smooth muscle cells, similar to what is observed in ADPKD [735]. While we observed ouabain-induced Ca^{2+} influx in NHK cells, we did not detect increased apoptosis in

NHK. Conversely, the lower $[Ca^{2+}]_i$ levels in ADPKD cells were not further decreased by ouabain, yet apoptosis was enhanced. Thus, while $[Ca^{2+}]_i$ may not be affecting ouabain-mediated apoptosis, organelle Ca^{2+} may still play a role. For example, both Ca^{2+} overload and depletion in the ER can result in protein misfolding and subsequent ER stress that might contribute to apoptosis [736]. While ER Ca^{2+} is decreased in ADPKD relative to NHK, we have not assessed whether ouabain affects ER stress proteins. We have, however, observed that ouabain-mediated apoptosis does rely on certain proteins which act on ER Ca^{2+}. For instance, BAX protein expression is increased by ouabain in ADPKD cells. Overexpression of BAX promotes Ca^{2+} translocation from the ER to the mitochondria and subsequent cytochrome c release for apoptosome activation [737]. This movement of Ca^{2+} from the ER into the mitochondria can overload the mitochondria, resulting in apoptosis [738]. In a related pathway involving Ca^{2+} and apoptosis, the ouabain-mediated decrease in BCL-2 protein observed in ADPKD would promote apoptosis via several mechanisms. First, BCL-2 reduces the filling of the ER Ca^{2+} store and diminishes store-operated Ca^{2+} entry [739, 740], thus preventing the overload of Ca^{2+} in the mitochondria. Alternatively, BCL-2 overexpression also increases the ability of the mitochondria to uptake Ca^{2+} without overload, and BCL-2 also prevents the release of cytochrome c by inhibiting the opening of the mitochondrial permeability transition pore [741-743]. Although ouabain-mediated alterations to ER or mitochondrial Ca^{2+} were not observed, this apoptosis-inducing movement of Ca^{2+} ions from the ER to mitochondria may still have occurred in response to the ouabain-mediated alteration in BAX and BCL-2 expression in ADPKD cells. This hypothesis is based on two facts. 1) Ca^{2+} alterations were assayed at earlier time points than the time points used in the apoptosis study, so changes in organelle Ca^{2+} due to ouabain-mediated apoptosis may not yet have taken place. 2) Additionally, both organelles (ER and mitochondria) contain many Ca^{2+}-binding proteins which may mask

actual increases in organelle Ca^{2+}, as the fluorescent Ca^{2+} probes utilized in Chapter 4 assess only free-Ca^{2+} levels. Further studies will be needed to determine the link between Ca^{2+} and apoptosis in ADPKD cells, however it is clear that ouabain enhances the characteristic apoptotic phenotype of ADPKD.

Specific Aim 2: Determine the role and mechanism of action of ouabain in inducing EMT changes in ADPKD.

The working hypothesis for Chapter 3 was that ouabain, via the activation of the TGFβ pathway, would induces EMT-related changes that cause upregulation of mesenchymal markers, relaxation of cell adhesion properties, and increase motility and fibrosis in APDKD cells. Cellular aspects affected by EMT-like processes, such as adhesion, migration, polarity and ECM deposition, are extremely important in both development and response to injury [553, 650]. Ouabain did not fully stimulate EMT; however, enhancement of the mesenchymal state was achieved. Also, ouabain induced a decrease in cell-cell adhesion and increased cell mobility, but it did not affect cell invasiveness. Taken together, these results agree with the characteristics required for cyst formation. The ADPKD epithelium cannot be fully dedifferentiated, since it would then loose the polarity needed to carry on basal-to-apical secretion of fluid into the cyst lumen. In addition, complete dedifferentiation would also lower adhesion between cells and decrease the transepithelial resistance necessary to impede leakage of fluid from the cyst. Finally, the inability of ouabain to promote invasiveness agrees with ADPKD as being a proliferative but not metastatic disease [95].

Ouabain was found to increase TGFβ expression as well as activate its downstream mediator, SMAD3. Elevated TGFβ signaling has been described in cystic cells [584], and my studies show ouabain further enhanced this ADPKD phenotype. Exposure of epithelial cells to TGFβ is a strong stimulus for EMT-signaling, causing upregulation of pro-mesenchymal E2A proteins (TCF3 and ITF1), and a concomitant downregulation of their inhibitors, the Id proteins [744]. Counterintuitively then, Id2 is upregulated in *PKD1*-null cells [745], probably via the transcriptional activities of c-Myc [746, 747]. Indeed, mutations in either polycystin lead to increased Id2 in the nucleus [748]. Thus, the upregulation of Id2 by c-Myc, despite increased TGFβ activity, may antagonize the classically observed EMT induced by TGFβ, causing the partial EMT in response to ouabain described in Chapter 3. The ouabain-mediated upregulation of TGFβ is expected to have a role in cystic progression rather than the induction of cysts, based on the literature [584, 749]. For example, in the Han:SPRD rat model of polycystic disease, expression of TGFβ and its target genes is low initially, but increases with the progression of the disorder [749]. Again, another study has found TGFβ signaling was not increased in initial stages of cyst formation but was increased during cyst expansion in models of polycystic kidney disease as well as in kidneys from ADPKD patients [584]. This role of ouabain in TGFβ cystic progression, therefore, favors ouabain as a factor responsible for the variability of cystic progression between individuals.

A plethora of data suggest that polycystins are important in proper tissue repair and are instrumental in the morphogenesis programs indispensable in development [750]. Indeed, PC1 expression increases after renal injury [71, 164], an event which has been suggested to provide the appropriate plasticity necessary during tissue repair [750]. Interestingly, SRC is also activated in response to renal injury and appears to participate in tubular regeneration [751], and PC1 has been

reported to interact with SRC and STAT3 in a complex that is speculated to facilitate tissue regeneration in response to normal renal insults [251, 752]. Interestingly, ouabain stimulates SRC activity in ADPKD cells [322, 491], and as NKA binds both PC1 CTT and SRC [582], it is tempting to speculate that ouabain may be contributing to the activity of the PC1-SRC-STAT3 pathway, promoting the futile repair pathways purported to increase cell proliferation and cystogenesis in ADPKD [95].

Specific Aim 3: Investigate the effect of ouabain on Ca^{2+} signaling in NHK and APDKD.

ADPKD cells have constitutively lower intracellular Ca^{2+} than NHK cells, which alters their response to many different stimuli. The working hypothesis for Chapter 4 was that ouabain would affect the intracellular Ca^{2+} concentration in NHK cells, but not in ADPKD cells, due to dysfunctional Ca^{2+} signaling in ADPKD cells. Indeed, ouabain was able to increase $[Ca^{2+}]_i$ in NHK cells, which was subsequently found to occur via LTCCs. Conversely, in ADPKD cells, LTCCs were inhibited by cleavage and by decreased localization into the membrane. While lower LTCC expression had been observed to cause renal cysts in animal models [719, 720], this had not been investigated in human samples prior to this thesis. Additionally, the observation of altered LTCC cleavage in ADPKD also led to the discovery of increased calpain activity in ADPKD cells, which also had not described prior to this thesis.

The third hit theory of ADPKD progression is based on the observation that cystic growth is accelerated by wounding stress [72]. Interestingly, activity of LTCCs can be altered by extracellular matrix proteins, via an interaction with integrins, which could possibly play a role in tissue injury responses [753]. Serendipitously, Ca^{2+} influx via LTCCs occurs in response to

myocyte injury and coordinates the movement of vesicles filled with calpain-induced cleavage products to the plasma membrane for injury repair [573]. Additionally, the activation of calpains may be another aspect of the wound-like phenotype of ADPKD cells. For instance, the self-sealing repair of damaged plasma membranes requires the activity of calpains, which may act to remodel the cortical cytoskeleton [754]. Additionally, the expression of calpains is increased during skin wound healing [755]. Indeed, in renal injury by ischemia-reperfusion, expression of calpastatin is decreased [756] and is perhaps a common mechanism used in renal injury to activate calpains for repair processes. Conversely, forced expression of calpastatin during skin wounding, delays scar formation [757], again showing the importance of calpain activation in response to injury. Therefore, in Chapter 4, the involvement of LTCCs, calpains, and calpastatin can be tied to an injury response and thus suggest a role for ouabain as a promoter of the third hit leading to ADPKD progression.

Physiologically important responses to ouabain between tubule cell types

The work outlined in this thesis consistently found differences between the response to ouabain that had been described in proximal tubules cells, and the response observed in ADPKD cells. For instance, ouabain was observed to protect proximal tubule (PT) cells from apoptosis, via translocation of p65 NFκB into the nucleus [403, 477, 558-560]. In collecting duct (CD) cells, this translocation was not observed to occur in either NHK or ADPKD. In fact, in ADPKD cells, induction of apoptosis was observed, not protection. Additionally, PT cells respond to ouabain with Ca^{2+} influx via IP_3Rs [403, 477, 558-560], however, in CD cells the ouabain-mediated Ca^{2+} influx occurred through LTCCs. Others have also observed a difference in how these two cell types respond to ouabain. For instance a 12-hour exposure to ouabain (100 nM) causes the endocytosis

of NKA in LLCPK1 (proximal) cells but not in MDCK (collecting duct) cells [758]. Thus, PT cells respond to ouabain in a different manner than ADPKD cells.

These two renal segments also respond to stress differently. For instance, following reductions of renal mass, mechanisms for sodium handling remain functional in the collecting duct, whereas in the proximal tubule, the handling of sodium is substantially altered and no longer respond [759]. This allows the "fine tuning" of sodium balance by the distal nephron to still proceed under stress [759]. As ouabain is involved in conditions of altered salt balance, as well as in renal stress, the differences between how proximal and collecting duct tubules respond to ouabain may have physiological importance.

Finally, PT and CD cells respond differently to polycystin mutations, as evidenced by the apparent collecting duct origin of the majority of cysts [131-133]. Interestingly, even during embryonic development, the two renal segments modify PC1 differently. For example, PC1 does not undergo GPS cleavage in the proximal tubule segments, while in the distal nephron segments (which includes the CD), cleaved PC1 products are important for normal tubulogenesis. An uncleavable PC1 mutant causes cyst formation only in the collecting duct [37, 127, 168]. Finally, the other polycystin, PC2, appears to be also differentially affected between the two cell types. In cultured PT cells, ouabain induces the translocation of PC2 from the ER to the plasma membrane [760]. Despite repeated attempts, (data not shown), ouabain has not been observed to cause a similar PC2 translocation in collecting duct cells, further emphasizing the physiologically important functional differences between how CD and PT cells respond to ouabain.

In conclusion this thesis provides new mechanistic evidence on the effects of the hormone ouabain in ADPKD. This work adds to other pro-cystogenic effects that ouabain has been shown

to trigger in ADPKD cells, and further supports the notion that ouabain is a circulating factor that can exacerbate the progression of the disease.

Future Directions

Interesting differences in the effect of ouabain in NHK and ADPKD cells have been observed in this thesis, however the mechanisms underlying the cause of the dissimilar response to ouabain in each cell type is still unclear. Previous results showed that ADPKD cells contain a subpopulation of NKA with an abnormally high affinity for ouabain, allowing them to respond to ouabain in an exacerbated manner [322]. However, the reason for the difference in ouabain affinity in these cells is not clear. In this regard, the NKA accessory protein FYXD2 may be of particular interest as FXYD2 has increased expression in ADPKD cells relative to NHK [322], and enhances ouabain sensitivity [342]. Investigations into this increased affinity of ouabain to NKA of ADPKD cells will help in developing pharmacological approaches to block the cystogenic effects of ouabain. Alternatively, it is possible that ouabain levels could be elevated in patients with ADPKD, especially at times in which cystic growth is accelerated, such as *in utero* [410] or during compression of the renal parenchyma by the cysts [385, 388, 396, 397]. Therefore, determining plasma levels of ouabain in ADPKD patients versus healthy controls could be instrumental in understanding the disease. In addition, it has yet to be determined if ouabain has any effect on polycystin activities. As mentioned previously, ouabain, via SRC activation could promote the activity of a PC1/SRC/STAT3 complex [251, 752], and may provide further insights into the mechanism by which ADPKD cells have a unique response to ouabain. Further, as ADPKD is a ciliopathy, determining if ouabain has a role in ciliary-mediated processes could also provide insight into the effect of ouabain on ADPKD cells. Future work could also include ADPKD mouse

models in which the effects of ouabain or ouabain inhibitors could be observed in an *in vivo* system. Perhaps further work could also be directed towards learning more about the calpain isoforms present in ADPKD cells and if these isoforms differ from that in normal renal epithelial cells.

The primary objective of this thesis was to better understand the mechanisms by which ouabain enhances several characteristics of ADPKD cells. A mechanistic understanding is a necessary first step for the development of pharmacological approaches to treat the disease. Eventually, methods to block ouabain, or downstream targets of ouabain, may be used clinically to limit cyst progression and prolong patient life. Additionally, this thesis has also revealed a new phenotype of ADPKD cystic cells, elevated calpain activity, which may prove to be especially pertinent to either the treatment or the understanding of this disease. Finally, this thesis has shed light on the differential ways ouabain affects proximal and collecting duct cells. Such findings are important due to the enigmatic systemic effects of ouabain. Any new knowledge pertaining to the action of ouabain may help explain the purpose of this incompletely understood hormone. Thus, the data presented in this thesis advances the knowledge of several fields and will hopefully be useful to subsequent researchers.

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