Endogenous concentrations of ouabain act as a cofactor to stimulate fluid secretion and cyst growth of in vitro ADPKD models via cAMP and EGFR-Src-MEK pathways

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Jansson K, Nguyen AT, Magenheimer BS, Reif GA, Aramadhaka LR, Bello-Reuss E, Wallace DP, Calvet JP, Blanco G. Endogenous concentrations of ouabain act as a cofactor to stimulate fluid secretion and cyst growth of in vitro ADPKD models via cAMP and EGFR-Src-MEK pathways. Am J Physiol Renal Physiol 303: F982–F990, 2012. First published August 1, 2012; doi:10.1152/ajprenal.00677.2011.—In autosomal-dominant polycystic kidney disease (ADPKD), renal cysts develop by aberrant epithelial cell proliferation and transepithelial fluid secretion. We previously showed that ouabain increases proliferation of cultured human ADPKD cells via stimulation of the EGFR receptor (EGFR)-Src-MEK/ERK signaling pathway. We examined whether ouabain affects fluid secretion and in vitro cyst growth of ADPKD cell microcysts cultured in a three-dimensional collagen matrix, and metanephric organ cultures from Pkd1<sup>m1Bei</sup> mice. Physiological concentrations of ouabain alone did not affect net transepithelial baso-apical fluid transport in ADPKD monolayers or growth of cultured ADPKD microcysts. In contrast, in the presence of forskolin or 8-bromo-cAMP, ouabain significantly enhanced ADPKD fluid secretion and microcyst expansion. Ouabain exerted this effect by enhancing cAMP-dependent Cl<sup>−</sup> secretion via the CFTR. Similarly, ouabain accelerated cAMP-dependent cyst enlargement in Pkd1<sup>m1Bei</sup> mice metanephiroi, with a more prominent response in homozygous than heterozygous mice. Ouabain had no effect on fluid secretion and cystogenesis of normal human kidney cells and caused only slight cystic dilations in wild-type mouse kidneys. The effects of ouabain in ADPKD cells and Pkd1<sup>m1Bei</sup> metanephiroi were prevented by inhibitors of EGFR (AG1478), Src (PP2), and MEK (U0126). Together, our results show that ouabain, used in physiological concentrations, has synergistic effects on cAMP-mediated fluid secretion and cyst growth, via activation of the EGFR-Src-MEK pathway. These data provide important evidence for the role of ouabain as an endogenous hormone that exacerbates ADPKD cyst progression.

Na-K-ATPase; polycystic kidney disease

OUABAIN IS A STEROIDAL HORMONE synthesized by the adrenal glands of several mammalian species, including humans (34, 35). Ouabain circulates in blood in nanomolar concentrations and, after reaching its target, triggers a variety of cell type-specific responses, including changes in cell metabolism, motility, and growth (3, 10, 27, 41). To exert its action, ouabain binds to its receptor, Na-K-ATPase, located on the surface of cells. Na-K-ATPase, a membrane-bound protein complex, is expressed in most animal cells and is highly abundant on the basolateral side of most tubular segments of the kidney. Similar to other hormone-receptor interactions, binding of ouabain to Na-K-ATPase initiates a series of intracellular events that include the scaffolding of Na-K-ATPase with neighboring proteins in a signaling apparatus that has been described as the Na-K-ATPase signalosome (32). Essential components of the Na-K-ATPase signaling system comprise activation of the kinase Src and stimulation of downstream members of the MAPK pathway (16, 20, 47).

Autosomal-dominant polycystic kidney disease (ADPKD) is a common inherited disorder characterized by formation and progressive growth of numerous fluid-filled cysts in the kidney and other organs with ductal structures (11). ADPKD is caused by mutations in Pkd1 or Pkd2, genes that encode polycystin (PC-1 and PC-2, respectively (17). The enlargement of cysts within the kidney disrupts the renal architecture and compromises organ function, resulting in end-stage renal failure in ~50% of affected individuals (14). Despite the genetic origin of ADPKD, cyst growth can be accelerated by a variety of nongenetic factors, including physiological and pharmacological agents (40, 46). For example, cAMP agonists, such as AVP and forskolin, and growth factors, such as EGF and insulin-like growth factor, promote the proliferation of human ADPKD cells (43, 48). In addition, cAMP itself induces human ADPKD cell proliferation and cyst growth in animal models of polycystic kidney disease (42, 48). Many of these cystogenic factors share a common mechanism of action, which involves stimulation of the MEK-ERK pathway.

Recently, we showed that ouabain, in concentrations similar to those circulating in blood, stimulates the proliferation of human ADPKD cyst-lining epithelial cells but has no significant effect in normal human kidney (NHK) cells (30). Ouabain binding to the Na-K-ATPase activates the EGF receptor (EGFR), the tyrosine kinase Src, and the MEK-ERK pathway, representing a novel circulating agent that has the ability to promote ADPKD cell growth (30). Increased proliferation of renal tubular cells is an essential step in the development of renal cysts (8, 31, 39). However, cyst expansion also requires the concomitant and continuous addition of fluid into the cavity.

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of the growing vesicles, which maintains the shape and turgidity of the cysts. This process depends on changes in the salt and fluid transport properties of the ADPKD cells, which favor fluid secretion over reabsorption in the cystic epithelium (37, 38). In ADPKD, fluid secretion is driven by transcellular Cl- secretion involving a basolateral Na-K-2Cl cotransporter (NKCC1) and apical CFTR Cl- channels (26). In the current study, we explored whether physiological amounts of ouabain can affect ADPKD fluid secretion and cyst growth. We show that ouabain can act as a cofactor enhancing the effects of forskolin and cAMP on fluid secretion and microcyst development of human ADPKD cells in culture and on cystlike dilations in metanephric organ cultures of Pkd1m1Bei mice. These effects of ouabain depend on enhanced transepithelial secretion of anions via apical CFTR Cl- channels and are mediated via the EGFR-Src-MEK pathway.

MATERIALS AND METHODS

Cell culture. Primary cell cultures from NHK epithelial cells and cyst-lining renal epithelial cells from patients with ADPKD were prepared from nephrectomy specimens by the Polycystic Kidney Disease 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. Cells were seeded and grown in DME/F12 medium supplemented with 5% heat-inactivated FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and insulin (5 μg/ml)-transferrin (5 μg/ml)-sodium selenite (5 ng/ml) (ITS), as described elsewhere (44).

Fluid secretion assay. Confluent NHK and ADPKD monolayers were established on 12-mm permeable Snapwell inserts (Corning, Corning, NY) in six-well tissue culture plates, as previously described (30). Cells were cultured for 1 wk, until a tight epithelium was established, as determined by the achievement of a transepithelial electrical resistance across the cell monolayer (30). Then cells were serum-starved in our basal DME/F12 medium for 24 h, as described elsewhere (30), and the basolateral side of the cell monolayers was treated with 3 mM ouabain, in the absence and presence of 5 μM forskolin or 100 μM 8-bromo-cAMP (8-Br-cAMP). Control monolayers were incubated in basal medium. Fresh medium (150 μl) was placed on the apical surface of the cells, and mineral oil was layered over the top of the medium to prevent evaporation. Cultures were incubated at 37°C for an additional 24 h, and the apical medium was collected and measured as described elsewhere (45). Fluid secretion data are expressed as microliters per hour per square centimeter, with positive values indicating fluid secretion and negative values indicating fluid absorption. For some experiments, monolayers were treated in the absence or presence of 2 μM tyrphostin AG1478 (an EGFR inhibitor), 10 μM 6-amino-5-(4-chlorophenyl)-7-(3-butyl)pyrazolo[3,4-d]pyrimidine (PP2, an Src inhibitor), or 1 μM U0126 (a MEK inhibitor).

Short-circuit current. Confluent monolayers of ADPKD cells were grown on 12-mm permeable Snapwell inserts (Corning), as previously described (30), and treated with or without 3 mM ouabain for 24 h. A dual voltage-clamp device (Warner Instruments, Hamden, CT) was used to mount cell monolayers in Ussing chambers for measurement of short-circuit current (Isc), as previously described (43). Once the monolayers were loaded in Ussing chambers, benzamil (10 μM) was added to the apical medium to block sodium reabsorption and ensure that any increase in Iac was due to apical anion secretion. Forskolin (5 μM) was added to the basolateral solution to stimulate cAMP production, and the change in Iac (ΔIac) was calculated as difference from baseline current, as previously described (2). To determine the involvement of CFTR, 10 μM CFTR (inh)-172, a selective CFTR inhibitor (Sigma Aldrich, St. Louis, MO), was added to the apical solution of the monolayers. Data are expressed as percentage of the maximum response to forskolin by the untreated monolayers.

Real-time RT-PCR analysis. NHK and ADPKD cells were treated with 8-Br-cAMP (100 μM) or ouabain (3 mM), alone or in combination, and total RNA was extracted using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using 1 μg of RNA with the Omniscript RT Kit (Invitrogen, Grand Island, NY) and oligo(dT) primers. Real-time quantitative PCR (RT-qPCR) was performed using ABI Prism 7900 (Applied Biosystems, Foster City, CA) and SYBR green PCR master mix (Warrington) following the manufacturer’s protocol. Briefly, the reaction mixture (25 μl total volume) contained 8 μl of a 1:10 dilution of cDNA [5′-GTAACCGGTGAAACC- CCATT-3′ (antisense) and 5′-CCATCCCAAATCGTGAGG-3′ (sense)] to amplify human 18S rRNA. NKCC1 and CFTR were amplified using 5′-CAATAGCGAATGGTACT-3′ (antisense) and 5′-CATGCGTTATCTTGGTAC-3′ (sense) for NKCC1 and 5′-GGAAAAAGGCACCAGTCTGC-3′ (antisense) and 5′-CAGGGCTGTCTGTATCCT-3′ (sense) for CFTR at a final concentration of 100 nM with 12.5 μl of 2× SYBR green PCR master mix. Target genes and 18S rRNA gene were amplified in the same reaction. All experiments were normalized to 18S rRNA. Comparative quantification is determined using the cycle threshold (2-ΔΔCT) method (23).

Immunoblot analysis. ADPKD cells (1×105) were treated with and without 3 mM ouabain or 100 μM 8-Br-cAMP, alone or in combination, and protein expression was analyzed by SDS-PAGE (7.5% gel) and immunoblotting, as previously described (29). CFTR and NKCC1 were identified using an anti-CFTR antibody (R&D Systems, Minneapolis, MN) and a NKCC1 antibody (Developmental Studies Hybridoma Bank, University of Iowa). Anti-β-tubulin (Sigma Aldrich) was used to determine tubulin as a control for protein loading. Horseradish peroxidase-conjugated secondary antibodies and chemiluminescence were used for detection. The images were scanned and quantified for band intensity using Gel-Pro software (Media Cybernetics, Silver Spring, MD).

Microcyst cultures. NHK and ADPKD cells were suspended at a density of 4,000 cells per well in a 96-well plate in ice-cold type I collagen (PoreCol, Advanced Biomatrix, San Diego, CA) in medium composed of 1:1 DME-F12 medium, 100 U/ml penicillin, 0.1 mg/ml streptomycin, ITS, 5×10−9 M hydrocortisone, and 5×10−12 M triiodothyronine (defined medium). After the cell/collagen suspension was seeded, the culture plates were warmed to 37°C to polymerize the gel. Cysts were induced to grow by pretreatment for 2–4 days with 5 μM forskolin and 5 ng/ml EGF. Once the cysts began to form, the agonists were removed, and cultures were treated with defined medium in the absence and presence of 3 mM ouabain or 5 μM forskolin, alone or together. Some experiments were also performed in the absence or presence of 2 μM tyrphostin AG1478, 10 μM PP2, or 1 μM U0126. Cysts were allowed to grow for 5–7 days in culture and then fixed with 0.5% buffered formalin in PBS. Cultures were photographed, and the diameters of individual cysts (≥100 μm diameter) were measured and used to calculate a total surface area using analySIS software (Lakewood, CO), as described previously (33). Data (means ± SE) are expressed as total surface area for each culture condition.

Embryonic organ cultures. All experimental protocols involving mice were approved by the KUMC Institutional Animal Care and Use Committee. Pkd1m1Bei mice were originally obtained from the Mutant Mouse Regional Resource Center (University of North Carolina, Chapel Hill, NC) (18) and stabilized on a C57BL/6 background (24). Mice heterozygous for Pkd1 were mated, and embryos from timed-pregnant females were removed at embryonic day 15.5. Embryo genotyping showed the Pkd1 allele to be inherited with the approximate expected Mendelian ratio. Metanephric kidneys were dissected under sterile conditions and transferred to Transwell filters (24-mm, 0.4-μm pore size; Corning) in a six-well culture plate. Metanephiroïdi were cultured at an air-fluid interface with serum-free medium containing equal volumes of DME-F12 medium supple-
**RESULTS**

Ouabain enhances cAMP-dependent fluid secretion by the ADPKD epithelium. Fluid secretion is an essential event in the formation and development of ADPKD cysts (12, 13). As a first approach to determine the effect of ouabain in cystogenesis, we determined the transepithelial movement of fluid across NHK and ADPKD cell monolayers. Cells were treated for 24 h without or with 3 nM ouabain, a concentration found to be optimal for stimulating ADPKD cell growth (30). Monolayers were also treated with 5 μM forskolin or 100 μM 8-Br-cAMP, alone or in combination with ouabain. After 24 h, the amount of fluid in the upper chamber of the cell monolayer was measured as described elsewhere (45). Untreated NHK monolayers transported fluid from the apical to the basolateral medium, as indicated by negative values of fluid secretion (Fig. 1A). Addition of forskolin or 8-Br-cAMP resulted in net fluid secretion; however, these values were not significantly different from untreated controls in the NHK monolayers (Fig. 1A). These results agree with the small stimulatory effects of cAMP agonists on apical fluid movement in normal dog and human kidney cells (25, 26). Addition of ouabain to NHK monolayers, alone or in combination with forskolin or 8-Br-cAMP, had no significant effect on fluid transport (Fig. 1A). When ADPKD monolayers were tested, they showed little fluid reabsorption in the absence and presence of ouabain alone (Fig. 1B). However, in contrast to NHK and in agreement with previous observations (26), forskolin and 8-Br-cAMP increased fluid secretion by ADPKD monolayers (Fig. 1B).

![Figure 1](http://ajprenal.physiology.org/)

**Fig. 1.** Ouabain increases forskolin- and cAMP-dependent fluid secretion by autosomal-dominant polycystic kidney disease (ADPKD), but not normal human kidney (NHK), cells. Confluent cell monolayers were treated with 3 nM ouabain, 5 μM forskolin, or 100 μM 8-bromo-cAMP (8-Br-cAMP), alone or in combination. Control monolayers were incubated in medium with no treatments added. After 24 h, fluid volume on the apical side of NHK (A) and ADPKD (B) cell monolayers was measured. Values are means ± SE of 3 (NHK) or 4 (ADPKD) experiments performed using cells obtained from different kidneys. *P < 0.01.

![Figure 2](http://ajprenal.physiology.org/)

**Fig. 2.** Ouabain increases forskolin-induced short circuit current (Isc) across ADPKD monolayers. ADPKD cell monolayers, treated in the absence or presence of 3 nM ouabain for 24 h, were mounted in Ussing chambers, and ISc was measured by a dual voltage-clamp device. Forskolin (5 μM) was added to the medium on the basolateral side of the cells to stimulate Isc, and CFTR(inh)-172 (10 μM), a specific CFTR inhibitor, was added apically to determine the contribution of CFTR. A: change in ISc (ΔIsc) from baseline for monolayers incubated in control medium or ouabain (3 nM) for 24 h prior to measurement of ISc. Baseline currents were calculated as an average of the ISc reading for 1–2 min prior to addition of forskolin. B: Isc response of ADPKD monolayers to forskolin before and after treatment with ouabain. Data are expressed as percentage of change in Isc from baseline after forskolin response in the absence of ouabain. Values are means ± SE of 9 control and 8 ouabain-treated monolayers from different ADPKD kidneys. *P < 0.05 vs. control.
Interestingly, this forskolin- and cAMP-dependent transepithelial fluid secretion of ADPKD cells was enhanced by the presence of ouabain (Fig. 1B). These results demonstrate that in ADPKD cells, but not in NHK cells, ouabain enhances cAMP-dependent transepithelial fluid secretion.

Ouabain increases forskolin-induced $I_{sc}$ by ADPKD cell monolayers via CFTR. Fluid secretion by ADPKD cells is dependent on transepithelial anion secretion (26, 42), and forskolin or other cAMP agonists have been shown to induce anion secretion by ADPKD monolayers through activation of the apical membrane CFTR (2, 4, 26, 36, 37). The stimulatory effect of ouabain on cAMP- and forskolin-induced fluid secretion may be mediated via forskolin-stimulated anion secretion. To test this, ADPKD monolayers were grown on permeable filter supports and treated with control medium or medium containing 3 nM ouabain for 24 h, and $I_{sc}$ was determined. $I_{sc}$ measurements have been commonly used as an indicator of anion secretion across ADPKD monolayers (2, 4, 26, 36, 37). As previously described (26, 37), forskolin was able to increase the $I_{sc}$ by ADPKD cell monolayers (Fig. 2A). Interestingly, addition of 3 nM ouabain to the monolayers increased the forskolin-induced $I_{sc}$ by ~23% (Fig. 2), indicating an increase in anion secretion across the monolayers. The forskolin-mediated increase in $I_{sc}$ in the absence and presence of ouabain was sensitive to the specific CFTR inhibitor CFTR(inh)-172 (Fig. 2A). These data suggest that ouabain enhances cAMP-dependent anion secretion by ADPKD monolayers, involving apical CFTR Cl$^-$ channels.

Ouabain does not affect the expression levels of CFTR or NKCC1 in ADPKD cells. The mechanism for anion secretion in ADPKD cells involves the uptake of Cl$^-$ across the basolateral membrane by NKCC1 and the efflux of Cl$^-$ via the apical CFTR channel (26). To determine whether ouabain stimulation of forskolin-dependent anion secretion is due to the ability of ouabain to regulate the expression of CFTR and NKCC1, ADPKD cells were treated in the absence or presence of ouabain and 8-Br-cAMP, alone or in combination, and the levels of CFTR and NKCC1 mRNA and protein were measured by real-time RT-qPCR and immunoblot. Ouabain, alone or with 8-Br-cAMP, had no effect on NKCC1 mRNA and protein levels of ADPKD cells (Fig. 3, A and B). While expression of transcripts for CFTR was slightly higher in the presence of 8-Br-cAMP alone or with ouabain, this was not statistically significant ($P = 0.31$ for 8-Br-cAMP alone and $P = 0.43$ for 8-Br-cAMP + ouabain compared with untreated controls; Fig. 3C), and addition of ouabain did not affect CFTR protein amounts in the cells (Fig. 3D). Together, these findings indicate that ouabain’s contribution to anion secretion in ADPKD cells does not involve upregulation of expression of CFTR or NKCC1 at the mRNA or protein level.

![Fig. 3. Ouabain does not affect expression of CFTR or Na-K-2Cl$^-$ cotransporter (NKCC1) in ADPKD cells. ADPKD cells were treated with 8-Br-cAMP (100 μM) or ouabain (3 nM), alone or in combination. Real-time quantitative PCR (RT-qPCR) and immunoblot were used to measure expression of NKCC1 (A and B) and CFTR (C and D) mRNA and protein, respectively. Transcript expression was normalized against expression of 18S rRNA for each sample. Protein levels were normalized to corresponding untreated controls. Values are means ± SE of 3 experiments performed in triplicate.](http://ajprenal.physiology.org/)

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Ouabain contributes to ADPKD microcyst development. When cultured in a three-dimensional matrix of polymerized collagen, ADPKD cells form microscopic cysts in the presence of EGF and forskolin (38, 44). These microcysts are lined by a polarized epithelium that secretes fluidlike native ADPKD cysts (50). We have used this in vitro model system to study the effects of ouabain on ADPKD cyst formation. NHK and ADPKD cells cultured within collagen matrices were first stimulated to form microcysts with EGF and forskolin. Once the cysts began to form, the agonists were removed and the cultures were treated without or with 3 nM ouabain and 5 μM forskolin, alone or in combination. Microcysts were allowed to grow for 5–7 days. Then changes in cyst size were quantified and expressed as average total surface area of the microcyst cultures. NHK cells showed little microcyst development in response to forskolin, and ouabain had no effect on NHK microcysts when used alone or with forskolin (Fig. 4A). ADPKD microcysts exhibited a more robust response to forskolin, or both. Microcysts were maintained for 5–7 days after treatment, and average total surface area of the formed microcysts per well was measured. Values are means ± SE of 6 experiments performed in sextuplicate in cells obtained from 6 different ADPKD kidneys. *P < 0.05.

Fig. 4. Ouabain increases forskolin-mediated microcyst growth in ADPKD cells. NHK (A) and ADPKD (B) cells were cultured within a 3-dimensional collagen gel and treated in the absence and presence of 3 nM ouabain, 5 μM forskolin, or both. Microcysts were maintained for 5–7 days after treatment, and average total surface area of the formed microcysts per well was measured. Values are means ± SE of 6 experiments performed in sextuplicate in cells obtained from 6 different ADPKD kidneys. *P < 0.05.

Ouabain exacerbates cAMP-induced cystic progression in metanephric organ cultures. Metanephric organ cultures of Pkd1m1Bei mice represent a useful tool for studying the mechanisms responsible for cystogenesis in an orthologous model of ADPKD. This mouse model carries a mutation in the Pkd1 gene that results in a nonfunctional PC-1 product. Renal tubules in embryonic Pkd1m1Bei mice secrete fluid and become dilated in response to 8-Br-cAMP (24). In the current study, we have used Pkd1m1Bei mice to explore the effects of ouabain on cyst growth in metanephric organs of this ADPKD mouse model. Embryonic kidneys from wild-type (Pkd1+/+), heterozygous (Pkd1+/−), or homozygous null (Pkd1−/−) mice were dissected on embryonic day 15.5 and cultured as previously described (24). Then the metanephroi were treated with 30 nM ouabain in the absence or presence of 100 μM 8-Br-cAMP, and the development of cystic dilations was followed for 4 days, when fractional cyst area was measured. Similar to our results in ADPKD monolayers and microcysts, ouabain alone did not have a significant effect on metanephric tubule dilation and cyst formation in the Pkd1+/−, as well as in the Pkd1+/− and Pkd1−/−, embryonic kidneys (Fig. 5). In agreement with previous work (24), addition of 8-Br-cAMP produced cystic dilations in the metanephroi. These cysts continued to expand during the course of the 4-day treatment of the cultures, becoming more marked in the mutated Pkd1 than wild-type mice (Fig. 5). While ouabain alone had no effect, the concomitant addition of ouabain and 8-Br-cAMP significantly exacerbated fractional cyst area in the metanephroi. This response was greatest in the Pkd1−/− and Pkd1+/− kidneys, reaching a threefold increase in fractional cyst area in the metanephroi from the more severe phenotype (Fig. 5, B, D, and F). Together, these results indicate that, in the presence of 8-Br-cAMP, ouabain stimulated metanephric cyst development, with the most dramatic effects in Pkd1−/− embryonic kidneys. This shows that the effects of ouabain on cyst development are not limited to ADPKD cells in culture but also occur in a system that maintains the architecture of the intact renal tissue.

Ouabain stimulates ADPKD fluid secretion and cyst growth through the EGFR, Src, and MEK pathway. Ouabain exerts its stimulatory effect on ADPKD cell proliferation via the Na-K-ATPase signalosome and its downstream mediators, EGFR, Src, and the MAPK pathway (32, 47). To explore whether the stimulatory effects of ouabain on ADPKD cystogenesis involve the Na-K-ATPase signaling machinery, we tested the effects of inhibitors of EGFR (tyrphostin AG1478), Src (PP2), and MEK (U0126) on ouabain- and forskolin-induced fluid secretion in ADPKD cell monolayers, ADPKD microcyst cultures, and Pkd1m1Bei metanephric organ cultures. The doses of tyrphostin AG1478, PP2, and U0126 were based on those used previously (29). In fluid secretion experiments, ADPKD cell monolayers were grown and treated with the inhibitors mentioned above in the presence of forskolin alone or forskolin with ouabain. Inhibition of EGFR, Src, and MEK, individually, blocked ouabain’s ability to increase forskolin-induced fluid secretion by ADPKD monolayers (Fig. 6A). In three-dimensional collagen microcyst experiments, cultures were treated with forskolin alone or forskolin with ouabain in the absence or presence of tyrphostin AG1478, PP2, or U0126. The inhibitors also prevented ouabain’s enhancement of forskolin-induced
ouabain pathway, involving EGFR, Src, and MEK.

and cyst growth through mediators of the Na-K-ATPase signalosome (29, 30). Here, we show that ouabain stimulates cAMP-dependent fluid secretion effect is most pronounced (Fig. 6C). Together, these results indicate that ouabain favored anion secretion by the cells. In addition, ouabain also affects fluid secretion by the ADPKD epithelium. Ouabain enhanced the vectorial movement of fluid delivery by the cells surrounding the lumen of the microcysts (15). Ouabain also promoted cAMP-dependent tubular dilations in metanephric organ cultures of \(Pkd1^{+/+}\) mice. These findings highlight the importance of ouabain not only as a cell-proliferating agent in ADPKD, but also as a factor that increases cyst volume content and growth. Therefore, ouabain influences two renal epithelial processes that are fundamental mechanisms for ADPKD cyst development.

Exogenous and endogenous ouabain has been shown to elicit a variety of effects on kidney cells and tissues: changes in cell proliferation (19), cell detachment and death (1, 9), regulation of sodium reabsorption (6, 7, 28), and protection of the kidney from the adverse effects of malnutrition and serum deprivation (21, 22). In the current study, we show that ouabain modulates the amplitude of cAMP-dependent fluid secretion and cyst growth of ADPKD microcysts and metanephric organ cultures, providing evidence for a novel effect of ouabain on renal cells. These effects take place at nanomolar concentrations of ouabain, which is an indicator of increased fluid delivery by the cells surrounding the lumen of the microcysts (15).

ADPKD microcyst growth (Fig. 6B). Finally, in metanephric organ cultures, metanephroi were treated with tyrphostin AG1478, PP2, or U0126 in the presence of 8-Br-cAMP alone or combined with ouabain. The EGFR, Src, and MEK inhibitors reduced the ability of ouabain to exacerbate cAMP-stimulated growth of tubular cystic dilations in embryonic kidneys from \(Pkd1^{-/-}\) mice, the phenotype in which ouabain’s effect is most pronounced (Fig. 6C). Together, these results show that ouabain stimulates cAMP-dependent fluid secretion and cyst growth through mediators of the Na-K-ATPase signaling pathway, involving EGFR, Src, and MEK.

**DISCUSSION**

In previous studies, we demonstrated that ouabain stimulates proliferation of ADPKD cells through activation of the Na-K-ATPase signalosome (29, 30). Here, we show that ouabain also affects fluid secretion by the ADPKD epithelium. Ouabain enhanced the vectorial movement of fluid from the basolateral to the apical side of ADPKD monolayers treated with 8-Br-cAMP or forskolin and increased the \(I_{sc}\) response of ADPKD monolayers to forskolin, which indicates that ouabain favored anion secretion by the cells. In addition, ouabain stimulated the forskolin-dependent growth of microcysts of ADPKD cells in three-dimensional collagen cultures, which is an indicator of increased fluid delivery by the cells surrounding the lumen of the microcysts (15). Ouabain also promoted cAMP-dependent tubular dilations in metanephric organ cultures of \(Pkd1^{+/+}\) mice. These findings highlight the importance of ouabain not only as a cell-proliferating agent in ADPKD, but also as a factor that increases cyst volume content and growth. Therefore, ouabain influences two renal epithelial processes that are fundamental mechanisms for ADPKD cyst development.

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While ouabain enhances fluid secretion and microcyst growth in ADPKD cells, it has no significant effect on normal human kidney cells, in the absence or presence of 8-Br-cAMP or forskolin. Similarly, ouabain differentially affects metanephric kidneys, causing only a small increase in fractional cyst area in wild-type metanephrro and a more pronounced, and progressively larger, increase in heterozygous and homozygous 
Pkd1

mutated metanephric kidneys. Therefore, cystic dilations induced by ouabain appear to correlate with the severity of the 
Pkd1

mouse phenotype. In agreement with these data, we previously showed that ouabain differentially impacts the growth of NHK and ADPKD cells (30). We speculate that the difference in the effect of ouabain may depend on the higher affinity of the Na-K-ATPase of ADPKD than NHK cells for ouabain (30). This characteristic property of ADPKD cells may make the cystic cells more susceptible to circulating levels of ouabain, allowing them to respond to ouabain in an exacerbated manner compared with normal cells.

Our previous and current observations suggest that the dual roles of ouabain in the ADPKD epithelium, i.e., cell proliferation and fluid secretion, are affected in different ways. Thus, while ouabain, by itself, enhances cell growth (29, 30), ouabain, together with other cyst-inducing factors, such as cAMP and forskolin, functions as an agonist to stimulate fluid secretion by the ADPKD epithelium. These effects of ouabain, acting alone or as a cofactor, suggest that different thresholds for the actions of ouabain may exist in the pathways or effector mechanisms, leading to cell proliferation and fluid secretion in ADPKD cells. Although further experiments are needed to prove these possibilities, our results show that ouabain has complementary effects on cell proliferation and fluid secretion to promote ADPKD cyst development.

Regarding its mechanisms of action, we have found that ouabain’s contribution to ADPKD fluid secretion and cystogenesis involves essential components of the Na-K-ATPase signaling machinery. In this manner, tyrphostin AG1478, PP2, or U0126 prevents the synergistic effect of ouabain on 8-Br-cAMP- and forskolin-induced fluid secretion and cystogenesis, suggesting that activation of EGFR, Src, and the MAPK pathway is necessary for ouabain’s action. In ADPKD cells, the inhibitory effect of tyrphostin AG1478, PP2 and U0126 was more robust on the growth of microcysts than on fluid secretion. As previously reported, phosphorylation and stimulation of activity of EGFR, Src, and the MAPK are also required for the ouabain-dependent proliferation of ADPKD cells (29). Therefore, the greater inhibition produced by tyrphostin AG1478, PP2, and U0126 in the microcyst cultures could be due to the additional effect of these inhibitors on cell proliferation, which will impact microcyst growth more than fluid secretion. Taken together, our previous (29) and current observations suggest that the proliferative and secretory effects

Fig. 6. Cystogenic effect of ouabain is mediated via the EGFR (EGFR)-Src-MEK pathway. A: monolayers of ADPKD cells were treated with the indicated inhibitors in the presence of forskolin (5 μM) or forskolin with ouabain (3 nM). After 24 h of treatment, apical fluid was collected and measured. Values are means ± SE of 4–7 determinations on samples from cells obtained from 2–4 different ADPKD kidneys. *P < 0.05 vs. forskolin + ouabain. B: ADPKD microcysts treated with the indicated inhibitors in the presence of forskolin (5 μM), with or without ouabain. After 5–7 days of growth, average total surface area of all microcysts per well was determined. Values are means ± SE of 6 determinations in cells obtained from 2 or 3 ADPKD kidneys. *P < 0.05 vs. forskolin + ouabain. C. 
Pkd1

mouse organ cultures were treated with the indicated inhibitors in the presence of 8-Br-cAMP (100 μM), with or without ouabain. At 4 days after treatment, images from the cultures were captured, and cyst surface area was measured for each condition. Values are means ± SE of average fractional cyst area for the different experimental conditions, using 2–3 different kidneys. *P < 0.05 vs. 8-Br-cAMP + ouabain.
of ouabain are mediated through common components of the Na-K-ATPase signalosome. Interestingly, forskolin and cAMP also exert their effects by direct activation of the MAPK pathway (38, 42). Therefore, it is possible that, acting through EGFR and Src, ouabain can provide another route for activation of the MAPK pathway, with ouabain, cAMP, and forskolin merging their effects at the level of the MAPK pathway.

Ouabain and cAMP appear to have common actions on the final regulators of fluid secretion and cyst growth in ADPKD. Fluid secretion induced by cAMP in the ADPKD epithelium is mediated by activation of Cl⁻ transport involving apical CFTR (26). Results from this work show that ouabain enhances the effect of 8-Br-cAMP or forskolin to induce fluid secretion by the human ADPKD cells and that ouabain has a synergistic effect with forskolin to increase Iₑ and apical secretion of Cl⁻ via CFTR, specifically in ADPKD cells. Therefore, the enhancement of forskolin- or 8-Br-cAMP-dependent fluid secretion and of ouabain-induced cyst growth may be due to its combined effects with CAMP on CFTR. The additive effect of ouabain on apical anion secretion is not dependent on changes in expression of CFTR or NKCC1 but, rather, on activation of CFTR. This ability of ouabain to directly or indirectly influence the function of other ion transport systems of the cell is supported by previous work, which showed that Na-K-ATPase-mediated ouabain signaling regulates the Na⁺/H⁺ exchanger (NHE3) at the apical membrane of pig kidney epithelial cell monolayers (7, 49). While additional studies in our laboratory will be directed to gain a more complete understanding of the mechanisms governing the ouabain-dependent increase in transepithelial apical fluid transport in ADPKD, our findings indicate that physiological concentrations of ouabain may exacerbate fluid secretion by the cystic epithelial cells in ADPKD, contributing to the overall expansion of renal cysts.

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

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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