cAMP Regulates Cell Proliferation and Cyst Formation in Autosomal Polycystic Kidney Disease Cells

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Abstract. Both epithelial cell proliferation and fluid accumulation are responsible for cyst growth in autosomal dominant polycystic kidney disease (ADPKD). It was previously reported that the cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in cysts from ADPKD patients and suggested that cAMP-stimulated Cl^- and fluid secretion occurs through CFTR. The purpose of this study was to investigate the role of cell proliferation in cyst formation in ADPKD and to explore further the role of fluid secretion in cyst growth. Primary cultures both of ADPKD epithelial cells and a mixed population of normal renal epithelial cells isolated from the cortex (HRCE cells) were used. This study tested whether cAMP was involved both in stimulating cell proliferation and formation of cysts *in vitro*. ^{3}H-Thymidine incorporation assays showed that epidermal growth factor stimulated proliferation both in ADPKD cells and HRCE cells. In addition, cAMP stimulated DNA synthesis and cell proliferation in ADPKD, but not HRCE, cells. The effects of cAMP and epidermal growth factor on cell growth in ADPKD cells were additive. cAMP also stimulated cyst enlargement and fluid secretion in ADPKD cells. By contrast, cyst formation and enlargement from HRCE cells occurred without cAMP. Fluid secretion into the cyst lumen was blocked by diphenylamine carboxylic acid (DPC) and glibenclamide in ADPKD cells but blocked only by DPC in HRCE cells. This study showed that ADPKD cells have unique characteristics; cAMP stimulates fluid secretion and cell proliferation, indicating cAMP plays a very important role in cyst growth during the course of ADPKD.

The formation of multiple cysts is a well characterized clinical feature of autosomal dominant polycystic kidney disease (ADPKD). In the early stages of ADPKD, numerous cysts begin to enlarge from many segments of the kidney (1,2). Later, the enlarged regions are disassociated from the original nephron, forming the actual cysts. The cysts continue to enlarge due to proliferation of epithelial cells and fluid secretion directed into the cyst lumen (2,3). Progressive renal cyst formation and enlargement results in a loss of renal function and hypertension, and culminates in renal failure in 50% of patients by the age of 60. Cysts also develop in other epithelia such as hepatic bile ducts, colon, and pancreas (4). ADPKD is also associated with several abnormalities, including alterations in the distribution and activation of the growth factor receptor EGFR, altered extracellular matrix, and incorrect polarization of essential membrane proteins (2,5–8).

Genes associated with the disease (PKD1 and PKD2) have been cloned (9–11), and proteins encoded by these genes (polycystins) have been investigated. Polycystins are widely expressed in fetal tissues (12,13). Recently, mouse models of PKD1 and PKD2 were developed and showed an embryonic lethal phenotype and massive cyst formation in the kidney (14,15). These results indicate that PKD1 and PKD2 probably play an important role during normal renal tubular development. However, their precise role in development and the cellular mechanisms of cyst formation induced by mutations in PKD1 and PKD2 is not fully understood.

Two key features associated with cyst formation in ADPKD are cell proliferation and fluid secretion, both of which are stimulated by cAMP. The effect of cAMP on cell proliferation varies among different cell types (16). For example, in smooth muscle cells, fibroblasts, and mesangial cells, elevation of intracellular cAMP blocks growth factor-stimulated cell growth by inhibiting the mitogen-activated protein (MAP) kinase cascade (16–18). On the other hand, in cell types such as thyroid cells, hepatocytes, and PC12 cells, cAMP activates cell proliferation (16,19). Although it is known that cAMP stimulates cyst enlargement, the exact mechanism of how cAMP stimulates cell proliferation in ADPKD is still unclear.

Maneuvers that raise intracellular cAMP such as addition of forskolin, which activates adenylate cyclase, addition of the membrane-permeable cAMP analogue 8-bromo-cAMP, and addition of prostaglandin E_2 stimulate fluid secretion both in monolayers of cultured epithelial cells established from ADPKD patients and in isolated ADPKD cysts (20–22). Increasing intracellular cAMP also enhances cyst formation and enlargement in an *in vitro* culture system (23,24). These studies have led to the hypothesis that fluid secretion into ADPKD cysts may occur via a secretory mechanism similar to that found in airway epithelia (25).

Because cell proliferation and fluid secretion are key determinants of cyst formation in ADPKD, we designed this study to investigate the role of cell proliferation in cyst formation in

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ADPKD and to further explore the role of fluid secretion in cyst growth.

Materials and Methods

Cell Culture

For control cells, mixed populations of normal renal epithelial cells derived from the cortex (HRCE cells) were obtained from Clonetics (San Diego, CA). The isolation procedure is as follows (as per the manufacturer). After the renal capsule was removed, pieces of renal cortex were isolated from normal human kidneys and minced and digested enzymatically. Primary cultures containing a mixture of proximal tubule, distal tubule, and cortical collecting duct cells were subcultured through a few passages as described below, and cryopreserved at the BioWhittaker cell culture facility (Walkersville, MD). Cells purchased from Clonetics were thawed immediately after arrival and cultured in the appropriate culture conditions. Similar cell types, designated as hCORT, were used by Wilson *et al.* (26). These were used for comparison studies with ADPKD cells.

Initially, HRCE cells were cultured in the culture media, REGM, with the following supplements: fetal bovine serum (FBS) (0.5%), human transferrin (10 mg/ml), hydrocortisone (0.5 mg/ml), insulin (5 mg/ml), triiodothyronine (5×10^{-12} M), epinephrine (0.5 mg/ml), epidermal growth factor (EGF) (10 mg/ml), and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, and 50 mg/ml gentamicin). Initally, HRCE cells were cultured either in Click-RPMI 1640 media or REGM media to check cell growth and cyst formation. No differences were noted in growth conditions using the two different media. Therefore, all experiments were conducted with Click-RPMI 1640 media.

Primary cultures were established from ADPKD cysts. Techniques used here have been described in detail elsewhere (27). In brief, 12 ADPKD cysts were isolated from four human ADPKD, end-stage kidneys. Each cyst was stripped clean of fibrous tissue, cut open, and chopped into 1-mm^2 pieces. Cyst-lining segments were plated onto type I collagen-coated plastic culture dishes and grown at 37°C in the culture media (Collaborative Research, Bedford, MA). Culture medium for ADPKD cells consisted of the following components: Click-RPMI 1640 media (Quality Biological, Rockville, MD) supplemented with FBS (1%; Mediatech, Herndon, VA), human transferrin (5 mg/ml), dexamethasone (5×10^{-8} M), insulin (5 mg/ml), triiodothyronine (5×10^{-12} M), and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin). All supplements described here except FBS were from Life Technologies-BRL (Gaithersburg, MD). When ADPKD cells and HRCE cells reached confluence, the cells were subcultured for the following experiments and incubated with the Click-RPMI 1640 media.

Thymidine Incorporation

DNA synthesis was measured as the amount of [^{3}H]-thymidine incorporated into TCA-precipitable material as described in detail previously (28). Cells were subcultured into 48-well culture plates at a density of 2×10^4/well and incubated with the culture media described above, Click-RPMI 1640 media with the supplements. When the cells reached 80% confluence, they were incubated with the hormone-free Click-RPMI 1640 media, which contained 0.2% FBS (basal medium) for 48 h, followed by exposure to the relevant agonist for 20 h. [^{3}H]-Thymidine (1 μCi/ml) (NEN Life Science Products, Boston, MA) was added 10 h before harvest. At the end of a 20-h exposure period, medium was removed by aspiration, washed twice with Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (Life Technologies-BRL), and exposed to 10% TCA. After the cells were incubated with TCA at 4°C for 1 h, TCA-precipitated material was washed twice with 5% TCA and solubilized with a mixture of 1N NaOH and 1% sodium dodecyl sulfate. Radioactivity was determined using liquid scintillation counting.

Cell Proliferation

To assess cell replication, ADPKD cells and HRCE cells were subcultured into 48-well culture plates with a concentration of 2×10^4/well and incubated with Click-RPMI 1640 media with the previously mentioned supplements. When the cells reached 60% confluence, the culture medium was replaced by basal medium, which is necessary to maintain cell viability for the experimental period. After 48 h, ADPKD cells were stimulated with the relevant agonists. ADPKD cells were counted at days 0, 4, and 8. Cells were washed with phosphate-buffered saline and then incubated in 0.05% trypsin (Life Technologies-BRL) with 0.53 mM ethylenediaminetetra-acetic acid for 15 min at 37°C. Detached cells were then suspended in the culture media containing 0.2% trypan blue (Life Technologies-BRL), and the number of viable cells was determined in a counting chamber.

Cyst Formation in Vitro

Before subculturing cells into a collagen gel, the gel mix was prepared and kept on ice. Gel mix was made from 0.7 vol of type I collagen, 0.2 vol of Click-RPMI 1640 media, and 0.1 vol of Hepes buffer solution. Cells were added into the gel mix with a concentration of 1×10^4/well and incubated at 37°C. When the gel mix became solid, culture medium was added and cells were cultured at 37°C with 5% CO_2. To promote cyst formation, normal culture medium with 1% FBS and hormones was used for 24 h. After cells were washed with Click-RPMI 1640 media three times, culture medium was changed to basal medium. After 24 h, relevant agents and/or blockers were applied into the culture medium and cyst size was determined with an inverted light microscope.

Materials

All of the cAMP analogues and antagonists, and 8-bromo-cGMP were purchased from Calbiochem (San Diego, CA).

Statistical Analyses

Data were presented as means ± SEM and analyzed by one-way ANOVA with the unpaired *t* test. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of cAMP on Thymidine Incorporation

To investigate whether cAMP could stimulate DNA synthesis in ADPKD cells isolated from patient cysts and HRCE cells, a mixed population of cells from the renal cortex was incubated with a basal medium containing 0.2% serum for 48 h before each experiment to minimize cell proliferation stimulated by factors present in serum. To maintain cell viability, 0.2% serum was necessary. After 48 h, cells were exposed to various experimental maneuvers for 20 h, and cell proliferation was measured. DNA synthesis was assessed by incorporation of [^{3}H]-thymidine into DNA during the last 10 h of the incubation period. Because EGF is a well known agent that stimulates cell proliferation in primary cultured renal tubular cells and ADPKD cells (7,29), cells were treated with EGF and 1% fetal calf serum (FCS) as a positive control. Sp-cAMP, a

membrane-permeable protein kinase A activator, stimulated thymidine uptake significantly (Figure 1 and Table 1). The effect of Sp-cAMP was most evident at 50 μM in ADPKD cells (Figure 1A). Another membrane-permeable cAMP analogue, CPT-cAMP, and forskolin also stimulated thymidine uptake (Table 1). When 50 μM Sp-cAMP was applied in addition to various concentrations of EGF, the effect on thymidine uptake was additive (Figure 1B). As indicated in Table 1, when protein kinase A was inhibited by either Rp-cAMP or H-89, no significant change in thymidine uptake was detected. When both Rp-cAMP and EGF were applied to ADPKD cells, the increase of thymidine uptake was similar to the cells only exposed to EGF. This suggests that stimulation of proliferation by protein kinase A and EGF occurs via separate signal transduction mechanisms.

Intracellular cGMP activates protein kinase G, another serine/threonine kinase. To check whether cGMP affects cell proliferation, 50 μM 8-bromo-cGMP, a membrane-permeable cGMP analogue, was tested (Table 1). The 8-bromo-cGMP did not change thymidine uptake, suggesting that an increase of cGMP does not play a role in stimulating thymidine uptake in ADPKD cells.

Although cAMP activated DNA synthesis in ADPKD cells, the response of HRCE cells to cAMP was quite different. As with ADPKD cells, addition of either 1% FBS or EGF stimulated thymidine uptake in HRCE cells by nearly twofold (Table 1). On the other hand Sp-cAMP did not change thymidine uptake in HRCE cells. We tested six different concentrations of Sp-cAMP (0.05 to 100 μM). In all cases, thymidine uptake was similar to that obtained from basal medium (Figure 1C). To exclude the possibility that Sp-cAMP could not effectively increase intracellular cAMP in HRCE cells, we also tested CPT-cAMP and forskolin. However, none of those agents changed thymidine uptake. Inhibiting protein kinase A by Rp-cAMP also did not change thymidine uptake (Table 1). Furthermore, the effect of EGF on thymidine uptake was not modified by adding either Sp-cAMP or Rp-cAMP (Figure 1D and Table 1). Thus, the HRCE cells respond quite differently to growth stimulation.

cAMP Activates Cell Proliferation

To confirm that the enhanced [^{3}H]-thymidine incorporation was associated with cell proliferation, we counted the number of cells at days 0, 4, and 8 after stimulation by cAMP and/or EGF (Figure 2 and Table 2). Hormone-free Click-RPMI 1640 basal media, which contained 0.2% FBS, did increase the cell

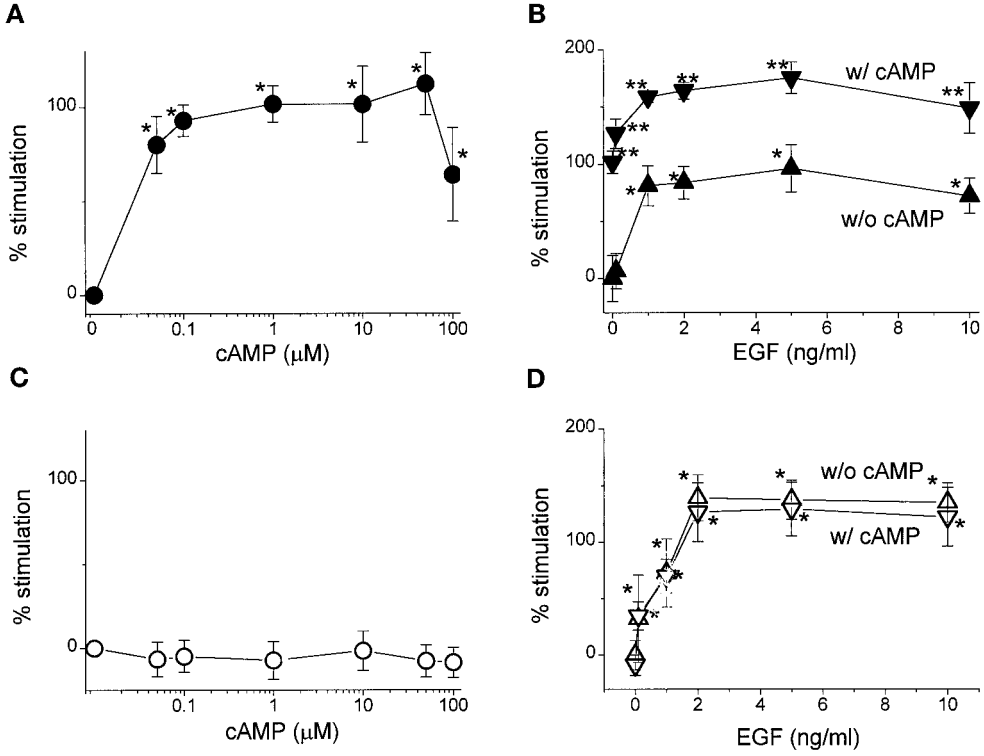

Figure 1. Effect of cAMP and epidermal growth factor (EGF) on thymidine uptake. Percent stimulation in ^{3}H-thymidine incorporation in response to increasing doses of Sp-cAMP in autosomal dominant polycystic kidney disease (ADPKD) cells (A) and in HRCE cells (C). Percent stimulation in ^{3}H-thymidine incorporation in response to increasing doses of EGF with and without 50 μM Sp-cAMP in ADPKD cells (B) and in HRCE cells (D). When the cells reached 60% confluence, the culture medium was replaced by basal medium. After 48 h, cells were stimulated with Sp-cAMP and/or EGF. Twenty hours later, DNA synthesis was measured by incorporation of [^{3}H]-thymidine. Percent stimulation was calculated according to the following equation: (([^{3}H]-thymidine uptake measured at each dose)/([^{3}H]-thymidine uptake measured in the basal medium) $-$ 1) $\times$ 100%. Results are given as mean $\pm$ SEM (n = 4 to 6 in each point). *$P < 0.05$ represents individual values of thymidine uptake compared to the basal medium; **$P < 0.05$ represents individual values of thymidine uptake stimulated by EGF in the presence of cAMP compared to absence of cAMP.

Table 1. Effect of cAMP analogues and inhibitors on ^{3}H-thymidine incorporation[a]

ADPKD Cells	Experimental Agents	HRCE Cells
0 ± 0	Basal medium (0.2% FBS)	0 ± 0
83.50 ± 15.08[b]	1% FBS	86.91 ± 22.68[b]
90.12 ± 11.32[b]	EGF (5 ng/ml)	139.14 ± 20.29[b]
120.83 ± 11.87[b]	Sp-cAMP (50 μM)	7.81 ± 9.67
170.81 ± 13.30[c]	Sp-cAMP + EGF	126.55 ± 25.97[b]
2.77 ± 2.77	Rp-cAMP (50 μM)	3.27 ± 8.09
87.06 ± 13.85[b]	Rp-cAMP + EGF	162.67 ± 24.39[b]
122.16 ± 14.57[b]	Forskolin	2.85 ± 7.56
88.73 ± 10.73[b]	CPT-cAMP (50 μM)	
0.94 ± 6.40	H-89 (1 μM)	
3.62 ± 5.04	Sp-cAMP + H-89	
5.48 ± 7.30	8-Br-cGMP (50 μM)	

[a] Percent stimulation in ^{3}H-thymidine incorporation in ADPKD and HRCE cells. [^{3}H]-Thymidine was measured after cells were stimulated by experimental agents, as described in detail in Materials and Methods. Percent stimulation was calculated according to the following equation: (([^{3}H]-thymidine uptake measured in the presence of the experimental agent)/([^{3}H]-thymidine uptake measured in the basal medium) − 1) × 100%. Results are given as mean ± SEM (n = 4 to 6 in each point. ADPKD, autosomal dominant polycystic kidney disease; FBS, fetal bovine serum; EGF, epidermal growth factor.

[b] $P < 0.05$ represents individual values of thymidine uptake measured in the presence of the agent compared to the basal medium.

[c] $P < 0.05$ represents individual values of thymidine uptake stimulated by EGF in the presence of cAMP compared to the absence of cAMP.

number of both ADPKD cells and HRCE cells. The effect of 0.2% FBS on the increase of cell number was much smaller than 1% FCS or 5 ng/ml EGF (Table 2).

ADPKD cells proliferated when exposed to 5 ng/ml EGF. When exposed to 50 μM Sp-cAMP, cell proliferation in ADPKD cells was stimulated to an extent comparable to 5 ng/ml EGF (Figure 2). The effects of EGF and Sp-cAMP were additive when applied together onto ADPKD cells. As shown in Table 2, 50 μM of the membrane-permeable cAMP antagonist Rp-cAMP alone or in the presence of EGF neither enhanced nor inhibited cell proliferation in ADPKD cells.

HRCE cells proliferated in the presence of either 5 ng/ml EGF or 1% FCS. In sharp contrast, 50 μM Sp-cAMP did not change the rate of cell proliferation in HRCE cells. Similar to ADPKD cells, 50 μM Rp-cAMP by itself did not show any effect on cell proliferation in HRCE cells (Table 2). These results demonstrate that cAMP activates cell proliferation in ADPKD cells but not in HRCE cells.

Cyst Formation and Effect of cAMP

We tested whether ADPKD cells and HCRE cells form *in vitro* cysts. In initial studies, cells (1×10^4/well) were cultured in type I collagen gel matrix (for details, see Materials and Methods) with basal medium; however, neither ADPKD cells nor HRCE cells formed a cyst (n = 12). When cells were incubated in Click-RPMI 1640 medium with a supplement of 1% FBS, HRCE cells formed a few microcysts in 24 h (10.4 ± 5.4/well at day 2; n = 8 wells). However, cyst formation could not be observed from ADPKD cells under this condition (Figure 3A). Addition of hormones, transferrin, dexamethasone, insulin, and triiodothyronine induced formation of cysts in ADPKD cells as well as in HRCE cells (Figure 3B for ADPKD cyst). At day 2, although average cyst volume was almost identical (0.0043 ± 0.0005 nl for ADPKD *versus* 0.0049 ± 0.0007 nl for HRCE; $P > 0.05$; n = 8 wells), the number of cysts was significantly different (125.4 ± 28.6/well for ADPKD *versus* 25.4 ± 8.3/well for HRCE; $P < 0.05$; n = 8 wells). Although both ADPKD and HRCE cells do form cysts, the number of cysts that are formed from HRCE cells is less. Because both 1% FBS and hormones were essential for cyst formation in ADPKD cells, in subsequent studies cells were cultured in the presence of 1% FBS and hormones for an initial 24-h period, followed by basal medium for 24 h.

After 2 d, the lumen diameter of ADPKD cyst was approximately 20 μm (Figure 3B). ADPKD cysts did not change their volume further when cultured only in basal medium for a period of 6 d (Figure 4A). In contrast, cyst volume increased when treated with 50 μM Sp-cAMP. Figure 3C shows a representative ADPKD cyst cultured with Sp-cAMP for 14 d. At 14 d the lumen diameter had increased to approximately 200 μm, indicating that when Sp-cAMP is applied, ADPKD cysts grow both by proliferation of cells lining the cyst wall and by fluid secretion into the cyst lumen. CPT-cAMP and forskolin also had a similar effect to increase cyst volume. Treatment of cysts with Rp-cAMP alone did not affect cyst volume. When H-89 was added into the culture medium, the effect of Sp-cAMP was completely inhibited. Additional acceleration of cyst enlargement was observed when both EGF and Sp-cAMP were applied (Figure 5). When FBS was removed from the culture medium, ADPKD cysts reduced in size by about 13% ($P < 0.05$; n = 4 wells) in 1 d. Beyond 1 d, cyst cells began to die in the absence of FBS. This demonstrates that cyst fluid is absorbed in the absence of stimulants in ADPKD cysts and that stimulation is needed to maintain cyst integrity.

After microcysts were formed from the control HRCE cells, cysts were cultured in basal medium or in the presence of 50 μM Sp-cAMP (Figure 4B). Unlike cysts formed from ADPKD cells, HRCE cysts continued to enlarge when treated with only basal medium lacking cAMP, but at a slower rate than ADPKD cysts activated by cAMP. The rate of HRCE cyst enlargement was not changed when 50 μM Sp-cAMP (Figure 4B) was added. HRCE cyst enlargement could be enhanced by higher concentrations of FBS or EGF (data not shown). Complete removal of FBS from culture medium reduced HRCE cysts in size by nearly 15% ($P < 0.05$; n = 4 wells) in 1 d.

We also tested purified proximal tubular epithelial cells (RPTEC) isolated from normal human kidney, for their ability to form cysts. RPTEC cells were purchased from Clonetics. Ninety-five percent of the RPTEC cells react to an antibody against γ-GTP, one of the markers of proximal tubule cells. When RPTEC cells were cultured in type I collagen gel matrix, the cells did not form any cysts in the presence of 1% FBS and

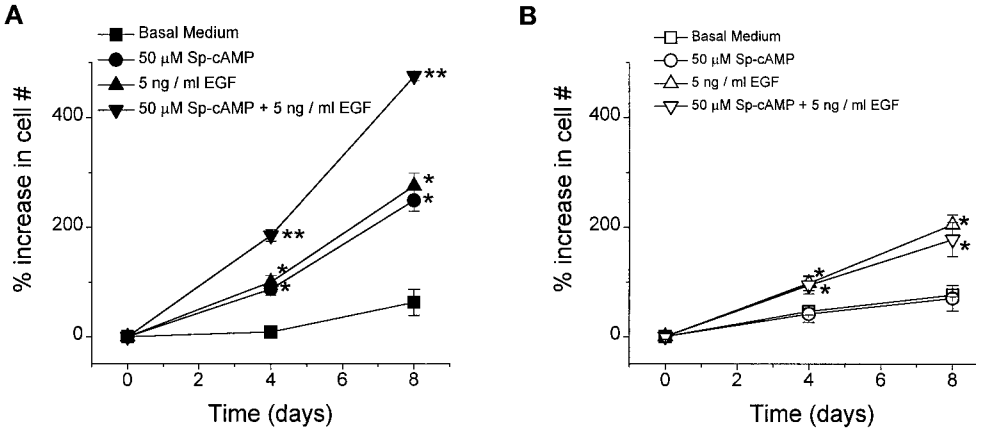

Figure 2. Effect of cAMP on cell proliferation. Percent increase in cell number *versus* time in days in ADPKD cells (A) and HRCE cells (B). When the cells reached 60% confluence, the culture medium was replaced by basal medium. After 48 h, cells were stimulated with 50 μM Sp-cAMP and/or 5 ng/ml EGF (this time was designated as day 0). Percent increase was calculated according to the following equation: ((cell number at either 4 or 8 d)/(cell number at day 0) $-$ 1) $\times$ 100%. Results are given as mean $\pm$ SEM (n = 4 to 6 in each point). *$P < 0.05$ represents a comparison of cell number measured in basal medium *versus* cell number after exposure to either to cAMP or EGF alone or to both cAMP and EGF; **$P < 0.05$ represents a comparison of cell number measured in EGF alone *versus* cell number after exposure to both cAMP and EGF.

Table 2. Effect of cAMP analogues and inhibitors on cell proliferation[a]

ADPKD Cells	Experimental Agents	HRCE Cells
62.2 ± 23.7	Basal medium (0.2% FBS)	76.6 ± 3.3
239.4 ± 23.4[b]	1% FBS	196.9 ± 13.4[b]
275.4 ± 23.1[b]	EGF (5 ng/ml)	205.3 ± 18.0[b]
248.9 ± 20.4[b]	Sp-cAMP (50 μM)	70.7 ± 23.3
474.3 ± 8.4[c]	Sp-cAMP + EGF	177.9 ± 31.0[b]
86.0 ± 16.9	Rp-cAMP (50 μM)	65.1 ± 2.3
274.0 ± 17.1[b]	Rp-cAMP + EGF	211.7 ± 38.6[b]

[a] Percent increase in cell number at day 8 in ADPKD cells and HRCE cells. Cell number was counted at day 8 after cells were stimulated by experimental agents, as described in detail in Materials and Methods. Percent increase was calculated according to the following equation: ((cell number at 8 d)/(cell number at day 0) $-$ 1) $\times$ 100%. Results are given as mean $\pm$ SEM (n = 4 to 6 in each point). Abbreviations as in Table 1.

[b] $P < 0.05$ represents a comparison of cell number measured in basal medium *versus* cell number after exposure to either to cAMP or EGF alone or to both cAMP and EGF.

[c] $P < 0.05$ represents a comparison of cell number measured in EGF alone *versus* cell number after exposure to both cAMP and EGF.

hormonal supplements (0 cysts were observed in 12 dishes). Unlike HRCE cells, higher concentrations of FBS did not enhance cyst formation. This suggests that cyst formation is not a universal property of all renal cells in culture.

Effect of Cl^- Channel Blockers

cAMP-activated Cl^- transport plays an important role in fluid transport in ADPKD cysts (27,30,31). To study whether channel blockers could inhibit cyst enlargement, cysts were cultured with 50 μM Sp-cAMP beginning at day 2, and different Cl^- channel blockers were added into the culture medium at day 4. Increase of cyst volume was then measured at day 8 (Figure 6). DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) is a drug that blocks several types of Cl^- channels and cotransporters but not cystic fibrosis transmembrane conductance regulator (CFTR) (32–34). A total of 200 μM DIDS had no effect on the volume of either ADPKD or HRCE cysts. Higher concentrations of DIDS (500 μM) were also examined, but cyst volume could not be measured because cells died during the experimental period. Diphenylamine carboxylic acid (DPC), an arylaminobenzoate, is also a potent Cl^- channel blocker and is known to block CFTR (32,33). A total of 200 μM DPC significantly inhibited cyst enlargement both in ADPKD and HRCE cysts. Glibenclamide, a sulfonylurea compound used for the treatment of diabetes mellitus, is known to block both ATP-sensitive K^+ channels (35) and CFTR Cl^- channels (32,36). Cyst growth was significantly inhibited in ADPKD cysts but not in HRCE cysts when 100 μM glibenclamide was applied. The inhibitory effect of glibenclamide on cyst enlargement in ADPKD cysts was reversible. When glibenclamide was taken out of the culture medium, ADPKD cysts again grew in the presence of 50 μM Sp-cAMP (data not shown). These results indicated that cAMP-activated cyst growth was decreased by ion channel blockers.

Discussion

cAMP and Cell Proliferation

In forming a cyst, cells in the kidney of ADPKD patients must proliferate to create large fluid-filled structures. To address the role of cAMP on cell proliferation, several investigators have studied the role of cAMP in activating MAP kinase activity, which is a signal transduction pathway often associated with stimulation of quiescent cells and increased cell proliferation. Heasley *et al.* showed that MAP kinase activity

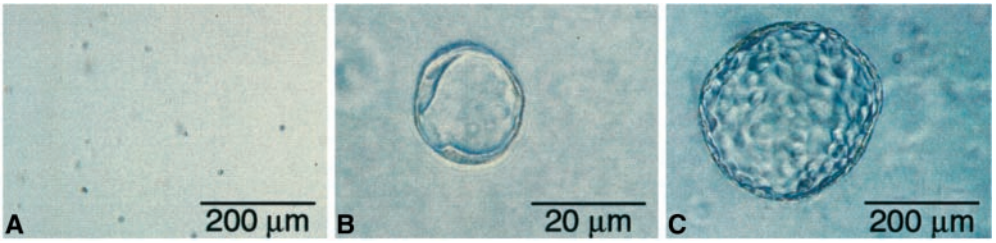

Figure 3. Cyst formation *in vitro*. When cells were cultured in basal medium for 2 d, no cysts formed (A). An example of a microcyst that formed after cells were cultured in 1% fetal bovine serum and hormones for 2 d is shown in Panel B. An example of a microcyst grown in the presence of 50 μM Sp-cAMP for 14 d is presented in Panel C. ADPKD cells were subcultured into gel matrix and incubated at 37°C.

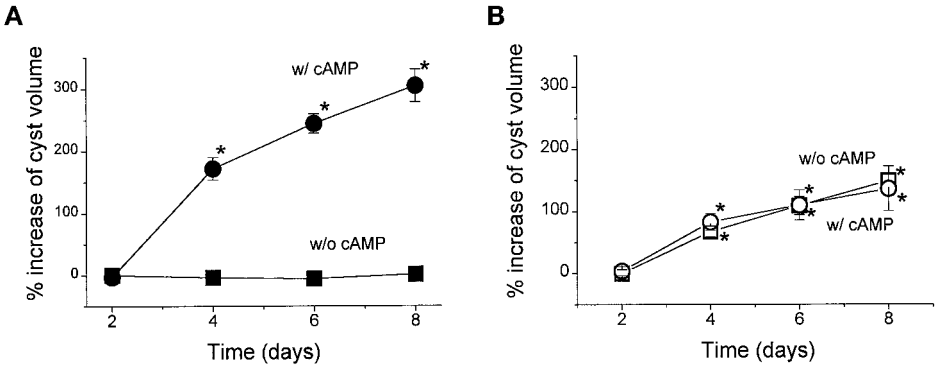

Figure 4. Effect of cAMP on cyst formation *in vitro*. Percent increase of cyst volume in ADPKD (A) and HRCE (B) cells grown in the presence or absence of 50 μM Sp-cAMP. Cells were embedded in a type I collagen gel at day 0, and in some experiments cAMP was added at day 2. Percent increase was calculated according to the following equation: ((cyst volume at each point)/(cyst volume at day 2) $-$ 1) $\times$ 100%. Results are given as mean $\pm$ SEM (n = at least 50 cysts in each point). *$P < 0.05$ represents a comparison of cyst volume at day 2 *versus* cyst volume at each time point.

in cultured rat inner medullary collecting tubule (RIMCT) cells is stimulated by carbachol, ATP, and EGF, whereas neither arginine vasopressin (AVP) nor β-adrenergic agonists, both of which increase intracellular cAMP, affect MAP kinase activity (37). These data suggest that there is no effect of increasing intracellular cAMP concentrations on MAP kinase activity in cultured RIMCT cells. On the other hand, Yamada *et al.* reported that AVP via cAMP inhibits cell proliferation in Madin-Darby canine kidney (MDCK) cells, whereas EGF activates the MAP kinase cascade and thymidine incorporation (38). AVP via an increase in intracellular cAMP inhibited MAP kinase activity and thymidine incorporation via the same MAP kinase cascade that is activated by EGF. However, another study also conducted in MDCK cells by Grantham and colleagues had a different result. Their study showed that agonists that increase intracellular cAMP such as forskolin activated thymidine incorporation in MDCK cells (39). Clearly, the exact nature of the role of cAMP in stimulating pathways associated with enhanced cell proliferation in normal renal tubular epithelia is still an unanswered question.

In our work, we measured both thymidine incorporation and cell proliferation directly. We show that only EGF activates proliferation in a mixed population of cells isolated from human renal cortex (Figures 1 and 2). Similar to the results of Heasley *et al.* in RIMCT (37), the proliferation of a mixed

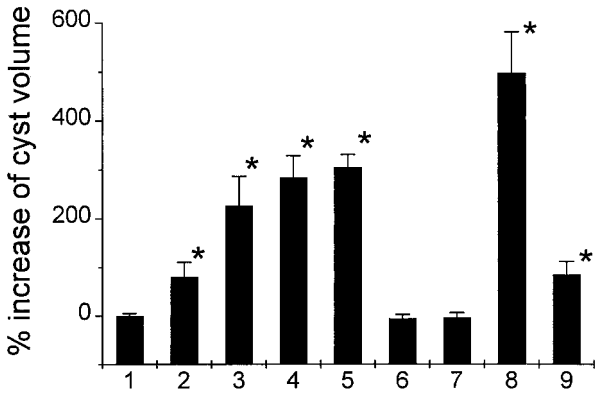

Figure 5. Comparison of various cAMP analogues and inhibitors. Percent increase of cyst volume measured at day 8 in ADPKD cysts. The following agents were applied at day 2: basal medium (1), 10 μM CPT-cAMP (2) or 100 μM CPT-cAMP (3), 1 μM forskolin (4), 50 μM Sp-cAMP (5), 50 μM Rp-cAMP (6), 50 μM Sp-cAMP + 1 μM H-89 (7), 50 μM Sp-cAMP + 5 ng/ml EGF (8), 5 ng/ml EGF (9). All agents were maintained in the culture medium throughout the 6-d period. Medium was changed daily to maintain the supply of agents. Percent increase was calculated according to the following equation: ((cyst volume at day 8)/(cyst volume at day 2) $-$ 1) $\times$ 100%. Results are given as mean $\pm$ SEM (n = at least 50 cysts in group). *$P < 0.05$ represents a comparison of cyst volume at day 2 *versus* cyst volume at day 8.

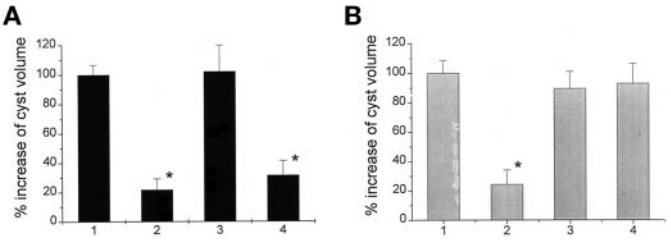

Figure 6. Effect of Cl^- channel blockers. Percent increase of cyst volume measured at day 8 in ADPKD (A) and HRCE (B) cysts. All cysts were cultured in the continuous presence of 50 μM Sp-cAMP starting at day 2. At day 4, the following agents were added: none (1), DPC (2), DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) (3), and glibenclamide (4). Percent increase was calculated according to the following equation: ((cyst volume at day 8)/(cyst volume at day 4) − 1) × 100%. Results are given as mean ± SEM (n = at least 50 cysts in group). *$P < 0.05$ represents a comparison of cyst volume at in the absence of a blocking agent (histogram 1) *versus* cyst volume after 4 d of treatment with the blocking agent.

population of human renal cortical cells is unaffected by cAMP. In contrast, however, we show that both EGF and cAMP activate cell proliferation in ADPKD cells grown in tissue culture and that the effect of both agonists is additive. We also show that EGF-stimulated cell proliferation is not inhibited by cAMP antagonists, whereas cAMP-activated proliferation is inhibited by these agents (Table 1 and 2). Thus, our results indicate that stimulation of proliferation by EGF and cAMP in ADPKD cells occurs via separate signal transduction cascades. Clearly, ADPKD cells have changed their ability to respond to signals that control cell proliferation when compared to a population of cells isolated from the cortex of a normal human kidney.

Cyst Formation

When a mixed population of normal human cells isolated from the renal cortex (HRCE) was cultured in collagen gels, cells formed cysts and secreted fluid into the cyst lumen in the presence of FBS and hormonal supplements (see Materials and Methods) (23,40). Although HRCE cells did form cysts under these conditions, the number of cysts formed was less than that noted for cells isolated from patients with ADPKD. In our experiments, cyst growth in HRCE cells was not dependent on cAMP. These results were consistent with the lack of effect of cAMP on thymidine incorporation and cell proliferation in these HRCE cells.

Although cells isolated from normal kidneys will form cysts albeit with properties different from those isolated from ADPKD patients, cyst formation is not a universal property of renal cells. For example, we showed that purified proximal tubular cells from normal human kidney, RPTEC cells, do not form cysts. Mangoo-Karim *et al.* reported that LLC-PK1 cells, derived from porcine proximal tubules, also do not develop cysts (23). Finally, Neufeld *et al.* conducted a detailed immunohistologic investigation of a mixed population of human cells isolated from the cortex and showed that most cysts grown in collagen gels were of distal tubular origin (40). Given that under our experimental conditions, HRCE cells can form cysts but a purer population of proximal tubule cells, RPTEC cells, cannot, suggests that most of the cysts grown from mixed populations of normal renal tubular cells *in vitro* are probably of distal tubular origin. Why can some cells isolated from normal kidneys form cysts and others cannot? One explanation is that the levels of expression of polycystin 1 or 2 may be altered in some cells in culture allowing them to form cysts.

In contrast to normal cells, cysts established from cells from ADPKD patients grow readily in three-dimensional culture and expand vigorously in the presence of cAMP (Figures 3 and 4). After cysts are formed, they require continued stimulation with hormonal supplements to maintain cyst growth (23,34), and without supplements fluid is actually absorbed (this study and reference (24). This suggests that the hormonal environment within the *in vivo* kidney must be conductive to the formation and growth of cysts. In cells isolated from patients, cAMP stimulates cell proliferation, but *in vivo* the precise mechanism of that stimulation is not understood. Yamaguchi *et al.* measured cAMP concentrations in cystic kidney tissue, urine, and cyst fluid in the murine polycystic kidney disease model, the *pcy* mouse (41). They showed that cAMP production and secretion into urine are increased compared with control animals, indicating that cAMP does play a role in cystic kidney *in vivo*. In normal human kidney, many hormones increase intracellular cAMP concentrations in different nephron segments. Given that cultured ADPKD cells enlarge in response to cAMP, it is feasible that hormones that operate by enhancing cAMP production, such as AVP, parathyroid hormone, and calcitonin, may play a role in enhancing cyst production in ADPKD *in vivo* (42). On the other hand, it is also possible that the enhanced cAMP production noted in cystic kidneys arises through an autocrine process, whereby each cyst stimulates its own growth.

Fluid Secretion

Fluid secretion also plays a key role in cyst formation in ADPKD. A key question is what mechanisms are involved in cyst formation? Cysts form from various nephron segments and are comprised of a heterogeneous population of cells that vary within and among individual cysts. Some cysts, especially those early in their development, may display some structural characteristics of proximal or distal tubule cells. These cysts may still transport solutes, using mechanisms similar to those found in the intact nephron. On the other hand, the majority of the cells within cysts do not have characteristics associated with their origin (6,43–45). These cysts are comprised of a heterogeneous population of cells. CFTR protein and channel activity can be detected in the apical cell membrane of renal cysts (27,30,31). Furthermore, there is some evidence that in patients with both cystic fibrosis and ADPKD, the severity of ADPKD is lessened. This suggests that fluid secretion into cysts may occur via a mechanism similar to that found for other CFTR-containing tissues (46). Although CFTR clearly plays a role, it is only expressed in about half of the cells in some cysts, and other cysts within a kidney do not express CFTR. This suggests that other ion channels and transporters are also

involved (27,31). Clearly, the mechanism and the rate of cyst enlargement may vary greatly among individual cysts, depending on the origin of the cells and the variety of transporters expressed in an individual cyst.

Grantham and colleagues have studied the effect of blockers of ion channels and transporters in ADPKD cells (20,24,34). In our study, we cultured ADPKD and HRCE cysts in the presence of channel blockers in the culture medium for 4 d and evaluated the effect of these agents. Both glibenclamide and DPC inhibited cAMP-activated enlargement of cysts grown from ADPKD cells, whereas only DPC inhibited growth in cysts established from HRCE cells. DIDS did not block cyst enlargement either in HRCE or in ADPKD cysts (Figure 6).

Glibenclamide is a known blocker of ATP-sensitive K^+ channels (35) and can also block CFTR (32,36). Although the presence of CFTR has been established, the existence of ATP-sensitive K^+ channels in ADPKD cells has not been determined. Given the importance of ROMK channels in renal K^+ transport, it is clearly possible that ATP-sensitive K channels, such as ROMK channels, exist and function during fluid secretion in ADPKD cells. Thus, it might be possible that glibenclamide is blocking both ROMK and CFTR and ultimately inhibiting fluid secretion in ADPKD cysts. The compound DPC is a general blocker of several types of Cl^- channels. We show that cyst enlargement is also inhibited by DPC. Because several transport pathways are involved in the formation of cysts and because of the propensity of blockers such as DPC and glibenclamide to block more than one channel, our experiments do not pinpoint the exact characteristics of ion channels present in the cysts. However, the demonstration that blocking transport pathways in cysts inhibits enlargement raises the possibility that this strategy may be a useful treatment for ADPKD patients suffering from renal dysfunction.

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