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Overexpression of the Polycystin-1 C-Tail Enhances Sensitivity of M-1 Cells to Ouabain

Kyle Jansson · Brenda S. Magenheimer · Robin L. Maser · James P. Calvet · Gustavo Blanco

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Abstract Cells derived from renal cysts of patients with autosomal dominant polycystic kidney disease (ADPKD) are abnormally sensitive to ouabain, responding to physiological ouabain concentrations with enhanced proliferation and increased forskolin-induced transepithelial fluid secretion. This requires activation of the epidermal growth factor receptor (EGFR), Src kinase and the extracellular signal-regulated kinases MEK and ERK. Here, we have determined if the ADPKD phenotype obtained in mouse cortical collecting duct cells by stable overexpression of the C-terminal domain of polycystin-1 (PC-1 C-tail) also elicits the ADPKD-like response to ouabain in the cells. M-1 C20 cells expressing the PC-1 C-tail and M-1 C17 cells lacking expression of this construct were treated with physiological concentrations of ouabain, and cell proliferation, activation of the EGFR-Src-MEK-ERK pathway, forskolin-induced transepithelial Cl^- secretion and the sensitivity of Na,K-ATPase to ouabain were explored. M-1 C20 cells responded to ouabain with increased cell proliferation and ERK phosphorylation. Ouabain also augmented forskolin-induced and cystic fibrosis transmembrane conductance regulator-mediated apical secretion of Cl^- in M-1 C20 cells. These effects required activation of EGFR, Src and MEK. In contrast, ouabain had no significant effects on M-1 C17 cells. Interestingly, approximately 20 % of the Na,K-ATPase from M-1 C20 cells presented an abnormally increased sensitivity to ouabain. Overexpression of PC-1 C-tail in M-1 C20 cells is associated with an ouabain-sensitive phenotype and an increased ability of the cells to proliferate and secrete anions upon ouabain stimulation. This phenotype mimics the ouabain sensitivity of ADPKD cells and may help promote their cystogenic potential.

Keywords Na,K-ATPase · Ouabain signaling · Cystic epithelia · Polycystic kidney disease · Polycystin-1

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K. Jansson · G. Blanco (✉)
Department of Molecular and Integrative Physiology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160, USA
e-mail: gblanco@kumc.edu

K. Jansson · B. S. Magenheimer · R. L. Maser · J. P. Calvet · G. Blanco
The Kidney Institute, University of Kansas Medical Center, Kansas City, KS, USA

B. S. Magenheimer · J. P. Calvet
Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS, USA

R. L. Maser
Department of Clinical Laboratory Sciences, University of Kansas Medical Center, Kansas City, KS, USA

Introduction

Ouabain is a steroidal hormone of mammals that is produced primarily in the adrenal glands and released to the circulation in nanomolar concentrations (Schoner 2002; Schoner and Scheiner-Bobis 2005). Ouabain has been shown to exert a variety of effects that are cell-specific, including increases in cell proliferation and apoptosis and changes in cell motility and metabolism (Allen et al. 2003; Chueh et al. 2001; Kometiani et al. 1998; Mohammadi et al. 2003). The effects of ouabain depend on its binding to Na,K-ATPase, a protein complex expressed in the plasma membrane of most animal cells. Classically, Na,K-ATPase is known for its ion transport functions and its ability to use the energy from ATP

hydrolysis to catalyze the exchange of intracellular Na^+ for extracellular K^+. This action of Na,K-ATPase is essential to preserve cell volume and osmolarity, maintain plasma membrane potential and allow the cotransport of ions, glucose and amino acids across the cell plasma membrane. In addition, Na,K-ATPase functions as the receptor for ouabain and as a transducer that activates intracellular signaling cascades. Ouabain–Na,K-ATPase binding initiates a series of molecular events that start with activation of the kinase Src, leading to the downstream phosphorylation of a series of intracellular pathways (Liang et al. 2007; Pierre and Xie 2006; Wang et al. 2004; Xie and Cai 2003).

Previous work in our laboratory has shown that ouabain has effects in autosomal dominant polycystic kidney disease (ADPKD). ADPKD is the most common, potentially lethal, inherited disorder of the kidney, affecting from 1 in 500 to 1 in 1,000 births worldwide (Grantham 2008). While clinical manifestations of the disease are systemic, the most prominent characteristic of ADPKD is the formation and development of numerous renal cysts (Grantham 1997). The progression and severity of ADPKD are variable; however, gradual growth of renal cysts eventually compromises renal architecture and function, with approximately half of affected individuals developing end-stage renal failure by the age of 60 (Grantham 2008). Although ADPKD is caused by mutations in either the *Pkd1* or the *Pkd2* gene, which encode for polycystin-1 (PC-1) or PC-2, respectively (Torres and Harris 2007), growth of renal cysts is highly influenced by circulating factors and exogenous pharmacological agents. Thus, arginine vasopressin, epidermal growth factor, forskolin and caffeine have been reported to exacerbate renal cyst growth via increases in cAMP in the cells (Wallace 2011). We have found that the Na,K-ATPase of human ADPKD cell primary cultures derived from the cyst-lining epithelium of kidneys with ADPKD has an abnormally high affinity for ouabain (Nguyen et al. 2007, 2011). In addition, we have shown that ouabain stimulates proliferation and contributes to enhancing fluid secretion of ADPKD monolayers and the growth of ADPKD microcyst cultures (Jansson et al. 2012; Nguyen et al. 2007, 2011). Increased cell proliferation of the cystic epithelium combined with fluid secretion into the growing cyst lumen are hallmark events required for ADPKD cystogenesis (Grantham et al. 1989). In this manner, ouabain is a circulating factor with the ability to promote ADPKD cyst formation and growth. The effects of ouabain in ADPKD are mediated through the Na,K-ATPase signaling apparatus, which involves the epidermal growth factor receptor (EGFR), Src kinase and the mitogen activated kinase (MAPK) pathway.

A great amount of work has been devoted to understanding the pathophysiology of ADPKD; however, the relationship between the defects in the polycystins and the cystic phenotype remains unclear. Different in vitro models and mouse models have been generated in which deletion, mutation and overexpression of the *Pkd1* gene have been performed. These have provided evidence connecting PC-1 function to known signaling pathways (Torres and Harris 2009). We have utilized a mouse cortical collecting duct cell model, in which inducible overexpression of the C-terminal cytosolic domain of PC-1 confers the cells with an ADPKD proliferative phenotype in response to cAMP. This behavior, typical of ADPKD cells, is not observed in normal kidney cells or in collecting duct cells lacking PC-1 C-tail overexpression, which respond to cAMP by decreasing cell growth (Sutters et al. 2001). The effect of PC-1 C-tail overexpression can be explained by a dominant-negative loss-of-function mechanism that causes disruption of the normal polycystin signaling pathway in the cells (Sutters et al. 2001).

In this study, we examined whether the ADPKD phenotype of these M-1 cortical collecting duct cells, overexpressing the PC-1 C-tail, affects the response of the cells to ouabain. We report that acquisition of the cystic phenotype by PC-1 C-tail overexpression increases the affinity of M-1 cortical collecting duct cells to ouabain, induces ouabain-stimulated intracellular signaling events in these cells and stimulates their ability to proliferate and secrete anions, all features typical of ADPKD cells.

Methods

Cell Culture

Two clonal cell lines, clone 17 (M-1 C17) and clone 20 (M-1 C20), which we previously developed (Sutters et al. 2001), were used in this study. These cell lines were prepared from mouse cortical collecting duct cells (M-1), after stable transfection with a dexamethasone-inducible construct containing the membrane-targeted C-tail fragment of PC-1. M-1 C20 cells express the PC-1 C-tail upon induction with dexamethasone, while M-1 C17 cells fail to express the PC-1 construct and, therefore, served as a negative control (Sutters et al. 2001). Cells were cultured in DME/F12 supplemented with penicillin/streptomycin and 5 % heat-inactivated fetal bovine serum (FBS) as described (Sutters et al. 2001). For induction of PC-1 C-tail expression, cells were treated with 1 μM dexamethasone and cultures were used 48 h later. Ouabain treatment was performed using different concentrations of ouabain on the induced cells after 24 h starvation in medium containing 0.002 % FBS and dexamethasone.

Na,K-ATPase Activity Assays

Na,K-ATPase activity was determined by measuring the initial rate of release of $^{32}P_i$ from $\gamma[^{32}P]$-ATP in

homogenates from M-1 C17 and M-1 C20 cells as previously described (Nguyen et al. 2007). Briefly, samples were incubated at 37 °C for 30 min in a final volume of 0.25 ml of medium containing 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 2 mM sodium azide and 30 mM Tris–HCl (pH 7.4) in the absence or presence of the indicated concentrations of ouabain and 3 mM ATP with 0.2 μCi γ[^{32}P]ATP. Specific hydrolysis of ATP, dependent on Na,K-ATPase, was determined as that sensitive to 1 mM ouabain. Curve fitting of the data for ouabain inhibition of Na,K-ATPase activity was performed using Marquardt least-squares nonlinear regression as previously described (Nguyen et al. 2007).

RT-PCR Analysis

Total RNA from M-1 C17 and M-1 C20 cells was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA was generated by reverse transcription of total RNA using SuperScript II reverse transcriptase (Invitrogen, Grand Island, NY) and oligo-dT primers (Invitrogen). Primers specific to the various α isoforms of mouse Na,K-ATPase were used to amplify segments of the corresponding cDNAs. These primers contained the following sense (S) and antisense (AS) sequences: α1, 5′-GG GGTTGGACGAGACAAGTAT-3′ (S) and 5′-CGGCTCA AATCTGTTCCGTAT (AS); α2, 5′-CCACCACTGCGGA AAATGG-3′ (S) and 5′-GCCCTTAGACAGATCCACTT GG-3′ (AS); α3 5′-GAAGAGGTCTGCCGGAAATAC-3′ (S) and 5′-GGTGGTGTGAGGGCGTTAG (AS); and α4, 5′-CAGTGGCGCATTTGTGGTTT-3′ (S) and 5′-CAGCG ATTCGAGCCAGGTAAA-3′ (AS). Amplification products were resolved on a 1 % agarose gel containing ethidium bromide.

Immunoblot Analysis

After the different treatment conditions, M-1 C17 and M-1 C20 cells were washed twice in ice–cold PBS and lysed in a solution containing 1 % NP-40, 0.25 % sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM NaF, 150 mM NaCl, 1 mM EDTA, 50 mM Tris (pH 7.4) and 1× protease inhibitor cocktail (Roche, Indianapolis, IN) (lysis buffer). Total protein content in the cleared lysates was determined using the dye-binding assay from Bio-Rad (Hercules, CA). Equal amounts of protein were resolved by SDS-PAGE (10 % gel) and blotted onto nitrocellulose membranes as previously described (Nguyen et al. 2007). Membranes were then probed for expression of the proteins of interest using antibodies specific to the different Na,K-ATPase α isoforms. The C46B/CB antibody (1:100), provided by M. Caplan (Yale University), was used to detect α1. For the α2 isoform, MCB2 antibody (1:100), provided by K. Sweadner (Massachusetts General Hospital), was applied. The α3 polypeptide was identified with MA3-915 antibody (1:500) from Affinity Bioreagents (Golden, CO). For α4, a monoclonal antibody (1:100), prepared at Covance Immunology Services (Denver, CO), was used (Sanchez et al. 2006). Horseradish peroxidase-conjugated secondary antibodies and chemiluminescence were used for detection.

Cell Proliferation Assay

The CellTiter 96 MTT Assay (Promega, Madison WI) was used to determine cell proliferation as previously described (Nguyen et al. 2011). This assay provides adequate estimates of cell proliferation, as validated through comparisons with direct counting of the cells. M-1 C17 and M-1 C20 cells (2×10^3 cells/well) were seeded onto a 96-well plate in culture medium supplemented with 1 % FBS and 1 μM dexamethasone for 24 h. Cells were starved overnight in culture medium containing 0.002 % FBS and 1 μM dexamethasone and then treated with ouabain, for 24 h. In some experiments, cells were also pretreated for 30 min without and with 2 μM tyrphostin AG1478, 10 μM PP2 or 1 μM U0126 to inhibit EGFR, Src or the mitogen extracellular kinase (MEK), respectively. Following pretreatment with inhibitors, cells were treated without and with ouabain for 24 h while maintaining the presence of the indicated inhibitors. Absorbance of each sample was determined using a multiwell plate reader. Data were expressed relative to the proliferation levels in the absence of ouabain.

Analysis of p-ERK Expression

M-1 C17 and M-1 C20 cells were seeded in six-well plates and treated in the absence or presence of different concentrations of ouabain for 30 min. In some experiments, cells were also pretreated without and with 2 μM tyrphostin AG1478, 10 μM PP2 or 1 μM U0126 prior to ouabain treatment. Following this, cells were washed twice with ice–cold PBS and lysed in lysis buffer supplemented with 1× phosphatase inhibitor cocktail (Roche). Samples were centrifuged at 10,000 rpm for 15 min. Equal amounts of total protein (5–10 μg) from the cleared lysates were subjected to SDS-PAGE (10 % gel) and blotted onto nitrocellulose membranes. Immunoblots were first probed using an antibody against the phosphorylated form of the extracellular signal-regulated kinase, ERK1/2 (p-ERK). Then, membranes were stripped as described previously and reprobed with an antibody against ERK1/2 (total ERK). Both anti-p-ERK and anti-total ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies and

chemiluminescence were used for detection. Following detection of bands by chemiluminescence, images were scanned and phosphorylated and total forms of each protein were quantified for band intensity using Gel-Pro software (Media Cybernetics, Silver Springs, MD). The ratios of p-ERK to total ERK levels were determined, and values were expressed as density units relative to the untreated controls.

Short Circuit Current Measurements

M-1 C17 and M-1 C20 cell monolayers were grown to confluence on 12-mm permeable Snapwell^{TM} inserts (Corning, Corning, NY) as previously described (Jansson et al. 2012). Following addition of dexamethasone and overnight starvation, monolayers were treated with or without 30 nM ouabain for 24 h. To study the involvement of Src, 10 μM PP2 was added to the cell monolayers 30 min before treatment with ouabain. At 24 h, cell monolayers were mounted in Ussing chambers and short circuit currents (I_{sc}) were measured using a dual voltage-clamp device (Warner Instruments, Hamden, CT) as previously described (Jansson et al. 2012). After short circuit currents were stabilized, benzamil (10 μM) was added to the apical side of the cells to block sodium reabsorption and ensure that any increase in short circuit current was due to apical anion secretion. Forskolin (5 μM) was added basolaterally to stimulate I_{sc}, and the forskolin response was calculated as a difference from baseline current, as previously described (Jansson et al. 2012). The cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor CFTR(inh)-172, at a concentration of 10 μM (Sigma-Aldrich, St. Louis, MO), was added apically to determine the involvement of CFTR activation in the observed I_{sc}. Data were expressed as percentage of the maximum response to forskolin in the untreated monolayers.

Data Analysis

Statistical significance was determined by ANOVA for multiple comparisons or Student's t test for individual comparisons. Statistical significance was defined as $p < 0.05$.

Results

Overexpression of PC-1 C-Tail Enhances Ouabain Proliferative Effects of M1 Cells

Previous work in our laboratory had shown that human ADPKD epithelial cell cultures derived from the cystic epithelium of ADPKD kidneys respond to nanomolar concentrations of ouabain by increasing cell proliferation. In contrast, human normal kidney cell growth is not significantly affected by ouabain (Nguyen et al. 2007, 2011). It has been shown that overexpression of the C-tail portion of PC-1 in M-1 cortical collecting duct cells provides these cells with changes in their proliferative response to agonists that increase intracellular cAMP levels, reminiscent of the behavior observed in ADPKD cystic cells (Sutters et al. 2001). We investigated if M-1 cells expressing the C-tail of PC-1 also responded to ouabain with exacerbated cell growth. For this, we treated M-1 C20 and M-1 C17 cells with dexamethasone and then incubated them in the absence or presence of various concentrations of ouabain for 24 h. While both M-1 C20 and M-1 C17 cells were transfected with a dexamethasone-inducible PC-1 C-tail construct, only the M-1 C20 clone expresses the PC-1 construct after induction. The lack of PC-1 C-tail expression in the M-1 C17 clone makes these cells an adequate control for studying the effects of the PC-1 construct. This property of the M-1 clones has been previously described (Sutters et al. 2001) and was confirmed in the present study (see Online Resource 1). Following ouabain treatment, cell proliferation was measured after 24 h. As shown in Fig. 1, ouabain stimulated proliferation of M-1 C20 cells in a dose-dependent manner. The maximal proliferative effect of ouabain was observed at concentrations between 3×10^{-9} and 10^{-7} M. At doses above 10^{-7} M, cell growth was progressively inhibited, agreeing with the notion that relatively high amounts of ouabain are toxic for the cells. In contrast to M-1 C20 cells, ouabain did not significantly affect proliferation of M-1 C17 cells. These results demonstrate that overexpression of the C-terminal portion of PC-1 confers an ouabain-dependent proliferative phenotype to M-1 cells.

M-1 C20 Cells Respond to Ouabain by Activating the ERK1/2 Pathway

ADPKD cells have been shown to respond to nanomolar concentrations of ouabain by activating the Na,K-ATPase signaling apparatus (Nguyen et al. 2011). One of the main downstream intracellular intermediates of this signaling system is the kinase ERK1/2. To determine whether ouabain utilizes signaling pathways in M-1 C20 cells common to those of ADPKD cells, we studied the ouabain-dependent phosphorylation of ERK1/2 in M-1 C20 and M-1 C17 cells. After induction of PC-1 C-tail expression with dexamethasone, cells were treated with different amounts of ouabain for 30 min and the total and phosphorylated forms of ERK1/2 were analyzed by immunoblot using lysates from the cells. As shown in Fig. 2a, the levels of phosphorylated ERK1/2 were not significantly changed by ouabain in M-1 C17 cells. In contrast, ouabain caused a

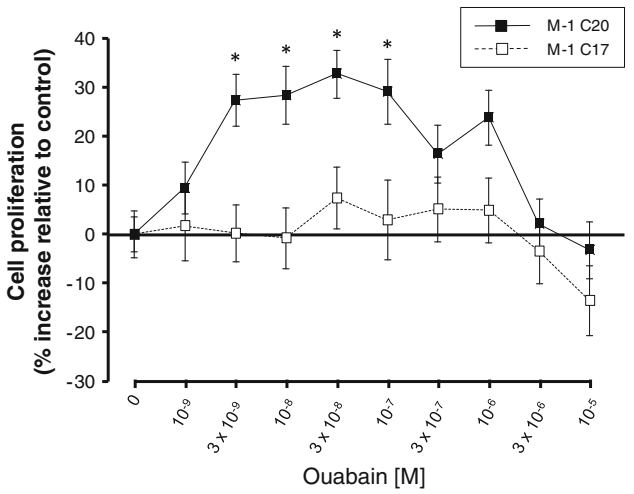

Fig. 1 Ouabain increases proliferation of M-1 C20 cells. Cells were plated in 96-well culture dishes. After 24 h in culture, cells were treated with 1 μM dexamethasone for 48 h. Following overnight starvation in medium with 0.002 % FBS, the indicated concentrations of ouabain were added for 24 h. Proliferation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

dose-dependent increase in ERK1/2 phosphorylation in M-1 C20 cells, with a maximal level of phosphorylation induced at 3×10^{-8} M ouabain (Fig. 2b). These results show that ouabain exerts effects on cells expressing the PC-1 C-tail by activating the ERK1/2 pathway.

Ouabain Activates the EGFR-Src-MEK-ERK Pathway in M-1 C20 Cells

Important intermediates upstream of ERK1/2 in the ouabain-induced, Na,K-ATPase-mediated pathway of ADPKD cells include Src, EGFR and MEK. To investigate whether this signaling pathway is activated by ouabain in M-1 C20 cells induced to express the PC-1 C-tail, we studied the phosphorylation of ERK1/2 as a downstream indicator of ouabain signaling in the absence and presence of EGFR, Src and MEK inhibitors. M-1 C20 cells were treated with and without 3×10^{-8} M ouabain in the absence or presence of tyrphostin AG1478, PP2 and U0126; and 30 min later, ERK phosphorylation was determined. The inhibitors were used at concentrations that have been shown to be optimal for blocking EGFR, Src and MEK activation of ADPKD cells (Nguyen et al. 2011). Figure 3a shows that ouabain-dependent phosphorylation of ERK1/2 in M-1 C20 cells is prevented by inhibition of EGFR, Src and MEK.

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

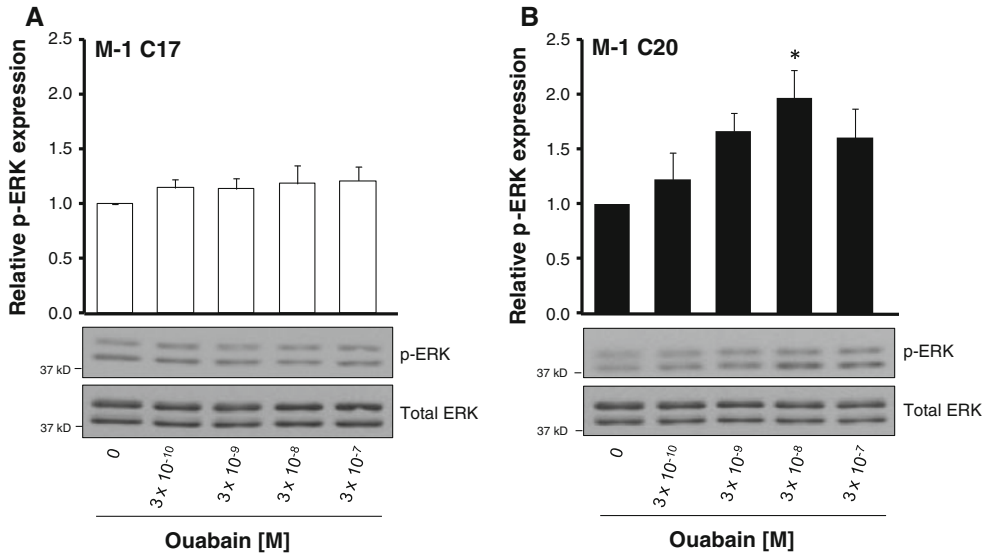

Fig. 2 Ouabain increases ERK1/2 phosphorylation in M-1 C20 cells. **a** M1-C17 and **b** M-1 C20 cells (1×10^5) were plated in six-well culture dishes. After 24 h, expression of PC-1 C-tail was induced with 1 μM dexamethasone for 48 h. Following overnight starvation in medium supplemented with 0.002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

ouabain-dependent activation of the EGFR-Src-MEK-ERK signaling pathway is necessary to cause cell proliferation.

Ouabain Enhances Forskolin-Dependent Anion Secretion in M-1 C20 Monolayers

Previous results indicated that overexpression of the C-terminal domain of PC-1 alters the ion transport properties of M-1 C20 cells, enhancing the ATP-stimulated apical Cl^- conductance of the cells (Hooper et al. 2003; Wildman et al. 2003). Measurement of the short circuit current, I_{sc}, has been used extensively as an indicator of anion fluid secretion in cystic cell monolayers (Grantham et al. 1989; Jansson et al. 2012; Mangoo-Karim et al. 1995; Wallace et al. 1996). We previously used this method and showed that ouabain augments a forskolin-induced increase of short circuit current in ADPKD monolayers (Jansson et al. 2012). Here, we determined if expression of the PC-1 C-tail is able to endow the cells with the ability to modify their ion transport properties in response to ouabain. For this, we studied the effect of ouabain on forskolin-induced anion secretion of M-1 C20 and M-1 C17 cells. Cells were grown on permeable filter supports until a tight monolayer was formed. Following dexamethasone treatment and overnight starvation in 0.002 % FBS, monolayers were treated with and without 3×10^{-8} M ouabain for 24 h and short circuit currents were measured. Ouabain had no significant effect on the forskolin-induced I_{sc} of M-1 C17 cells (Fig. 4a, c).In contrast, ouabain treatment of M-1 C20 monolayers resulted in an approximately 40 % augmentation of the forskolin-induced increase in I_{sc} (Fig. 4b, d). The forskolin-induced increase in I_{sc} of M-1 C20 cells was inhibited by addition of CFTR(inh)-172, a specific inhibitor of CFTR. To assess whether the effect of ouabain on forskolin-induced anion secretion was mediated via the Na,K-ATPase signaling cascade, we tested the sensitivity of the ouabain-dependent increase of short circuit currents to the Src kinase inhibitor PP2. PP2 prevented the ouabain-dependent increases in forskolin-stimulated anion secretion of M-1 C20 cells. In contrast PP2 did not significantly affect the basal I_{sc} in the absence of ouabain, indicating that this inhibitor specifically interfered with ouabain–Na,K-ATPase signaling (Fig. 4b, d). Altogether, these data demonstrate that ouabain is able to enhance forskolin-induced, CFTR-dependent I_{sc} in cells overexpressing the C-tail of PC-1. This effect is mediated by activation of the intracellular tyrosine kinase Src.

Overexpression of the PC-1 C-Tail Increases the Ouabain Affinity of M1 Cells

In ADPKD cells, a fraction of Na,K-ATPase has an abnormally high sensitivity to ouabain. This is indicated by a biphasic response to ouabain inhibition, with approximately 20 % of the Na,K-ATPase of ADPKD cells presenting a ouabain inhibition constant in the nanomolar

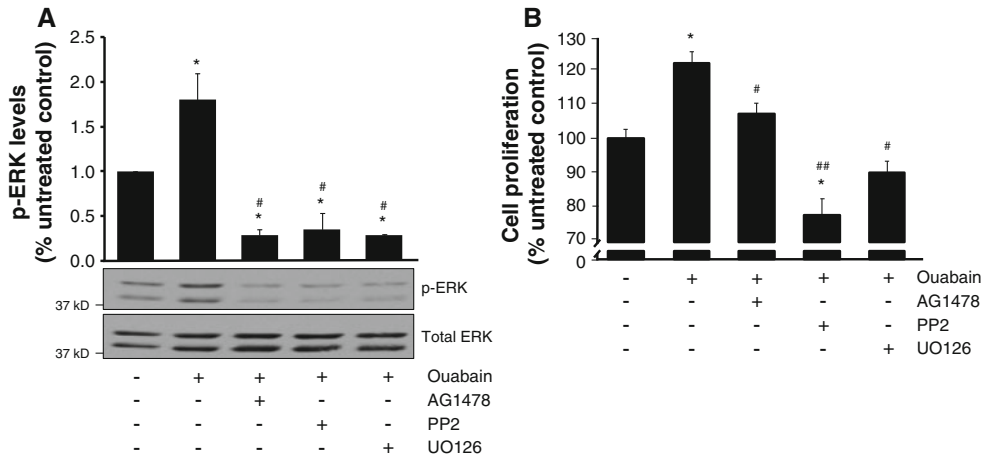

Fig. 3 Ouabain increases ERK1/2 phosphorylation and proliferation of M-1 C20 cells via the EGFR-Src-MEK pathway. **a** Dependence of ERK phosphorylation on the EGFR-Src-MEK pathway. Expression of PC-1 in M-1 C20 cells was induced with 1 μM dexamethasone for 48 h, and then, after overnight starvation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

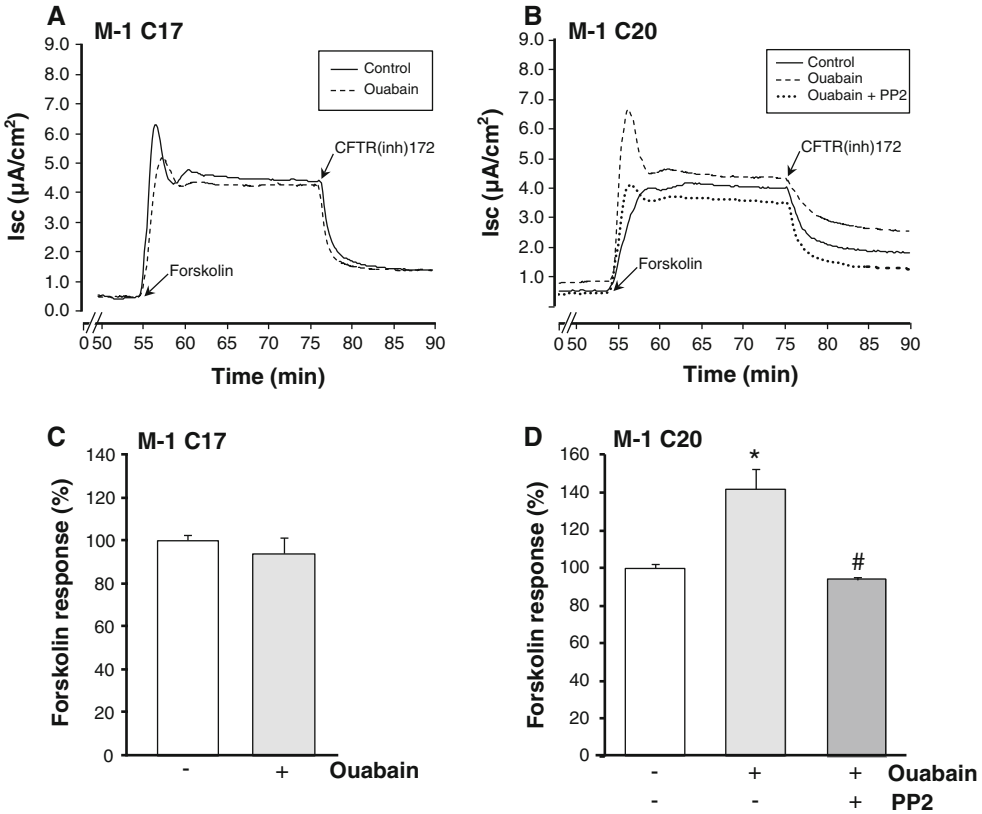

Fig. 4 Ouabain augments forskolin-induced increases in short circuit current of M-1 C20 cell monolayers. Confluent cell cultures were grown on permeable filter supports, and PC-1 C-tail expression was induced with 1 μM dexamethasone for 48 h. After overnight starvation, cells were treated with and without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

range. The remaining Na,K-ATPase activity of ADPKD cells retains a relatively lower sensitivity to ouabain inhibition, which is characteristic of normal kidney cells (Nguyen et al. 2007). We determined whether overexpression of the PC-1 C-tail in cortical collecting duct cells, which confers an ADPKD phenotype, was able to affect the sensitivity of these cells to ouabain. For this, the ouabain inhibition profile of Na,K-ATPase activity was tested on homogenates of M-1 C20 cells after induction of overexpression of the PC-1 C-tail with dexamethasone. As a control, M-1 C17 cells, which do not express the PC-1 construct, were used. As shown in Fig. 5a, Na,K-ATPase from M-1 C20 cells exhibited a bimodal dose–response curve to ouabain, presenting components with high and low sensitivity to ouabain. In contrast, M-1 C17 cells only presented the Na,K-ATPase fraction with low sensitivity to ouabain, which is typical for normal rodent kidney cells (Blanco and Mercer 1998). The calculated inhibition constant (K_i) values for ouabain inhibition of Na,K-ATPase activity and the relative amounts of each Na,K-ATPase population are depicted in Table 1. As shown, approximately three-fourths of the Na,K-ATPase of M-1 C20 cells had a K_i for ouabain in the millimolar range, similar to that of M-1 C17 cells. The remaining one-fourth of the Na,K-ATPase of M-1 C20 cells had a K_i for ouabain in the nanomolar range. These results indicate that a portion of the Na,K-ATPase of M-1 C20 cells exhibits an abnormally increased sensitivity to ouabain, which is not found in the M-1 C17 cells not expressing the PC-1 C-tail.

One of the mechanisms that dictates the response of Na,K-ATPase to ouabain is the presence of different isoforms of its catalytic α-subunit. Four different Na,K-ATPase α polypeptides (α1, α2, α3 and α4) are expressed in mammalian cells. These isoforms are characterized by distinct kinetic properties, among which is their particular response to ouabain (Blanco and Mercer 1998). Normally, renal epithelial cells largely express the α1 isoform of Na,K-ATPase, which is relatively insensitive to ouabain.

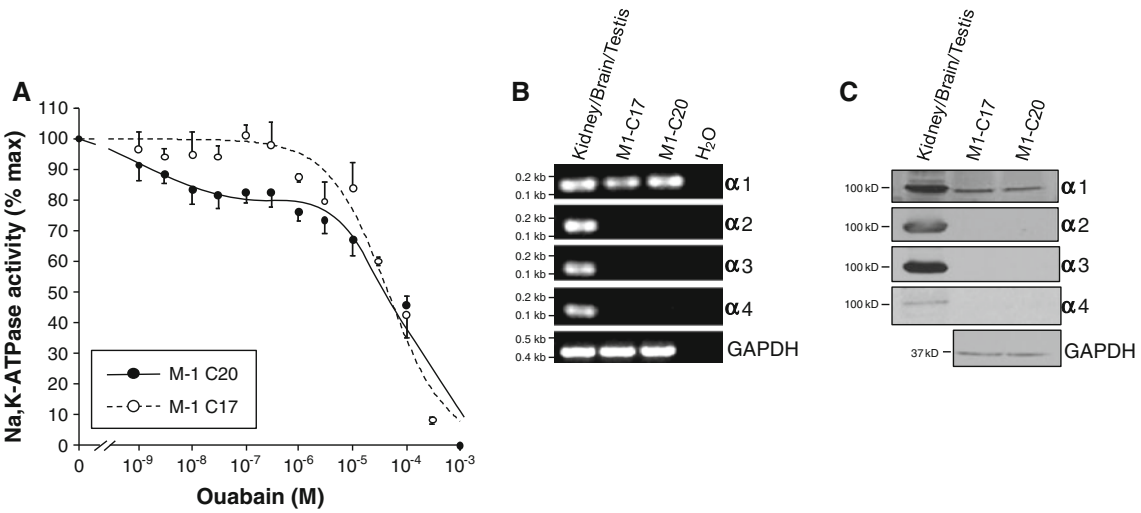

Fig. 5 Na,K-ATPase from M-1 C20 cells exhibits increased sensitivity to ouabain, despite a normal expression pattern of Na,K-ATPase α isoforms. **a** Dose–response curves for the inhibition of Na,K-ATPase by ouabain. Cells were treated with 1 μM dexamethasone for 48 h, harvested and homogenized. Na,K-ATPase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

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

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

The presence of this population of Na,K-ATPase with high sensitivity to ouabain suggests that M-1 C20 cells may be misexpressing one of the Na,K-ATPase isoforms that has a high affinity for ouabain. To investigate this, expression of the different Na,K-ATPase α isoforms was determined at the mRNA and protein levels. As shown in Fig. 5b, c, both M-1 C20 and M-1 C17 cells only expressed the α1 isoform of Na,K-ATPase. This demonstrates that the increased ouabain sensitivity observed in M-1 C20 cells is not due to an altered expression of α isoforms of the Na,K-ATPase.

Discussion

Results from our studies show that overexpression of a PC-1 C-tail construct in M-1 cells induces a phenotypic change with respect to the affinity and response of the cells to ouabain. This was reflected by increased ouabain-dependent cell proliferation and enhancement of forskolin-induced transepithelial anion secretion, as well as an augmented sensitivity of Na,K-ATPase activity to ouabain. Overexpression of the C-terminal domain of PC-1 has been shown to confer M-1 cells with phenotypic characteristics of ADPKD cells, likely through a dominant-negative mechanism (Sutters et al. 2001). In particular, M-1 cells expressing an excess of the PC-1 cytosolic segment exhibit aberrant cell proliferation and enhanced transepithelial Cl^- secretion (Hooper et al. 2003; Sutters et al. 2001). Both of these characteristics are typical of the ADPKD phenotype. Our present data suggest that increased ouabain sensitivity is another distinctive property of these cells. This ouabain-sensitive phenotype mimics our previous findings in human ADPKD cells and allows ouabain to stimulate both cell proliferation and apical anion secretion, which are two essential components of ADPKD cystogenesis (Jansson et al. 2012; Nguyen et al. 2007, 2011). Moreover, these effects take place at ouabain concentrations that are similar to those normally circulating in plasma. Therefore, our

current findings further support the importance of ouabain as a cystogenic factor in ADPKD. Additionally, the ouabain-responsive phenotype makes the cells more prone to respond to ouabain and may be relevant to the progression of renal ADPKD cysts.

In M-1 cells expressing a PC-1 C-tail construct, ouabain was able to induce cell proliferation through activation of the Na,K-ATPase signaling pathway. Similar to ADPKD cells, the effects of ouabain on M-1 C20 cell proliferation are abolished by tyrphostin AG1478, PP2 and U0126, suggesting that the mechanism of action of ouabain in M-1 C20 cells requires activation of EGFR, Src, MEK and ERK1/2. Therefore, ouabain-induced signaling in M-1 C20 cells is mediated through components that are common to those of the Na,K-ATPase signalosome of other cells. In addition, these intracellular messengers constitute important mediators of the cystic ADPKD phenotype in response to cAMP (Wallace 2011). Moreover, aberrant overexpression of the C-terminal cytosolic tail of PC-1 has been implicated in dysregulation of several signaling pathways, including the response of the MAPK pathway to cAMP stimulation (Sutters et al. 2001). In this manner, it is possible that ouabain, acting independently through EGFR-Src-MEK-ERK, converges on the same, already hyperactive, cAMP-stimulated pathway of cystic cells.

While previous work has shown that overexpression of the PC-1 C-tail enhances ATP-stimulated Cl^- secretion, our work is the first to investigate the effect of the C-tail construct on forskolin-induced anion secretion. In our studies, forskolin-stimulated increases in I_{sc} were comparable between M-1 C17 and M-1 C20 cells; however, ouabain treatment enhanced the forskolin-induced anion secretion only in M-1 C20 cells. Additionally, the ouabain enhanced, forskolin-stimulated I_{sc} resulting from PC-1 C-tail overexpression in M-1 C20 cells was sensitive to the specific CFTR inhibitor CFTR(inh)-172. This indicates that the acquired cystic phenotype of the cells follows molecular mechanisms for anion secretion that are similar to those described in native ADPKD epithelium. However, in contrast to our studies in human ADPKD cells (Jansson et al. 2012), inhibition of CFTR did not completely abolish the ouabain-dependent and forskolin-induced Cl^- current in these monolayers. The small remaining I_{sc} fraction, resistant to CFTR(inh)-172, suggests that M-1 C20 cells have an additional pathway for the apical movement of Cl^- that is independent from CFTR. On the other hand, the similar degree of I_{sc} inhibition achieved by CFTR(inh)-172 in M-1 C20 monolayers treated with and without ouabain suggests that the component of the forskolin-induced increase in I_{sc} that was augmented by ouabain treatment is due to specific activation of the CFTR Cl^- channel. The dependence of I_{sc} on the activity of CFTR agrees with that previously reported in ADPKD cells and supports the role of ouabain in the regulation of ion transport systems of the cell plasma membrane (Yan et al. 2012). The ouabain-enhanced, forskolin-stimulated Cl^- current in the M-1 C20 cells overexpressing the PC-1 C-tail was blocked by PP2. This suggests that both proliferation and Cl^- secretion induced by ouabain in M-1 C20 cells involve Src, a key component of the Na,K-ATPase signaling machinery.

At present, the links between abnormalities in expression of PC-1 and the final effectors involved in ADPKD cystogenesis are not clear. The exacerbated ouabain-responsive phenotype resulting from overexpression of the PC-1 C-tail in M1 cells is also poorly understood. However, we found that M-1 C20 cells have a population of Na,K-ATPase that has increased sensitivity to ouabain. Our observation that M-1 C20 cells, similar to M-1 C17 cells, only express the α1 isoform of Na,K-ATPase indicates that the molecular basis for the change in ouabain sensitivity is not based on misexpression of one of the ouabain-sensitive α isoforms of Na,K-ATPase. Similar results were obtained in ADPKD cells, which express the Na,K-ATPase α1 subunit that is normally found in kidney epithelial cells. Alternatively, the increase in ouabain affinity may depend on the interaction of Na,K-ATPase with other proteins. Through the use of the yeast two-hybrid system, the cytosolic tail of PC-1 has been shown to interact with the Na,K-ATPase (Zatti et al. 2005). We previously found that exogenous coexpression of the transmembrane and C-tail domains of PC-1 and Na,K-ATPase in Sf-9 insect cells augments the sensitivity of Na,K-ATPase to ouabain (Nguyen et al. 2007). Currently, additional experiments are being conducted to confirm this possibility.

In conclusion, we show that overexpression of a membrane-anchored C-tail domain of PC-1 induces an ouabain-responsive phenotype in mouse cortical collecting duct cells. The similarity of the ouabain effects in M-1 C20 cells with those of primary ADPKD cells is important since M-1 C20 cells represent a valuable system to study the role and mechanisms of action of ouabain in a cell model for ADPKD in which PC-1 function, specifically, has been rendered abnormal.

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Conflicts of interest No conflicts of interest, financial or otherwise, related with this work are declared by the authors.

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