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Cyclic AMP promotes growth and secretion in human polycystic kidney epithelial cells[1]

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Background. Progressive cyst enlargement, the hallmark of autosomal-dominant polycystic kidney disease (ADPKD) and autosomal-recessive (ARPKD) polycystic kidney disease, precedes the eventual decline of function in these conditions. The expansion of individual cysts in ADPKD is determined to a major extent by mural epithelial cell proliferation and transepithelial fluid secretion. This study determined if common receptor-mediated agonists and an anonymous lipid stimulate the production of 3′ 5′-cyclic monophosphate (cAMP) in mural epithelial cells from the two major types of human cystic diseases.

Methods. cAMP responses to maximally effective concentrations of renal agonists were determined together with measurements of transepithelial anion current and cellular proliferation and extracellular signal-related kinase (ERK 1/2) expression in primary cultures of epithelial cells from human ADPKD and ARPKD cysts.

Results. The rank orders of responses to ligands for ADPKD and ARPKD cells were identical: epinephrine > desmopressin (DDAVP) ≈ arginine vasopressin (AVP) > adenosine > prostaglandin E_2 (PGE_2) > parathyroid hormone (PTH). cAMP concentrations elevated by epinephrine, DDAVP, adenosine, and PGE_2 were diminished by receptor-specific inhibitors. Pools of cyst fluid collected individually from 16 of 19 ADPKD kidneys increased, to varying degrees, cAMP levels in ADPKD and ARPKD cells. PGE_2, β-adrenergic and AVP antagonists partially inhibited cAMP accumulation in response to fluids from three kidneys, but a large portion of the endogenous activity was attributed to yet-to-be identified bioactive lipid, designated cyst activating factor (CAF). CAF stimulated cAMP production in ADPKD and ARPKD cells, activated $ERK_{1/2}$, and increased cellular proliferation in ADPKD cells. CAF increased positive short circuit current (I_{SC}) in polarized ADPKD and T-84 monolayers, indicating stimulation of net anion secretion.

Conclusion. Endogenous adenylyl cyclase agonists promote cell proliferation and electrolyte secretion of human ADPKD and ARPKD cells in vitro. We suggest that increased levels of cAMP may accelerate cyst growth and overall renal enlargement in patients with PKD.

[1]**See Editorial by Torres, p. 1283.**

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The growth of individual cysts in polycystic kidney disease (PKD) is determined primarily by epithelial cell proliferation within cyst walls in conjunction with transmural fluid secretion [1]. Evidence based on studies of cyst epithelial cells in vitro points strongly to a role for adenosine 3′ 5′-cyclic monophosphate (cAMP) in determining the rate of cyst epithelial cell growth and fluid secretion [2–7]. Thus, natural receptor-mediated agents that activate adenylyl cyclase [arginine vasopressin (AVP), prostaglandin E_2 [PGE_2], catecholamines, and adenosine] and inhibitors of phosphodiesterase (caffeine and theophylline) have the potential to accelerate the rate of renal enlargement in PKD [8, 9]. A novel neutral lipid isolated from cyst fluids, named cyst activating factor (CAF), can be added to this conventional list of agonists [10, 11].

Recently, Gattone et al [12, 13] found that inhibition of vasopressin V_2 receptor coupling to adenylyl cyclase was sufficient to dramatically diminish the formation and growth of renal cysts in animals with three different hereditary renal cystic disorders. Renal function was preserved in all instances. This compelling body of work places cAMP in a final common pathway in situ for the regulation of growth in most, if not all, hereditary renal cystic disorders, opening opportunities for specific treatment with agents that block the activation of adenylyl cyclase by hormones and autacoids.

The regulation of cell proliferation and fluid secretion by cAMP has been examined in human autosomal-dominant PKD (ADPKD) but the relative roles of hormones and autacoids that activate adenylyl cyclase are incompletely understood. In the current study we used a standard protocol to evaluate more rigorously the responses of both human ADPKD and autosomal-recessive

PKD (ARPKD) cyst epithelial cells to physiologically important adenylyl cyclase agonists and CAF. The expanded list of agonists under examination and the inclusion of human ARPKD cells are important new features of this study. We also sought to determine if cyst fluids harbor any of these agonists, thereby providing a potential reservoir for sustained activation of adenylyl cyclase. The results indicate that receptor-mediated agonists and an anonymous lipid derived from human ADPKD cysts have the capacity to stimulate cAMP production in both human ADPKD and ARPKD cells in vitro.

METHODS

Cultures of human ADPKD and ARPKD cells

Mural cells were retrieved from renal cysts of two adult patients with ADPKD and two infants with ARPKD who underwent elective nephrectomy as part of their treatment plan. The diagnosis was verified by anatomic examination. Explicit genotypic data were not available. The discarded but viable kidneys were packed immediately in ice (4°C) and shipped by expedited delivery to the laboratory. Cells removed from cortical cysts were placed in primary culture as described previously [14, 15]. Primary cultures were used for immediate studies or frozen in 10% dimethyl sulfoxide (DMSO) for future use. ADPKD cells retained the capacity to form a differentiated epithelium for at least three passages; by contrast, ARPKD cells could be used for more than ten passages. The protocol was approved by the Institutional Review Board at the University of Kansas Medical Center.

Cells maintained in liquid nitrogen were thawed and grown in plastic flasks containing a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (DMEM/F12) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite (ITS) (Collaborative Biomedical Products, Bedford, MA, USA). Then cells were seeded onto 24-well plates (Costar, Cambridge, MA, USA). Media were replaced every 2 days.

Cyst fluid

Fluids were collected, as described previously [16, 17], from the cysts of 19 individuals with advanced ADPKD. The kidneys were handled as noted above and shipped by expedited delivery to the laboratory. Surface cysts containing clear liquid were aspirated, and the fluids removed from a single kidney or a pair were mixed together to form a single large pool. Sediment was removed by centrifugation and the supernatant was stored at –20°C.

Measurements of intracellular cAMP

Two $\times 10^5$ cells were seeded into individual chambers (1.9 cm^2) of 24-well plates in DMEM/F12 containing 5% FBS, ITS, and penicillin/streptomycin. Near confluence, the medium was changed to 1% FBS to reduce the rate of basal growth. Twenty-four hours later, cells were rinsed in fresh DMEM/F12 containing 1% FBS for 15 minutes. The rinsing procedure ensured the removal of autocrine cAMP agonists that may have accumulated in the conditioned medium. Stock solutions (1:100 to 1:1000 dilutions into buffered saline) of desmopressin (DDAVP), AVP, PGE_2, epinephrine, and adenosine were added directly to the incubation medium. Each sample was determined in quadruplicate and statistically significant stimulation above baseline ($P < 0.05$) was determined by unpaired *t* test.

Stock solutions of the following were added to medium: AVP-V_2R antagonist (β-mercapto-β, β-cyclopentamethylene propion-*O*-methyl-Tyr^2, Arg^8), vasopressin, indomethacin, and a β_2 adrenergic receptor inhibitor (ICI-118551). An EP4 PGE_2 receptor antagonist (L-161982) was dissolved in DMSO. Forskolin and a β_1 inhibitor (atenolol) were dissolved in ethanol. Equivalent concentrations of water or other carriers were added to control media. AVP, PGE_2, epinephrine, adenosine, atenolol and forskolin were purchased from Sigma (St. Louis, MO, USA). DDAVP was purchased from Rhone Poulenc Rorer Pharmaceuticals (Collegeville, PA, USA). ICI-118551 was purchased from Biomol (Butler Pike, PA, USA). L-161982 was a gift of Merck-Frosst Canada and Co. (Kirkland, Quebec, Canada).

The cells were harvested for cAMP determinations 15 minutes after adding the respective agonists. Inhibitors were added at least 30 minutes before the agonists. Intracellular cAMP was extracted from epithelial cells in the individual wells (area 1.9 cm^2) into 80% methanol, reconstituted in 0.05 mol/L sodium acetate and quantified by an enzyme immunoassay system (Amersham Pharmacia Biotech, Buckinghamshire, UK) as described previously [9].

Bioelectric measurements

Polarized, confluent monolayers of T-84 and ADPKD cells, grown on permeable supports (Snapwell 1.1 cm^2 area) (Costar), were mounted in modified Ussing chambers as described previously [14, 15, 18]. The device contained 4 mL of media in each of two chamber halves separated by the monolayer and the media were gassed and stirred with a low stream of 5% CO_2/95% O_2. Medium was an isotonic Ringer's solution that contained no serum or serum components maintained at 37°C. CAF samples stored in chloroform:methanol (1:1) were dried under liquid nitrogen and dissolved in 100 μL

DMEM/F12 + 0.1% decomplimented FBS. The transepithelial potential difference (Vte), short-circuit current (Isc), and transepithelial resistance (Rte) were determined as described previously [15, 18]. The monolayers were maintained in the short-circuited state, and the open-circuit voltage was determined at 1- to 5-minute intervals. After a period of equilibration, sequential Isc measurements were recorded. Steady-state Isc was recorded, typically 15 to 20 minutes after the addition of agonists or CAF extracts to the basolateral medium.

Immunoblot analysis

Methods identical to those reported recently were used [4, 5]. Cells (10^5) were seeded onto 100 mm diameter plastic dishes in DMEM/F12 with 5% FBS, ITS, and penicillin/streptomycin. As the cells approached confluency, FBS was reduced to 1% for 24 hours. Prior to incubation with agonists, cells were washed in 1% FBS for 15 minutes. Forskolin, DDAVP, or extracts of CAF were added for 15 minutes. Cells were lysed in 500 μL of ice-cold buffer (TLB) [20 mmol/L Tris (pH 7.4), 137 mmol/L NaCl, 25 mmol/L β-glycerophosphate, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L sodium orthovanadate, 2 mmol/L $NaHPO_4$, 1%. Triton X-100, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 5 μg/mL aprotinin, 5 μg/mL leupeptin, 2 mmol/L benzamidine, and 0.5 mmol/L dithiothreitol (DTT)]. Cell lysates were centrifuged to obtain soluble fractions, aliquots of which were quantified for protein. Aliquots (20 μg protein) were then heated (95 to 100°C) in sodium dodecyl sulfate (SDS) sample buffer, separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose membranes (Hybond ECL) (Amersham, Buckinghamshire, UK). After transfer, the membranes were blocked with 5% milk in TBS-T (20 mmol/L Tris-HCl, 137 mmol/L NaCl, and 0.05% Tween 20, pH 8.0) for 1 hour at room temperature and then incubated with primary antibody in 5% milk in TBS-T for 2 hours at room temperature or overnight at 4°C. Membranes were next washed (3×) with TBS-T and incubated with secondary antibody with 5% milk in TBS-T for 1 hour and finally were washed (3×) with TBS-T. Proteins were visualized using an enhanced chemiluminescence system (ECL) (Amersham Life Science, Arlington Heights, IL, USA). Resolved protein bands were detected and quantified by a Fluor-S Max multi-imager system (Bio-Rad, Hercules, CA, USA).

Antibodies specific for ERK1 (C-14), ERK2 (C-16), and phospho-ERK (E-4) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies were derived from antirabbit, antimouse, or antirat IgG-conjugated horseradish peroxidase (HRP) (Santa Cruz Biotechnology).

Cell proliferation

The Promega Cell Titer 96 MTT assay method (Madison, WI, USA) was used to determine the relative rates of cell proliferation under different experimental conditions [19]. This method, which measures the optical density of a proliferation-dependent reaction product, was found to correlate directly with determinations of cell number using a direct cell-counting technique. To determine the effects of agonists on the rate of proliferation, approximately 4000 cells were seeded into individual chambers of a 96-well plate. The cells were incubated initially in DMEM/F12 medium supplemented only with penicillin, streptomycin, ITS, and 1% FBS. After 24 hours, the FBS was reduced to 0.002% (ITS-deleted) to arrest growth. Specific agonists were added for another 48 to 72 hours. Preliminary studies were examined for desquamated cells, and no consistent effects of agonists were detected on the number of cells in the supernatant. Inhibitors of protein kinase A activity, H-89 and Rp cAMP, were added for 30 minutes before the agonists.

CAF extraction and separation

We employed a two-phase system with two washes to extract 10 to 50 mL samples of human cyst fluid [20]. Following published procedures, crude lipids were separated by thin-layer chromatography on plates of silica gel (K6) (60 Å particle size, 250 μm thickness) (Whatman, Clinton, NJ, USA), with development in chloroform/acetone/methanol/acetic acid/water (5:2:1:1:0.5, by volume) [21]. Lipids were recovered from bands 0.5 to 1.0 cm in width and labeled 1 to 10. In this system polar lipids generally exhibit R_f values <0.5 and neutral compounds R_f values >0.7. Reference lipids were included at the edges and marked after brief development in iodide vapor.

Active fractions, determined by cAMP or Isc assays in ADPKD and T-84 cell monolayers, were then subjected to normal-phase, isocratic high-performance liquid chromatography (HPLC) [22]. The instrumentation was an LC-6A system (Shimadzu, Columbia, MD, USA) controlled by Class-VP software, columns were μPorasil, 3.9 × 300 mm (Waters, Milford, MA, USA), and the elution solvent was hexane/2-propanol/acetic acid (100:10:0.02, by volume). Column fractions were monitored at 206 nm, but CAF levels were too low to be detected by ultraviolet or light spectroscopy. An inactive neutral lipid, 2-oleoylglycerol, was employed as an internal standard. Crude and purified lipid fractions were taken to dryness in vacuo, reconstituted in chloroform/methanol (1:1, by volume), and stored at −20°C under N_2.

Lipid standards, including free fatty acids, 1-acylglycerol, 1,2-diacylglycerol, several glycerophospholipids, sphingosine, ceramide, and several eicosanoids, were obtained from Avanti Polar Lipids (Alabaster, AL,

USA), Cayman Chemical (Ann Arbor, MI, USA), and Sigma-Aldrich (St. Louis, MO, USA). HPLC grade solvents and other chemicals were purchased from Fisher (Pittsburgh, PA, USA).

RESULTS

cAMP agonist effects on ADPKD and ARPKD cells

Recent studies demonstrated that inhibition of AVP V_2 receptor blockade of adenylyl cyclase activation strikingly reduced the enlargement of renal cysts in three strains of rodents with hereditary cystic disorders. These findings have important therapeutic implications; however, before direct application to humans it is important to confirm that cAMP has a central role in the target cells derived from human renal cysts.

In the current in vitro study, we determined the relative capacities of several agonists to increase cAMP in mural epithelial cells derived from the cysts of patients with two major types of hereditary polycystic disease, ADPKD and ARPKD. ARPKD cells were used for preliminary screening studies since the yield from primary cultures was greater than for ADPKD cells. The effects of key agonists were then directly compared between the ADPKD and ARPKD genotypes.

Highly specific inhibitors were used to confirm that increases in cAMP caused by the different ligands were receptor-mediated. We also searched for anonymous cAMP agonists by adding to ADPKD and ARPKD cells cyst fluids collected from ADPKD patients. Receptor blockers were used to determine if common adenylyl cyclase agonists accounted for increases in cAMP caused by cyst fluid.

The cAMP responses of ADPKD and ARPKD cells to maximally effective concentrations of the respective agonists were compared (Table 1). Concentrations sufficient to maximally increase cellular cAMP were determined in preliminary studies (data not shown). Forskolin (10 μmol/L), which directly activates adenylyl cyclase to a maximal extent, was used as a positive control. The effect of each agonist to raise cell cAMP above baseline levels was determined as a percent of the maximal forskolin response in cells from the same preparation. In this calculation, given in the legend to Table 1, the baseline level of cAMP is subtracted from the response to experimental agonist and to forskolin. The calculated change, therefore, reflects the percent elevation of cAMP above the baseline in relation to a maximal response to forskolin. In Table 1 forskolin increased cAMP in ADPKD from baseline of 1.4 to 26.8 ± 3.2 pmol/sample ($N = 4$); in ARPKD forskolin increased cAMP from baseline 1.3 to 34.3 ± 6.6 pmol/sample ($N = 12$). In Figure 1, forskolin increased cAMP in four preparations derived from an ARPKD kidney from baseline of 0.7 ± 0.2 pmol/sample to 21.5 ± 2.0 pmol/sample, $N = 4$. In Figure 2, forskolin increased cAMP levels in 11 ARPKD preparations from baseline of 0.7 ± 0.2 pmol to 19.6 ± 3.2 pmol and nine ADPKD preparations from baseline of 0.7 ± 0.1 pmol to 12.0 ± 1.3 pmol.

Table 1. Effect of receptor-mediated agonists and antagonists on 3′ 5′ cyclic monophosphate (cAMP) production by autosomeal-dominant polycystic kidney disease (ADPKD) and autosomal-recessive polycystic kidney disease (ARPKD) cells

	cAMP level (relative to forskolin)%[a]	
	ADPKD cells	ARPKD cells
Epinephrine (1 μmol/L)	95.8 ± 4.4[b] (4)	94.7 ± 3.0[b] (4)
Epinephrine (1 μmol/L) + atenolol (10 μmol/L) + ICI-118551 (10 μmol/L)	0.5 ± 0.1 (4)	0.0 ± 0.0 (4)
DDAVP (100 mU/mL)	64.7 ± 2.6[b] (12)	86.6 ± 0.9[b,c] (12)
DDAVP + AVP V_2 receptor antagonist (1 μmol/L)	0.0 ± 0.0 (4)	2.2 ± 0.6 (4)
AVP (100 mU/mL)	54.8 ± 3.2b (12)	81.6 ± 4.4[b,c] (4)
Adenosine (100 μmol/L)	63.1 ± 7.0[b] (8)	71.2 ± 2.3[b] (4)
Dimethyl-propargyl (10 μmol/L) + adenosine	24.5 ± 2.9 (4)	39.7 ± 1.3 (4)
PGE_2 (0.4 μmol/L)	45.7 ± 1.8[d] (7)	51.4 ± 15.7[b] (8)
L-161982 (1μmol/L) + PGE_2	20.3 ± 4.1 (3)	5.3 ± 0.2 (4)
Parathyroid hormone (10 nmol/L)	20.6 ± 1.1[d] (4)	5.7 ± 0.4[c,d] (4)

Abbreviations are: DDAVP, desmopressin; AVP, arginine vasopressin; PGE_2, prostaglandin E_2.

[a]Experimental − baseline/(forskolin − baseline) × 100. Mean forskolin response, ADPKD 26.8 ± 3.2 pmol/sample ($N = 4$); ARPKD 34.3 ± 6.6 pmol/sample ($N = 12$). Baseline, ADPKD 1.4 pmol/sample; ARPKD 1.3 pmol/sample. Results are expressed as the mean ± SE for the indicated number of measurements.

[b]$P < 0.001$, differences from baseline levels by analysis of variance (ANOVA).

[c]$P < 0.001$, difference between ADPKD and ARPKD cells by unpaired t test.

[d]$P < 0.01$, differences from baseline levels by ANOVA.

Surprisingly, epinephrine was the most potent receptor-mediated agonist tested in both ADPKD and ARPKD cells (Table 1). The effect of epinephrine to raise cAMP levels was completely inhibited by a combination of β_1 (atenolol) and β_2 (ICI-118551) adrenergic receptor blockers.

AVP and the V_2-specific agonist, DDAVP, raised cAMP levels in ARPKD cells to a greater extent than in ADPKD cells (Table 1). The difference in the degree of stimulation is probably related to the fact that ARPKD cells were derived primarily from cortical collecting ducts, whereas ADPKD cells represent a broader mixture of progenitor cells from the cortex, including proximal tubule, distal tubule, and collecting ducts [5, 23]. A specific AVP V_2 receptor antagonist (1 μmol/L) virtually eliminated the elevation of cAMP caused by DDAVP in both ADPKD and ARPKD cells. The observation that the cAMP responses to DDAVP and AVP were nearly identical within each cell line suggests that the V_1 receptor did not alter cAMP production or metabolism to a detectable extent.

PGE_2 (0.4 μmol/L) stimulated the accumulation of cAMP in ADPKD and ARPKD cells to about the same

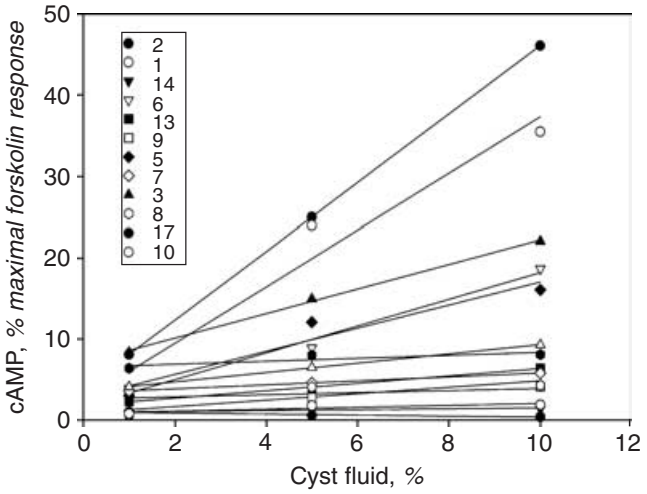

Fig. 1. Concentration-dependent effects of 12 pools of autosomal-dominant polycystic kidney diseae (ADPKD) cyst fluid on 3′ 5′ cyclic adenosine monophosphate (cAMP) levels in autosomal-recessive polycystic kidney disease (ARPKD) cells. Boxed symbols indicate cyst fluids from individual patients. Cyst fluids were diluted in medium and added for 15 minutes. cAMP expressed in relation to the maximal forskolin response in each experiment. Eleven of 12 cyst fluids showed significant stimulation above baseline ($P < 0.05$) for 1%, 5%, and 10% concentrations when evaluated by unpaired *t* test. Forskolin increased cAMP in four preparations derived from an ARPKD kidney from baseline of 0.7 ± 0.2 pmol/sample to 21.5 ± 2.0 pmol/sample ($N = 4$).

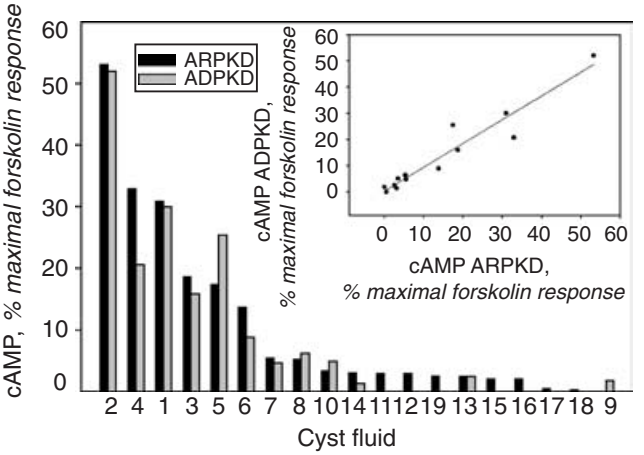

Fig. 2. Capacity of autosomal-dominant polycystic kidney disease (ADPKD) cyst fluid to stimulate 3′ 5′ cyclic adenosine monophosphate (cAMP) accumulation in ADPKD and autosomal-recessive polycystic kidney disease (ARPKD) cells in vitro. A 10% concentration of fluid from each of 19 ADPKD cysts was added for 15 minutes to 19 monolayers of ARPKD cells (■) and 13 monolayers of ADPKD cells (▣) ($N = 4$ each). cAMP (% of maximal forskolin response) was increased in the ARPKD cells by all but three cyst fluids determined by nonpaired *t* test. The inset shows the linear correlation between responses to cyst fluid in ARPKD in comparison to ADPKD cells. $cAMP_{ADPKD} = 0.38 + 0.907\ cAMP_{ARPKD}$, $r^2 = 0.921$. Forskolin increased cAMP levels in 11 ARPKD preparations from baseline of 0.7 ± 0.2 pmol to 19.6 ± 3.2 pmol and 9 ADPKD preparations from baseline of 0.7 ± 0.1 pmol to 12.0 ± 1.3 pmol.

degree (Table 1). The EP_4 specific antagonist, L-161982 (1 μmol/L), reduced the effect of PGE_2 to a greater degree in ARPKD than in ADPKD cells. Adenosine potently stimulated cAMP levels in both ADPKD and ARPKD cells (Table 1). This effect was partially inhibited by an adenosine A_2 receptor antagonist.

Parathyroid hormone (PTH) increased cAMP in ADPKD to a greater level than in ARPKD cells (Table 1). This is opposite to the effect of AVP noted above, but like vasopressin, is probably a reflection of the fact that the ADPKD preparations are comprised of a higher proportion of proximal tubule segments than ARPKD preparations. A PTH receptor inhibitor was not available.

These results establish that both ADPKD and ARPKD cells responded to cAMP agonists in the same order: epinephrine > DDAVP ≈ AVP > adenosine > PGE_2 > PTH.

Evaluation of unfractionated cyst fluid agonists

To determine if cysts harbored potential adenylyl cyclase agonists, we determined the capacity of fluids retrieved from human cysts to elevate cAMP in ARPKD cells. Cyst fluids were diluted with DMEM/F12 to working concentrations of 1%, 5%, and 10%. The increase in cell cAMP content stimulated by cyst fluid was concentration-dependent (Fig. 1). Concentrations higher than 10% were not tested in order to minimize the potential effects of uremic toxins that may have accumulated in the azotemic patients.

In subsequent studies, a 10% concentration was used to determine the relative cAMP responses of ARPKD and ADPKD cells to individual pools of cyst fluid. Sixteen of 19 cyst fluids selected for this study stimulated cAMP levels above the baseline in ARPKD cells ($P < 0.05$) (Fig. 2). Thirteen of these fluids were added to ADPKD cells, and 11 of them increased cAMP accumulation to about the same extent as they did in ARPKD cells (Fig. 2). The relation between the cAMP content of ADPKD in comparison to ARPKD cells was linear ($[cAMP]_{ADPKD} = 0.38 + 0.907 \times [cAMP]_{ARPKD}$, $r^2 = 0.921$). Taken together, this set of experiments shows that agonists in ADPKD cyst fluid have the capacity to stimulate cAMP accumulation in both ADPKD and ARPKD epithelial cells.

To reveal the identities of the putative agonists, nine cyst fluids with the highest capacities for stimulating cAMP accumulation were added to PKD cells together with specific β-adrenergic, AVP-V_2, or PGE_2 receptor blockers, or with indomethacin, which inhibits eicosanoid synthesis. An effect of an antagonist to significantly diminish the increase in cAMP caused by the cyst fluid was taken to indicate the presence of the ligand in the cyst fluid. β-adrenergic, AVP-V_2, and PGE_2 receptor blockers diminished the cAMP response of ADPKD cells to cyst fluid; however, only three fluids appeared to contain significant quantities of the agonists (Table 1) (Fig. 3).

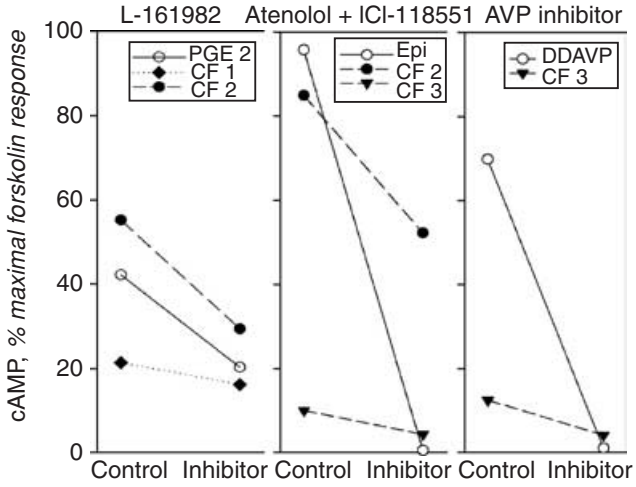

Fig. 3. 3′ 5′ cyclic adenosine monophosphate (cAMP) agonists detected in autosomal-dominant polycystic kidney disease (ADPKD) cyst fluids (CF). Nine cyst fluid pools were screened and three were found to contain appreciable quantities of common cAMP agonists. L-161982, an EP_4 receptor antagonist, reduced the effect of prostaglandin E_2 (PGE_2) on cAMP cells by 52%. The inhibitor reduced the capacities of CF 1 and CF 2 to increase cAMP levels by 25% and 47%, respectively. Atenolol and ICI-118551, agents that block β1 and β2 adrenergic receptors, respectively, completely inhibited the effect of epinephrine. The β blockers reduced the cAMP levels elevated by CF 2 and CF 3 by 38% and 57%, respectively. The arginine vasopressin (AVP) V_2 receptor antagonist completely inhibited the effect of desmopressin (DDAVP). This inhibitor reduced by 67% the capacity of CF 3 to elevate cAMP.

The adenosine A_2 antagonist evaluated in Table 1 had no effect on cAMP accumulation caused by cyst fluids with high intrinsic activity, and this was confirmed by direct measurements of adenosine with a sensitive HPLC method (data not shown). Thus, a large component of the agonist activity in the high capacity fluids of Figure 2 was unaccounted for.

Evaluation of partially purified cyst fluid lipids

We had previously shown that fluids from certain cysts contained a relatively potent neutral lipid that stimulated cAMP production in renal epithelial cells [10, 11]. The cyst fluids examined in this study were screened for the presence of CAF by determining the capacity of a chloroform:methanol (1:1) extract to increase Isc in monolayers of T-84 cells grown on permeable supports (Fig. 4A). T-84, an immortal cell line derived from a human colon adenocarcinoma laden with a high density of cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels within apical membranes, has been used extensively to study receptor-mediated transepithelial chloride secretion mediated by cAMP [24]. CAF extracts, reconstituted in medium and applied to these cells, caused a sharp rise in positive Isc (Fig. 4A), indicative of an in-

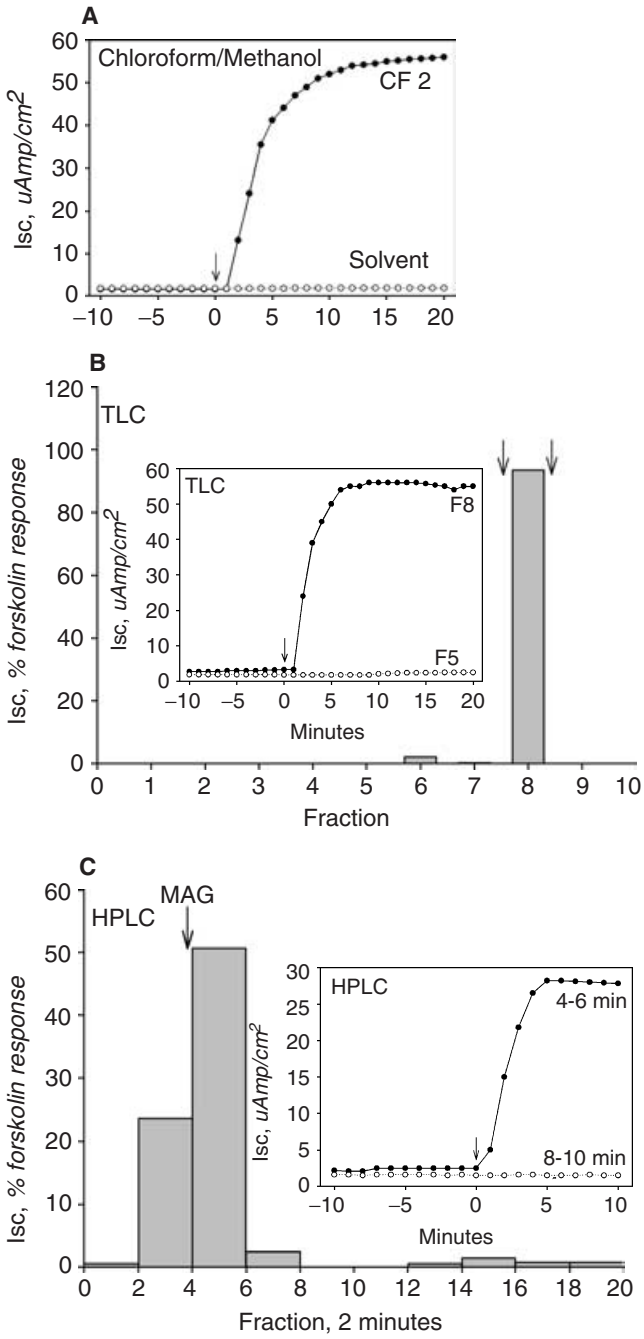

Fig. 4. Effect of cyst activating factor (CAF) in cyst fluid 2 on short-circuit current (Isc) in T-84 cells. (*A*) Time course of Isc after adding (arrow) a chloroform:methanol (1:1) extract of cyst fluid (CF 2) in Dulbecco's modified Eagle's medium (DMEM)/F12 solvent. (*B*) Thin-layer chromatography (TLC), CF 2 CAF activity was confined to fraction 8, between monoacylglycerol (MAG) and diacylglycerol (DAG). Inset, Time course of active fraction 8 and inactive fraction 5. (*C*) High-performance liquid chromatography (HPLC), CF 2 CAF activity was confined to the 2- to 6-minute collections. MAG indicated by arrow. Inset, Time course of the 4-to 6-minute and 8-to 10-minute fractions. In this series of studies, forskolin alone increased Isc from a baseline of 1.5 ± 0.3 μAmp/cm^2 to 52.9 ± 3.1 μAmp/cm^2 ($N = 4$).

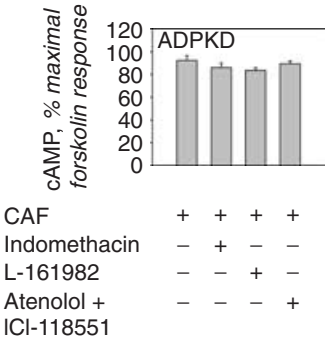

Fig. 5. Effect of cyst activating factor (CAF) on 3′ 5′ cyclic adenosine monophosphate (cAMP) accumulation in autosomal-dominant polycystic kidney disease (ADPKD) cells. Thin-layer chromatography (TLC)- and high-performance liquid chromatography (HPLC)-purified CAF fractions were pooled from cyst fluids (CF) 1, 3, 6, and 8. CAF addition approximated the active lipid in a ~25% concentration of the original cyst fluid. Indomethacin (10 μmol/L), L-161982 (1 μmol/L), atenolol (10 μmol/L) + ICI – 118551 (10 μmol/L) were added 30 minutes before CAF. Values are means ± SE ($N = 3$). None of the inhibitors had an effect. Forskolin (10 μmol/L) increased cAMP to 13.4 ± 0.03 pmol/sample ($N = 3$).

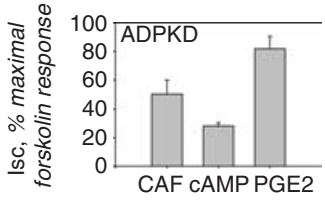

Fig. 6. Effect of cyst activating fluid (CAF), 8-Br-3′ 5′ cyclic adenosine monophosphate (cAMP) and prostaglandin E_2 (PGE_2) in autosomal-dominant polycystic kidney disease (ADPKD). Short-circuit current (Isc) determined in monolayers of ADPKD on permeable supports. Values are means ± SE ($N = 3$) of steady-state Isc after 15 to 20 minutes. Additions were CAF (~25% concentration of original cyst fluid), PGE_2 (0.4 μmol/L), and 8-Br cAMP (100 μmol/L). *P for all values vs. baseline < 0.001.

crease in net chloride secretion. Relatively high levels of CAF activity were found in cyst fluids 1 to 6; activity was detected in fluids 7 to 16; and activity was undetectable in fluids 17 to 19 (see Fig. 2).

CAF was further purified by thin-layer chromatography (TLC) into a discreet band that migrated between mono-and diacylglycerols (Fig. 4B) [10, 11]. Crude chloroform/methanol extracts of cyst fluids with high capacities to stimulate Isc in T-84 cells also had high levels of CAF in specific TLC band 8 (Fig. 4B, inset). By contrast, the other TLC fractions were relatively inactive. CAF in the high activity TLC band was further separated by normal-phase HPLC into a relatively narrow region of high activity (Fig. 4C and inset). This activity did not coelute with PGE_2 or arachidonic acid, other lipids known to activate adenylyl cyclase.

Because cyst fluids 1, 3, 6, and 8 shared identical TLC and HPLC characteristics (data not shown), aliquots of these extracts were pooled in order to have an adequate supply of highly active common source material for the remainder of the experiments. In each experiment a constant amount of CAF was applied approximating the amount of active lipid in a 25% solution of the original cyst fluid.

CAF stimulated cAMP accumulation in ADPKD cells (Fig. 5). Indomethacin pretreatment of the cells did not alter the subsequent effect of CAF to increase the cAMP content, demonstrating that the effect of CAF is not mediated through the generation of an eicosanoid. An EP_4 PGE_2 receptor blocker had no effect on CAF in either ARPKD or ADPKD cells. Blockade of β-adrenergic receptors also had no effect on the response of cells to CAF. Thus, CAF appears to be an uncommon, if not unique, bioactive lipid.

Three confluent ADPKD monolayers grown on permeable supports developed stable baseline Vte (−0.7 ± 0.07 mV), Rte (282 ± 26 ohm/cm^2), and Isc (2.4 ± 0.4 μÅ/cm^2). CAF added to the basolateral medium significantly stimulated Isc (Fig. 6). PGE_2 and an analogue of cAMP, 8-Br-cAMP, also increased steady-state Isc significantly in the same preparation of ADPKD cells.

In ADPKD cells, cAMP activates the mitogen-activated protein (MAP) kinase pathway and complements the action of epidermal growth factor (EGF) [4, 5] to stimulate cell proliferation. In the current study, CAF increased phospho-ERK levels 11-fold above the baseline (Fig. 7). Phospho-ERK levels were also increased by PGE_2, forskolin, and 8-Br-cAMP.

Although CAF was extracted from ADPKD cyst fluids, it also stimulated the accumulation of cAMP in ARPKD cells (71.9 ± 5.6% maximal forskolin response, $N = 4$). CAF also activated P-ERK in these cells (data not shown).

In previous studies the activation of ERK in ADPKD cells was linked to the increased rate of proliferation caused by the activation of B-Raf by cAMP [4, 5]. CAF, which elevated cAMP, also stimulated the proliferation of ADPKD cells to the same extent as EGF (Fig. 8). H-89 and Rp cAMP, two inhibitors that inhibit protein kinase A (PKA), eliminated the effect of CAF on proliferation as did PD98059, an inhibitor of MAP kinase kinase (MEK). Although MEK inhibition completely blocked the effect of EGF, inhibition of PKA had no effect on the cytokine. Taken together, these results suggest that the effect of CAF on the proliferation of ADPKD and ARPKD cells is probably mediated through the action of cAMP to activate B-Raf, and in turn to stimulate the MAP kinase pathway.

DISCUSSION

Kidney enlargement, often to grotesque proportions, is the hallmark of most hereditary polycystic disorders

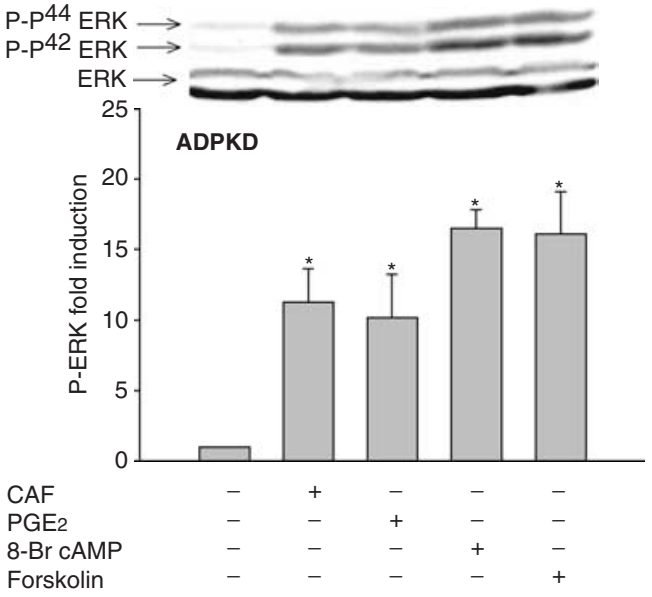

Fig. 7. Effect of cyst activating factor (CAF) on phospho-extracellular-related protein kinase (ERK) expression in autosomal-dominant polycystic kidney disease (ADPKD) cells. CAF and other agonists were added for 15 minutes. Additions were CAF (~25% concentration of original cyst fluid), prostaglandin E_2 (PGE_2) (0.4 μmol/L), 8-Br-3′ 5′ cyclic adenosine monophosphate (cAMP) (100 μmol/L) and forskolin (10 μmol/L). Upper two rows of a representative immunoblot show phospho-ERK1 and -ERK2 above two rows from a parallel study showing total ERK1 and ERK2 abundance. Fold increases of phospho-ERK vs. control determined in three separate experiments shown below the immunoblots. Values are means ± SE. $^*P < 0.001$ vs. baseline.

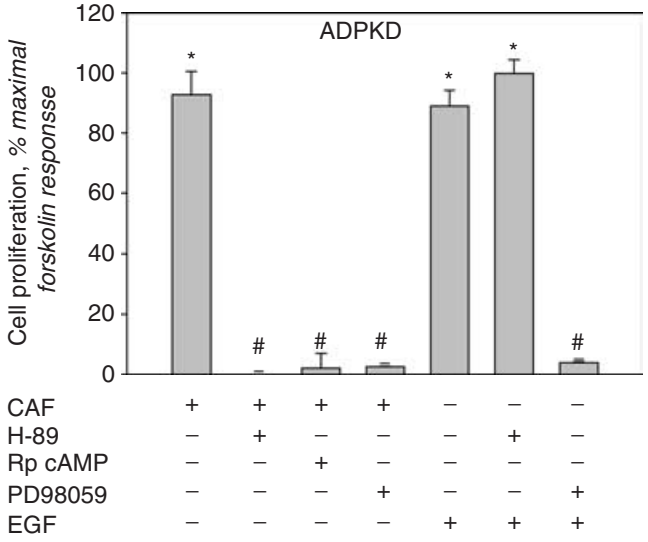

Fig. 8. Effect of cyst activating factor (CAF) on proliferation of autosomal-dominant polycystic kidney disease (ADPKD) cells. Additions were CAF (~25% concentration of original cyst fluid), epidermal growth factor (EGF) (25 ng/mL), H-89 (10 μmol/L), Rp 3′ 5′ cyclic adenosine monophosphate (cAMP) (250 μmol/L), and PD98059 (50 μmol/L). Values are means ± SE ($N = 12$). Forskolin (10 μmol/L) increased ADPKD cell count 1.56 ± 0.10-fold above baseline. $^{\#}P < 0.01$ vs. CAF; $^*P < 0.001$ vs. baseline.

[25, 26]. The basis for this enlargement lies in the dilation of individual renal tubules to diameters that may reach 1000 times greater than normal. In the ADPKD, renal enlargement proceeds progressively over several decades, culminating in renal failure in approximately one half of cases. By contrast, in ARPKD, the enlargement is usually much more rapid, often leading to profound renal enlargement and failure in the neonatal period.

Abnormal proliferation of renal tubule epithelial cells underlies the renal enlargement in both ADPKD and ARPKD [27, 28]. Consequently, polycystic renal disorders are now regarded as benign neoplastic conditions [29]. Like other growth disorders, the rate of cellular proliferation may be controlled by genetic and environmental factors. cAMP has been identified as perhaps one of the most important regulators of cyst growth [4, 6, 7]. The nucleotide stimulates both the proliferation of cyst mural cells and the transepithelial secretion of chloride and water [2, 3]. Thus, cAMP accelerates two biologic processes that are of fundamental importance in the expansion of renal cysts.

In the current study we demonstrated that mural epithelial cells representing the two major genotypes of PKD generated increased amounts of cAMP in response to epinephrine, AVP, adenosine, PGE_2, and PTH, agonists known to have important physiologic and pathophysiologic roles in the regulation of renal function (Table 1) (Figs. 1 and 2). cAMP is generated normally within renal tubule cells in response to one or more of these agonists day and night. The locus of stimulation might vary within the kidney depending upon hydration status. Extremes of adenylyl cyclase activation would be anticipated in antidiuretic states created by water restriction (increased AVP and PGE_2 production) or extracellular volume contraction (increased AVP, epinephrine, PGE_2, and adenosine production). Consequently, the rate of cell proliferation and fluid secretion by cyst epithelial cells could be increased by exposure to agonists arriving in the blood or generated locally as a consequence of normal homeostatic regulatory events.

The current study demonstrates that cyst epithelial cells from two genetically different kinds of PKD bear receptors for conventional hormones and autacoids that increase cAMP levels in a similar pattern in each type of cell. It is important to note, however, that the relative strengths of stimulation noted in these cultured cells may not hold forth in situ. For instance, we did not determine the relative effects of the agonists from the apical versus the basolateral locus of application nor were we able to control for the possible influence that acquired cystic disease forces might have had on these cells prior to their removal from the azotemic patient.

The current data seem consistent with the view that cells derived from cysts of the cortical collecting ducts

are the primary locus of the cAMP effects. Lectin binding studies revealed that most of the cells in ADPKD and ARPKD cortex cultures originate from collecting ducts [5, 30–32]. In the current study, ARPKD preparations had higher cAMP responses to AVP and DDAVP than ADPKD cells; conversely, ADPKD preparations had higher responses to PTH than ARPKD cells. In light of the well-known tubular actions of AVP and PTH these findings are consistent with a predominance of cysts derived from collecting ducts in both types of PKD, with the ARPKD preparation containing a slightly higher proportion of collecting duct cysts than ADPKD. This distribution of cysts is in accord with in situ histochemical studies of lectin binding in ADPKD and ARPKD [33, 34].

It is conceivable that certain cAMP agonists might be synthesized and/or secreted by the cyst epithelia and retained within cysts, thereby creating a reservoir of adenylyl cyclase stimulants. In the current study highly specific receptor blockers identified putative effects of PGE_2, adrenergic agonists, and AVP in three pooled cyst fluids with the highest capacities to stimulate cAMP accumulation (Fig. 3). Although these common agonists may accumulate within cysts under the conditions of the current study, other compounds appeared to account for a substantial portion of the agonist effects of cyst fluid on cAMP accumulation within PKD cells (Figs. 1 to 3). One of these compounds is a neutral lipid (CAF). At first glance, the potential impact of CAF might be underappreciated since the samples of raw cyst fluid added to the cells were only one tenth as concentrated as that of the original cyst fluid (Figs. 1 and 2). Extraction and purification of CAF permitted the application of larger quantities of the active material that increased cellular cAMP in T-84. ADPKD cells to levels approaching that of forskolin (Figs. 4 to 6). Similar to AVP, PGE_2, epinephrine, adenosine, and PTH (Table 1), CAF increased cAMP levels in ARPKD and ADPKD cells.

CAF strikingly increased Isc in polarized monolayers of ADPKD epithelial cells, consistent with the stimulation of chloride secretion (Fig. 6) and activated ERK1/2 in both ADPKD (Fig. 7) and ARPKD cells (data not shown). Accordingly, it appears that the increase in cAMP caused by CAF activated the MAP kinase pathway as reported previously for common receptor-mediated agonists [4, 5]. The activation of ERK 1/2 by CAF appeared to be linked to an increase in cellular proliferation since the effect could be specifically blocked by inhibitors of PKA and MEK (Fig. 8). Thus, CAF, like the other cAMP agonists examined in this study, has the capacity to stimulate both cellular proliferation and electrolyte secretion and thereby accentuate the enlargement of renal cysts to a greater extent than agents that act only on one of these important determinants of cyst enlargement.

The chemical identity of CAF remains an enigma. Inhibitors of PGE_2 did not interfere with the stimulation of cAMP by CAF excluding PGE_2 as a candidate (Fig. 5). A hypotensive, natriuretic neutral lipid has been isolated from dog renal medulla. This material, called medullipin, is synthesized in the medulla and activated by passage through the liver [35, 36]. Another anonymous neutral lipid with chemical properties similar to CAF is generated by the renal tubular catabolism of albumin [37]. That lipid is chemotaxic and stimulates renal adenylyl cyclase; however, it does not co-localize with CAF by TLC. Thus far, the molecular properties of all of these potent renal bioactive lipids have eluded identification, largely due to the vanishingly small quantities of the recovered compounds that can be isolated.

The capacity of PKD cells to generate cAMP within cyst cells in situ will also be determined by rate at which the nucleotide is metabolized by phosphodiesterase. Recent evidence indicates that inhibitors of this enzyme increase the levels of cAMP in ADPKD cyst epithelial cells [9]. Consequently, the use of phosphodiesterase inhibitors such as theophylline and caffeine may accentuate the effects of common endogenous adenylyl cyclase agonists and CAF.

The clinical relevance of the current studies depends upon the extent to which cultured renal epithelial cells reflect the function of the in situ precursor cells. It is important to note in this regard that the stimulation of transepithelial fluid secretion by cAMP agonists has been demonstrated in intact renal cysts in situ and in in vitro studies of cells cultured from cysts [2, 3, 17, 23, 38]. Experiments utilizing rodent models of PKD have also shown that renal and urine levels of cAMP are abnormally increased [12, 13, 39]. In a rat model of ADPKD, activation of ERK has been demonstrated in whole kidney extracts by western blot and in individual cysts by immunocytochemistry [40]. Activated ERK has also been detected in the epithelial cells lining ADPKD cysts [41]. Thus, the data from cell culture studies, though indirect, support a role for cAMP in fluid secretion and cell proliferation in PKD.

CONCLUSION

The experiments reported in this study support the view that cAMP is an important determinant of the rate at which individual cysts enlarge within polycystic kidneys. Moreover, there is a rich array of adenylyl cyclase receptors that may be activated by common and unexpected agonists such as CAF.

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