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ATP Release Mechanisms in Primary Cultures of Epithelia Derived from the Cysts of Polycystic Kidneys

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Abstract. Autosomal dominant polycystic kidney disease (ADPKD) cyst enlargement is exacerbated by accumulation of fluid within the lumen of the cyst. Extracellular nucleotides and nucleosides stimulate fluid and chloride (Cl^-) secretion across epithelia and are potent autocrine and paracrine agonists within tissues. This study tests the hypothesis that ATP may be released by ADPKD epithelial cells. Once released, extracellular nucleotides and their metabolites may become "trapped" in the cyst lumen. As a consequence, extracellular ATP may augment ADPKD cyst enlargement through stimulation of salt and water secretion across ADPKD epithelia that encapsulate ADPKD cysts. To test this hypothesis, bioluminescence detection assays of ATP released from primary cultures of human ADPKD epithelial cells were compared with non-ADPKD human epithelial primary cultures. ADPKD cultures release comparable or greater amounts of ATP than non-ADPKD cultures derived from proximal tubule or cortex. ATP release in both ADPKD and non-ADPKD primary epithelial monolayers was directed largely into the apical medium; however, basolateral-directed ATP release under basal and stimulated conditions was also observed. Hypotonicity potentiated ATP release into the apical and basolateral medium in a reversible manner. Reconstitution of isotonic conditions with specific osmoles or inhibition with mechanosensitive ion channel blockers dampened hypotonicity-induced ATP release. "Flash-frozen" cyst fluids from ADPKD cysts, harvested from multiple donor kidneys, were screened by luminometry. A subset of cyst fluids contained as much as 0.5 to 10 μM ATP, doses sufficient to stimulate purinergic receptors. Taken together, these results show that ADPKD and non-ADPKD human epithelial primary cultures release ATP under basal and stimulated conditions and that ATP is released *in vitro* and into the cyst fluid by cystic epithelial cells in concentrations sufficient to stimulate ATP receptors. It is hypothesized that extracellular nucleotide release and signaling may contribute detrimentally to the gradual expansion of cyst fluid volume that is a hallmark of ADPKD.

Autosomal dominant polycystic kidney disease (ADPKD) is an inherited, multigenic renal disorder caused by defects in as many as three genes (1–3). ADPKD has several phenotypic hallmarks. Kidneys that are afflicted with ADPKD have dilation of renal tubules and the progressive formation, budding, and enlargement of cysts derived from multiple segments of the nephron (1,2). Once cysts develop, they enlarge due to an increased rate of growth and proliferation and expression of a "fetal-like" epithelial phenotype (1,2). This is due to abnormal polarity of multiple ion transporters and growth factor receptors, most notably the Na^+,K^+-ATPase pump and the epidermal growth factor (EGF) receptor (1,4,5). Cyst enlargement is also exacerbated by a detrimental combination of salt and water secretion into the cyst lumen and increased cystic epithelial proliferation (1,2,6,7). The CF gene product, the cystic fibrosis transmembrane conductance regulator (CFTR), contributes somewhat ironically to this unwanted Cl^- and fluid secretion (8–11).

Extracellular nucleotides and nucleosides of the ATP, ADP, 5′-AMP and adenosine axis or "purinergic" axis are potent autocrine and paracrine agonists that act within tissues to regulate cell function (12,13). Cells of nonepithelial origin release ATP for specialized physiologic purposes such as self-aggregation, neurotransmission, pain perception, and cell volume regulation (12,14–17). Epithelial cells release ATP for autocrine and paracrine regulation of epithelial Cl^- and Na^+ channels (18–25), autocrine and paracrine regulation of fluid secretion (26), and cell volume regulation (17). These effects on cellular function are transduced by two classes of purinergic receptors, the G protein-coupled, seven transmembrane P2Y receptors and the ATP-gated, calcium-permeable P2X receptor channels (14–16).

Because extracellular ATP is a potent autocrine and paracrine agonist and because extracellular ATP is a potent Cl^- and fluid secretagogue, we hypothesized that extracellular nu-

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cleotides are released by ADPKD epithelial cells that line ADPKD cysts. To test this hypothesis, a highly sensitive bioluminescence detection assay of ATP released by epithelial cells grown as less polarized cell cultures on collagen-coated dishes or as polarized monolayers on collagen-coated permeable filter supports was used to characterize ATP release from ADPKD and non-ADPKD human renal epithelial primary cultures. Luminometry was also used to detect ATP in the cyst fluid harvested from ADPKD cysts of multiple donor kidneys. Results described herein will show that ADPKD cells (as well as non-ADPKD cells) release significant quantities of ATP under basal and stimulated conditions. ATP was also detected in concentrations sufficient to stimulate ATP receptors. As such, extracellular ATP signaling may cause fluid accumulation in ADPKD cysts and exacerbate the progression ofADPKD.

Materials and Methods

Human Renal Epithelial Cell Primary Culture

Human proximal tubule epithelial cell primary cultures (hPCT; Clonetics™, San Diego, CA) and human mixed renal cortical epithelial cell primary cultures (hCORT; Clonetics™) were grown on diluted Vitrogen (collagen types I and IV diluted 1:15 in Dulbecco's phosphate-buffered saline (PBS); CelTrix, Santa Clara, CA)-coated 35-mm culture dishes (Corning) and 25- and/or 75-cm^2 culture flasks (Falcon, Oxnard, CA) or on filter supports (Millicell 12-mm diameter insert; Millipore, Bedford, MA) in a defined, reduced serum medium (renal epithelial basal medium [REBM]) supplemented with an REBM BulletKit™ containing 0.5% fetal bovine serum (FBS), human EGF (0.5 ml), insulin (0.5 ml), hydrocortisone (0.5 ml), epinephrine (0.5 ml), tri-iodothyronine (0.5 ml), transferrin (0.5 ml), gentamycin (0.5 ml), and 1× penicillin/streptomycin (diluted in 500 ml of REBM medium).

Human ADPKD epithelial primary cultures (coded "hADPKD") and normal human kidney proximal tubule epithelial cells (coded "NHK-PST") were grown in a supplemented serum-free Click's/RPMI as described previously (27), but with the same BulletKit of additives listed above. A few cultures of human ADPKD epithelial cells were also obtained as gifts from two other laboratories (Drs. Kazushige Hanaoka and William Guggino at Johns Hopkins University School of Medicine; Drs. Darrin Wallace and Lawrence Sullivan at University of Kansas Medical Center). Cells from the Guggino laboratory were grown as described above in supplemented REBM medium. Cells from the Sullivan laboratory were grown in DMEM/F12 (Life Technologies-BRL) supplemented with 10% FBS (Life Technologies-BRL) and 2 ml of insulin/transferrin/selenium stock (Life Technologies-BRL). These cells had lower ATP release magnitudes than cells derived from the other two laboratories and were grown in medium with ≤1% FBS. All media were also supplemented with 1× penicillin/streptomycin solution (Life Technologies-BRL) and 2 ml of fungizone stock solution (Life Technologies-BRL).

ATP Bioluminescence Release Assays of Epithelial Cells Grown to Confluence on Culture Dishes

Specific details concerning the development and optimization of this assay have been described previously (28). ADPKD and non-ADPKD epithelial cell primary cultures were grown to confluence on collagen-coated dishes. Cells were washed twice in PBS, and an OptiMEM-I serum-free medium (Life Technologies-BRL) was added to the cells containing 2 mg/ml luciferase-luciferin reagent (lyophilized reagent; Sigma, St. Louis, MO). The dish bathed in the ATP bioluminescence detection medium was placed on a platform, lowered into a chamber in complete darkness within a simplified model Turner TD20/20 luminometer, and studied immediately within the luminometer in real time. Background luminescence (cells and medium without luciferase-luciferin reagent) was less than 0.1 arbitrary light unit (ALU).

ATP Bioluminescence Release Assays of Epithelial Cells Grown as Confluent Monolayers

Again, specific details concerning the development and optimization of this assay have been described previously (28). ADPKD and non-ADPKD epithelial cell primary cultures were seeded at high density (at least 10^5 cells) onto collagen-coated permeable supports (Millicell 12-mm diameter insert; Millipore). Monolayers were grown for 4 to 6 d until no fluid leaked from the basolateral space into the apical side or filter cup. This reflected a transepithelial resistance of 200 Ω/cm^2 or higher depending on the cell type (after subtracting the resistance of the filter itself), as measured with an epithelial voltohmmeter (World Precision Instruments, Sarasota, FL), and a monolayer tight to fluid for at least 24 h. Cells were then fed with fresh medium and incubated overnight for subsequent experimentation the next day. This also served to wash away any cells that had been shed by the monolayer. Monolayers were washed twice in PBS on both sides, and an OptiMEM-I serum-free medium was added to the cells containing 2 mg/ml luciferase-luciferin reagent to one side of the monolayer (*i.e.*, the side on which ATP is detected) and 200 μl of OptiMEM-I medium without detection reagent on the contralateral side. The filter was placed on a lid of a 35-mm culture dish in a 200-μl drop of medium on the basolateral side, and a 200-μl volume was added into the filter cup on the apical side. The filter on the lid was placed on a platform, lowered into a chamber in complete darkness within a simplified model Turner TD20/20 luminometer, and studied immediately within the luminometer in real time.

Analysis of ATP in Cyst Fluids by Luminometry

Standard curves of ATP (ATP-Mg^{2+} salt; Sigma) at known concentrations were performed with 2 mg/ml luciferase-luciferin reagent in OptiMEM-I medium by serial dilution from a 0.5 M ATP stock (made fresh at the time of performing standard curves) to approximate the concentrations of ATP released from cells. This curve was performed on the day that cyst fluid samples were studied. The same mixture of luciferase-luciferin reagent was mixed in a 1:1 volume with cyst fluid, and luminescence was assayed in triplicate. Luminescence was corrected for the total volume of fluid collected from the individual cyst, and the concentration of ATP in a given cyst fluid sample was determined through comparison to the standard curve.

Materials

Once every month, standard curves were performed to authenticate the detection reagent. The Sigma detection reagents were consistent from vial to vial. Measurements at each dose of ATP were performed in triplicate; luminescence values were stable among those three measurements (*i.e.*, no "bleaching" or other instability in the signal was observed). All inhibitors or osmoles were tested for their effect on luciferase activity; $GdCl_3$ or the osmoles used to reconstitute isotonic osmolality did not affect luciferase activity significantly (28).

Statistical Analyses

Bioluminescence (in arbitrary light units, or ALU) was measured continuously from 15-s photon collection intervals by the luminometer and recorded in a laboratory notebook. Data values were compiled into Microsoft Excel spreadsheets in which the mean ± SEM was calculated for each time point in each set of experimental time courses. Data were then plotted in SigmaPlot for Windows using the same arbitrary light unit values. Statistics were performed using SigmaStat for Windows. Paired *t* tests were performed. A P value <0.05 was considered significant.

Results

To compare ADPKD primary cultures to non-ADPKD primary cultures, primary cultures of hPCT and hCORT were studied in parallel with hADPKD cells. hCORT cells are a heterogeneous mixture of renal epithelial cells from the cortex that may include proximal tubule cells, distal tubule cells, and cortical collecting duct cells. There is no ideal renal cell culture that controls for the heterogeneity of origin of hADPKD cells; however, hPCT cells and hCORT cells provide a close approximation.

Primary Renal Epithelial Cells Release ATP

Initially, renal primary cultures grown to confluence in 35-mm culture dishes were studied for their capacity to release ATP in comparison to a standard curve of ATP bioluminescence detection (Table 1). Human ADPKD cells released twofold more ATP than hPCT cells and threefold more ATP than hCORT cells under basal conditions (Table 1). The approximate amount of released extracellular ATP, when correlated with the standard curve, is also shown in Table 1. Released ATP concentration was approximately 350 nM and 500 nM for hCORT and hPCT cells, respectively. In hADPKD cells, the released ATP concentration achieved under basal conditions was approximately 2 μM. These results suggest that human ADPKD cells release significantly more ATP than non-ADPKD primary cultures under basal conditions and that both ADPKD and non-ADPKD epithelial cells release quantities of ATP sufficient to stimulate purinergic receptors.

Dynamics of ATP Release in Primary Cultures of ADPKD and Non-ADPKD Epithelial Cells

Time courses of ATP release from confluent cell cultures *without stimulation* are shown in Figure 1. In each case, a basal magnitude of ATP is released by cells that is consumed immediately by a molar excess of luciferase-luciferin enzyme in the medium. Again, more ATP is released by human ADPKD cell cultures than the non-ADPKD cultures (Figure 1, A through C). In each culture, the luminescence signal decays over time. This is due to the expression and/or secretion of ecto-ATPases or ecto-apyrases by epithelial cells (12). Degradation of ATP dampens the luminescence signal in these assays. The luminescence signal decays more rapidly in hCORT primary cultures, from 886.7 ± 97.47 ALU at the beginning of the time course to 399.0 ± 28.93 ALU immediately before addition of inhibitors (Figure 1B), suggesting that these cells express more ecto-ATPase activity. There is also loss of signal from the hPCT primary cultures, from 578.0 ± 88.64 ALU to 358.7 ± 53.45 ALU (Figure 1A), as well as hADPKD primary cultures (1575 ± 188.3 ALU to 1273 ± 84.56 ALU); however, the decay was not statistically significant (Figure 1C). Nevertheless, this does not interfere with the study of epithelial cell ATP release mechanisms or their regulation (see below). Moreover, nucleotide metabolites also have biologic activity, and their significance will be discussed below. Interestingly,

Table 1. Basal luminescence detection of released ATP from cultures or monolayers of ADPKD and non-ADPKD human renal epithelial primary cultures[a]

ATP Release Assays of Epithelial Cell Cultures Grown on 35-mm Diameter Dishes		
Renal Epithelial Cell Cultures	Total ATP Release	Approximate [ATP]
hPCT	1245 ± 87.98 (79)	~500 nM
hCORT	828.2 ± 66.62 (30)	~350 nM
hADPKD	2592 ± 197.0 (34)[b]	~2μM
ATP Release Assays of Polarized Epithelial Monolayers Grown on 12-mm Diameter Membrane Supports		
Renal Epithelial Monolayers	Apical Release	Basolateral Release
hPCT	19.27 ± 3.15 (16)	6.275 ± 1.399 (14)
hCORT	10.22 ± 1.74 (17)	2.454 ± 0.804 (17)
hADPKD	31.18 ± 3.63 (30)[b]	9.304 ± 1.736 (28)[c]

[a] Peak luminescence values in arbitrary light units (ALU) are shown from ATP release assays of confluent cell cultures in 35-mm diameter dishes or ATP release assays from polarized monolayers grown on 12-mm diameter filters. For the latter, both apical and basolateral release values are shown. These luminescence values are total units and are not corrected for volume or for apical *versus* basolateral membrane estimated surface area. Experimental number (n) is shown in parentheses. Approximate [ATP] concentration relative to a standard curve is also shown (100 nM ATP = 303 ALU; 1 μM ATP = 2184 ALU; and 10 μM ATP = 7259 ALU). Data are expressed as mean ± SEM. ADPKD, autosomal dominant polycystic kidney disease primary cultures; hPCT, human proximal tubule epithelial cell primary cultures; hCORT, human mixed renal cortical epithelial cell primary cultures; hADPKD, human ADPKD epithelial primary cultures.

[b] $P < 0.005$ by unpaired ANOVA.

[c] $P < 0.05$ by unpaired ANOVA.

gadolinium chloride ($GdCl_3$, 100 μM), a broad specificity inhibitor of mechanosensitive ion channels and Ca^{2+} entry channels (29), inhibits ATP release partially in both human ADPKD and non-ADPKD primary cultures (Figure 1, A through C). The remaining luminescence signal was abolished by the ATP scavenger, apyrase, verifying that the bioluminescence signal was generated by ATP (Figure 1, A through C). Background was less than 0.1 ALU (Figure 1, A through C). These results show that human ADPKD cells release significantly more ATP than non-ADPKD primary cultures under basal conditions. Moreover, basally active ATP release mechanisms are partially sensitive to the ion channel blocker $GdCl_3$, suggesting that epithelia express $GdCl_3$-sensitive and $GdCl_3$-insensitive ATP release mechanisms.

Hypotonic Challenge Potentiates ATP Release from Renal Epithelial Primary Cultures

Hypotonicity is a potent stimulus of ATP release in hepatocytes (17,30) and in airway epithelial cells (28). We tested the hypothesis that hypotonicity may trigger ATP release from ADPKD and non-ADPKD epithelial cells. After an initial period of monitoring ATP release under isotonic conditions, stepwise dilutions of the medium osmolality were performed with distilled water (Figure 1, A through C). As little as 24% dilution of the osmolality caused a significant increase in ATP release (Figure 1). Additional dilutions of the osmolality of 33 and 41% triggered additional increases in ATP release (Figure 1). Addition of isotonic medium had no significant effect on ATP release (data not shown; but see Figure 3 for example), ruling out mechanical artifact as a stimulus (31). Hypotonicity stimulated ATP release to concentrations that approached or exceeded a concentration of approximately 1 μM in non-ADPKD cultures and that exceeded 5 μM in ADPKD cultures (compare Figure 1 values with the standard curve values in Table 1 legend). Subsequent addition of $GdCl_3$ at concentrations of 100 and 200 μM inhibited hypotonicity-induced ATP release to a level below isotonic luminescence values (Figure 1, A through C). Apyrase abolished the remaining signal. These results show that hypotonicity stimulates ATP release in human ADPKD and non-ADPKD primary cultures and that ATP release mechanisms active under isotonic conditions and potentiated by hypotonicity are sensitive to the ion channel blocker $GdCl_3$. These results agree with previous studies in hepatocytes (17,30) and in airway epithelia (28), showing that hypotonic challenge stimulates ATP release and extracellular ATP signaling.

Differential ATP Release Across the Apical and Basolateral Membranes of Renal Epithelial Monolayers

These initial studies of renal primary cultures grown in culture dishes were informative with respect to the relative amounts of ATP released in isotonic and hypotonic conditions and the identification of hypotonicity as a stimulus for ATP release. However, to study the epithelial cell biology and polarity of ATP release more precisely, primary cultures of human ADPKD and non-ADPKD cells were grown on collagen-coated permeable filter supports to study the sidedness of ATP release and signaling in the apical and basolateral media bathing renal epithelial monolayers. Table 1 also shows the magnitude of ATP released into the apical and basolateral medium for each primary culture grown as monolayers. Again, ADPKD epithelial monolayers release significantly more ATP under basal conditions into the apical and basolateral media when compared with non-ADPKD epithelial monolayers (hPCT and hCORT) (Table 1). Importantly, in all normal and ADPKD primary cultures, apical-directed ATP release outweighed basolateral-directed ATP release three- to fourfold (Table 1 and Figure 2). Figure 2 illustrates the time courses of bioluminescence detection of ATP released into the apical or basolateral medium surrounding hPCT (Figure 2A), hCORT (Figure 2B), and hADPKD (Figure 2C). Similar results and magnitudes of ATP release were also observed in monolayers of normal human kidney proximal tubules (NHK-PST cells) generated originally in the Wilson laboratory (data not shown). $GdCl_3$ (100 μM) inhibited 50% of apical-directed ATP release in ADPKD and non-ADPKD monolayers (Figure 2, A through C). Intriguingly, $GdCl_3$ inhibited more than two-thirds of basolateral-directed ATP release, when added at the same 100 μM dose (Figure 2, A through C). Figure 3 shows pooled results from all ADPKD primary cultures. These results mirror release assays of primary cultures grown in culture dishes showing that ADPKD cells release more ATP than non-ADPKD cells. Importantly, these results also show that epithelial ATP release is directed largely across the apical membrane and that extracellular nucleotide signaling is more prominent on the apical side of the epithelium.

Hypotonic Challenge Potentiates ATP Release Across Apical and Basolateral Membranes of ADPKD and Non-ADPKD Epithelial Monolayers

Hypotonicity was also tested as a stimulus for ATP release across the apical and basolateral membranes of renal epithelial monolayers. In hPCT monolayers, stepwise dilutions of the medium osmolality of 10% (“mild”), 33% (“intermediate”), and 55% (“robust”) were performed, and ATP release was potentiated by each hypotonic challenge across both the apical and basolateral membranes (Figure 2, A through C). Similar additions of isotonic medium had no effect on ATP release (data not shown; but see Figure 3 for example), ruling out mechanical or hydrostatic pressure stimuli as artifactual stimuli for ATP release in this assay (31). For apical-directed ATP release in hPCT monolayers, faithful and incremental increases in ATP release were observed (Figure 2A). In contrast, basolateral-directed ATP release began at a much lower level under isotonic conditions and during mild or intermediate hypotonicity; however, 55% dilution of the medium osmolality triggered a profound and rapid increase in ATP release that equaled or even exceeded apical-directed and hypotonicity-induced ATP release (Figure 2A). Monolayers of hCORT cells also responded to stepwise increases of hypotonicity with incremental potentiation of ATP release across both the apical and basolateral membranes (Figure 2B). In contrast to hPCT monolayers, however, hCORT monolayers did not respond as robustly to the most significant hypotonic challenge with basolateral

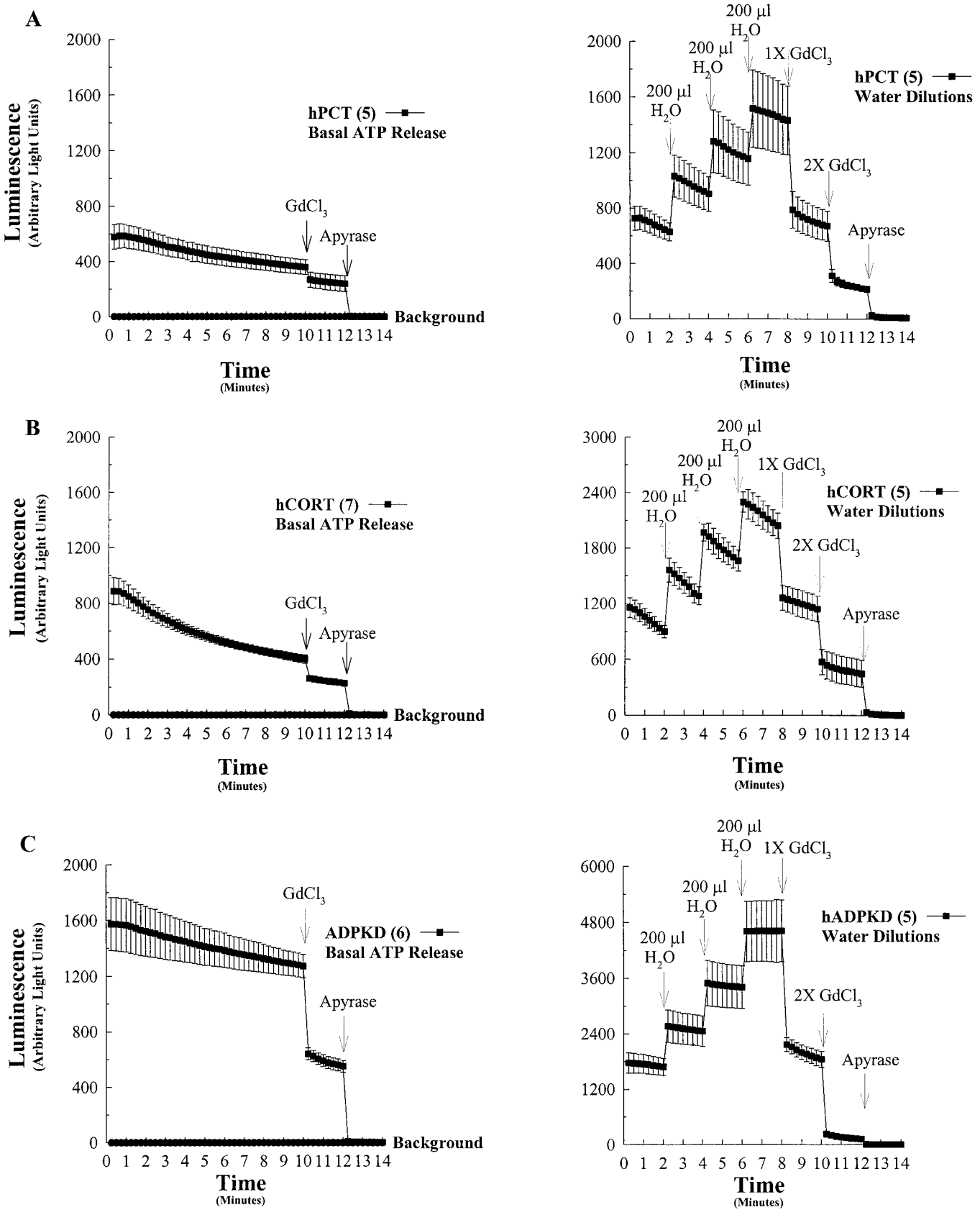


Figure 1. Autosomal dominant polycystic kidney disease (ADPKD) and non-ADPKD epithelial primary cultures release ATP under isotonic and hypotonic conditions. Time courses of ATP bioluminescence detection under basal conditions (Left) and ATP bioluminescence detection upon hypotonic challenge (Right) for human proximal tubule epithelial cell primary cultures (hPCT; A), human mixed renal cortical epithelial cell primary cultures (hCORT; B), and human ADPKD epithelial primary cultures (hADPKD; C). Luminescence (in arbitrary light units

ATP release (Figure 2B). Bioluminescence values in the basolateral medium remained less than in the apical medium after all hypotonic challenges (Figure 2B). Similar results and magnitudes of ATP release were also observed in monolayers of normal human kidney proximal tubules (NHK-PST cells) generated in the Wilson laboratory (data not shown).

Summarized results from all ADPKD monolayers are also shown in Figure 2C. Hypotonic challenge stimulated ATP release in a stepwise manner similar to that observed in hCORT monolayers but in magnitudes two- to fourfold greater than observed in non-ADPKD monolayers (Figure 2, A and B). Like hCORT results, ATP release values in the basolateral medium remained less than in the apical medium after all hypotonic challenges (Figure 2C). Hypotonicity-induced ATP release at the most robust dilution was as great or greater in hPCT monolayers than in hADPKD monolayers (Figure 2C). Taken together, these results suggest that hypotonicity potentiates ATP release across both the apical and basolateral membranes of ADPKD and non-ADPKD epithelial monolayers in primary culture. As with ATP release under isotonic or basal conditions, hADPKD monolayers released more ATP under isotonic states and most, but not all, hypotonic states.

Gadolinium Chloride Inhibits Hypotonicity-Induced ATP Release into the Apical and Basolateral Media Bathing ADPKD and Non-ADPKD Epithelial Monolayers

As with isotonic ATP release without stimulation, $GdCl_3$ (100 μM) inhibited approximately 67% of hypotonicity-induced ATP release across the apical membranes of ADPKD and non-ADPKD epithelia (Figure 2, A through C). Increasing $GdCl_3$ to 200 μM lowered ATP release to basal levels in all cases (Figure 2, A through C). Hypotonicity-induced ATP release into the basolateral medium was blocked completely by 100 μM $GdCl_3$; 200 μM $GdCl_3$ had little additional inhibitory effect (Figure 2, A through C). These results show that hypotonicity-induced ATP release in ADPKD and non-ADPKD epithelial monolayers is sensitive to the ion channel blocker $GdCl_3$. Basolateral ATP release mechanisms under isotonic and hypotonic conditions were more sensitive to $GdCl_3$ than apical release pathways. $GdCl_3$ may be a useful tool in the identification and characterization of ATP release mechanisms in epithelia, both $GdCl_3$-sensitive and $GdCl_3$-insensitive pathways.

Readdition of Specific Osmoles Attenuates Hypotonicity-Induced ATP Release

Hypotonicity elicited by dilution of the medium osmolality with distilled water may stimulate ATP release by at least two mechanisms. First, hypotonicity may cause increased epithelial cell volume ("hypotonic cell swelling"), triggering the release of nucleotide agonists by a mechanically driven mechanism. Alternatively, dilution of the medium osmolality may also dilute an inhibitor of ATP release or a constituent of the medium sensed by the epithelial cell as a measure of osmolality. To discriminate between these two possibilities, a battery of different osmoles was added to the preparation after hypotonic challenge in a manner similar to the ion channel blocker $GdCl_3$. Figure 3 illustrates these results. Readdition of consecutive boluses of 50 mM NaCl reversed fully hypotonicity-induced ATP release in hPCT, hCORT, and hADPKD epithelial monolayers (Figure 3, A through C). Interestingly, 50 and 100 mM NaCl added to isotonic medium to induce a hypertonic environment reduced basal ATP release in ADPKD and non-ADPKD monolayers (Figure 3, A through C). These results show that hypotonicity-induced ATP release is fully reversible and rule out cellular lysis as an artifactual contributor to the bioluminescence signal. Moreover, establishment of a hypertonic environment dampens ATP release detected under isotonic conditions.

Does any osmole work to reverse hypotonicity-induced ATP release? If ATP release were caused merely by osmotically induced, physical swelling of the epithelium, any osmole should reverse the response. To our surprise, addition of sucrose in 100 mM aliquots failed to reverse the response in hCORT monolayers (Figure 3, C and D). A similar lack of effect was observed with mannitol (data not shown). These data suggested that Na^+ and/or Cl^- ions were important in hypotonicity-induced ATP release: Dilution of salt would stimulate release and increasing the concentration of salt should inhibit release. To test this concept, sodium gluconate and *N*-methyl-D-glucamine (NMDG)-Cl were also tested for their relative ability to reverse hypotonicity-induced ATP release. Sodium gluconate inhibited the response poorly, whereas NMDG-Cl had similar efficacy to NaCl in blocking hypotonicity-induced ATP release (Figure 3, C and D). A summary of the effects of mechanosensitive ion channel blockers, $GdCl_3$ and a related trivalent cation, lanthanum chloride ($LaCl_3$), as well as sucrose, NaCl, NMDG-Cl, and sodium gluconate, is given in Figure 3D. Taken together, these results show that dilution of extracellular anions is essential for the hypotonicity-induced ATP release response. Moreover, adding greater than isotonic amounts of NaCl inhibits the release of ATP observed under basal conditions, illustrating that hypotonicity augments ATP release, whereas hypertonicity reduces ATP release. Thus, extracellular Cl^- concentration may be sensed by the epithelium and is an indicator of the external osmotic environment.

[ALU]) is shown on the y-axis, and time (in minutes) is shown on the x-axis in this and all luminescence graphs. Background luminescence was less than 0.1 ALU. Medium controls where isotonic medium was added in lieu of distilled water had no significant effect on the luminescence signal (data not shown). $GdCl_3$ ($1\times$ = 100 μM and $2\times$ = 200 μM) inhibited ATP release measured as luminescence. Apyrase abolished the remaining signal in all experiments. Number of experiments is shown in parentheses. Mean ± SEM is plotted in this and all figures.

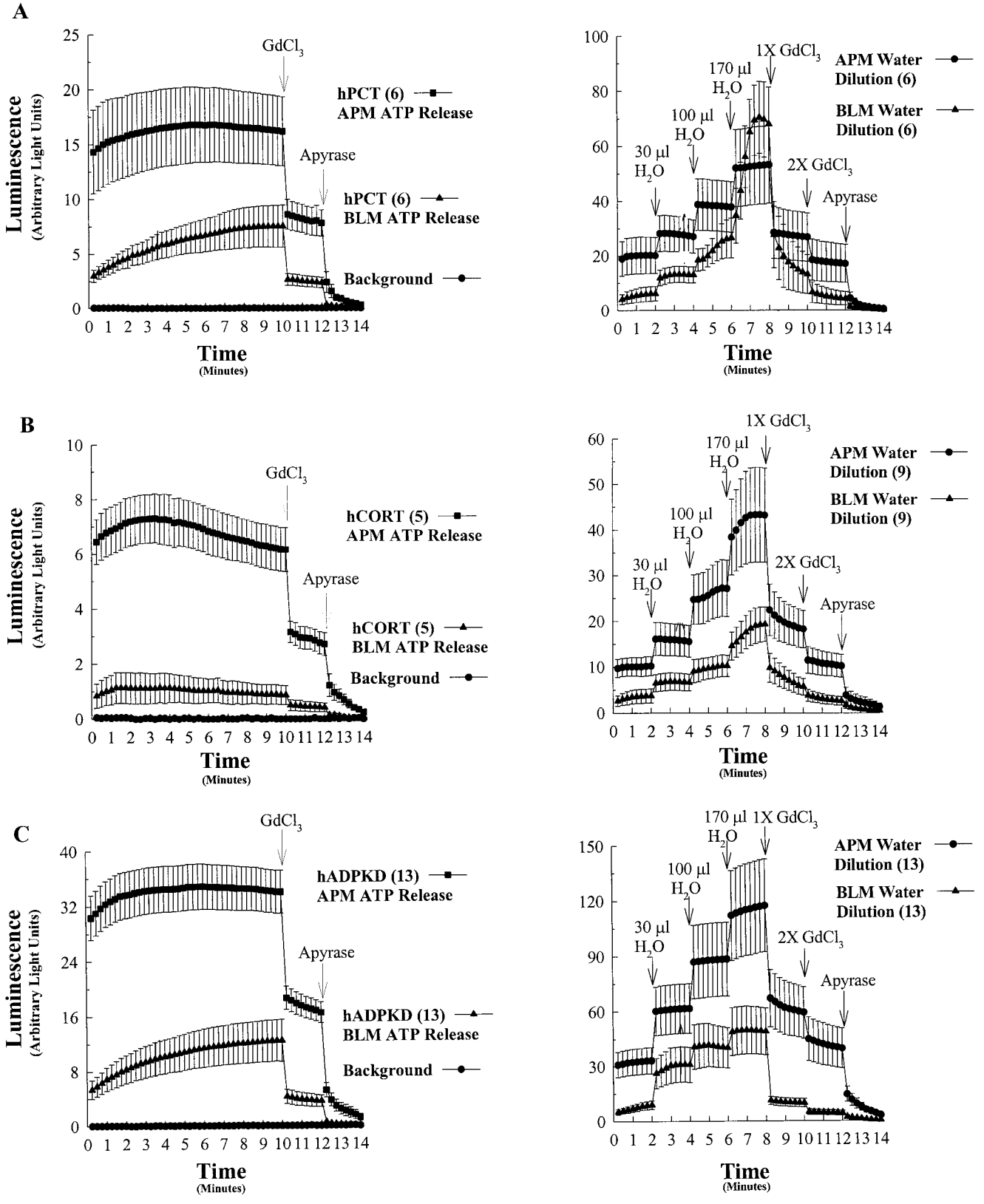


Figure 2. ADPKD and non-ADPKD epithelial monolayers release ATP under isotonic and hypotonic conditions. Time courses of isotonic (Left) and hypotonicity-induced (Right) ATP release into the apical or basolateral medium (measured as bioluminescence) for hPCT monolayers (A), hCORT monolayers (B), and hADPKD monolayers (C). Medium controls where isotonic medium was added in lieu of distilled water had no significant effect on the luminescence signal (data not shown). $GdCl_3$ ($1\times = 100\ \mu M$ and $2\times = 200\ \mu M$) inhibited ATP release measured as luminescence. Apyrase abolished the remaining signal in all experiments. Number of experiments is shown in parentheses. Mean ± SEM is plotted in this and all figures.

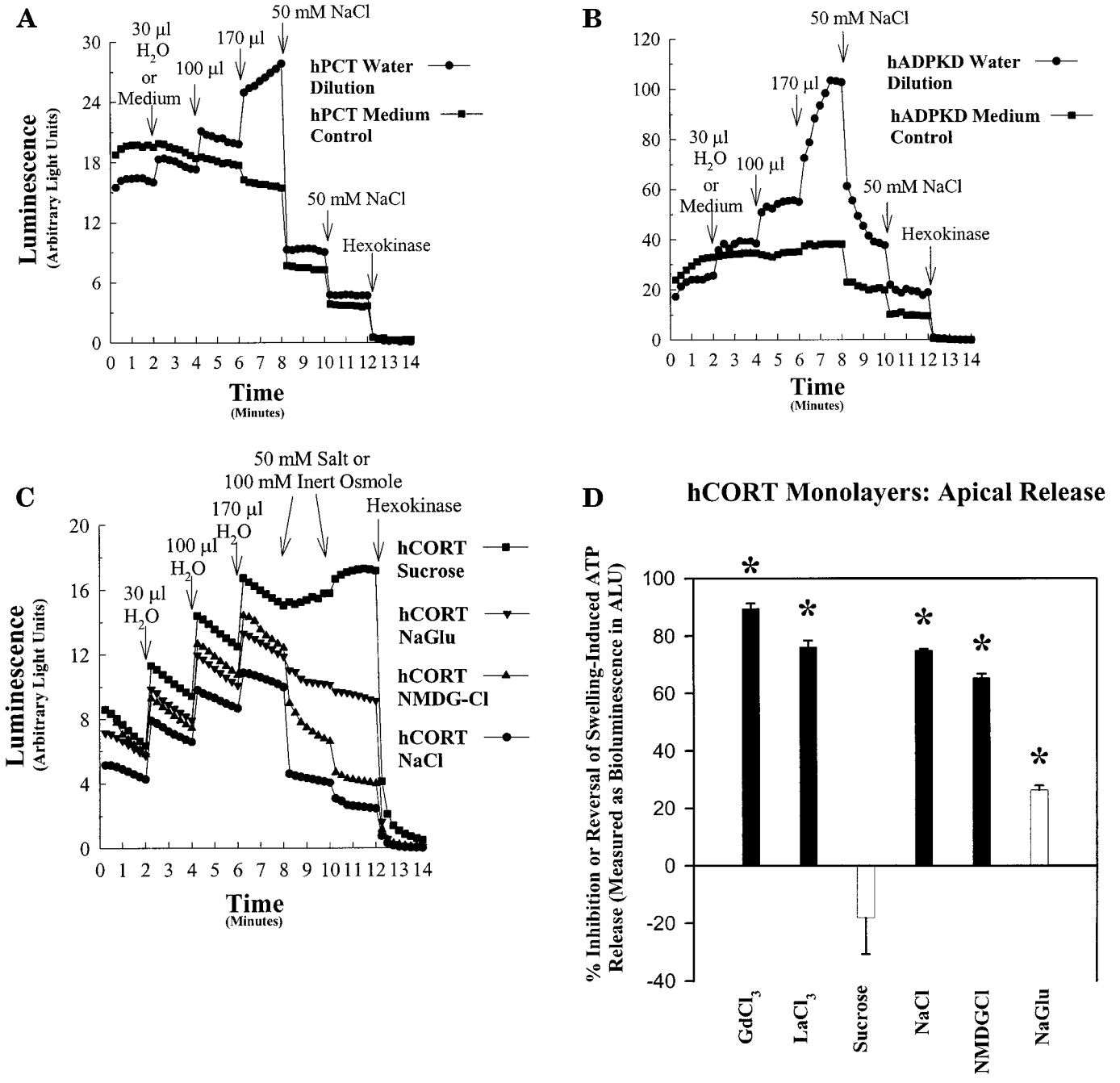


Figure 3. Hypotonicity-induced ATP release is attenuated by readdition of specific osmoles. Representative apical-directed and hypotonicity-induced ATP release time courses are shown for an hPCT monolayer (A) and an hADPKD monolayer (B) (●). Representative medium controls are also shown (■). Consecutive additions of 50 mM NaCl inhibited hypotonicity-induced release of ATP and attenuated isotonic ATP release in each case. In C, a comparison of representative experiments on hCORT monolayers with sucrose (■), sodium gluconate (▼), NMDG-Cl (▲), and NaCl (●) is shown. Hexokinase abolished the signal in all cases. In D, a summary of the inhibitory effects of ion channel blockers, $GdCl_3$, and $LaCl_3$ (200 μM each), and the battery of different osmoles (total amount of 100 millimoles) are shown ($n = 3$ each in this series). Here, percentage inhibition of hypotonicity-induced ATP release is shown. *$P < 0.05$, statistically significant inhibition by paired t test.

ATP Is Detectable in Significant Amounts in ADPKD Cyst Fluid

If ATP release and extracellular nucleotide signaling is detrimental to cyst volume expansion in ADPKD, then ATP should be present in the cyst fluid. Cyst fluid samples aspirated from the cysts of multiple ADPKD kidneys at the time of initial dissection were flash-frozen in liquid nitrogen for later analysis by luminometry. Total cyst fluid volume was measured at that time. An aliquot of each cyst fluid was mixed with an equal volume of reduced serum medium containing luciferase-lucife-

rin reagent, and the luminescence was measured in triplicate. The luminescence values were correlated with an ATP standard curve constructed on the day of cyst fluid luminometry (see Table 1 for an example) to estimate ATP concentration in the cyst fluid. The results of cyst fluid samples derived from at least 10 different donor kidneys chosen at random are shown in Figure 4. Concentrations of ATP in cyst fluid clustered into two groups. Cluster A had ATP concentrations that ranged from 500 nM to 10 μM (Figure 4). These concentrations are sufficient to stimulate all purinergic receptor subtypes. Cluster B had lower ATP concentrations that ranged from 3 nM to 50 nM. Again, these data provide another example of the heterogeneity of ADPKD cysts. Nevertheless, these results show that ATP is present in significant quantities in all ADPKD cysts and in amounts sufficient to stimulate ATP receptors in a subset of ADPKD cysts. These data also provide an estimate of the ATP concentration in cyst fluids *in vivo*. Ecto-ATPase activity may decrease the amount of ATP measured in this analysis and, therefore, ATP concentration may be underestimated in this analysis. These data also show, although indirectly, that ATP metabolites (ADP, 5′ AMP, and adenosine) are likely generated in cyst fluid in similar or even higher concentrations due to the degradation of ATP. It is interesting to speculate that adenosine as well as ATP may also stimulate detrimentally Cl^- and fluid secretion into the ADPKD cyst lumen.

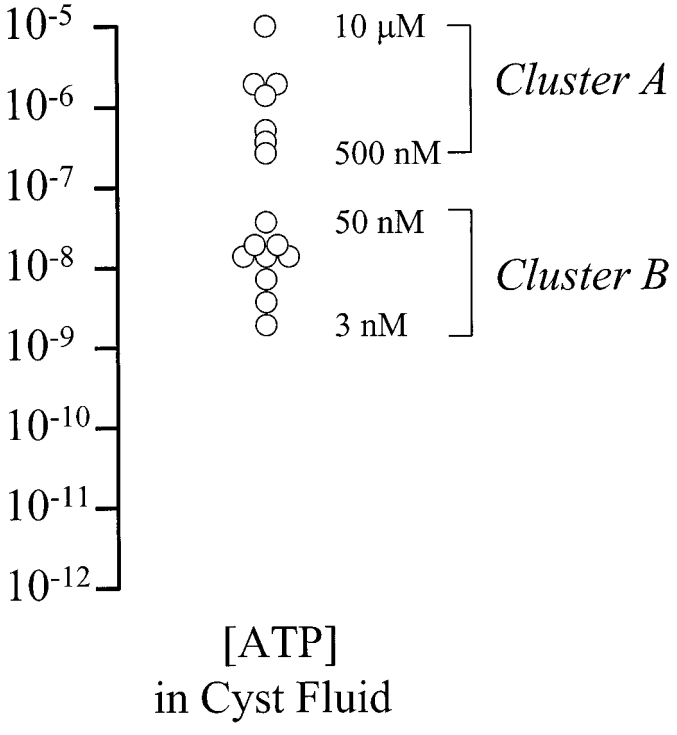
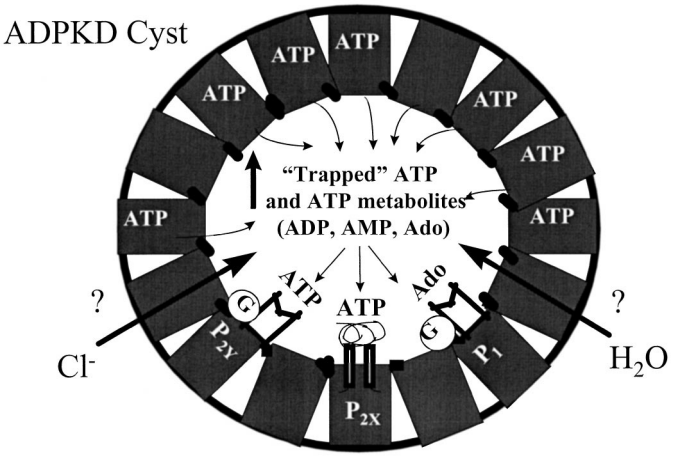


Figure 4. ATP is present in significant concentrations in ADPKD cyst fluids. A cyst fluid luminometry scatterplot of [ATP] detected in different cyst fluids. The data fell into two groups, shown as clusters A and B. Each cyst fluid was measured in triplicate. In cluster A, [ATP] measured are sufficient to stimulate P2Y and P2X purinergic receptor subtypes. No ATP controls had a luminescence that was less than 0.1 ALU (falls at or below 10^{-11} M ATP).

Discussion

Nucleotide Release and Signaling in ADPKD Cysts May Be Detrimental in the Pathogenesis of PKD

Because ADPKD epithelial cell cultures and monolayers grown in primary culture release significant amounts of ATP under isotonic conditions and greater quantities of ATP upon hypotonic challenge, and because ATP is detectable in nanomolar to micromolar concentrations in a subset of ADPKD cysts, we have formed the following working hypothesis concerning the possible detrimental consequences of ATP release and extracellular or "cyst interior" ATP or ATP metabolite signaling in ADPKD cysts. This working hypothesis is shown in Figure 5. ATP released into the lumen of an ADPKD cyst would be trapped in the cyst because it has a negative charge and because the gradient for ATP transport is >100,000-fold in favor of secretory transport out of cells as opposed to absorptive transport into cells (inversely proportional to the gradient for Ca^{2+} entry into cells). Continual release of ATP under basal conditions or stimulation of higher ATP release rates would concentrate that ATP into doses sufficient to elicit autocrine and/or paracrine stimulation of purinergic receptors expressed by ADPKD epithelia. Moreover, metabolites of ATP may be present in even higher concentrations, such as ADP, 5′AMP, and adenosine.



"Trapped" ATP and ATP metabolites, as fluid and Cl^- secretagogues, may exacerbate fluid accumulation and volume expansion in ADPKD cysts.

Hypothesized Result: Accelerated Cyst Expansion

Figure 5. Extracellular nucleotide signaling may be detrimental to cyst volume expansion in ADPKD. Working hypothesis to explain how autocrine and paracrine nucleotide and nucleoside signaling, normally present in epithelia derived from many tissues (including the kidney) and essential for epithelial cell volume regulation, may contribute detrimentally to fluid accumulation in ADPKD cysts and, as a consequence, expansion of volume in each ADPKD cyst in an ADPKD kidney. Although not illustrated for reasons of clarity, basolateral release of ATP may also affect cystic epithelial function or influence extracellular matrix formation through interaction with purinergic receptors that may be expressed on the basolateral surface of the ADPKD cyst.

Extracellular Nucleotide Stimulation of Purinergic Receptors in ADPKD Cysts May Be Detrimental in the Pathogenesis of PKD

The physiologic ligand for P2 purinergic receptors is ATP. However, ADP, a metabolite of ATP, binds to and stimulates P2Y1, P2Y3, and P2Y5 receptors as well or better than ATP (14,15). It is not known whether these P2Y isoforms are expressed on renal epithelial cells. Our laboratory is currently performing degenerate PCR for the P2Y receptor gene family in renal and other epithelial cells to determine which P2Y isoforms are expressed by ADPKD and non-ADPKD renal epithelial cells. UTP and ATP stimulate a broadly expressed P2Y2 or P2U isoform that has been found by Knepper and coworkers initially in collecting duct epithelia and more recently in multiple segments along the nephron (32). Preliminary results gathered in a mouse inner medullary collecting duct cell line (mIMCD-K2) (33) with specific and degenerate reverse transcription-PCR show that this cell line expresses two P2Y receptor isoforms (P2Y1 and P2Y2), as well as two ion channel-forming or ionotropic P2X purinergic receptor isoforms (P2X3 and P2X4) (E. M. Schwiebert, L. M. Schwiebert, B. A. Kudlow, A. L. Taylor, D. C. McCoy, B. A. Stanton, unpublished observations). Filipovic *et al.* have shown recently by molecular and physiologic methods that a novel P2X receptor isoform is expressed by LLC-PK1 porcine kidney epithelial cells (34). Takeda *et al.* showed recently by PCR that a mouse early proximal tubule cell line and an outer medullary collecting duct cell line express P2Y1, P2Y2, and P2X4 purinergic receptor isoforms, and that ATP increased intracellular free calcium and ^{3}H-thymidine uptake in these renal cell lines (35). Ishikawa and colleagues demonstrated that extracellular ATP stimulates cell growth in renal inner medullary collecting duct cells via P2Y2 or P2U purinoceptors (36). Indeed, preliminary results beyond the scope of this study show that mRNA for P2Y2, P2X4, and P2X5 purinergic receptors are expressed by ADPKD epithelial primary cultures (L. M. Schwiebert, E. M. Schwiebert, unpublished observations). ATP, via ATP receptors, could then promote Cl^- and fluid secretion across the ADPKD cystic epithelia that encapsulate the ADPKD cyst and, as a consequence, increase the volume of the cyst. Therefore, there is substantial precedence for P2 purinergic receptor expression on renal epithelial cells and for nucleotide agonist effects on renal function. This study provides novel evidence for epithelial cell release of ATP as a source in the kidney for extracellular nucleotide signaling.

Because metabolism of ATP is likely (but not as robust in hADPKD cultures *versus* non-ADPKD cultures), ATP metabolites such as adenosine may also affect cystic epithelial function. Adenosine, another potent autacoid, binds to its own subclass of P1 purinergic receptors, subclassified as A1, A2, and A3. The renal effects of the ATP metabolite adenosine are extensive, are mediated largely by A1 and A2 adenosine receptors in kidney, and were reviewed extensively by Spielman and Arend (13). As such, adenosine, via adenosine receptors, may also contribute to this hypothesized signaling cascade which can also promote Cl^- and fluid secretion across epithelia.

ATP Release Phenotype of ADPKD Cells Predicts a Hybrid or Progenitor Cell When Compared with Non-ADPKD Controls

As noted above, the basal ATP release phenotype of ADPKD cells was more similar to hPCT cells than to hCORT cells. However, the hypotonicity-induced ATP release responses of ADPKD monolayers were more similar to hCORT cells than to hPCT cells. Although not readily apparent from the data presentation, there was heterogeneity in the ATP release phenotype expressed by subsets of ADPKD primary cultures. Human ADPKD cells obtained from Wallace and Sullivan at Kansas were grown in 10% FBS-containing medium suggested by this laboratory, which was vastly different from the more defined $\leq$1% FBS-containing medium used for all other primary cultures. These results led us to consider three hypotheses concerning the cell biology of ADPKD cells. First, ADPKD cells may revert to a "progenitor cell" with an undifferentiated or fetal-like phenotype and assume a heterogeneous phenotype as to ATP release mechanisms, expression, and polarity of transporter proteins, and expression and polarity of receptors. Second, ADPKD cells may be a type of "hybrid" renal epithelial cell that displays characteristics of both proximal and distal nephron segments. Third, ADPKD cells and cysts may truly derive from multiple nephron segments and maintain characteristics of that segment after cyst formation. These hypotheses require further investigation; however, the comparison of ATP release and signaling in different renal tubules or from renal epithelial cells derived from different parts of the nephron may provide a physiologic end point with which to identify the origin of ADPKD cysts. This heterogeneity in the origin of ADPKD cysts provides a challenge for the future study of the physiology of ADPKD.

Components of the Autocrine and Paracrine Nucleotide Signaling System in ADPKD Epithelia: Importance in Control of Epithelial Cell Volume

Extracellular ATP signaling has been implicated recently in the control of basal cell volume and in the stimulation of regulatory volume decrease following a hypotonic challenge (17,28,30). Such a basally active signaling system inside and outside ADPKD cysts to maintain epithelial cell volume could exacerbate volume expansion of ADPKD cysts. Even worse, in a state of diuresis and positive free water clearance when the tubular fluid in the distal tubule and collecting duct is hypotonic, ATP release would be potentiated and could promote Cl^- and fluid secretion not only across normal collecting duct, but also into ADPKD cysts. Like ATP and its metabolites, other additional autacoids or growth factors have been implicated in the pathogenesis of PKD. EGF along with other growth factors have been found in PKD cyst fluid (4–7). Moreover, the EGF receptor is mislocalized to the apical membrane of ADPKD epithelia, at least in a subset of ADPKD cystic epithelia studied (4–7). Genetic cross of a PKD mouse model with a mouse transgenic for a mutant EGF receptor attenuated the progression of PKD cyst formation (5). This work is a helpful analogy to the hypotheses posed here for related nucleotide and nucleoside autacoids. If proven that

autocrine and paracrine ATP signaling is detrimental in ADPKD disease progression following cyst formation, strategies to dampen this signaling system such as inhibitors of ATP release mechanisms, ATP scavengers, or ATP receptor antagonists could be explored as therapies to slow the progression of ADPKD.

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