GENETIC DISORDERS–DEVELOPMENT

Cyclic AMP activates B-Raf and ERK in cyst epithelial cells from autosomal-dominant polycystic kidneys

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Cyclic AMP activates B-Raf and ERK in cyst epithelial cells from autosomal-dominant polycystic kidneys.

Background. The proliferation of mural epithelial cells is a major cause of progressive cyst enlargement in autosomal-dominant polycystic kidney disease (ADPKD). Adenosine 3′, 5′ cyclic monophosphate (cAMP) stimulates the proliferation of cells from ADPKD cysts, but not cells from normal human kidney cortex (HKC), through the activation of protein kinase A (PKA), mitogen-activated protein kinase kinase (MEK), and extracellular signal-regulated kinase (ERK/MAPK). In the current study, we examined the signaling pathway between PKA and MEK in ADPKD and HKC cells.

Methods. Primary cultures of human ADPKD and HKC cells were prepared from nephrectomy specimens. We determined the effects of cAMP and epidermal growth factor (EGF) on the activation of ERK, B-Raf and Raf-1 in ADPKD and HKC cells by immune kinase assay and Western blot.

Results. 8-Br-cAMP increased phosphorylated ERK (2.7- ± 0.6-fold, $N = 7$), and B-Raf kinase activity (3.6- ± 1.1-fold, $N = 5$) in cells from ADPKD kidneys; levels of phosphorylated Raf-1 were not changed. Inhibition of PKA by H89 strikingly decreased cAMP-stimulated phosphorylation of ERK and B-Raf, and MAPK inhibition by PD98059 blocked the effect of the nucleotide to activate ERK. By contrast, in HKC cells 8-Br-cAMP did not activate B-Raf and ERK. EGF stimulated the phosphorylation of ERK and Raf-1 in both ADPKD and HKC cells, but had no effect on B-Raf. 8-Br-cAMP and EGF conjointly increased ERK activation above that of either agonist alone in ADPKD cells, and this combined effect was abolished by PD98059, indicating that ERK was activated by EGF- and cAMP-responsive cascades that converge at MAPK.

Conclusion. cAMP activates ERK and increases proliferation of ADPKD epithelial cells, but not cells from normal human kidney cortex, through the sequential phosphorylation of PKA, B-Raf and MAPK in a pathway separate from, but complementary to, the classical receptor tyrosine kinase cascade. Consequently, cAMP and EGF have great potential to accelerate the progressive enlargement of renal cysts.

Autosomal-dominant polycystic kidney disease (ADPKD) is the most common monogenetic hereditary renal disorder in adults and is caused by the mutation of *PKD1* or *PKD2* genes in most cases [1–3]. Fluid-filled cysts derived from renal tubular epithelia are the hallmarks of ADPKD. Though not widely appreciated, ADPKD is a neoplastic condition. The progressive expansion of kidneys is due to the accelerated proliferation of cells lining cyst cavities created by radial extension of the mural epithelium in conjunction with the accumulation of fluid within the expanding cysts [4]. Although the initiation of cyst formation is controlled by genetic events, the rate of cell proliferation within cysts is modulated by external factors, including epidermal growth factor (EGF), together with hormones and autocoids that activate adenylyl cyclase. Recently, adenosine 3′, 5′ cyclic monophosphate (cAMP), an intracellular mediator of adenylyl cyclase agonists, was shown to stimulate the proliferation of cells derived from the cysts of human polycystic kidneys by activating the extracellular signal-regulated kinase (ERK) [5].

ERK is the last of three serine-threonine kinases that are serially activated in response to extracellular growth-factor stimulation of the small GTPase protein, Ras.

For example, EGF binding to receptor-tyrosine-kinase activates Ras, which, in turn, directly phosphorylates Raf-1 followed by sequential downstream activation of mitogen-acitvated protein kinase kinase (MEK) and ERK. In several types of cells, including those from normal kidneys, cAMP inhibits this receptor-tyrosine-kinase signaling pathway [6–10]. Thus, the mitogenic effect of cAMP we observed in concert with the activation of ERK in ADPKD epithelial cells was unexpected [5]. In as much as the mammalian kidney is a target of numerous hormones (vasopressin, parathyroid hormone, secretin, and vasoactive intestinal polypeptide), and autocoids

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(prostaglandins and adenosine) that stimulate adenylyl cyclase to elevate intracellular levels of cAMP, the molecular mechanisms by which renal epithelial cells switch from a nonmitogenic to a mitogenic response to the nucleotide takes on added significance in a proliferative disorder such as ADPKD.

Cross talk between the cAMP and the MAPK signaling pathways in the regulation of cell proliferation has been explored recently in cells from normal and cancerous tissues [11]. The extent to which cAMP may inhibit or stimulate cell proliferation appears to be highly cell-specific. For example, ERK is activated by cAMP in neuronal cells, whereas phosphorylation and activation of this kinase is inhibited by cAMP in astrocytes [6]. The propensity of cAMP to stimulate or to inhibit ERK activation may depend upon the relative abundance of B-Raf, one of the three Raf proteins that act to transmit extracellular-mediated signals [6–8]. B-Raf has a higher affinity and a stronger stimulation toward MEK than A-Raf or C-Raf (Raf-1). Further in this regard, B-Raf is differentially regulated by protein kinase A (PKA) activation through Rap1 GTPase, which activates B-Raf but not C-Raf [12, 13].

In the current study 8-Br-cAMP, a membrane permeable analog of cAMP, was found to activate kinases ERK and B-Raf in cyst-derived cells, but not in tubule cells from normal human kidney cortex (HKC). Moreover, EGF, which activates receptor tyrosine kinase, and cAMP were found to have complementary effects on the activation of ERK and upon the stimulation of cell proliferation. It appears, therefore, that in renal tubules the phenotypic transformation from a normal to a cyst-forming cell modifies the receptor-tyrosine-kinase and adenylyl cyclase signaling pathways in such a way that their respective activation by different agonists would accelerate the progressive enlargement of polycystic kidneys.

METHODS

Cell culture

Kidney cortex tissue was obtained from viable, although discarded, nephrectomy specimens [ADPKD (five) and normal (three), age 45 to 64 years old of either gender] and placed in primary culture. The detailed methods have been published previously [5, 14, 15]. The protocol for retrieving surgically discarded kidney tissue was approved by the Human Subjects Committee at the University of Kansas Medical Center. Cells from primary cultures were stored in liquid nitrogen until they were thawed and recultured in a flask containing Dulbecco's modified Eagle's medium/Ham 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 Prod-

ucts, Bedford, MA, USA) until the monolayers became approximately 75% to 80% confluent. Cells were released from the plastic with trypsin-ethylenediaminetetraacetic acid (EDTA) solution, centrifuged, and the pellet rinsed twice and resuspended in DMEM/F12 supplemented with 5% FBS, penicillin/streptomycin and ITS.

Lectin staining

ADPKD and HKC monolayers grown in 8-well chamber slides were examined using two distal nephron specific lectins, *Arachis hypogaea* (PNA) and *Dolichos biflorus agglutinin* (DBA), and a proximal tubule lectin, *Tetraglonolobus purpeas* (LTA) [16–20]. The lectins were conjugated to horseradish peroxidase (HRP) and visualized with 3, 3′-diaminobenzidine (DAB). The concentration of each lectin was 50 μg/mL. Specificity of binding was evaluated by preincubating the lectins with (200 mmol/L) galactose (PNA), *N*-acetyl-galactosamine (DBA), or fucose (LTA), respectively.

Intracellular cAMP measurement

Approximately 5×10^4 cells were seeded into individual chambers of a 12-well plate in DMEM/F12 containing 1% FBS, ITS, and penicillin/streptomycin. At 75% to 80% confluence, ITS was deleted from the media and the FBS was reduced to 0.002% to reduce cell growth. After 24 hours, forskolin, desmopressin (DDAVP), prostaglandin E_2 (PGE_2), or EGF were added to the medium for 15 minutes. Forskolin, PGE_2, and EGF were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and DDAVP from Rhone Poulenc Rorer Pharmaceuticals (Collegeville, PA, USA). Intracellular cAMP was extracted into 80% methanol, reconstituted in 0.05 mol/L sodium acetate for quantitation using an enzyme-immunoassay system (Amersham Pharmacia Biotech, Buckinghamshire, UK). In a parallel set of culture plates, cell protein content was measured by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). cAMP content was expressed in picomoles per milligram protein.

Antibodies

Anti-ERK1 (C-16), anti-ERK2 (C-14), anti-phospho-ERK (E-4), anti-B-Raf (C-19) and anti-Raf-1 (C-12) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-phospho-Raf-1 antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Secondary antibodies, antirabbit, antimouse, or antirat immunoglobulin G (IgG)-conjugated HRP antibodies were purchased from Santa Cruz Biotechnology.

Western blot analysis

Cells (10^5) were seeded in 100 mm diameter plastic dishes containing DMEM/F12 with 5% FBS, ITS, and penicillin/streptomycin. At 75% to 80% confluence, ITS

was deleted and FBS was reduced to 0.002% for 24 hours. Then, 8-Br-cAMP (Sigma Chemical Co.) or EGF were added for 15 minutes with or without 30 minutes pretreatment with the PKA inhibitor, H89 (Calbiochem, San Diego, CA, USA), or the MEK inhibitor, PD98059 (New England Biolabs, Beverly, MA, USA). Cells were lysed in 500 μL of ice-cold Tris lysis buffer [TLB; 20 mmol/L Tris (pH 7.4), 137 mmol/L NaCl, 25 mmol/L β-glycerophosphate, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 2 mmol/L Na_2HPO_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]. Insoluble particles in the cell lysate were removed by centrifugation. Aliquots of soluble cellular protein were measured by BCA Protein Assay Kit. Cell lysate (20 μg protein) was then heated (95°C to 100°C) in sodium dodecyl sulfate (SDS) sample buffer, separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose membrane (OPTITRAN™; Midwest Scientific, Valley Park, MO, USA). After transfer, the membranes were blocked with 5% powdered milk in TBS-T, pH 8.0 (20 mmol/L Tris-HCl, 137 mmol/L NaCl, and 0.05% Tween 20) for 1 hour at room temperature or overnight at 4°C. Blocked membranes were incubated with primary antibody in 5% powdered milk in TBS-T for 2 hours at room temperature or overnight at 4°C. Membranes were then washed three times with TBS-T and incubated with secondary antibody with 5% milk in TBS-T for 1 hour. The membranes were washed three times with TBS-T, and proteins visualized using an enhanced chemiluminescence system (ECL; Amersham Life Science, Arlington Heights, IL, USA). Intensity was detected by x-ray film and blots were scanned and quantitatively analyzed by Fluor-S MAX Multi Imager System (Bio-Rad, Hercules, CA, USA).

B-Raf kinase assay

We modified the B-Raf kinase assay method by Erhardt et al [8]. This assay utilizes activated B-Raf, obtained from cells, to phosphorylate exogenous MEK substrate. Soluble cellular extract was obtained as described for the Western blot method. 500 μg of soluble cellular extract were immunoprecipitated for 2 hours with gentle rotation at 4°C with anti-B-Raf antibody covalently coupled to protein A/G PLUS agarose beads (Santa Cruz Biotechnology). Immunoprecipitates were washed and resuspended in 20 μL of [0.5 mmol β-glycerophosphate (pH 7.3), 1.5 mmol ethylene glycol-bis (2-aminoethyl)-N, N, N',N'-tetraacetic acid (EGTA), 1 mmol/L dithiothreitol, 0.03% Brij 35]. The phosphorylation of MEK was performed in reaction mixtures containing 20 μL of kinase reaction solution [16 μL of 50 mmol/L $MgCl_2$, 1μL of 1 mmol ATP (Sigma Chemical Co.), 10 μCi of γ-(^{32}P)-ATP (6000 Ci/mmol, Perkin-Elmer, Bos-

ton, MA, USA) and 0.5 μg of human MEK-1 fusion protein (full-length, Santa Cruz Biotechnology)] at 30°C for 30 minutes and stopped with SDS sample buffer. Samples were heated (95°C to 100°C), beads were sedimented and supernatant separated on 10% SDS-PAGE. Activated B-Raf stimulated phosphorylation of MEK with radioactive (^{32}P) that was detected by x-ray film and quantified by Fluor-S MAX Multi Imager System.

Statistics

Mean and SEM were calculated, and levels of significant difference ($P < 0.05$) were determined by unpaired *t* test.

RESULTS

Origin of cultured cells

The cells used in this study were obtained from the cortex of normal human kidneys and the epithelium lining the cavities of cysts on the surfaces of ADPKD kidneys. Cells from several cysts in a single kidney were pooled together. HKC cells were obtained from cortical tubule fragments of individual kidneys. The collagenase digestion and other preparative steps were identical for ADPKD and HKC.

Cells cultured from both ADPKD and HKC sources were epithelial, as demonstrated previously in our laboratory [5, 15, 21]. Although the primary cultures of confluent HKC and ADPKD cells derive from heterogeneous cells, they establish relatively tight junctional complexes, and monolayers of them develop transmembrane electrical potential differences and actively transport salt and fluid [15, 21–23]. The cells comprising the monolayers were typically pleomorphic (Fig. 1). They stained with PNA and DBA, lectins that bind with high affinity to distal convoluted and collecting tubule cells. A majority of the HKC and ADPKD cells stained with either PNA or DBA (Fig. 1 A and B) and this staining was eliminated with the respective competing sugars, galactose (Fig. 1D) or *N*-acetyl-galactosamine (Fig. 1E). The heterogenous composition of the cultures was confirmed by the staining of a relatively few HKC and ADPKD cells with LTA, a proximal tubule marker (Fig. 1C). This staining was eliminated with the competing sugar, fucose (Fig. 1F).

cAMP levels in ADPKD and HKC cells

cAMP content was measured in cells from three ADPKD kidneys (kidneys ## 136, 138, and 159) and three kidneys from individuals without ADPKD (kidneys ## 129, 144, and 149). A maximally effective concentration of forskolin (10 μmol/L) increased cellular cAMP above control levels in ADPKD and HKC cells (Fig. 2). The difference between the forskolin responses of ADPKD and HKC cells was not significant. The recep-
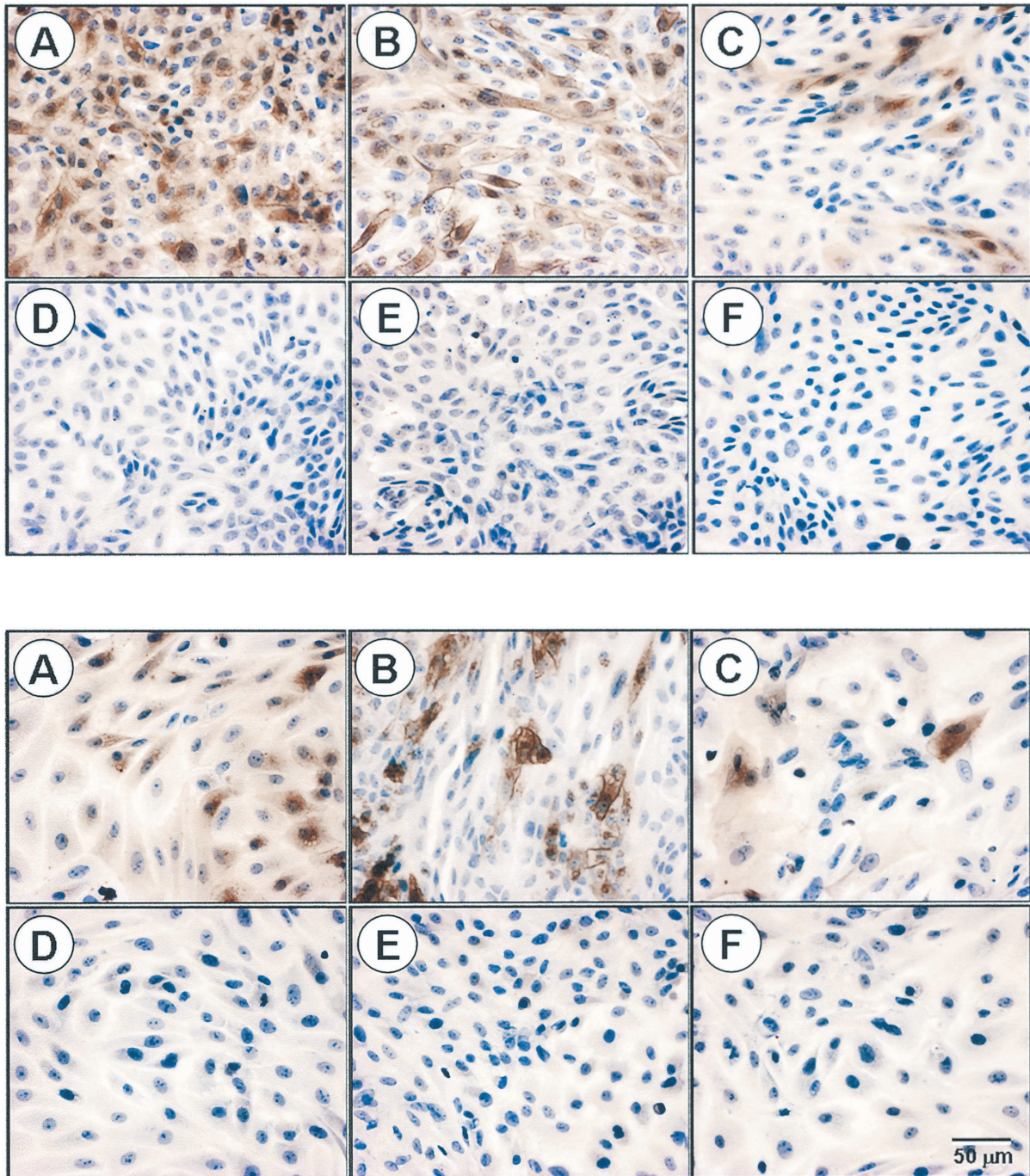
Fig. 1. Lectin staining of cells cultured from normal [human kidney cortex (HKC)] and autosomal-dominant polycycstic kidney disease (ADPKD) kidney cortex. Confluent cultures on Transwell permeable supports. Upper Panel, HKC cells. (*A*) HKC cells stained with *Arachis hypogaea*. (*B*) HKC cells stained with *Dolichos biflorus*. (*C*) HKC cells stained with *Tetragonolobus purpeas*. (*D*) *Arachis hypogaea* plus 200 mmol/L galactose. (*E*) *Dolichos biflorus* plus 200 mmol/L *N*-acetyl galactosamine. (*F*) *Tetragonolobus purpeas* plus 200 mmol/L fucose. Lower Panel, ADPKD cells. (*A*) ADPKD stained with *Arachis hypogaea*. (*B*) ADPKD cells stained with *Dolichos biflorus*. (*C*) ADPKD cells stained with *Tetragonolobus purpeas*. (*D*) *Arachis hypogaea* plus 200 mmol/L galactose. (*E*) *Dolichos biflorus* plus 200 mmol/L *N*-acetyl galactosamine. (*F*) *Tetragonolobus purpeas* plus 200 mmol/L fucose. Magnification marker, 50 μm.
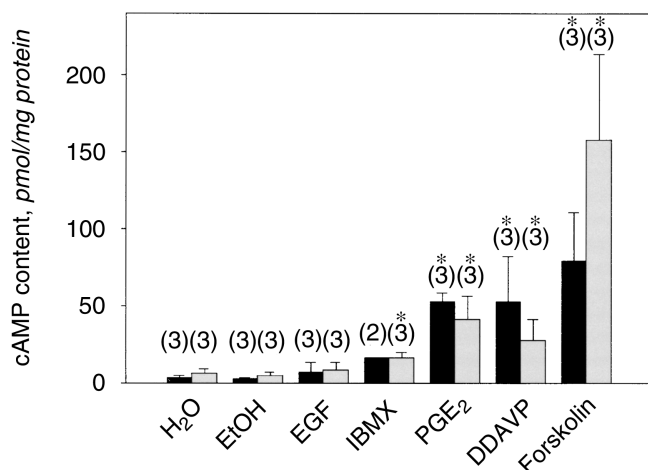
Fig. 2. Adenosine 3′, 5′ cyclic monophosphate (cAMP) levels in autosomal-dominant polycystic kidney disease (ADPKD) (■) and human kidney cortex (HKC) (▨) cells. Cells from three subjects with ADPKD and three subjects with normal kidney cortex (HKC) were placed in minimal growth media for 24 hours, and then agonists were added for 15 minutes [forskolin, desmopressin (DDAVP), prostaglandin E_2 (PGE_2) and epidermal growth factor (EGF) or for 30 minutes [isobutyl-methylxanthine (IBMX)]. cAMP content expressed in picomoles per milligram protein. Optimum concentrations of agonists had been determined in preliminary experiments. Forskolin (10 μmol/L); DDAVP (100 mU/mL); PGE_2 (25 ng/mL); IBMX (100 μmol/L); EGF (25 ng/mL); H_2O, solvent for DDAVP, PGE_2, EGF, and IBMX; and ethanol (EtOH) (0.1%), solvent for forskolin. The number of different kidneys is shown in parenthesis. Bars represent means ± SEM. *Difference from carrier treated cells, $P < 0.05$.
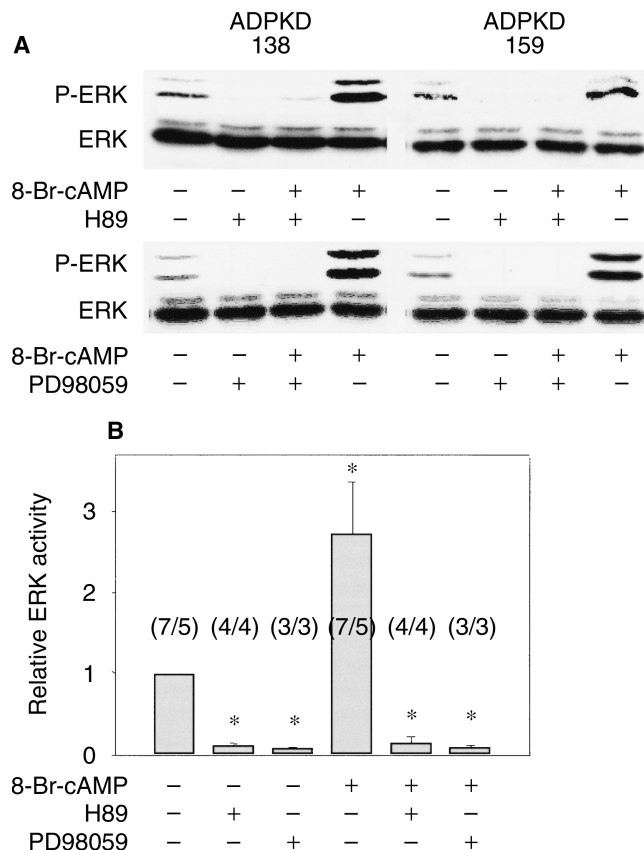

Fig. 3. Effect of 8-Br-cAMP on phosphorylated extracellular-regulated kinase (P-ERK) levels in autosomal-dominant polycystic kidney disease (ADPKD) cells. Cells from ADPKD kidneys were placed in minimal growth media for 24 hours, and then 8-Br-cAMP (100 μmol/L) was added for 15 minutes with or without 30 minutes pretreatment with H89 (10 μmol/L) or PD98059 (50 μmol/L). (*A*) P-ERK intensity and total ERK intensity in cells from two ADPKD kidneys. (*B*) Relative ERK activity (P-ERK intensity/total ERK intensity) compared with control. Numerator in parenthesis is the number of determinations and the denominator is the number of different ADPKD kidneys. Bars represent means ± SEM. *Differences from control, $P < 0.05$.

tor-mediated agonists DDAVP (100 mU/mL) and PGE_2 (25 ng/mL) also increased intracellular cAMP content of ADPKD and HKC cells. Pretreatment with the phosphodiesterase inhibitor, IBMX (100 μmol/L), increased the cAMP content of cells in basal media, and increased further the cAMP levels in response to forskolin, DDAVP, and PGE_2 (data not shown). By contrast, EGF (25 ng/mL) did not significantly increase intracellular cAMP content nor did IBMX potentiate the response (data not shown).

ERK activation by cAMP in ADPKD cells

Utilizing an immune-kinase activity assay, we previously demonstrated in cells from ADPKD kidneys (kidneys ## 136 and 138) that cAMP agonists stimulated cellular proliferation and ERK phosphorylation [5]. In the current study, we quantified the activation of ERK in cells from these and three additional kidneys (kidneys ## 158, 159, and 163) based on the intensity of phosphorylated-ERK (P-ERK) expression in Western blots. Addition of 8-Br-cAMP (100 μmol/L) for 15 minutes confirmed the previous findings [5]. Figure 3A shows representative blots from kidneys ## 138 and 159, whereas Figure 3B shows quantitative analysis from all five ADPKD kidneys in which P-ERK1 and P-ERK2 were determined together. We also quantified the effect of 8-Br-cAMP on the activation of ERK1 and ERK2, individually. P-ERK1/

ERK levels increased 267% ± 62% and P-ERK2/ERK2 levels increased 275% ± 65%, which were not different. Pretreatment with the PKA inhibitor, H89 (10 μmol/L), or the MEK inhibitor, PD98059 (50 μmol/L), completely blocked the effect of 8-Br-cAMP to increase P-ERK levels, indicating that the activation of this kinase was PKA- and MEK-dependent.

It is interesting to note that H89 and PD98059 decreased the basal levels of P-ERK to nearly undetectable levels. This observation suggests that in the control condition the cells may have produced an autocrine factor that stimulated the production of cAMP during the 24-hour period in the growth-arrest medium. Preliminary unpublished data from our laboratory suggest this may be due in part to the accumulation of small amounts of PGE_2 and possibly adenosine, both of which can activate adenylyl cyclase in these cells. On balance, these experi-
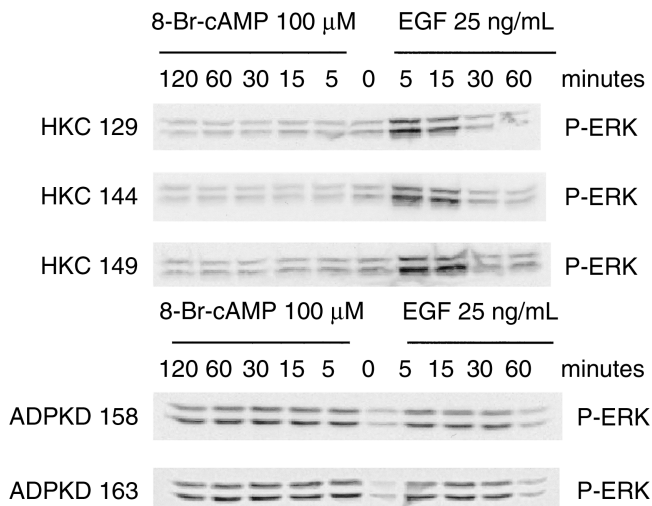
Fig. 4. Time course of 8-Br-cAMP and epidermal growth factor (EGF) effects on phosphorylated extracellular-regulated kinase (P-ERK) expression in human kidney cortex (HKC) and autosomal-dominant polycystic kidney disease (ADPKD) cells. Cells from three HKC and two ADPKD kidneys were placed in minimal growth media for 24 hours, and then 8-Br-cAMP (100 μmol/L) or EGF (25 ng/mL) were added. Levels of activated ERK (P-ERK) were determined by Western blot at specified intervals. EGF transiently increased P-ERK in both HKC and ADPKD. By contrast, 8-Br-cAMP increased P-ERK levels in ADPKD cells for as long as 120 minutes, but had no effect on HKC.

Fig. 5. Effect of 8-Br-cAMP on B-Raf activity in autosomal-dominant polycystic kidney disease (ADPKD) cells. Cells from ADPKD kidneys were placed in minimal growth media for 24 hours and 8-Br-cAMP (100 μmol/L) was added for 15 minutes with or without 30 minutes pretreatment with H89 (10 μmol/L). (*A*) B-Raf-activated (^{32}P)-MEK and total B-Raf intensities in cells from three ADPKD kidneys. (*B*) Relative B-Raf activity (B-Raf-activated (^{32}P)-MEK intensity/total B-Raf intensity) compared with control. Numerator in parenthesis is the number of determinations, and the denominator is the number of different ADPKD kidneys. Bars represent means ± SEM. *Differences from control, $P < 0.05$.

ments show that cAMP activates ERK through PKA and MEK-dependent mechanisms.

Time dependence of P-ERK activation response to cAMP and EGF

Experiments were performed in three HKC and two ADPKD cell lines to determine the time course of ERK phosphorylation induced by cAMP and EGF (Fig. 4). P-ERK levels of ADPKD cells were increased within 5 minutes after adding 8-Br-cAMP to the medium and remained elevated for 120 minutes. On the other hand, P-ERK levels remained low in HKC cells examined over the same length of time.

P-ERK levels of both ADPKD and HKC cells were increased within 5 minutes after adding EGF to the medium, but in contrast to cAMP, P-ERK levels returned to baseline after 60 minutes. These studies rule out the possibility that maximal rates of stimulation may have occurred at different times after adding the agonists to account for the difference responses of ADPKD and HKC cells to cAMP.

Effect of 8-Br-cAMP on B-Raf activation in ADPKD cells

In the classical MAPK/ERK cascade leading to proliferation, activation of Raf-1 by Ras leads to sequential phosphorylation of MEK and ERK [24]. Ordinarily, increased levels of cAMP lead to inhibition of Raf-1,
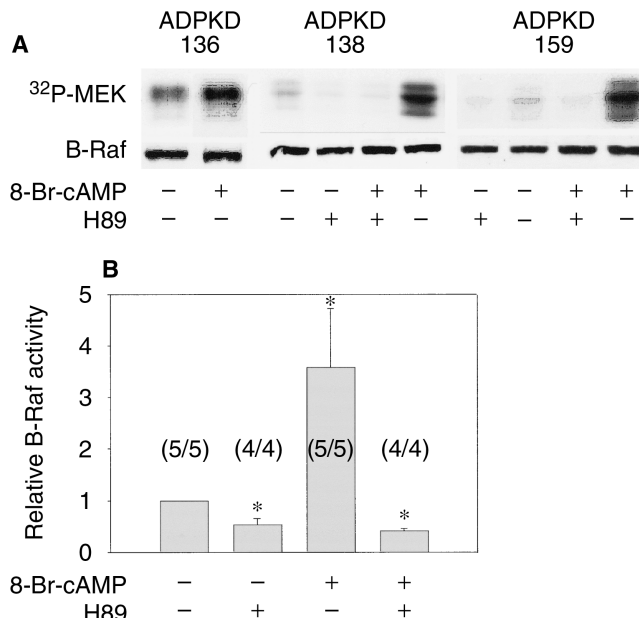
thereby blocking receptor-mediated activation of MEK and ERK. However, recent studies have shown that cAMP can activate MEK and ERK in certain types of cells utilizing a Ras-independent pathway. In these cells PKA leads to the activation of the serine-threonine-kinase B-Raf, which in turn activates MEK and ERK [11]. Thus, in cells enriched in B-Raf PKA may stimulate, rather than inhibit, MEK and ERK. We, therefore, employed an immune complex kinase assay using MEK as a substrate to determine B-Raf kinase activity after the addition of 8-Br-cAMP to the medium for 15 minutes (Fig. 5). In cells from all five ADPKD kidneys (three of them shown in Fig. 5A), 8-Br-cAMP (100 μmol/L) increased B-Raf phosphorylation of MEK substrate 358% ± 114%, $P < 0.05$. In the four kidneys that were tested (kidneys ## 138, 158, 159, and 163), we found that the stimulatory effect on B-Raf activity was completely blocked by pretreatment with 10 μmol/L H89 (Fig. 5). B-Raf kinase activity was clearly increased by 8-Br-cAMP, and its striking inhibition by H89 indicated that the effect of PKA induced by cAMP was a key factor in the activation process. On the basis of these observations, we conclude that the activation of ERK by cAMP is mediated by B-Raf.
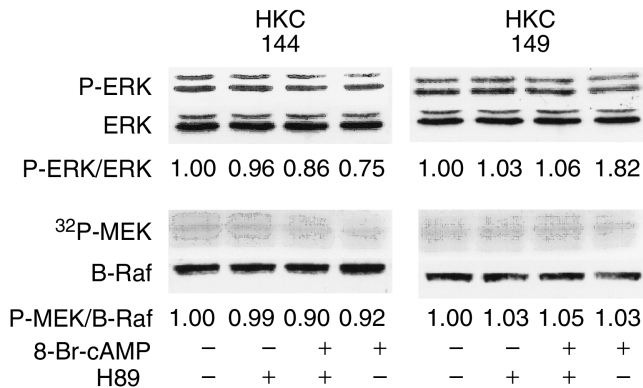
Fig. 6. **Effect of 8-Br-cAMP on phosphorylated extracellular-regulated kinase (P-ERK) and B-Raf activity levels in human kidney cortex (HKC) cells.** Cells from two HKC subjects were placed in minimal growth media for 24 hours and 8-Br-cAMP (100 μmol/L) was added for 15 minutes with or without 30 minutes pretreatment with H89 (10 μmol/L). The extent of ERK activation (P-ERK intensity/total ERK intensity) and B-Raf activation (B-Raf activated (^{32}P)-MEK intensity/ total B-Raf intensity) divided by the control ratio for each kinase is shown beneath each lane.

Fig. 7. **Effect of 8-Br-cAMP on P-Raf-1 and Raf-1 levels in autosomal-dominant polycystic kidney disease (ADPKD) and human kidney cortex (HKC) cells.** Cells from two ADPKD and two HKC kidneys were placed in minimal growth media for 24 hours and 8-Br-cAMP (100 μmol/L) was added for 15 minutes with or without 30 minutes pretreatment with H89 (10 μmol/L). The intensity ratios of Raf-1 (P-Raf-1/ total Raf-1) compared with control are shown beneath each lane.

It should be noted that, as for P-ERK expression (Fig. 3), H89 decreased basal B-Raf activity (Fig. 5), suggesting that B-Raf activity was dependent to some extent upon the endogenous activation of PKA.

Effect of 8-Br-cAMP on B-Raf and ERK activation in noncystic HKC cells

Previously, we found that cAMP did not increase the rate of cell proliferation or increase ERK activity in epithelial cells obtained from normal kidney cortex [5]. In the current study, we used one of the primary cultures from the previous study (kidney # 129) and two additional normal kidney cortex culture preparations (kidneys ## 144 and 149) to explore the effect of cAMP on Raf kinases.

In contrast to ADPKD cells, 8-Br-cAMP (100 μmol/L) did not increase the activity of ERK (88% ± 9% of control) or B-Raf (99% ± 2% of control) in cells from the three HKC kidneys lacking polycystin mutations (two of the blots are shown in Fig. 6). In cells from two subjects (kidneys ## 144 and 149), treatment with H89 (10 μmol/L) had no apparent effect on the baseline activity of either ERK or B-Raf (Fig. 6).

Effect of 8-Br-cAMP on Raf-1 activation in ADPKD and HKC cells

We examined the effect of 8-Br-cAMP (100 μmol/L) on the activation of Raf-1 in both ADPKD and HKC cells. 8-Br-cAMP did not increase P-Raf-1 levels in cells from two ADPKD subjects, although it appeared to slightly decrease the activation of this kinase in cells
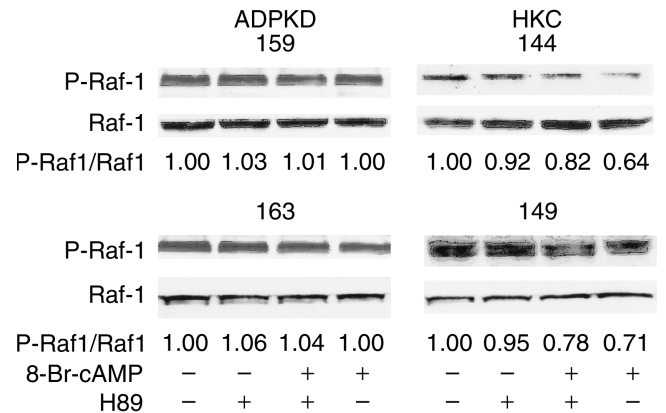
from two normal kidneys (Fig. 7). H89 (10 μmol/L) did not affect the baseline activity of P-Raf-1 in either ADPKD or HKC cells (Fig. 7). These findings indicate that Raf-1 is not activated by PKA in either ADPKD or HKC cells.

EGF effect on Raf-1 and ERK in ADPKD and HKC cells

Previously we showed that EGF stimulated the proliferation of both ADPKD and HKC cells [5]. EGF (25 ng/mL) activated ERK in both types of cells and this effect was blocked by the MEK inhibitor, PD98059. In the current study, EGF increased the expression of P-Raf-1 and P-ERK in cells from two ADPKD and two HKC kidneys, whereas the activity of B-Raf was not increased (Fig. 8A). In HKC cells, 8-Br-cAMP may have diminished the expression of P-Raf-1 slightly (Fig. 8B) but H89 had no effect on EGF-stimulated P-Raf-1 expression (Fig. 8A). Although HKC did not respond to cAMP by activating Raf-1, the cells were normally responsive to activation by EGF. It appears, therefore, that Raf-1, but not B-Raf, is activated by EGF whereas B-Raf, but not Raf-1, is activated by cAMP.

Combined effects of EGF and cAMP on ERK activity

When forskolin, which activates adenylyl cyclase, and EGF were added conjointly in our previous study, the rate of ADPKD cell proliferation increased greater than when either agonist was added alone [5]. By contrast, forskolin diminished the effect of EGF to stimulate proliferation in HKC cells. In the current study, we examined the effect of 8-Br-cAMP and EGF on P-ERK expression in comparison to the effects of each agonist alone. In ADPKD cells, EGF and 8-Br-cAMP increased
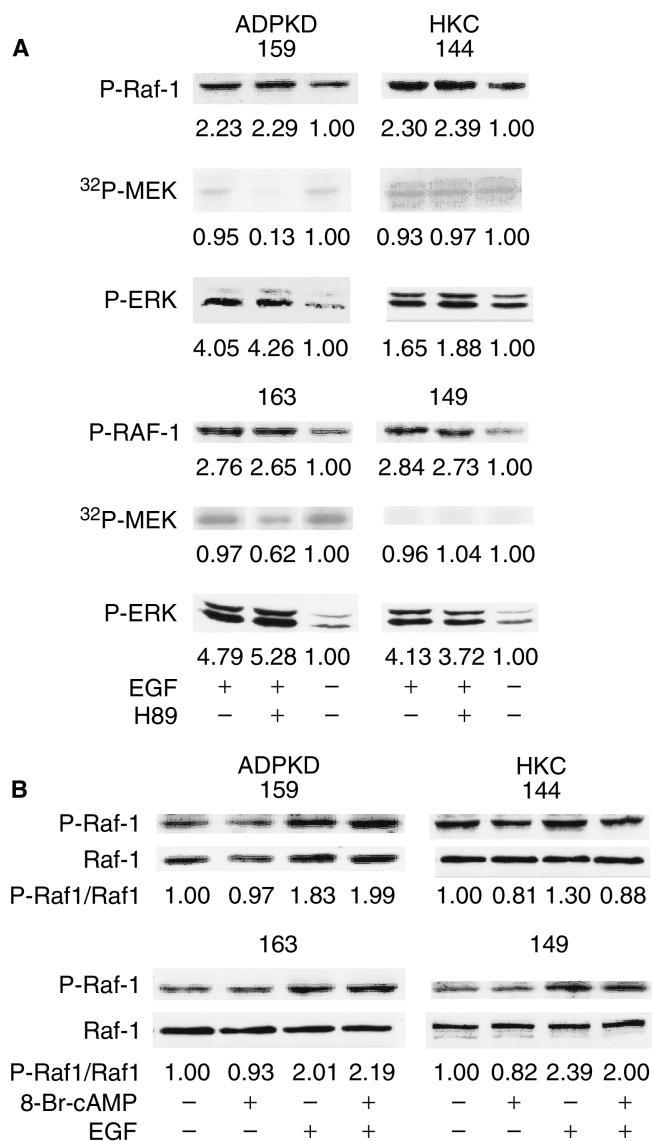
Fig. 8. Effect of epidermal growth factor (EGF) on Raf-1, B-Raf and extracellular-regulated kinase (ERK) activity levels in autosomal-dominant polycystic kidney disease (ADPKD) and human kidney cortex (HKC) cells. (*A*) Cells from two ADPKD and two normal HKC were placed in minimal growth media for 24 hours and EGF (25 ng/mL) was added for 15 minutes with or without 30 minutes pretreatment with H89 (10 μmol/L). P-Raf-1/Raf-1 intensity-, B-Raf-activated (^{32}P)-MEK intensity/B-Raf- and P-ERK/ERK intensity ratios compared with control are shown beneath each lane. (*B*) Cells from two ADPKD and two normal HKC were placed in minimal growth media for 24 hours and 8-Br-cAMP (100 μmol/L) or EGF (25 ng/mL) were added for 15 minutes. The intensity ratios of Raf-1 (P-Raf-1/total Raf-1) compared with control are shown beneath each lane.

P-ERK levels above that of either agonist alone (Fig. 9 A and B). PD98059 completely blocked the effect of 8-Br-cAMP or EGF when administrated separately or in combination. On the other hand, H89 had no effect on the activation of ERK by EGF, although the PKA inhibitor diminished P-ERK expression when EGF and 8-Br-cAMP were added conjointly (Fig. 9 C and D). These

results are interpreted to indicate that the adenylyl cyclase and the receptor-tyrosine-kinase pathways converge at MEK to activate ERK. By contrast, in HKC cells the activation of ERK by EGF was not accentuated by 8-Br-cAMP; rather, the nucleotide decreased the expression of P-ERK to a small extent (Fig. 9 E and F).

DISCUSSION

Renal tubule epithelial cells utilize intracellular cAMP, generated by a host of endocrine and autocrine agonists, to regulate an array of physiologic and pathophysiologic functions. Under ordinary circumstances, the day-to-day generation of renal epithelial cAMP is directed primarily to the regulation of water and solute transport, there being no effect of the nucleotide to stimulate proliferation in these normally quiescent cells [9, 10] (Fig. 10B). By contrast, in cyst-derived cells bearing a mutated polycystin gene, cAMP is clearly mitogenic, an aberrant behavior that appears to depend on an alteration in B-Raf signaling [5, 25] (Fig. 10A).

In each ADPKD kidney used in this study, several cortical cysts were used and presumably contained epithelial cells that harbored both germ-line and somatic mutations in polycystin-1 or polycystin-2 [26, 27]. By contrast, it is reasonable to assume that cells derived from normal kidneys had normal complements of polycystins. Normal human cortex cells (HKC) may have derived from proximal, distal and collecting tubules, and glomeruli. Thus, is it possible that the qualitative differences in proliferation [5, 25] and ERK activation [5] responses to cAMP of HKC and ADPKD cells could be reflections of different types of progenitor cells that may have been more abundant in one culture than in the other. To address this issue we examined HKC and ADPKD cells with lectin markers that preferentially stain distal convoluted and collecting tubules, or proximal tubule cells [16–20]. A majority of the cells in the HKC and ADPKD cultures stained with distal nephron markers PNA and DBA (Fig. 1 A and B) suggesting that a significant fraction of the cells in the respective cultures may have arisen from distal convoluted and collecting tubules. Relatively few cells stained with the proximal tubule lectin marker, LTA (Fig. 1C). While these studies do not prove that cultures of HKC and ADPKD cells derive exclusively from distal nephron segments, they establish that the cultures are similar in respect to their lectin-binding profiles. Thus, it seems unlikely that the clear-cut qualitative differences in the proliferation and ERK-activation responses of HKC and ADPKD cells to cAMP can be ascribed to the predominance of one progenitor cell type over another. Rather, this phenotypic difference is most likely due to the fact that HKC cultures were derived from normal renal epi-
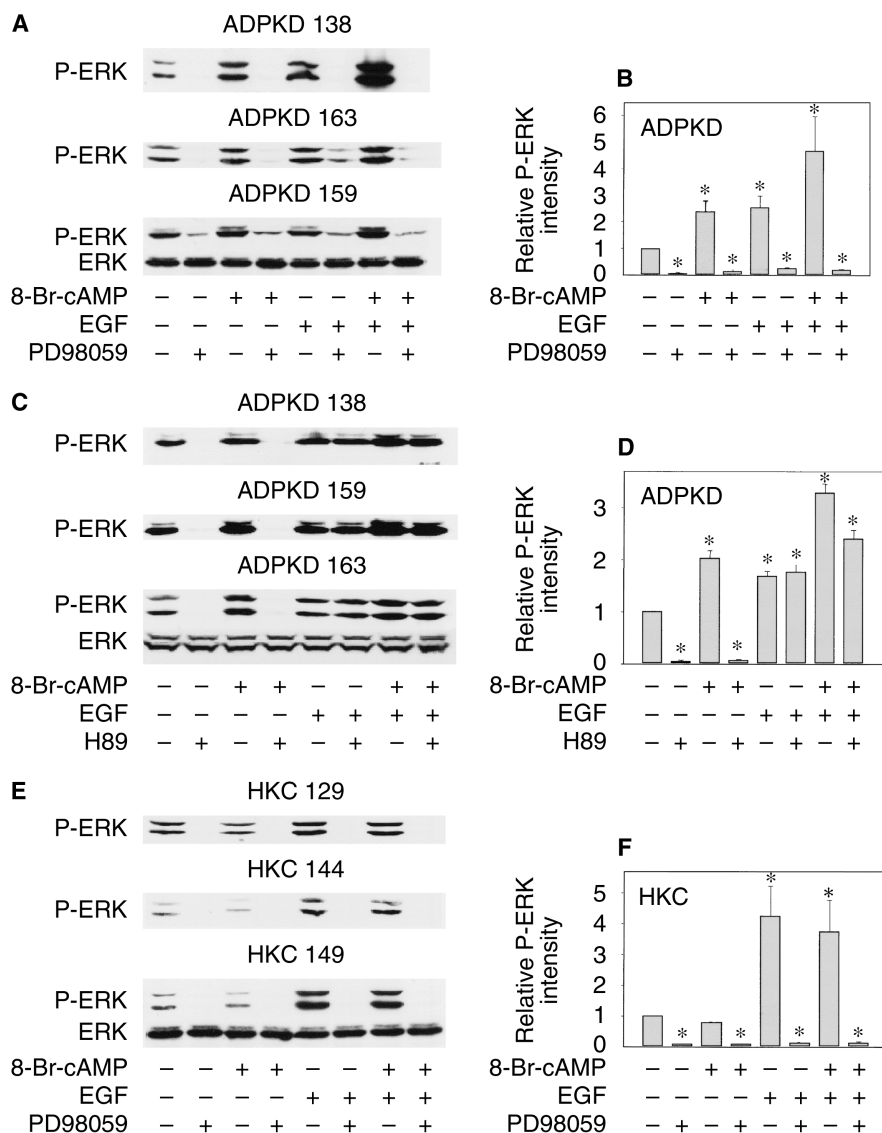
Fig. 9. Combined effect of epidermal growth factor (EGF) and adenosine 3′, 5′ cyclic monophosphate (cAMP) on phosphorylated extracellular-regulated kinase (P-ERK) expression in autosomal-dominant polycystic kidney disease (ADPKD) and human kidney cortex (HKC) cells. Cells from ADPKD and normal HKC were placed in minimal growth media for 24 hours and 8-Br-cAMP (100 μmol/L) or EGF (25 ng/mL) were added for 15 minutes with or without 30 minutes pretreatment with PD98059 (50 μmol/L), or H89 (10 μmol/L; used only in ADPKD cells). (*A*) Western blot of P-ERK expression in cells from three ADPKD kidneys and total ERK in cells from one ADPKD kidney. (*B*) Relative P-ERK intensity compared with control. (*C*) Western blot of P-ERK in cells from three ADPKD kidneys and total ERK in one kidney. 8-Br-cAMP and EGF were added individually and conjointly with or without H89 pretreatment. (*D*) Relative P-ERK activity compared to control. (*E*) Western blot of P-ERK in cells from three HKC kidneys and total ERK in cells from one HKC kidney. (*F*) Relative P-ERK intensity compared with control. Bars represent means ± SEM. *Differences from control, *P* < 0.05.

thelial cells and ADPKD cultures from mural cells lining cysts.

It had previously been shown that cells in intact human ADPKD cysts react with distal nephron lectin markers [17] as do cells cultured from normal human renal cortex (HKC) [21]. Studies of electrolyte transport in polarized monolayer cultures of ADPKD and HKC cells prepared in our laboratory indicate they are comprised of relatively "tight" epithelia that have the capacity to generate substantial transepithelial electric potentials and short-circuit currents [22, 23]. Net chloride secretion was stimulated by cAMP in both cell lines, and, as shown in the current study, HKC and ADPKD cultures generated cAMP in response to several physiologic agonists. Recent studies have established that cAMP stimulates chloride and net fluid secretion in monolayers of cells cultured from normal rat or human inner medullary

collecting ducts [18, 28]. Thus, the primary cultures established from cortical nephrons and cysts exhibit transport processes indistinguishable from those found in collecting ducts of the renal medulla, findings that further support the view that the similar transport mechanisms observed in the HKC and ADPKD monolayers reflect dominance by cells with a common functional phenotype.

ERK was activated by EGF in both ADPKD and HKC cells, whereas cAMP activated ERK only in ADPKD cells (Figs. 3 to 6, 8, 9). Thus, the consistency of the mitogenic and ERK-activating responses to cAMP in numerous ADPKD cell preparations and the lack of an effect in HKC cells in this and preceding studies suggests that the response to cAMP is most likely related to changes imposed by cystic transformation, which affects all of the cells in the ADPKD cultures, rather than
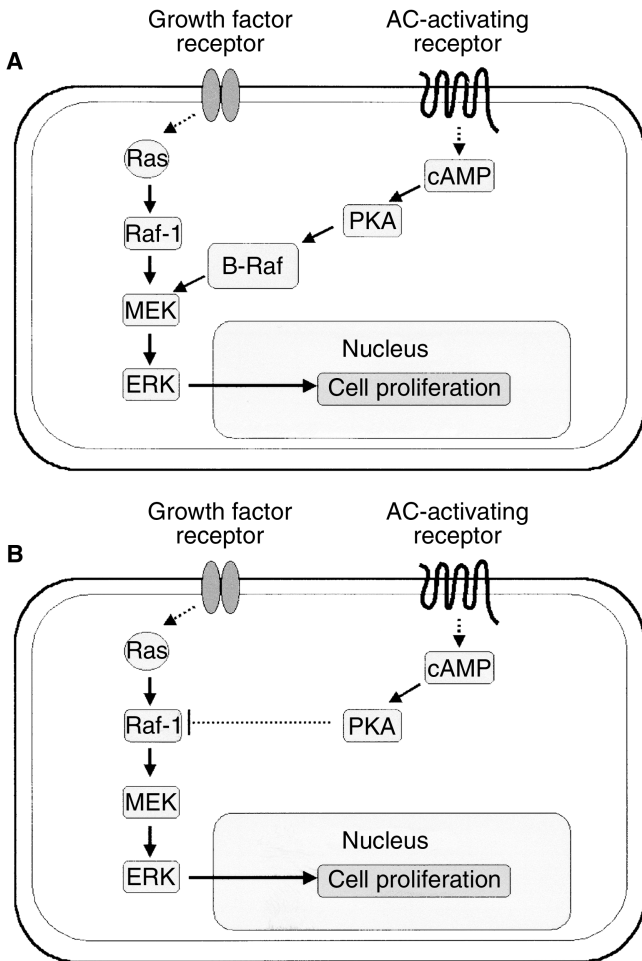
Fig. 10. Signaling pathways in autosomal-dominant polycystic kidney disease (ADPKD) and human kidney cortex (HKC) cells emphasizing differences at the point of B-Raf and Raf-1 activation. (*A*) ADPKD. Adenylyl cyclase (AC) is stimulated by extracellular ligands, increasing the intracellular concentration of adenosine 3′,5′ cyclic monophosphate (cAMP) and the activation of protein kinase A (PKA). PKA, probably through some intermediary steps remaining to be defined, activates B-Raf, and thence MEK and extracellular-regulated kinase (ERK). Epidermal growth factor (EGF) activates receptor-tyrosine-kinase, Ras, and Raf-1 to also phosphorylate MEK and ERK. (*B*) Normal renal tubule cells. EGF activates Ras, Raf-1, MEK and ERK, as in ADPKD cells, only in this case PKA inhibits EGF signaling to ERK and diminishes cellular proliferation in response to growth factor stimulation.

selection of subpopulations of responsive or nonresponsive cells. This view is supported further by our finding that the response of an immortalized culture of murine cortical collecting duct cells to cAMP could be switched from a negative to a positive proliferative response to cAMP by altering the function of polycystin-1 [29].

The molecular basis of this phenotypic difference between normal and polycystic kidney cells is brought into clearer focus by the current study. The capacity of cAMP to provoke mitogenesis appeared to reside in the abnormal susceptibility of B-Raf to activation (Figs. 5 and 6). cAMP led to the activation of B-Raf and phosphoryla-

tion of MEK in ADPKD cells (Fig. 5), but there was no discernible effect on the levels of phosphorylated Raf-1 (Figs. 7 and 8B). Since PD98059, a specific MEK inhibitor, blocked the activation of ERK and decreased the rate of cellular proliferation caused by cAMP [5], it seems reasonable to conclude that B-Raf promotes MEK activation in response to the cyclic nucleotide. The activation of ERK by B-Raf was completely inhibited by the PKA inhibitor, H89, thus cAMP stimulates B-Raf through the intermediate effect of activated PKA. Far less likely is a role for PKA-independent mechanisms in which cAMP directly activates Rap-1 or B-Raf via an adaptor-like molecule [30].

It is interesting to consider that in the cAMP to ERK pathway, small G proteins, such as Rap-1, may be interposed between PKA and B-Raf. In many cells Rap-1 activates B-Raf through the intermediate action of Src [31]. We have detected Rap-1 in ADPKD cells by Western blot in preliminary studies and we have found in preliminary studies that Src is activated by cAMP (T. Yamaguchi, unpublished observations, 2002). Additional studies are needed to further implicate these and other potential intermediate proteins that may lie between the activation of PKA by cAMP and the phosphorylation of B-Raf.

We have confirmed that EGF activation of the classical receptor-tyrosine-kinase pathway leads to strong phosphorylation of Raf-1 and ERK in both ADPKD and HKC cells (Fig. 8). Thus the proximal limb of the receptor-tyrosine-kinase pathway extending to Raf-1 appears to operate in a similar mode in both HKC and ADPKD cells (Fig. 10). On the other hand, the Raf-1 and B-Raf kinases of normal and ADPKD cells appear to differ to a remarkable extent in the nature of their interactions with PKA. cAMP did not increase the expression of P-Raf-1 in HKC and ADPKD cells (Fig. 7), although the nucleotide led to strong activation of B-Raf in ADPKD, but not in HKC cells (Figs. 5 and 6). Interestingly, EGF did not activate B-Raf kinase in either ADPKD or HKC cells, suggesting that the pathway to ERK from Ras through Raf-1 and MEK is reserved for agonists linked to receptor-tyrosine-kinase activation (Fig. 10). Thus, our studies indicate that EGF activates ERK through the sequential stimulation of Ras, Raf-1 and MEK, whereas PKA activates ERK through its capacity to activate B-Raf and MEK (Fig. 10).

Yet this does not completely explain the complementary actions of EGF and cAMP on ERK activation and the relatively high rate of cell proliferation in ADPKD in contrast to normal cells [5] (Figs. 9 and 10). The simultaneous addition of EGF and 8-Br-cAMP led to a striking increase in ERK activation, greater than either agonist alone (Fig. 9 A to D). Thus, in contrast to HKC cells in which cAMP diminished the activation of Raf-1 and ERK, in ADPKD cells the nucleotide did not inhibit the activation of Raf-1 by Ras. Thus, the phenotypic

response of ADPKD cells to cAMP reflects an increase in the sensitivity of B-Raf to activation by PKA as well as relief from the inhibition of Raf-1 (Fig. 10).

How the Raf-1/B-Raf functional response to cAMP is switched in the conversion of normal epithelial cells to abnormal cyst cells remains a mystery. It is tempting to speculate that there may be fundamental changes in the structure or the function of B-Raf. This kinase has two sites that are phosphorylated by PKA. At one site PKA activates B-Raf to phosphorylate MEK and at the other PKA inhibits B-Raf activation [12, 13, 32]. Access to the inhibitory site may be controlled by 14-3-3, a ubiquitous protein that appears to bind to the PKA-inhibitory site in B-Raf, leaving the PKA activation site unopposed [32]. Thus, the absolute abundances of B-Raf and 14-3-3, and the degree to which they interact could determine how extensively B-Raf is activated by PKA [6, 8].

The role of intracellular Ca^{++} levels in B-Raf activation is also of considerable interest. Polycystin–2, a non-selective cation channel that is highly permeable to Ca^{++} could be abnormally regulated in cyst epithelial cells [33–37]. Dysfunctional polycystin-2 could lead to transient or steady-state changes in cell Ca^{++} levels and secondary effect on kinases and phosphatases within the cells. In this regard, we have observed that calcium channel blockers (nifedipine, verapamil, gadolinium) will convert M-1 (cortical collecting tubule) cells from a basal state in which cAMP inhibits proliferation and P-ERK expression, to one in which the nucleotide stimulates proliferation and increased P-ERK expression (absract; Yamaguchi T, et al, *J Am Soc Nephrol* 12:549A, 2001).

In summary (Fig. 10), the capacity of cAMP to stimulate cell proliferation in ADPKD cyst epithelial cells is mediated through the combined interaction of the adenylyl cyclase and MAPK signaling pathways. In ADPKD but not in HKC cells, cAMP activates PKA thence B-Raf, MEK, and ERK. The classical receptor-tyrosine-kinase pathway appears to operate as usual in ADPKD cells except that after its stimulation by EGF, cAMP does not inhibit the downstream Ras–Raf-1 step as it ormally does. The parallel operation of these two classic signaling processes in a positive mode renders renal cysts unusually vulnerable to ordinary physiologic stimuli mediated by arginine vasopressin (AVP), secretin, parathyroid hormone (PTH), β-agonists, PGE₂, and EGF-like proteins and growth factors. The adenylyl cyclase pathway, in particular, has the potential to stimulate cyst enlargement in two different ways: (*1*) by stimulating the proliferation of cyst mural epithelial cells and thereby creating more potential space for the accumulation of fluid, and (*2*) by stimulating the secretion of solutes and fluid into that potential cavity created by accelerated cellular proliferation. Pharmacologic interruption of adenylyl cyclase and receptor-tyrosine-kinase pathways at strategic sites may prove to be useful measures to diminish the rate at which cysts enlarge in ADPKD.

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