cAMP-dependent protein kinase and proliferation differ in normal and polycystic kidney epithelia

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Marfella-Scivittaro, Carmela, Andrea Quiñones, and Stephanie A. Orellana. cAMP-dependent protein kinase and proliferation differ in normal and polycystic kidney epithelia. Am J Physiol Cell Physiol 282: C693–C707, 2002. First published October 17, 2001; 10.1152/ajpcell.00122.2001.—Developmental control of cell proliferation is crucial, and abnormal principal cell proliferation may contribute to cystogenesis in polycystic kidney disease. This study investigates roles of cAMP and its primary effector, cAMP-dependent protein kinase (protein kinase A; PKA), in control of cell proliferation in filter-grown nongenecytic (NC) and cystic (CY)-derived principal cell cultures. These cultures had similar cAMP pathway characteristics upstream of PKA subunit distribution but differed in predicted PKA subtype distribution. Functionally, cultures were proliferative before polarization, with constitutively higher proliferation in CY cultures. NC cultures achieved levels similar to those of CY cultures on pharmacological manipulation of cAMP production or PKA activation or inhibition of PKA subtype I activity. Inhibition of overall PKA activity, or of PKA subtype II anchoring, diminished cAMP/PKA-mediated proliferation in NC cultures but had no effect on CY cultures. Polarized CY monolayers remained proliferative, but NC monolayers lost responsiveness. No large proliferation changes resulted from treatments of polarized cultures; however, polarized NC and CY cultures differed in poststimulation handling of PKA catalytic and type IIα regulatory subunits. Our results support PKA subtype regulation of prepolarization proliferation in NC principal cells and altered regulation of PKA in CY cells and suggest that differences at or downstream of PKA can contribute to altered proliferation in a developmental renal disease.

epidermal growth factor receptor; polycystic kidney disease; protein kinase subtypes; immunolocalization

CELL PROLIFERATION is a normal physiological component of development. In the kidney increased proliferation can result in cancers (reviewed in Ref. 40) or cysts (reviewed in Ref. 39), whereas decreased proliferation can result in loss or diminished size of renal structures (2). Study of disease models with abnormal cell proliferation can help to define specific regulatory mechanisms by identifying differences from normal. The intracellular signaling molecule cAMP has been implicated in regulation of proliferation and differentiation in many cell types (reviewed in Ref. 64). Effects are either mediated by cAMP-dependent protein kinase (protein kinase A, PKA; Refs. 4, 12, 15, 26, 44) or are independent of PKA and involve small guanine nucleotide binding (G) proteins (46, 56, 67). Both positive and negative modulation has been reported, which appeared to depend on cell type, culture conditions, or the presence of other stimuli.

In addition to roles in regulating renal fluid and ion transport, cAMP and/or PKA modulate proliferation in cell cultures derived from throughout the kidney: human proximal tubular cells (24, 28); proximal tubular epithelial cell lines MCT (71), LLC-PK1 (5, 71), and PKSV-PCT (68); glomerular mesangial cells (7, 33); Madin-Darby canine kidney (MDCK) cell lines (21, 24, 72, 73); immortalized human kidney epithelial cell line IHKE-1 (3); human embryonic kidney cell line HEK293 (56); and renal cancer cells (8). Because cAMP can induce proliferation and formation of renal epithelial cysts in vitro (21), and abnormally increased principal cell proliferation is a factor in collecting duct cyst formation and expansion in a developmental renal disease, autosomal recessive polycystic kidney disease (ARPKD) (39), cAMP could play a role in cell proliferation in normal developing principal cell epithelium. Earlier ureteric bud and collecting duct proliferation is regulated by bone morphogenetic protein (BMP)/7 (47), but a later developmental switch to collecting duct cyst formation and progressive expansion occurs in ARPKD (39).

The inherited polycystic kidney diseases (PKDs) autosomal dominant PKD (ADPKD) and ARPKD make up the most common life-threatening genetic disease family (reviewed in Ref. 49). The family is characterized by progressive fluid accumulation in renal tubules to form cysts that eventually lead to renal failure. Genes responsible for ADPKD1 and ADPKD2 subtypes, and a gene associated with murine ARPKD in orpk mice, were identified (reviewed in Refs. 35, 36, 49).
and 69). Their gene products (polycystin-1, polycystin-2, and polars) may form a membrane-associated signaling complex involved in nephrogenesis, and mutations may contribute to PKD by altering the complex's function (reviewed in Refs. 18 and 35). Positional cloning studies identified chromosomal loci linked to ARPKD and several murine ARPKD models (reviewed in Refs. 39 and 40), but specific genes for these PKDs have not been found. All known PKD genes and genetic loci are on distinct chromosomes, and cellular mechanisms for initiation and progression of cystic disease are still unclear.

All PKDs have a consistent molecular phenotype of abnormal overexpression of epidermal growth factor (EGF) receptor (EGFR) on apical surfaces of cystic collecting tubular (CT) principal cell epithelium, whereas noncystic receptor expression is basolateral (reviewed in Ref. 61). EGFR activation promotes increased CT mitogenesis, cyst formation, and progressive cystic enlargement that is inhibited by blocking EGF-activated EGFR tyrosine kinase activity (48, 53, 61, 63). In addition to characteristic altered fluid transport and abnormal extracellular matrix, EGFR-mediated cell proliferation is very likely a mechanism for cyst formation and expansion in PKD (reviewed in Refs. 39 and 60). However, because the EGFR phenotype occurs downstream from several different primary gene mutations, other signal transduction systems present in principal cells could also modulate abnormal cell proliferation.

In classic cAMP signaling, a receptor family exerts its effects by activating an intracellular signaling cascade of heterotrimeric G proteins, adenylyl cyclase (AC), cAMP synthesis, and cAMP-dependent protein kinase (PKA) activation. The PKA is a tetrameric holoenzyme of two cAMP-binding regulatory (R) and two catalytic (C) subunits. Holoenzymes with different pairs of R subunit isoforms (RI or RII) are classified as type I and type II PKA subtypes, respectively (reviewed in Ref. 19). Activation is accomplished when cAMP binding to R occurs (reviewed in Refs. 19 and 23). Activated PKA is believed to move throughout the cell as free C subunits, although holoenzyme may be capable of activation without dissociation (76). Activation mediates serine or threonine phosphorylation, an effect reversible by specific protein phosphatases. Phosphodiesterases (PDEs) degrade cAMP (14) and promote the return of PKA to its inactive, tetrameric state. Any C subunits sent to the nucleus on activation are inhibited and shuttled out by the endogenous PKA-specific protein kinase inhibitor (PKI) (reviewed in Ref. 19). Subcellular distribution of PKA holoenzyme is mediated partly by A kinase anchoring proteins (AKAPs) (Refs. 6 and 75; reviewed in Ref. 11) that bind R subunits and allow assembly of signaling complexes (57, 70). Therefore, R binding to AKAPs may regulate subtype activity distribution. Isoforms of each cAMP pathway component have been identified, and their diverse expression patterns probably contribute to the range of cellular functions credited to cAMP (reviewed in Refs. 19 and 23).

Exactly how these isoforms act to regulate cellular function is a central question in cAMP research. A physiological model for investigating the role of PKA subtypes in cell proliferation can be found in the abnormal cell proliferation of cancer. Predominant type II activity is found in normal, nonproliferating tissues and in growth-arrested cells, whereas type I activity predominates in tumor cells (reviewed in Ref. 9). Human mammary epithelial cells grow in serum-free medium when they overexpress transfected RIIα (66). Specific inhibition of type I activity by cAMP analogs 8-chloro-cAMP (8-ClcAMP) (38, 54) and the Rp diastereomer of adenosine 3′,5′-cyclic monophosphothioate (Rp-cAMPS; Ref. 20) inhibits growth in human cancer cell lines, and EGFR and type I PKA inhibition cooperatively limits proliferation (reviewed in Ref. 9). Therefore, coordinate cAMP/EGFR regulation of cancer cell proliferation may occur. PKD is another physiological model in which coordinate cAMP/EGFR regulation of abnormal cell proliferation may occur. Mechanistic similarities between cyst formation and the development of cancerous tumors could exist, because increased CT cell proliferation and altered extracellular matrix properties might be expected to result in tumors in the absence of the altered fluid transport required for cyst formation.

cAMP pathway regulation of epithelial cyst formation was demonstrated in three-dimensional collagen gel culture, where MDCK cells form spherical cysts and enlarge after stimulation with cAMP analogs (21, 30, 31, 34). MDCK cell clones with comparable capacities for cAMP production despite differences in responsiveness to cAMP agonists have unique distributions of RIIα and RIIβ subunits that probably reflect PKA anchoring differences. This is likely to allow PKA subtype regulation of the clones' distinct tubule and cyst morphologies (41, 42).

Does cAMP regulate proliferation in normal and ARPKD principal cells? Because the cellular balance of PKA type I and type II activities, their subcellular locations, their preferred targets, and their ultimate functional effects may vary depending on the cell's unique complement of R and C subunits, the present study was undertaken to characterize cAMP signaling and PKA expression in normal murine principal cells and in cells derived from a murine model of ARPKD.

**EXPERIMENTAL PROCEDURES**

**Cell isolation and culture.** An animal model of ARPKD, the C57BL/6j(pkpk) mouse (55), arose from a spontaneous mutation in the C57BL/6j(C57+/C57) strain. The murine disease phenotype and its progression have been described extensively, and it has been shown to mimic the stages and tubular profile of the human disease ARPKD (reviewed in Refs. 1 and 60). As previously described (43), cystic cplk mice were killed to obtain kidney tissue at postnatal days 8–14. Collecting tubular epithelial cells were isolated from cystic kidneys with a selective enrichment method that resulted in an isolated cell population comprised predominantly of principal cells (60). The enriched population of principal cells was cultured and passaged six times without loss of the apical EGFR phenotype. Control, noncystic cells
were obtained in the same way from C57 kidneys at corresponding ages.

Cell cultures were maintained as previously described (60). Stock cultures were seeded on 10-cm plastic culture plates (Corning Costar, Cambridge, MA) at \(1.2 \times 10^4\) cells/cm\(^2\) and were passaged every 7 days. For experiments, cells were seeded at \(1.2 \times 10^4\) cells/cm\(^2\) on 0.4-µm pore Transwell-Clear filter inserts (no. 3460, Corning Costar) unless otherwise noted. In defined growth medium (60), cells formed confluent monolayers by 1–2 wk. Conditions for preparing subconfluent and confluent filter-grown cultures were standardized by consistent seeding and growth conditions, followed by visual inspection with a standard phase-contrast microscope and correlation with ZO-1 immunostaining to determine when polarized monolayers were established (see Fig. 1). The physiological tightness of the confluent monolayers under these growth conditions was established previously by measuring \(^{3}H\)-labeled inulin leakage (60). Medium changes for experiments are described individually.

**Immunological reagents.** Primary antibodies used were anti-EGFR-catalytic subunit-α (sc-903), a rabbit IgG against human Ca (Santa Cruz Biotechnology, Santa Cruz, CA); anti-PKA-regulatory subunit type I (P19920), a mouse IgG against mouse RI (Transduction Laboratories, Lexington, KY); anti-PKA-regulatory subunit type IIα (sc-909), a rabbit IgG against mouse RIIa (Santa Cruz Biotechnology); anti-EGFR (sc-003), a rabbit IgG against human EGFR (Santa Cruz Biotechnology); and anti-ZO-1 (61–7300), a rabbit IgG against human ZO-1 (Zymed Laboratories, San Francisco, CA). The fluorescence-tagged secondary antibodies used were Oregon green 488 goat anti-mouse IgG conjugate (O-6380, Molecular Probes, Eugene, OR), Oregon green 488 goat anti-rabbit IgG conjugate (T-6391, Molecular Probes), Texas red-X goat anti-mouse IgG conjugate (T-6390, Molecular Probes), and Texas red-X goat anti-rabbit IgG conjugate (T-6391, Molecular Probes). Peroxidase-linked secondary antibodies used were goat IgG against rabbit IgG (sc-2005, Santa Cruz Biotechnology), goat IgG against mouse IgG (sc-2004, Santa Cruz Biotechnology), and mouse IgG against biotin (200-032-096, Jackson Immunoresearch Laboratories, West Grove, PA).

**Western blot analysis of protein expression.** To characterize protein expression in cultured cells, samples grown on filter inserts were harvested and 10 µg of protein (unless otherwise noted in individual figures) from each sample was subjected to SDS-PAGE, immunoblotting, and signal detection as previously described (41). Biotinylated ECL protein molecular weight markers (RPN2107; Amersham Pharmacia Biotech, Piscataway, NJ) were included in each gel and were detected by immunoblotting with anti-biotin 200-032-096 at 1:40,000. Primary antibodies used were anti-Ca sc-903 at 1:2000, anti-RI P19920 at 1:25, anti-RII sc-909 at 1:500, and anti-EGFR sc-003 at 1:500. Peroxidase-linked secondary antibodies were used at 1:500–2,000 dilutions. Specific primary and secondary antibody combinations are indicated in Figs. 1, 4, 8, and 9.

**cAMP-dependent protein kinase assays.** Cells were seeded onto 24-mm diameter Transwell-Clear filter inserts (Corning Costar) and maintained in culture as described in Cell isolation and culture. Samples were prepared (10) for assay, and total cAMP-dependent protein kinase activity toward the specific substrate kemptide was measured as previously described (41). Sample activity was compared with a standard curve generated with 0.0063–0.1 units of purified bovine heart PKA C subunit (no. 29538; Pierce, Rockford, IL). A unit is equivalent to 1 pmol of phosphate transferred to substrate per minute. Values were normalized per milligram of protein assayed. In the present study, activity of cAMP plus the specific PKA inhibitor peptide PKA inhibitor 6–22 amide (PKI 6–22, 100 nM; no. 539684, Calbiochem-Novabiochem, La Jolla, CA); or in the absence of cAMP, was similar (data not shown).

**Localization studies.** Confocal laser scanning microscopy was used to localize specific proteins identified by immunofluorescence. Cells were seeded onto 12-mm diameter Transwell-Clear filter inserts and maintained in culture as described in Cell isolation and culture. For monolayers treated before fixation, growth medium was removed, the confluent cell monolayers were washed three times with phosphate-buffered saline (PBS; 10 mM NaPO\(_4\), 150 mM NaCl, pH 6.9) and cells were incubated at 37°C in defined medium containing additions indicated in Fig. 6. All subsequent steps were performed as previously described (41). Specific primary and secondary antibody combinations used are listed in Figs. 1, 5, and 6. The specificity of antibody immunoreactivity was demonstrated with controls lacking primary antibodies, which in all cases resulted in no detectable fluorescent signal (data not shown). Anti-selective PKA immunolabeling was identified after immunofluorescent labeling was completed with an Oregon green 514-phalloidin conjugate (O-7465; Molecular Probes) according to the manufacturer’s protocol. All samples were examined with a scanning laser confocal microscope, by using a 100× objective and software to optically section the cell monolayer every 0.5 µm, as previously described (41).

**Intracellular cAMP determinations.** Cells were seeded onto 12-mm-diameter Transwell-Clear filter inserts (Corning Costar) and maintained in culture as described in Cell isolation and culture. Growth medium was removed, and confluent cell monolayers were washed three times with PBS. Defined medium with additions indicated in Fig. 2 was added, and cultures were replaced in the tissue culture incubator for 1 h. Monolayers were then assayed for cAMP as previously described (41). Values were normalized for protein content. Fold changes in accumulation were calculated as follows: [(stimulated) − (control)]/ [(IBMX + rolipram) − (control)].

**Cell proliferation assays.** The proportion of proliferating cells in cell cultures was determined by seeding cells onto Transwell-Clear filter inserts, as described in Cell isolation and culture. Cultures were expanded to the desired confluence (subconfluent cultures at \(\leq 20\,000\) cells/insert, 100% confluence at \(\geq 100,000\) cells/insert) and then were treated with agents added to standard growth medium for an additional 16 h as described in Figs. 7–9. Proliferation was assayed using the 5-bromo-2'-deoxyuridine (BrdU) labeling and detection kit (no. 1–296–736; Roche Molecular Biochemicals) according to the manufacturer's protocol. Briefly, immunofluorescent fluorescent labeling was used to detect BrdU incorporated into cellular DNA. All samples were then stained with 500 nM propidium iodide (Molecular Probes) in PBS for 5 min to detect nuclei according to the manufacturer’s protocol. Filter inserts were mounted on standard microscope slides as described in Localization studies. Samples were viewed with an Optiphoto-2 fluorescence microscope (Ni- kon, Japan) with FITC HQ and Texas red HQ filter sets (Chroma Technologies, Brattleboro, VT), and three rectangular fields (270 × 210 µm) in each sample were photographed with a SPOT digital color camera (Diagnostic Instruments, Sterling Heights, MI). Semitransparent layered images in Adobe Photoshop 5.5 software (Adobe Systems, San Jose, CA) were scored for the number of nuclei showing BrdU immunostaining and the total number of nuclei observed (propidium iodide staining). The ratio of these values was used to reflect BrdU incorporation into cells. Higher values reflected...
increased levels of BrdU uptake and incorporation into cellular DNA, an indication of relatively greater levels of cell proliferation. In a tightly packed confluent monolayer, the number (mean ± SE; n = 4) of propidium iodide-stained nuclei per well was equal to the number of trypan blue excluding live cells removed by trypsinization (567,000 ± 42,438 vs. 583,875 ± 48,549, respectively). If cultures were allowed to overgrow, more cells were removed by trypsinization than were scored visually. However, because cysts enlarge progressively, producing and maintaining an epithelial monolayer without overgrowth, cultures were used and scored only if cells or monolayers were present without overgrowth.

Other information. Statistical determinations were calculated with InStat3 v3.05 software (GraphPad Software, San Diego, CA). Protein was quantitated with either Bio-Rad (Bio-Rad Laboratories, Hercules, CA) or bicinchoninic acid (Pierce, Rockford, IL) protein assay reagents, according to the manufacturers’ protocols. The EGFR tyrosine kinase inhibitor EKI-785 (13, 61) was obtained from Dr. Philip Frost (Wyeth-Ayerst Research, Pearl River, NY) for the Rainbow Center for Childhood PKD. The AKAP binding inhibitor InCELLect AKAP St-Ht31 inhibitor peptide was obtained from Promega (Madison, WI). Reagents not specifically listed were either cell culture or molecular biology grade and were obtained from Sigma (St. Louis, MO), Life Technologies, and Calbiochem-Novachem.

RESULTS
cAMP accumulation was normal in cultured cells that expressed the cystic phenotype. Selectively enriched populations of isolated CT principal cells used in this study maintained characteristic phenotypes associated with either PKD cysts or unaffected collecting tubules (43, 60). Apical and basolateral EGFR immunostaining was observed in filter-grown cystic cpk cell cultures (Fig. 1). Only lateral EGFR immunostaining was visible in noncystic C57 cells (Fig. 1). Unlike the overexpression observed in tissue samples harvested from murine cystic kidneys (43), EGFR protein expression was similar in cell cultures grown in the absence of EGF (Fig. 1). Immunostaining for the tight junction protein ZO-1 was identical for both culture types (Fig. 1). Therefore, both isolated cystic and noncystic cells formed epithelial layers in filter culture, and phenotypic differences were maintained.
To determine whether the cystic phenotype included alterations in the initial cAMP signaling step, intracellular cAMP levels in cystic cpk and noncystic C57 CT cells were measured. Basal levels of intracellular cAMP were similar in the two cell types (Fig. 2). Similar low levels of cAMP accumulated in the two cell types in response to the PDE inhibitors IBMX and rolipram. The two inhibitors likely inhibit the majority of PDE isoform activity present in these cells (59). To maximize the likelihood that cAMP-mediated responses would be detected and to allow direct comparisons between treatments, PDE inhibitors were included for all subsequent treatment conditions and experiments unless specifically noted. In response to EGF (in the presence of PDE inhibitors, as noted), no intracellular cAMP accumulation occurred in noncystic cells and no consistent accumulation was observed in cystic cell cultures. Therefore, EGF, which was required to maintain cysts in murine metanephric organ culture (48, 61), did not promote increased cAMP production. Forskolin, a direct activator of AC, induced similar large increases in cAMP accumulation in both C57 and cpk cells, although the absolute amount of cAMP present in cpk cells was smaller. Therefore, the cell types had similar, normal capabilities for cAMP production.

Subcellular distribution of PKA subunits differed between noncystic and cystic cell cultures. Because the primary effector of cAMP action is PKA, protein kinase activity was measured in confluent cultures of each cell type to determine whether both cystic and noncystic cell cultures expressed similar overall levels of PKA activity. Total cAMP-dependent protein kinase activity levels were similar in both cell types (Fig. 3), but this assay does not distinguish between type I and type II subtypes. CAMP-independent basal activity and activity in the presence of cAMP plus PKI 6–22 were similar (data not shown).

To address the potential for PKA subtype differences, expression of PKA proteins was determined in filter-grown cultures of each cell type. Both cystic cpk and noncystic C57 confluent cell cultures expressed the ubiquitous PKA subunit proteins Cα, RIα, and RIIα (Fig. 4). Slightly higher relative expression of cpk RIα and Cα was observed, although no difference in overall PKA activity was detected (Fig. 3). All subunits were expressed at similar levels regardless of the state of confluence of the culture type (data not shown). Therefore, confluent and subconfluent cultures of both cell types had the components necessary for expression of PKA type I and type II activities.

If PKA subunits are located in specific subcellular compartments, subtypes could be individually regulated or have preferential access to substrates. There-
cAMP-DEPENDENT PROTEIN KINASE IN CYSTIC EPITHElia

Fig. 4. Both cystic and noncystic cell cultures express PKA subunit proteins (C, catalytic; R, regulatory). Western blot analysis of PKA subunit expression was described as detailed in EXPERIMENTAL PROCEDURES in unstimulated confluent cultures. Antibody combinations for Western analyses were anti-Cα sc-903 (1:2,000), anti-RIIα P19920 (1:25), or anti-RIIα sc-909 (1:500), each with the appropriate peroxidase-linked secondary antibody, sc-2004 (1:2,000), sc-2005 (1:500), or sc-2004 (1:2,000), respectively. Protein loaded per lane was 5 μg RIIα, 10 μg RIα, and 2 μg Cα. Apparent molecular weights are indicated. Data are representative of 5–7 experiments.

Therefore, the location of each PKA subunit was mapped in confluent cultures of each cell type (Fig. 5). In noncystic C57 cells, RI expression was cytoplasmic and diffuse, with slightly stronger staining intensity nearest the nucleus. Cystic cpk cells had a distinct pattern of RI expression throughout the cytoplasm and outside the nucleus, with stronger expression near the apical surface. Expression of the RIIα subunit in C57 cell cultures was strong in the perinuclear region and diffuse in the cytoplasm. Staining for RIIα in cpk cells also occurred throughout the cytoplasm and was very strong in specific locations around the nucleus. However, cpk RIIα expression overall was reticulated rather than diffuse as for RI or for cytoplasmic RIIα in C57 cells. Staining for Cα, in both cpk and C57 cells, was both cytoplasmic and strongly perinuclear. In cpk, there was some reticulated Cα immunostaining. Therefore, each PKA subunit had a unique localization pattern, and the subcellular distribution of subunits in noncystic cultured cells differed from that in cystic cells.

Because RIα and RIIα distributions predict the resting locations of inactive type I and type II PKA holoenzymes, local alterations in cellular signaling could affect PKA subtype activation. Elevations of intracellular cAMP can induce dissociation of the PKA holoenzyme, so with a change in the location of Cα as an indicator of PKA activation, the effects of pharmacological stimuli were evaluated in each cell type. A time course of Cα subunit localization after stimulation was obtained (Fig. 6). PDE inhibitors alone produced an initial diffuse immunostaining pattern that probably reflected movement of Cα subunit throughout the C57 cell cytoplasm and nucleus. After 30 min, C57 cell Cα subunit immunostaining returned to nearly the same restricted locations as before the inhibitors were applied. Incubation of C57 cells with forskolin produced a more pronounced diffuse staining pattern that rapidly returned to basal, even in the presence of PDE inhibitors. Although C57 cells showed diffuse Cα redistribution at early time points that returned to primarily cytoplasmic as time progressed, this was not the case for the cystic cpk cells. The relatively low levels of cAMP accumulation induced by PDE inhibitors alone (Fig. 2) that were sufficient to induce transient redistribution of Cα subunit in C57 cells produced a prolonged location change in cpk cells (Fig. 6). Larger cAMP increases due to forskolin (Fig. 2) that caused transient Cα relocation in C57 cells also led to prolonged redistribution in cpk cells. Neither cell type showed a complete shift to nuclear Cα immunostaining in response to short-term stimuli. Application of EGF (with PDE inhibitors), either to both cell surfaces together or separately, produced diffuse staining that was similar to that of PDE inhibitors alone in both cell types (data not shown).

Regulation of proliferation differed between noncystic and cystic cell cultures. Progressive enlargement of CT cysts during the disease course is a hallmark of ARPKD and murine models for it and is postulated to be due partly to increased cell proliferation, although expanding cysts maintain a single layer lining of cells (60). Proliferation rates are low in normal collecting tubules, so why proliferation persists in cystic collecting tubules is a central question (37). In organ culture, EGF stimulated cystic kidney cell proliferation and was required for maintenance of cysts (48, 60, 61), but the organ culture model includes many cell types other than the principal cells that express the characteristic cystic phenotype of apical EGFR expression. The mechanism responsible for apical targeting of the EGFR in PKD is unknown. Because it is unlikely that new principal cells appear fully epithelialized, any new cells produced in the normal tubule or in a cyst as the result of proliferation are likely to transition from unpolarized to polarized and to acquire the epithelial phenotype as they develop. Therefore, subconfluent cultures were postulated to model regions of growing collecting tubule before establishment of a polarized epithelium, whereas confluent cultures were shown to express a polarized phenotype (Fig. 1).

To determine whether proliferation in the cell cultures reflected proliferation observed in the developing PKD kidney (48), cell proliferation was measured in both subconfluent and confluent cultures of CT cells. Control, or basal, levels of cell proliferation were higher in subconfluent cpk cell cultures than in C57 cell cultures (Table 1; Fig. 7A). Relative proliferation leveled off to minimum detectable values as C57 cultures reached confluence. Cystic cultures maintained high relative proliferation as confluence increased, with control proliferation levels similar to those for subconfluent cultures. To determine whether cAMP and PKA activity regulated proliferation in principal cells, effects of the cAMP analog 8-bromo-cAMP (8-BrcAMP) in the absence or presence of the PKA activity inhibitor H-89 were tested in the proliferation assay. As shown, 8-BrcAMP stimulated proliferation in subconfluent C57 cultures and H-89 blocked 84% of the 8-BrcAMP-induced increase (Table 1). This overall PKA activity inhibitor had no significant effect on control levels of proliferation in any culture condition.
Fig. 5. Localization of PKA subunits is different in cystic and noncystic cultured cells. Confocal immunolocalization of PKA subunits in unstimulated, confluent cultures was performed as described in EXPERIMENTAL PROCEDURES. Antibodies used for CLSM were anti-Cα sc-903, anti-RI P19920, and anti-RIIα sc-909, each with Oregon green conjugate O-6380 or O-6381, depending on host specificity. Square panels are single x-y scans, and rectangular panels are cross sections reconstructed from a series of z scans. Data are representative of 2–5 experiments. Bar, 25 μm.
Fig. 6. Stimulation alters the cellular location of C57. Experiments were performed on confluent cultures as described in EXPERIMENTAL PROCEDURES, incubated with either 100 µM IBMX + 5 µM Ro (1+Ro) or 10 µM forskolin + 100 µM IBMX and 5 µM Ro (Forsk) for 5, 15, or 30 min. Control, cultures incubated in defined medium alone. Immunostaining and CLSM were performed as described in Fig. 5. Data are representative of 2 experiments. Bar, 25 µm.

Table 1. Effects of cAMP on proliferation are mediated by PKA

<table>
<thead>
<tr>
<th>Condition</th>
<th>C57 Subconfluent</th>
<th>C57 Confluent</th>
<th>cpk Subconfluent</th>
<th>cpk Confluent</th>
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<tbody>
<tr>
<td>Control</td>
<td>33.9 ± 5.3 (14)</td>
<td>14.9 ± 2.7 (12)</td>
<td>78.6 ± 6.3 (11)</td>
<td>72.2 ± 4.6 (11)</td>
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<tr>
<td>8-BrcAMP</td>
<td>72.3 ± 4.7 (3)</td>
<td>30.0 ± 2.0 (3)</td>
<td>89.0 ± 8.5 (3)</td>
<td>53.7 ± 1.8 (3)</td>
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<tr>
<td>H-89</td>
<td>34.3 ± 3.8 (3)</td>
<td>26.3 ± 6.2 (3)</td>
<td>85.0 ± 12.6 (3)</td>
<td>59.0 ± 6.1 (3)</td>
</tr>
<tr>
<td>8-BrcAMP + H-89</td>
<td>40.0 ± 6.3 (3)</td>
<td>27.3 ± 9.3 (3)</td>
<td>85.3 ± 11.8 (3)</td>
<td>62.3 ± 4.1 (3)</td>
</tr>
</tbody>
</table>

Values reflect relative proliferation (% of cells that incorporated 5-bromo-2'-deoxyuridine into the nucleus) and are means ± SE of indicated no. of experiments (in parentheses) performed in duplicate. Proliferation assays were performed on confluent cultures as in the legend to Fig. 7, with defined medium alone (Control), 100 µM 8-bromo-cAMP (8-BrcAMP), 10 µM cAMP-dependent protein kinase (PKA) activity inhibitor H-89 (H-89), or 100 µM 8-BrcAMP + 10 µM H-89. Treatments were applied to both surfaces in the absence of phosphodiesterase inhibitors. Significant differences identified, within the C57 subconfluent culture conditions, with 1-way ANOVA with the Student-Newman-Keuls Multiple Comparisons test are indicated as follows: *P < 0.05, between 8-BrcAMP and Control, H-89, or 8-BrcAMP + H-89. No significant differences were found between values within each of the other culture types. Significant differences identified between all Control values with 1-way ANOVA with the Tukey-Kramer Multiple Comparisons test are indicated as follows: †P < 0.05, between subconfluent and confluent C57 cultures; ‡P < 0.001, between subconfluent C57 and subconfluent or confluent cpk cultures, and between confluent C57 and subconfluent or confluent cpk cultures. No significant difference was identified between subconfluent and confluent cpk cultures. Inhibition of the 8-BrcAMP effect by H-89 (84.3%) was calculated only where significant differences were identified (C57 subconfluent cultures), as follows: 100 - ([(8-BrcAMP+ H-89) - H-89]/(8-BrcAMP - Basal)) × 100.
Specific agonists and inhibitors were then used to determine pharmacologically whether components of the proliferative responses (in C57) or the apparent constitutive proliferation (in cpk) were mediated by cell type differences in PKA subtypes or EGFR tyrosine kinase activity (Fig. 7). The PDE inhibitors IBMX and rolipram had little effect on proliferation in either C57 or cpk cells and were included with all other treatments, as previously noted (Fig. 7A). Proliferation was stimulated by forskolin, as well as by EGF, in subconfluent C57 cell cultures. The combination was no more effective than either agent alone (data not shown). Confluent C57 cultures were unaffected by any stimulus tested. In cpk cultures, the already high basal proliferation levels were unchanged by the addition of forskolin, EGF, or their combination (data not shown).

To determine the involvement of a particular PKA subtype, two approaches were used. First, type I PKA activity was specifically inhibited with the cAMP analog Rp-8-BrCAMP (20). Although use of a single cAMP analog inhibitor in intact cells is unlikely to clearly demonstrate involvement of a particular PKA subtype because some analogs may act as inhibitors of one subtype and partial activators of another, Rp-8-BrCAMP was shown to have greater specificity for type I than other analogs. Second, type II PKA action, but not kinase activity, was inhibited by blocking anchoring/binding of RII to AKAPs with InCELLect St-Ht31 inhibitor peptide, an agent demonstrated to interfere with vasopressin/PKA-mediated translocation of aquaporin-2 to cell membranes in principal cells (27). Most examples of AKAP binding to R subunits involve RII and thus type II PKA, although examples of RI binding have been reported (16). To determine whether EGFR tyrosine kinase activity was involved in mediating responsiveness or the constitutive proliferation, a specific inhibitor previously demonstrated to effectively inhibit EGFR tyrosine kinase activity and cyst formation in in vitro organ culture and in vivo was utilized: Wyeth-Ayerst EKI-785 (13, 61, 62). Autophosphorylation and activation of cultured cell EGFR by EGF alone were demonstrated previously (60). All inhibitors were cell permeant.

In subconfluent cultures of noncystic C57 cells, the PKA type I-specific inhibitor Rp-8-BrCAMP enhanced proliferation levels under all conditions tested (Fig. 7B). In contrast, only control proliferation levels were modestly inhibited by Rp-8-BrCAMP in subconfluent cpk cells, and in the presence of other stimuli, Rp-8-BrCAMP inhibited proliferation modestly, while not affecting proliferation levels under all conditions tested (Fig. 7C). To further assess the involvement of type I PKA, the PKA type I-specific inhibitor Rp-8-BrCAMP was assayed in subconfluent cell cultures in the presence of forskolin, EGF, or their combination. Results are means ± SE of n = 3–11 experiments, except for values without error bars, where n = 2. Major significant differences identified using 1-way ANOVA: *P = 0.025 vs. basal and 1-Ro of same culture condition; **P < 0.01 vs. corresponding C57 conditions; #P < 0.05 vs. corresponding subconfluent conditions; •P < 0.001 vs. corresponding C57 condition; †P < 0.001 vs. corresponding culture conditions without inhibitor; **P < 0.05 vs. corresponding culture conditions without inhibitor; @P = 0.09, not quite significantly different from corresponding culture conditions without inhibitor.
BrcAMPS had little effect. In subconfluent cultures of both types, control and PDE-inhibited proliferation levels were unaffected by the inhibitor of RII subunit binding to AKAPs, St-Ht31 inhibitor peptide (Fig. 7C). However, this inhibitor of type II PKA action clearly diminished the ability of forskolin, and the combination of EGF and forskolin (data not shown), to stimulate proliferation in subconfluent C57 cell cultures. The effect on C57 cell responsiveness to EGF appeared insignificant. The inhibitor was completely ineffective in constitutively proliferating subconfluent cpk cells. In both subconfluent cell types, the EGFR tyrosine kinase inhibitor EKI-785 had no effect on control proliferation or proliferation in the presence of PDE inhibitors alone (Fig. 7D). However, EKI-785 completely blocked responses to all stimuli in C57 cells. It also partially inhibited the apparent constitutive proliferation observed in cpk cells but only under conditions in which forskolin, EGF, or EGF plus forskolin (data not shown) were present, even though no stimulation of proliferation had been observed.

In confluent noncystic cultures, the low levels of proliferation observed under all conditions (Fig. 7A) were unaffected by the presence of any inhibitor tested (Fig. 7, B–D). In confluent cpk cultures that still demonstrated apparently constitutive proliferation levels (Fig. 7A), only the type I PKA activity inhibitor Rp-8-BrcAMPS modestly inhibited proliferation if EGF was present (Fig. 7B). Pharmacological inhibition of type II PKA action or EGFR tyrosine kinase activity did not affect proliferation (Fig. 7, C and D).

**Pharmacological stimulation of cystic and noncystic cultures resulted in differential protein expression changes.** The proliferation assay results (Fig. 7, B and C) suggest that PKA subtype differences exist between cell types and confluence states, but there were no major differences in expression of any PKA subunits when subunit expression was compared in subconfluent and confluent (Fig. 4) cultures and overall PKA activity was the same (Fig. 3). However, when confluent cultures were treated to determine whether stimulation affected expression of specific subunits, an expression difference was found between cystic and noncystic cells (Fig. 8). Stimuli that elevated intracellular cAMP levels (PDE inhibitors, with and without forskolin) or were expected to activate EGFR (EGF in the presence of PDE inhibitors) resulted in either no changes or very modest changes in Cα or RI expression. In C57 cells, a shift in RIα expression from an apparent molecular weight of 51,000 to 54,000 was observed only in the presence of forskolin. The same stimuli resulted in the appearance of an immunoreactive RIαa doublet in cpk cultures whenever PDE inhibitors were present. Therefore, only a large increase in intracellular cAMP (Fig. 2) produced an RIα shift in C57 cells, whereas any increase over control (Fig. 2) resulted in the appearance of the doublet pattern in cpk cells. Unlike RI, which contains a pseudosubstrate motif lacking a PKA-phosphorylatable serine residue, RI may be autophosphorylated in its pseudosubstrate region. The apparent molecular weight shift observed (Fig. 8) suggests endogenous autophosphorylation of the RIα subunit by C (reviewed in Ref. 25).

Results of the proliferation assay (Fig. 7D) also suggested that forskolin exerted some of its effects on cell proliferation through activation of EGFR tyrosine kinase activity, because EKI-785 was inhibitory. Possible explanations for this finding include effects of EKI-785 on PKA activity, forskolin/PKA effects on EGFR expression, or forskolin/PKA effects on EGFR tyrosine kinase activity or activation. Subsequent steps in the EGFR activation pathway could be affected by forskolin but would not be expected to be inhibited by EKI-785, an ATP-binding site inhibitor specific for initial EGFR, and to a lesser degree c-erbB-2, tyrosine autophosphorylation (13). However, in vitro, the PKA serine kinase activity of purified C subunit was inhibited by EKI-785 (Fig. 9A) with the same approximate half-maximal value reported for in vitro inhibition of EGFR-mediated phosphorylation of a tyrosine-containing peptide substrate. No cellular effects of EKI-785 on PKA activity have been reported (13), and the doses that affected PKA activity extended over at least 3 log units, so the inhibitory effect on C subunit may occur only in vitro and may not be relevant in the cell. To test this possibility, the phenomenon of possible RIi auto-phosphorylation (Fig. 8) was used as a cellular assay for the effects of EKI-785 on C subunit activity. As shown (Fig. 9B), EKI-785 did not block the RIα molecular weight shift in response to forskolin, IBMX, and rolipram, suggesting a lack of inhibitory effect on cellular PKA.

To test for cAMP-mediated effects on EGFR protein expression, confluent filter-grown cultures of noncystic
Protein loaded per lane was 5/experiments. ° min at 4 C before assay. Values are means substrate in 1 min). Inhibitor was incubated with C subunit for 10 enzyme required to catalyze transfer of 1 pmol phosphate into DURES (Fig. 9 EGFR antibody as described in EXPERIMENTAL PROCEDURES with 0.1 unit of purified PKA C subunit (1 unit = amount of enzyme required to catalyze transfer of 1 pmol phosphate into substrate in 1 min). Inhibitor was incubated with C subunit for 10 min at 4 C before assay. Values are means + SE; n = 3 except for points at 3 × 10⁻¹⁴ (n = 1), 3 × 10⁻¹² (n = 2), 10⁻¹¹ (n = 2), and 10⁻⁸ (n = 1). Values reflect activity measured in a 10-min incubation. B: experiments were performed as described in Fig. 7, but instead of samples being processed for the proliferation assay, they were prepared for Western blot analysis as described in Figs. 4 and 8. Note that PDE inhibitors were included in all conditions except Control. Data are from cpk cultures but are representative of 2 experiments each performed in filter cultures of C57 (no change in single band shift) and cpk cells (no change in doublet). Arrowheads, migration of basal and shifted immunoreactive RIIa bands. C: experiments were performed as described in Fig. 8, and Western blot analysis for the EGFR was performed as described in EXPERIMENTAL PROCEDURES. Protein loaded per lane was 5 μg. Data are representative of 2 experiments.

C57 and cystic cpk cells were treated overnight with forskolin or EGF and then whole cell lysates were subjected to standard Western blot analysis with an EGFR antibody as described in EXPERIMENTAL PROCEDURES (Fig. 9C). Expression of EGFR was comparable in filter-grown C57 and cpk cells cultured in defined medium without EGF (Figs. 1 and 9) despite the localization differences observed (Fig. 1). Expression differences were modest in confluent C57 cell cultures. In contrast, in confluent cpk cultures, although EGFR expression stayed at control levels in the presence of PDE inhibitors with and without forskolin, ligand-induced loss of EGFR occurred. Because similar renal epithelial cell downregulation of EGFR expression in response to EGF has been reported (50), these data suggest altered regulation of receptor metabolism by EGF in cystic cells. Although forskolin did not affect equilibrium levels of EGFR protein expression, the results do not rule out the possibility that cAMP/PKA affects EGFR activity or activation.

DISCUSSION

The results presented in this study suggest that an appropriate balance of PKA distribution and activity is necessary for normal proliferation in principal cells. The results demonstrate that PKA type I activity limits proliferation to low, basal levels in normal principal cells before polarization. The results also demonstrate that cAMP-stimulated proliferation, observed only in noncystic, expanding cell cultures, is mediated by PKA activity and AKAP anchoring. On reaching confluence, normal cells proliferate only at very low levels and proliferation is unresponsive to agonists or inhibitors. In contrast, both unpolarized and polarized cystic principal cells are highly proliferative and also mostly unresponsive to agonists and inhibitors, despite normal cAMP accumulation, PKA activity, and subunit expression. Striking differences are observed in the subcellular distribution of both RI and RIIa PKA subunits and in the distinctive redistribution of catalytic subunit and altered expression of RIIα subunit in response to cAMP-elevating agents. In summary, these studies suggest that differences in the predicted location of both PKA type I and type II activities result in loss of normal PKA-regulated control of proliferation so that nearly constitutive proliferation is observed in cystic principal cells (Fig. 10).

Proliferative activity of cyst epithelium in human cystic diseases was evaluated in a previous report. Approximately 32- and 8-fold greater proliferative indexes were measured in ARPKD and ADPKD epithelia, respectively, compared with normal kidney (37). The results of the present study suggest that cultured cpk principal cells maintain a proliferative phenotype similar to that observed in the cyst epithelium of human ARPKD. The double enrichment method used is selective for principal cells and was used with kidneys from animals at early stage to midstage of the disease, when CT cysts are forming and expanding.
rapidly. These results are in contrast to the low proliferation levels reported for *Dolichos biflorus* lectin-expressing collecting duct cells derived from the same animal model (52). However, the two culture systems were dissimilar because both collecting duct principal and intercalated cells express the lectin and lectin-expressing cells were obtained from 21-day-old animals at the end stage of the disease, near the end of the animals’ life span.

There are few data in the literature about PKA subtype activation in PKD. In the pcy mouse model of ARPKD, kidney cAMP content increased over the disease time course whereas cAMP content in normal murine kidney remained constant, and a lipid found in pcy cyst fluid stimulated cellular cAMP production, fluid transport, and cell proliferation in renal cell lines (73). In two previous reports, activation of the cAMP pathway regulated cell proliferation in ADPKD cyst cells (cultured on plastic or on collagen) but not in human kidney cortex cells (22, 74). In the former report, cAMP-mediated proliferation occurred by activation of the extracellular signal-regulated kinase (ERK)1/2 kinase pathway at a step distinct from that stimulated by EGFR activation (74). The type I PKA inhibitor used in that study was as inhibitory as the general PKA inhibitor H-89, suggesting that cAMP-induced proliferation through the ERK pathway required type I PKA activity. The latter report suggested that effects of EGF and cAMP on ADPKD cell proliferation were independent (22). In contrast to the ADPKD studies, the results of the present study demonstrate a role for PKA-mediated proliferation in normal murine principal cells and suggest that high levels of cell proliferation in murine ARPKD cells assayed on permeable filter supports could result from a shift in the balance and distribution of PKA subtype activities, that the shift may contribute to altered EGFR activity, and that differences exist even before a polarized epithelium is firmly established. The ability of H-89 to inhibit all but 16% of the cAMP-stimulated effect on proliferation in noncystic cultures suggests that normal principal cells have a small PKA-independent mechanism regulating cAMP effects on proliferation. Perhaps this becomes a major factor in murine ARPKD cells in the absence of normal PKA distribution.

Although individual subtype activities were not measured in the present study, the functional effects of the two subtypes were addressed pharmacologically. Type I PKA was responsible for maintaining basal proliferation at low levels in expanding cultures of noncystic cells and was diffusely distributed within the cell in polarized cultures. In contrast, perhaps the distinct apical distribution of type I in cystic cells prevented its inhibition and contributed to the cell’s constitutive proliferation levels. Binding of RIα to the Grb2 protein recruited to autophosphorylated EGFR in cancer cells has been reported (65), and inhibition of EGFR and PKA cooperatively inhibits cancer cell growth (reviewed in Ref. 9). If a similar interaction occurred between apical EGFR and apical RIα in PKD cells, this might contribute to the persistent proliferation observed, although this is pure speculation.

Type II PKA anchoring to AKAPs was required for forskolin-mediated proliferation in subconfluent, noncystic cells. In contrast, in expanding and confluent cystic cell cultures, normal anchoring appeared to be absent or dysfunctional. With the RIα distribution differences in unstimulated confluent cells, and the prolonged redistribution of Cα in stimulated *cpk* cells, these results support the hypothesis that cystic cells have a defect in PKA anchoring via AKAPs or redistribution mechanisms via PKI or slightly elevated RIα and Cα expression. Some AKAPs are associated with the cytoskeleton via actin, and phalloidin labeling demonstrated that although both C57 and *cpk* cells had similar patterns of lateral actin staining, only C57 cells showed basal stress fiber staining (Fig. 1). Basal actin in the *cpk* cells appeared to be disorganized (Fig. 1), supporting the likelihood of anchoring defects. However, use of the anchoring inhibitor does not address possible type II activity differences. Evidence that type II activity is altered in *cpk* is provided by the observation that immunoreactive RIα bands of higher apparent molecular weight appeared in a distinct pattern on cAMP stimulation of the two cell types. Such shifts have been accounted for by RIα “autophosphorylation” by C, a modification that can result in the appearance of additional bands of higher apparent molecular weight in polyacrylamide gels, and have been linked to cell cycle differences, cell proliferation, apical endocytic recycling, and altered subcellular location of RIα (Refs. 17, 32, and 58; reviewed in Ref. 25). Autophosphorylation in vitro shifted RIα from 51,000 to 54,000, decreased the affinity between RIα and C (51), and promoted a more efficient association with AKAPs (77, 78). If the presence of apparent molecular weight differences in CT cell RIα observed in the present study reflects RIα autophosphorylation, then these data suggest that Type II PKA activity, or phosphorylation/dephosphorylation mechanisms, differed between confluent C57 and *cpk* cultures.

Because EGF and EGFR were previously observed to play roles in ARPKD proliferation and cystogenesis (reviewed in Refs. 39 and 60), and because EGFR and PKA type I appear to regulate proliferation coordinately in cancer (reviewed in Ref. 9), EGF was included in the present study. In contrast to tissue and organ culture models, cultured cystic principal cells were constitutively proliferative rather than hyperresponsive to EGF. The cell culture system lacks normal organotypic architecture and other cell types that may contribute endogenous growth controls. However, the characteristic EGFR phenotype was maintained. Principal cells are the affected cells that line cysts, so regardless of the mechanism, EGF may activate apical EGFR and provide activated receptors access to cellular molecules with which they might not normally communicate. This concept is supported by the clear difference seen in ligand-induced loss of EGFR protein expression in cystic compared with noncystic cells. Although membrane-associated proteins other than
the EGFR were not found to be clearly mislocalized in PKD (reviewed in Ref. 61), the observation that basal actin appeared disorganized in the cystic cph cells suggests that cytoskeletal anchoring or signal transduction mechanisms may differ in cystic compared with noncystic cells.

The EGFR autophosphorylation/tyrosine kinase activity inhibitor was included because an effect on EGFR-mediated proliferation was expected, the inhibitor blocked cyst progression in vivo, and it has been considered as a potential therapeutic agent (49, 61). Unexpectedly, the experimental results suggested a role for PKA regulation of the EGFR. The observation that the inhibitor blocked the proliferative effects of both EGFR and forskolin in noncystic cells suggested that cAMP/PKA regulated EGFR expression or activity to regulate proliferation and that this was a normal CT cell process. However, elevation of intracellular cAMP did not increase equilibrium EGFR protein expression, so EKI-785 was unlikely to be acting on cAMP-induced EGFRs. Possible PDE inhibitor, or PKA, stimulation of EGFR activity was not measured in the present study. Because the inhibitor has some effects on c-erbB-2 tyrosine autophosphorylation (13), questions remain about the mechanism of action of EKI-785. The inhibitor was reported to have greater efficacy at early stages of cystic disease (62, 63), perhaps corresponding to its effect on the subconfluent cystic cultures, as a potential model for the growing collecting duct, and supporting the idea that there is a therapeutic “window of opportunity,” because confluent cultures were unaffected. If cellular PKA inhibition by EKI-785 did occur, this result would confirm PKA regulation of proliferation in these cells. Perhaps EKI-785 was effective at inhibiting PKD cystogenesis because of its combined effects on activated EGFR and PKA.

The ability of normal principal cells to cease proliferation appropriately after reaching confluence although cystic cells that express and handle PKA differently do not suggest that appropriate PKA action is crucial for normal principal cell proliferation. Type I PKA may maintain low basal proliferation levels, whereas AKAP anchoring may mediate proliferation in response to elevated cAMP. In cystic principal cells, perhaps unusual coupling of type I activity could mediate altered proliferation and variable type II anchoring or activation could mediate altered endocytic recycling or activation of the EGFR, making PKA abnormalities an upstream step in the process of PKD pathogenesis. Preferential access of PKA subtypes to polarized signaling complexes or substrates could regulate specific functions or phenotypes. For example, polycystin-1, the transmembrane protein product of the PKD1 gene, was a PKA substrate in vitro (29, 45), although the functional significance of this finding is unknown. If a PKD protein complex regulates normal nephrogenesis, then mutations that alter its regulation by PKA subtypes could contribute to cyst formation. Murine ARPKD may be an example of a disease in which cAMP pathway variations have serious physiological consequences. This study is the first demonstration of PKA subunit localization in PKD. Further study is required to identify and pinpoint direct subtype links to PKA substrates and to demonstrate phosphorylation-specific regulation of proliferation in normal cells as well as proliferation and cystogenesis in cystic disease.

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