Chapter 8

The Role of Calcium and Cyclic AMP in PKD

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Abstract

Cyclic AMP (cAMP)-driven mechanisms are central to the pathogenesis of polycystic kidney disease (PKD). Cyclic AMP stimulates both fluid secretion and cell proliferation, making abnormal cAMP-regulated pathways key targets for PKD therapy. The success of vasopressin receptor blockade in lowering cAMP levels and ameliorating disease in murine models of PKD and in a recent clinical trial, argues that cAMP-regulated mechanisms are fundamental to cyst formation and disease progression. This chapter focuses on why cAMP is important to the disease process, and how the primary abnormality in PKD is the abnormal response of cells to cAMP rather than high levels of cAMP *per se*. This abnormal

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cAMP response is a consequence of the calcium environment being disrupted in PKD from loss of polycystin function. We have identified signaling mechanisms by which decreased intracellular calcium levels can transform normal cells into PKD-like cells. By treating normal renal epithelial cells with calcium channel blockers it has been possible to de-repress B-Raf, allowing its activation by cAMP and subsequent MEK/ERK activation to stimulate cell proliferation. Autosomal dominant PKD (ADPKD) cells can also be switched back to a normal phenotype by raising intracellular calcium. The abnormal response to cAMP is made worse by mechanisms that further raise intracellular cAMP, causing cAMP to stimulate cyst-filling fluid secretion in a cystic fibrosis transmembrane conductance regulator (CFTR)-dependent fashion. The abnormal PKD-like phenotype is likely a result of misregulated gene expression as well as disruption of a number of signaling pathways and altered cell cycle control, all resulting in a change in the phenotypic state. It is hypothesized that disruption of the calcium/calcineurin/nuclear factor of activated T-cells (NFAT) pathway would contribute to this phenotypic change by altering gene expression, and activating and upregulating CDK4 causing loss of cell cycle control, events that would cause cyst initiation, and that would promote cyst growth and enlargement.

Key words: B-Raf; Calcineurin; CDK4; ERK; NFAT

Introduction

Polycystic kidney disease (PKD) is characterized by the abnormal growth of epithelial-lined cysts from the nephrons and collecting ducts of affected kidneys (1-3). PKD is associated with dramatic increases in kidney size, starting before birth, which results from the unrelenting growth of thousands of fluid-filled cysts, many undergoing massive enlargement. As cysts grow, they compress neighboring tubules and capillary circulation, causing functional nephron loss and promoting the development of fibrosis, destroying the surrounding renal parenchyma and interstitium (4, 5).

PKD is inherited as either an autosomal dominant condition (ADPKD) or an autosomal recessive condition (ARPKD). ADPKD is common, with a frequency of 1 in 400-1000 individuals, and results in 7-10% of all end-stage kidney disease (6). ARPKD is much less prevalent but has many features in common with ADPKD (7). Renal failure can occur in newborns or early childhood in the case of ARPKD or later in adulthood in the case of ADPKD. Mutations in the PKD1 gene or PKD2 gene are responsible for ADPKD and mutations in the PKHD1 gene are responsible for ARPKD. The products of these genes, polycystin-1 (PC1), polycystin-2 (PC2), and fibrocystin/polyductin are membrane proteins that are thought to regulate intracellular calcium in response to external stimuli (8, 9). PC2

is a transient receptor potential channel subunit (TRPP2) that forms complexes with PC1 and fibrocystin/polyductin, although the relevant cellular locations of these proteins and their specific functions at these sites are still being investigated.

Cyst formation occurs with loss of PKD gene function and a subsequent disruption in calcium homeostasis (10). In ADPKD, most cells appear to function normally in the heterozygous state and cysts form only sporadically throughout the kidney (Figure 1). As such, it is likely that there is a second initiating event – either a second somatic mutation or threshold event causing haploinsufficiency. As ADPKD cysts grow in size, they often remodel and pinch off from the tubule, and become isolated, self-contained structures (Figure 1, Right). These cysts continue to enlarge over years through a slow proliferative process, and they fill as they enlarge by secretion of fluid into the cyst lumen.

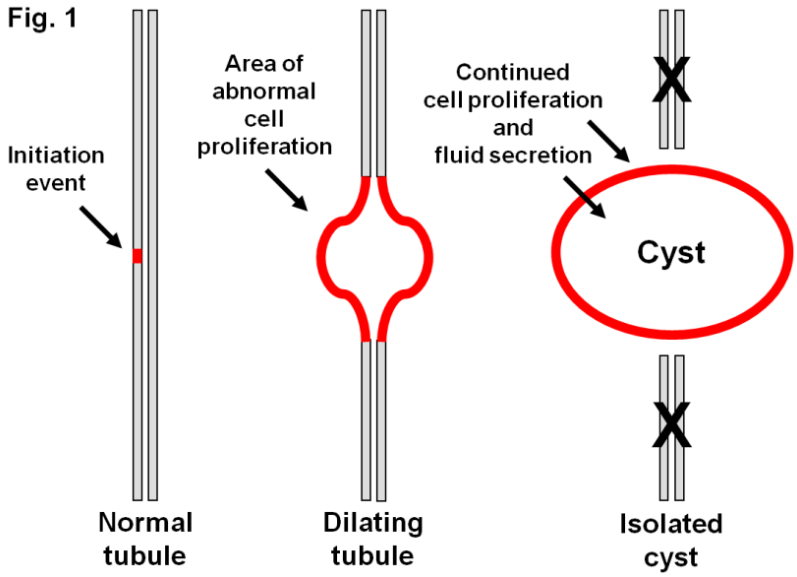

Figure 1. The process of cyst formation in autosomal dominant polycystic kidney disease. Each cyst is thought to initiate from a single cell, as the result of either a second somatic mutation or from a haploinsufficiency or threshold effect. Once that has occurred, it is thought that the abnormally proliferating cells, being incapable of forming a normal tubule, would cause a small region of the tubule to begin to dilate. As the cyst grows in size, it would become isolated by pinching off from the nephron. The cyst would then continue to enlarge over decades, through continued abnormal cell proliferation, and it would fill as it enlarges through a process of cystic fibrosis transmembrane conductance regulator -dependent fluid secretion. Cyclic AMP is important in driving both cell proliferation and fluid secretion.

In ARPKD, every tubule cell is homozygous for the PKHD1 mutation and thus is poised to initiate cell proliferation and cyst formation. It appears that large groups of cells or whole nephron segments lose their normal tubule morphology causing tubule dilatation along the axis of the nephron, in contrast to the focal cyst formation seen in ADPKD that is thought to arise initially from the transformation of a single cell. These cyst-like tubule dilatations in ARPKD may expand initially through glomerular filtration, although it is likely that they, too, depend on net fluid secretion as they enlarge.

However, in both ADPKD and ARPKD, loss of gene function alone is not sufficient to trigger abnormal cell proliferation. There is also an important role for cAMP. This chapter will examine the primary mechanisms in the cyst forming process: how normal cells become abnormal through loss of calcium homeostasis and cyclic AMP-driven cell proliferation and fluid secretion, mechanisms that are fundamental to cyst development and the progression of PKD.

A brief history of cAMP in cystic disease

Early studies carried out in cell culture demonstrated that cAMP is the key driver of cyst growth and expansion (11-20). Some of the earliest research recognized that ADPKD cysts enlarge in association with an accumulation of fluid within cysts as they form by the abnormal proliferation of tubule epithelial cells. In the initial stages of the disease (Figure 1), cysts appear as isolated structures throughout the kidney. At end-stage, polycystic kidneys are typically very large and packed full of fluid-filled cysts of various sizes. Cyclic AMP has been implicated in all aspects of the disease, from initiation, to progression, to end-stage (21).

There is no question that the growth and expansion of renal cysts in ADPKD is driven by cell proliferation. This idea came from early studies that showed the abnormal expression of genes involved in regulation of cell proliferation, including in particular, the oncogene c-myc (22-25). Renal cysts have been described as benign neoplastic growths, which unlike typical tumors are filled with fluid rather than being solid (26, 27).

Cyclic AMP was implicated in the growth of renal cysts using cell culture systems, with the demonstration that cAMP accelerates the enlargement of microcysts derived from MDCK and ADPKD cyst-lining epithelial cells growing in three-dimensional (3D) collagen gels (12, 14). These studies demonstrated increased microcyst growth, increased cell proliferation, and stimulation of transepithelial fluid secretion by prostaglandin E1 (PGE1), arginine vasopressin (AVP), cholera toxin, forskolin, 8-Br-cyclic AMP, and the phosphodie-

sterase inhibitor 1-methyl-3-isobutylxanthine (IBMX), in both established renal cell lines and primary cultures of normal human kidney and human cystic cells (11-20). All of these treatments would be expected to raise intracellular cAMP.

These studies further showed that fluid secretion by these cells is dependent on cAMP-mediated chloride secretion (18-21, 28, 29). As would be expected, it was found that in the absence of cAMP-based secretagogues, fluid was measurably *absorbed* from the microcyst cavity made up of ADPKD cells in collagen matrix (28). In contrast, 8-Br-cAMP plus IBMX induced a *reversal* in the net transport of fluid causing *secretion* into the lumen of the microcysts (28). Cellular chloride was also monitored, and changes in short circuit current (ISC) induced by forskolin in monolayers in the presence and absence of external chloride showed that cultured ADPKD cells can transport fluid in either direction, and that cAMP stimulates secretion dependent on the presence of chloride (28). Importantly, it has been demonstrated that CFTR-dependent chloride secretion drives net fluid secretion by ADPKD cyst epithelial cells (20, 29-31). Thus, cyst-filling fluid secretion is driven by cAMP.

The importance of cAMP and chloride secretion was further demonstrated in metanephric organ culture using embryonic kidneys from Pkd1$^{-/-}$ mice (32). These kidneys thrive in culture over a 4-5 day period and can be treated with various agonists and inhibitors to determine their effects on the formation of cyst-like tubule dilations. As was shown in Magenheimer et al. (32), embryonic kidneys from Pkd1$^{-/-}$ mice responded to the addition of 8-Br-cAMP to the culture medium by forming tubule dilatations that grew in size over several days. These dilatations were reduced by treatment with a protein kinase A (PKA) inhibitor, and were completely eliminated by genetic deletion of the CFTR gene, supporting the essential role of CFTR-dependent chloride secretion in this model system.

In summary, these studies identified that there are two essential cAMP-dependent components to cyst growth – cell proliferation and fluid secretion. Cyst growth cannot occur without both processes (see Figure 1). Cell proliferation is necessary to start the process, dilate the tubule, and enlarge the cyst from microscopic size to macroscopic. However, cell proliferation alone would not produce a cyst – only a relatively small solid clump of cells or adenoma, which would be much more benign than a cyst. Fluid secretion is also required, to fill the dilating structure and to enable it to swell to its enormous dimensions. However, fluid secretion alone would not produce a cyst. Without the formation of an actual enclosed cyst, increased secretion would only drain more fluid down the nephron. Thus, cAMP was shown to have two vital and essential roles in cyst formation, growth, and expansion, to stimulate both cell proliferation and cyst-filling fluid secretion.

High cAMP levels are associated with PKD

Yamaguchi et al. (33) found abnormally increased levels of cAMP in the kidneys and urine of homozygous pcy/pcy mice, which have a slowly progressive form of nephronophthisis (NPHP3). The cyst fluid from these mice and also from humans with ADPKD (34, 35) contained a lipid compound, later identified as forskolin (36), that stimulated both cAMP accumulation and cell proliferation of MDCK monolayers and increased transepithelial fluid secretion by these cells. Other studies have also shown that PKD kidneys have higher than normal levels of cAMP. Gattone et al. (37) showed higher renal cAMP in both pcy/pcy mice and PCK (PKHD1) rats, late in disease progression. Cyclic AMP levels were also measured in the kidneys of juvenile cystic kidney (jck/jck) mice, which have a mutation in the Nek8 gene, during early cyst formation at 26 postnatal days and later at 50 days (38). While there were no significant increases in the early phase of disease, there was very significant cAMP upregulation in the jck/jck cystic samples late in disease progression at 50 days. Large increases in cAMP were also seen in the urine of male Han:SPRD Cy/+ rats (39), and in a Pkd1 conditional null model in advanced-stage disease (40). As such, the results are consistent – that cAMP levels increase significantly in parallel with the degree of cystic disease.

What is the cause of the high cAMP?

Several potential causes for the increases in cAMP in PKD have been proposed. Gattone et al. showed that vasopressin receptor (V2R) mRNA was increased dramatically in early-stage postnatal cpk/cpk disease (41). Activation of V2R stimulates an increase in cAMP. This observation served as the basis for successful attempts to decrease cAMP and slow the development of cystic disease using the V2R antagonist OPC31260 in the cpk/cpk mouse and in later experiments using another OPC V2R antagonist Tolvaptan in other cell culture and animal models of PKD and in clinical trials (42-44). Consistent with these experiments, Wang et al. (45) showed that cyst formation could be effectively inhibited in PCK (PKHD1$^{-/-}$) ARPKD rats by crossing them with AVP deficient (AVP$^{-/-}$) Brattleboro rats, and that treatment of these rats with the AVP analog desmopressin (DDAVP) initiated cyst growth, clearly demonstrating the requirement for cAMP as an essential cyst-promoting factor in this genetically cystic model. The successful use of V2R blockade to lower cAMP and significantly slow or prevent cyst growth confirms the central importance of cAMP-driven mechanisms in PKD and is consistent with the major site of cyst formation being the collecting duct. However, the fact that cysts also form in other tubule segments, albeit with less overall impact, suggests that there are other mechanisms, as well, for increasing cAMP in addition to V2R stimulation.

Because of the calcium-impaired environment in PKD mutant cells, it has been suggested that calcium-regulated adenylate cyclases (ACs) and calcium-regulated phosphodiesterases (PDEs) may contribute to increasing cAMP. Pinto et al. (46) have shown that there is increased expression of the calcium-inhibited ACs 5 and 6 in ADPKD cells (but also an abnormally increased dependence on the calcium-stimulated AC3). Increased levels of ACs 5 and 6 and loss of their calcium-inhibition would be expected to generate increased cAMP. Indeed, this was supported by studies in which collecting duct conditional knockout of the AC6 gene ameliorated collecting duct-specific cystic disease (47). There was little or no impact of targeted AC6 knockout on overall kidney or urinary cAMP, indicating that decreasing cAMP specifically in the cyst cells *per se* was sufficient to reduce cyst growth. Recently, Pinto et al. (48) showed evidence for compartmentalized phosphodiesterase isoform regulation, where PDE4 appears to have a global role in regulating cAMP and cAMP-dependent fluid secretion, whereas the calcium-dependent PDE1 has a major role in regulating cAMP-dependent cell proliferation. Chebib et al. (49) have proposed an interesting hypothetical model in which increased cAMP in PKD cells results from a combination of events that ultimately causes the dysregulation of cAMP, including increased calcium-inhibited AC activity, decreased calcium-activated phosphodiesterase activity, and decreased calcium-dependent ATP release leading to decreased purinergic Gi-signaling that would normally limit vasopressin-dependent cAMP production. The result of this combination of events would be spiraling increases in cAMP in response to normal levels of vasopressin (49).

It is also quite possible that other mechanisms play a role in increasing cAMP. Of note is the observation that cyst fluid was found to contain a cyst-activating factor that promoted both cell proliferation and fluid secretion (33-35). When this factor was purified and identified by mass spectrometry, it was found to be a forskolin-like molecule (36). If it can be shown that forskolin, ordinarily thought to be specific to plants, is synthesized by animal cells, and particularly cyst-lining epithelial cells, this would provide a new mechanism for non-receptor-mediated upregulation of cAMP by direct stimulation of AC.

Pharmacological activation of the somatostatin receptor with somatostatin analogs has been used in clinical trials for ADPKD (50-52). These compounds activate Gi signaling, which down-regulates AC activity and lowers cAMP, indicating that Gi signaling has a significant impact on cAMP levels in PKD kidneys. Thus, since this is possible, it may also be the case that decreased Gi signaling by loss of PC1 activation of heterotrimeric G-protein signaling (53, 54) could lead to increased AC activity and higher cAMP. Finally, as an additional mechanism, GSK3β has been shown to upregulate vasopressin-induced cAMP, promoting cyst growth in PKD through a positive feed-forward mechanism (55, 56). Thus, abnormal GSK3β activation in PKD (57) could contribute to high levels of cAMP in the collecting duct.

Thus, there are many possible mechanisms for increasing cAMP, and for maintaining high levels in the collecting ducts and elsewhere in the kidney. These observations support the view that cAMP-driven mechanisms are important in PKD. However, it should be recognized that cAMP levels are often normal early in the disease process. Furthermore, cAMP itself is not cystogenic in wild-type animals (45), and there is no evidence that elevated cAMP alone can convert a normal cell to a cystic cell. Therefore, while cAMP is an essential (or critical) cyst-promoting factor, abnormally elevated cAMP alone cannot be the sole cyst-forming determinant.

Why high cAMP alone is not the main disease culprit?

Hanaoka et al. (31) and Yamaguchi et al. (58) demonstrated that cAMP directly stimulates ADPKD cell proliferation, and Yamaguchi et al. (58) went on to demonstrate that this involved activation of the mitogen-activated protein kinase (MAPK) pathway (Figure 2). In these studies, primary epithelial cells from cysts of ADPKD kidneys and from normal human kidney cortex were studied in culture. The effects of agonists and inhibitors on cell proliferation and activation of the extracellular signal-regulated kinase (ERK1/2) pathway were determined. Direct stimulation with 8-Br-cAMP was seen to increase the proliferation of the ADPKD cells (58), and this proliferation was inhibited by PKA inhibitors. The cAMP-generating agonists AVP, DDAVP, secretin, vasoactive intestinal polypeptide (VIP), forskolin, and prostaglandin E2 (PGE2) also stimulated proliferation. The MEK (mitogen/extracellular signal-regulated kinase) inhibitor PD98059 effectively inhibited ADPKD cell proliferation in response to cAMP agonists, whereas genistein, a receptor tyrosine kinase inhibitor, did not block cAMP-dependent proliferation (58). Importantly, cells from normal human kidneys responded in an opposite fashion to cAMP agonists by showing decreased cell proliferation (Figure 2, Left vs. Right) (31, 58).

Thus, it was evident from these studies that cAMP agonists stimulate the proliferation of ADPKD but not normal epithelial cells through PKA activation of the ERK pathway through a B-Raf dependent mechanism and that cAMP may play a critical role in ADPKD by driving cell proliferation (58, 59). Indeed, it appeared that cAMP could stimulate the proliferation specifically of ADPKD cyst cells, while not affecting the surrounding normal renal tubule cells, resulting in focal cyst growth. These experiments showed that it was not the level of cAMP, but the *response* to cAMP that characterized the cyst-promoting activity of cAMP, and this being the case that higher levels of cAMP would certainly exacerbate this pathogenic process.

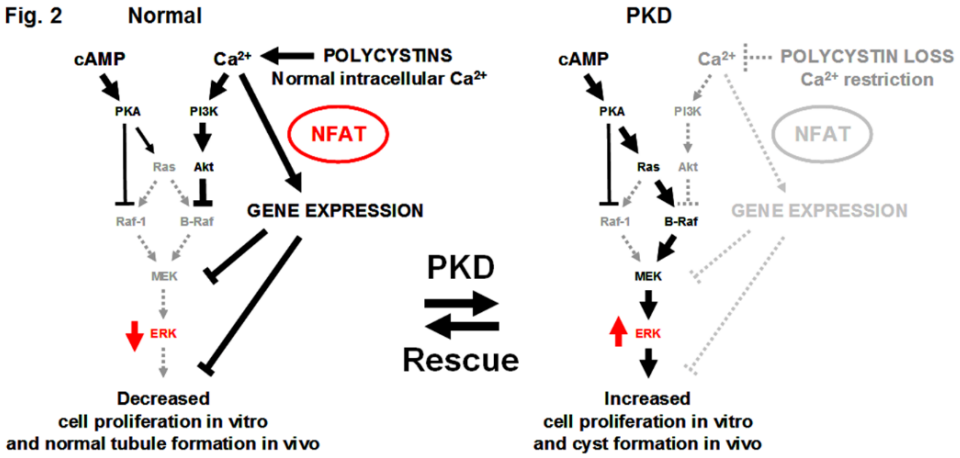

Figure 2. Cyclic AMP signaling in normal and PKD cells. Signaling pathways are shown from cAMP and calcium to extracellular signal regulated protein kinase (ERK) and cell proliferation. Left. Normal signaling pathways. Right. Signaling pathways in PKD due to loss of polycystins and/or disrupted intracellular calcium and NFAT-regulated gene expression. Solid lines = active pathways. Dotted lines = diminished pathways. PKD cells, shown on the right, differ phenotypically from normal cells (on the left) in part because loss of polycystin signaling in ADPKD disrupts the PI3K/Akt pathway and NFAT signaling, converting the cellular response to cAMP from anti-mitogenic to mitogenic. The same effect is seen by stably transfecting normal M-1 cells with a dominant negative polycystin-1 C-tail construct. ADPKD cells or PKD-like cells can be rescued by treatment with a calcium ionophore. Adapted from Figure 11 in Yamaguchi et al. (61).

The preceding experiments were instrumental in showing that ADPKD cyst-derived cells have a demonstrable phenotypic abnormality – their abnormal response to cAMP made worse by high levels of the cyclic nucleotide. However, it was not clear from these experiments, which used human ADPKD cystic cells cultured from end-stage kidneys, whether this abnormal responsiveness to cAMP was due only to PKD mutation and loss of polycystin function, or to other secondary events resulting from decades of accumulated genomic mutations and/or epigenetic changes. In principle, a second-hit initiation event could set off a cascade of secondary mutagenic events whose accumulated effects might lead to an abnormal, transformed cellular phenotype manifested by an altered sensitivity to cAMP.

Sutters et al. (60) answered this question by the development of a cell line that stably expressed a dominant-negative Pkd1 construct in immortalized M-1 renal collecting duct

cells. Cells expressing the mutant PC1 C-tail construct (Clone 20) were dexamethasone inducible and could be directly compared to cells transformed with a control construct (Clone 17) that while integrated did not express the mutant PC1 C-tail. Thus, comparable cell lines, induced to overexpress a mutant PC1 construct or no PC1 construct, could be grown in culture and their responses to cAMP examined. In proliferation assays, the mutant PC1 clone grew somewhat faster than the control clone, due presumably to low levels of endogenous cAMP, and when exogenous cAMP was added to the culture medium, the differences in their growth rates were pronounced, with Clone 20 being stimulated to proliferate, and Clone 17 being inhibited. The behavior of other clones was also examined, and in every case, cells carrying these dominant-negative PC1 constructs responded like the ADPKD cyst cells while the cells carrying control constructs behaved like normal renal epithelial cells (and the parental wild-type M-1 cells). These mutant PC1 clones responded in the same way to 8-Br-cAMP and other cAMP agonists, including forskolin, AVP, DDAVP, VIP, secretin, and PGE2, and this response could be blocked by PKA inhibitors but not by the tyrosine kinase inhibitor, genistein. The fact that the MEK inhibitor PD98059 was effective in blocking proliferation suggested that activation of the MAPK pathway was necessary for the cAMP stimulated cell proliferation. As such, these experiments showed that cells could be made to change their phenotype by overexpressing the PC1 C-tail to mimic the cAMP-responsive cyst-forming phenotype of ADPKD cells.

Expression of a dominant-negative PC1 construct changed the phenotype of these cells from one that was inhibited by cAMP to one that was stimulated by cAMP. The mutant PC1 C-tail construct did not act simply to alter the degree of cAMP-responsiveness of these cells, but acted by switching cells from one state to another. This phenotypic change was not caused by a changing level of cAMP, since both the control cell line and the mutant cell line increased their cAMP levels when treated with cAMP agonists. As such, it is not the high levels of cAMP but the opposite *responses* to cAMP that *distinguish and define* the normal and PKD phenotypes.

What is the primary abnormality?

While there is general consensus that the PKD genes regulate intracellular calcium, there is uncertainty as to where and under what conditions the gene products function, and what the calcium signal actually does to the cell. Despite this critical lack of knowledge, we reasoned that if the polycystin proteins regulate calcium, and since there is loss of the polycystins in PKD, then perhaps a decrease in intracellular calcium is the primary causative abnormality in PKD, converting the cell proliferation phenotype from cAMP-inhibited to cAMP-stimulated. If so, it might be possible to model the PKD phenotype in

cell culture by artificially reducing the levels of intracellular calcium in genetically normal cells (see Figure 2, Left vs. Right).

This experiment was accomplished by Yamaguchi et al. (61) using primary normal human kidney cells and immortalized M-1 cells treated in various ways to lower intracellular calcium, and then assayed for cell proliferation and ERK activation. Both M-1 cells and normal HKC (human kidney cortex) cells showed decreased cell proliferation when treated with 8-Br-cAMP, but increased cell proliferation when pre-treated with the calcium channel blockers, Nifedipine, Gadolinium, or Verapamil, prior to treatment with 8-Br-cAMP. Lowering free extracellular calcium with EGTA also resulted in cAMP stimulation. Thus, normal cells could be made to switch their phenotype to PKD-like cells simply by lowering intracellular calcium. Of interest was the observation that the above-mentioned stably transfected M-1 Clone 20 cells, which behaved like PKD cells (60, 62), could be normalized with respect to their cAMP-responsiveness by treatment with the calcium ionophore A23187, further validating the PC1-mutant Clone 20 cells as a faithful model of ADPKD cells and underscoring the importance and relevance of calcium in controlling the cAMP-response.

Cyclic AMP stimulation of the calcium-restricted HKC and M-1 cells was further analyzed and found to function through activation of the Ras/MAPK pathway in a PKA and Src-dependent fashion (61). This analysis revealed how the phenotypic switch is controlled by calcium. Normally growing cells in culture, in response to serum and other autocrine or paracrine growth factors in the medium, have an active Ras/MAPK pathway leading to ERK activation and cell proliferation. The absence of cAMP allows signal transduction through the MAP3K, Raf-1. If these cells are treated with 8-Br-cAMP or a cAMP agonist (Figure 2, Left), the growth factor signal will be inhibited by PKA phosphorylation and inactivation of Raf-1, and there will be decreased ERK phosphorylation and decreased cell proliferation. Under conditions of calcium restriction, as in PKD (Figure 2, Right), there is decreased intracellular calcium, which inhibits calcium-dependent PI3K and deactivates its downstream target Akt. These events result in the loss of an inhibitory Akt phosphorylation of B-Raf, causing its de-repression, allowing the cAMP signal to bypass inactive Raf-1 to upregulate ERK phosphorylation and increase cell proliferation. In other words, the ADPKD state is caused by cAMP-activated mitogenic stimulation in a cellular context made permissive by decreased calcium, as can be modeled simply by lowering intracellular calcium levels and then treating cells with cAMP agonists. These studies are the first to describe a phenotypic switch of this nature, in which a genetically normal cell is transfigured to adopt an abnormal phenotype simply by lowering tonic intracellular calcium levels.

The primary ADPKD cells described above (58) have an approximately 20 nM lower intracellular calcium concentration than their normal counterparts (63), which can explain their mitogenic response to cAMP. Importantly, these primary human ADPKD cells and primary human ARPKD cells (63) can be switched back to normal simply by raising intracellular calcium using the calcium channel activator Bay K8644, or the calcium ionophore A23187 (Figure 2, Rescue). Of significance is that these experiments take a genetically-programmed cell with an abnormal phenotype and reestablish its normal behavior with increased cellular calcium, which overrides the genetic damage (63).

Animal studies have supported and extended these observations. Using the Cy/+ Han:SPRD rat model of dominant PKD, it was possible to show that treatment of cystic rats with Verapamil dramatically exacerbated cystic disease, as determined by kidney weight and cystic index (39). A dose of Verapamil was used sufficient to normalize blood pressure increases in these animals. Increased cyst growth was accompanied by increased cell proliferation, and phosphorylation and activation of ERK, and was presumed to be in response to the endogenous cAMP. These responses were not seen in wild-type kidneys. Thus, in a non-orthologous model in which cyst formation occurs predominantly in proximal tubules, calcium appears to play a role in the disease state, consistent with the cell culture models. Experiments using the calcimimetic R-568 to raise intracellular calcium in pcy/pcy mice reduced cyst enlargement and renal fibrosis (64). Interestingly, the Chinese herbal active ingredient Triptolide, which acts by increasing intracellular calcium, has been shown to slow cell proliferation and ameliorate cystogenesis in fetal mouse kidneys when administered during pregnancy, and in a postnatal conditional Pkd1 mouse model (65, 66). Calcium has also been shown to be important in 3D microcyst cultures in which knockdown of PC2 or the inositol 1,4,5-trisphosphate receptor supported cyst growth (67); and in metanephric organ culture in which cystic dilations were reduced by treatment with a calcium ionophore (68).

In the experiments by Yamaguchi et al. referred to earlier (61), it was evident that lowering intracellular calcium using a variety of approaches was effective in converting normal cells to PKD-like cells (see Figure 2). However, in these experiments, it was noted that calcium restriction required hours-long treatments. With EGTA, a minimum of 3 hours was required, and with Verapamil, a minimum of 5-8 hours was required, depending on the dose, with 16 hours treatment being most effective. It was also demonstrated that once the phenotypic switch was established with an 8-hour Verapamil treatment, it remained in place following up to a 12-hour washout period in which cells were no longer exposed to Verapamil. These observations were inconsistent with a simple model in which the sole effect of decreased calcium is inhibition of the PI3K/Akt signaling pathway since Verapamil treatment is likely to affect intracellular calcium within minutes. A requirement

for hours-long treatments is suggestive of a need to alter gene expression through changes in gene transcription, protein translation, and/or turnover of existing mRNAs and proteins to bring about a change in the differentiated state of the cell.

In thinking about a role for the protein products of the PKD genes in regulating gene expression, the following should be considered. As mentioned earlier, PC1 and PC2 are thought to regulate intracellular calcium, probably in response to a ligand-mediated event or a mechanosensory stimulus (69). While PC2 is known to be a calcium-regulated cation channel (70), PC1 has also been shown to be capable of elevating intracellular calcium through a heterotrimeric G protein-coupled mechanism (53, 54, 71). Thus, it is likely that PC1 and PC2 proteins function together as part of a multi-protein membrane complex to regulate a number of calcium-dependent signaling pathways that ultimately regulate gene expression. Importantly, among the downstream targets regulated by polycystin-1 is the calcium-dependent phosphatase calcineurin and its immediate substrate, the transcription factor NFAT (Figure 2, NFAT) (71).

What causes the phenotypic switch? An hypothesis

We suggest that calcium-restriction does two things to renal epithelial cells. In the short term, it inhibits PI3K/Akt thus de-repressing B-Raf, but then, in the long term, it alters calcium-dependent gene expression. It is proposed that both are needed to bring about the switch to the PKD state. As shown in Figure 2, it is suggested that the calcium-dependent transcription factor NFAT is involved in mediating this phenotypic switch. The significance of this idea is that polycystin-dependent calcium signaling, acting to regulate the phenotypic state, may protect cells from the effects of normal fluctuations of cAMP, or to increases in cAMP above normal levels. Loss of polycystin-regulated calcium signaling would render cells inappropriately vulnerable to this cAMP.

There are four calcium/calcineurin-regulated NFAT family members NFAT1 (p, c2), NFAT2 (c, c1), NFAT3 (c4), and NFAT4 (x, c3), all expressed in the kidney (72-79). The NFAT proteins are maintained in an inactive state in the cytosol as phosphoproteins (P-NFAT) (Figure 3). Various phosphatases and kinases regulate the nuclear (active) or cytoplasmic (inactive) localization of NFAT. Regulation of NFAT involves calcium-dependent activation of the serine threonine phosphatase, calcineurin (Caln, PP2B, Ppp3ca), which dephosphorylates NFAT resulting in its translocation to the nucleus (Figure. 3, Left). Once in the nucleus, NFAT can bind DNA elements in target promoters often in association with other co-induced nuclear proteins, such as AP-1, GATA, and NF-κB, to regulate gene expression. The continuous maintenance of NFAT in the nucleus

requires sustained, oscillatory, calcium increases (80) to keep calcineurin in an activated form. This calcineurin-dependent signal is sensitive to inhibition by calcium channel blockers such as Verapamil or calcineurin inhibitors such as Cyclosporin A (CSA) or Tacrolimus (FK-506) (Figure 3, Right) (81-83). A number of protein kinases, in particular, GSK3β, act to phosphorylate nuclear NFAT, driving it back to the cytoplasm (84). As such, there is a continuous, calcium-regulated 'push-pull' of phosphorylation-dephosphorylation affecting active nuclear NFAT levels.

The NFAT proteins are highly homologous and have partially overlapping functions. Interestingly, calcineurin A-α knockout results in impaired kidney growth, consistent with a role for NFAT in kidney development (85, 86). Additionally, calcineurin knockout alters trafficking of aquaporin-2 (AQP2) and causes diabetes insipidus (87), suggesting that NFATs are involved in adaptive responses in the kidney. Recent data have demonstrated that renal development is absolutely calcium-dependent (88) requiring the non-canonical calcium/NFAT Wnt signaling pathway (86). A role for NFAT in the kidney is consistent with the known nephrotoxicity of Cyclosporin A (89). Additionally, the Cox-2 gene is an NFAT target in the kidney (90), and disruption of the Cox-2 gene gives rise to cysts (91), suggesting that there may be a connection between NFAT and cystic disease through Cox-2.

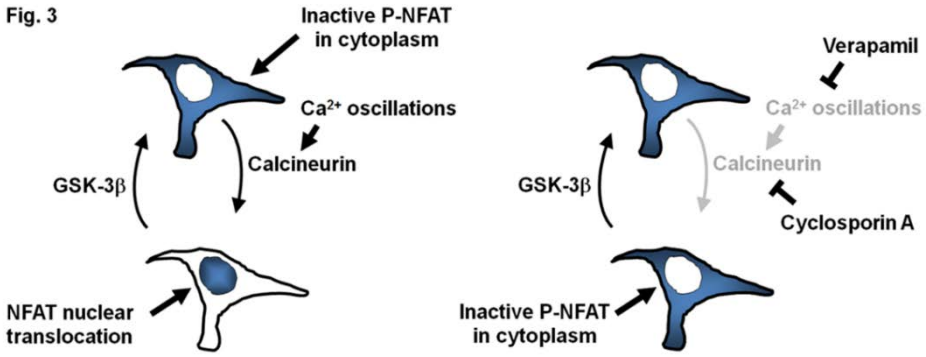

Figure 3. Mechanism of nuclear factor of activated T-cells (NFAT) regulation. Shown are cells containing cytosolic or nuclear NFAT (blue shading). (Left) The inactive hyperphosphorylated NFAT (P-NFAT) which resides in the cytosol, is dephosphorylated by calcineurin following activation by calcium oscillations, allowing NFAT to translocate to the nucleus where it activates or represses genes together with other transcription factors. NFAT is then re-phosphorylated by cellular kinases, including GSK3β, returning it to the cytoplasm. (Right) NFAT activation can be inhibited by treating cells with calcium channel blockers, such as Verapamil, or with calcineurin inhibitors, such as Cyclosporin A. It is hypothesized that decreased intracellular calcium resulting from polycystin loss inhibits NFAT activation, contributing to the PKD phenotypic switch.

Importantly, the calcium/calcineurin/NFAT pathway also has a cell cycle role that may be directly relevant to PKD. The cyclin-dependent kinase CDK4 has been shown to be a key player in cell proliferation associated with PKD (92-96). CDK4 is under the regulation of cAMP (97) and other pathways relevant to PKD (94), but also calcineurin and NFAT through different mechanisms. NFAT transcriptionally down-regulates the CDK4 gene (98, 99). As such, decreased nuclear NFAT activity would be expected to increase CDK4 protein. For its activation, CDK4 requires phosphorylation of Threonine-172 (T172) by an ill-defined cyclin-activating kinase, which is regulated indirectly by cAMP (97). T172 phosphorylation is reversed by calcineurin phosphatase activity (100), causing its inactivation. Therefore, decreased calcium/calcineurin signaling in PKD would lead to increased CDK4 levels and activity, promoting cell cycle entry, a requirement for cyst growth and enlargement. As such, impaired calcium signaling in PKD could bring about a phenotypic switch at multiple levels, both upstream and downstream of ERK (Figure 2).

Conclusions

There is ample evidence demonstrating that PKD is associated with abnormally high levels of cAMP. However, it is also evident that the essential abnormality in PKD is not high cAMP *per se*, but the *response* of cells to cAMP. The basis for this abnormal response to cAMP is that calcium regulation is disrupted in PKD as a consequence of abnormal polycystin function, resulting in a number of signaling and gene expression changes that cause cells to respond abnormally to cAMP regardless of whether the levels are physiologically normal or elevated. One of the abnormal signaling events is the loss of calcium inhibition of B-Raf, which allows cAMP to activate B-Raf, MEK, and ERK. However, it is likely that additional abnormalities are required to transform the phenotype of a cell. It is hypothesized that one such abnormality results directly from decreased calcium, which would impair calcineurin function and decrease the activity of the NFAT transcription factor, affecting the differentiated state of these cells and their cell cycle control, and leading to the transformation of normal tubular epithelial cells to cystic epithelial cells that would continue to divide unchecked as they grow into massive fluid-filled cysts.

Conflict of interest

The author declares that he has no conflicts of interest with respect to research, authorship and/or publication of this book chapter.

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