Autocrine extracellular purinergic signaling in epithelial cells derived from polycystic kidneys

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Departments of 1Physiology and Biophysics, 2Cell Biology, and 3Nephrology, University of Alabama at Birmingham, Birmingham, Alabama 35294; 4Kidney Institute, University of Kansas Medical Center, Kansas City, Kansas 66160; and 5Department of Physiology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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Schwiebert, Erik M., Darren P. Wallace, Gavin M. Braunstein, Sandi R. King, Janos Peti-Peterdi, Kazushige Hanaoka, William B. Guggino, Lisa M. Guay-Woodford, P. Darwin Bell, Lawrence P. Sullivan, Jared J. Grantham, and Amanda L. Taylor. Autocrine extracellular purinergic signaling in epithelial cells derived from polycystic kidneys. Am J Physiol Renal Physiol 282: F763–F775, 2002. First published August 8, 2001; 10.1152/ajprenal.0337.2000.—ATP and its metabolites are potent autocrine agonists that act extracellularly within tissues to affect epithelial function. In polycystic kidneys, renal tubules become dilated and/or encapsulated as cysts, creating abnormal microenvironments for autocrine signaling. Previously, our laboratory has shown that high-nanomolar to micromolar quantities of ATP are released from cell monolayers in vitro and detectable in cyst fluids from microdissected human autosomal dominant polycystic kidney (ADPKD) cysts. Here, we show enhanced ATP release from autosomal recessive polycystic kidney (ARPKD) and ADPKD epithelial cell models. RT-PCR and immunoblotting for P2Y G protein-coupled receptors and P2X purinergic receptor channels show expression of mRNA and/or protein for multiple subtypes from both families. Assays of cytosolic Ca2+ concentration and secretory Cl− transport show P2Y and P2X purinergic receptor-mediated stimulation of Cl− secretion via cytosolic Ca2+-dependent signaling. Therefore, we hypothesize that autocrine purinergic signaling may augment detrimentally cyst volume expansion in ADPKD or tubule dilation in ARPKD, accelerating disease progression.

polycystic kidney disease; adenosine 5′-triphosphate; autocrine; epithelia; purinergic receptors

POLYCYSTIC KIDNEY DISEASE (PKD) exists in at least two genetic forms, autosomal recessive (ARPKD) and autosomal dominant (ADPKD), that can be affected further by additional modifier genes (43). In both forms, tubules are altered in their architecture, leading to dilated tubules or cysts encapsulated by a single monolayer of renal epithelia (43). This abnormal structure creates abnormal microenvironments in which autocrine and paracrine agonists such as growth factors, nucleotides, and inflammatory mediators could affect cystic epithelial function abnormally.

Recently, our laboratory showed that ATP is also present in high-nanomolar to micromolar quantities in fluids extracted from the interior of a subset of ADPKD cysts, the abnormal microenvironment alluded to above (50). Nucleotides in extracellular fluid act as autocrine and paracrine hormones to regulate a variety of physiological processes under normal physiological conditions (1, 14, 28, 37, 39). Nucleotides inhibit Na+ absorption and stimulate Cl− secretion across epithelia derived from airway, gastrointestinal, and other tissues (10, 17, 21, 38). Recently, McCoy et al. (30) found that nucleotide agonists stimulate Cl− secretion and inhibit Na+ absorption in an inner medullary collecting duct (IMCD) cell line (mIMCD-K2). Kishore and co-workers (22, 23) have shown that ATP and UTP inhibit vasopressin-induced water flow across IMCD. Once released from cells, nucleotides activate purinoceptors that are divided into two classes: P1 (activated by adenosine) and P2 (activated by ATP and ADP) (1, 28, 37, 39). P2 purinoceptors have been subdivided into two families, P2X and P2Y. The P2X purinoceptor family contains at least eight distinct subtypes (P2X1–P2X7 and P2XM), each of which is a two transmembrane-spanning, ion channel-forming protein (1, 28, 37, 39). Ionotropic P2X receptor channels bind ATP and form Ca2+-permeable, nonselective cation channels that, when activated, increase intracellular Ca2+ (1, 28, 37, 39). The P2Y purinoceptor family is a seven transmembrane-spanning, G-protein-coupled, metabotropic receptor family containing at least six distinct receptor subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y8, and P2Y11) (1, 18, 28, 37, 39). P2Y purinoceptors are

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coupled to effectors through heterotrimeric G proteins. P2Y receptors stimulate phospholipase activity and, in turn, increase intracellular Ca²⁺. In addition, P2Y purinoreceptors enhance cAMP production through receptor-mediated prostaglandin release.

In the kidney, potential roles of purinergic signaling have been postulated (7). However, few studies have documented P2Y and P2X receptor expression. Knepper, Kishore, and co-workers (23) have documented expression of the P2Y2 receptor protein in IMCD and other nephron segments. UTP, via the P2Y2 receptor, inhibits vasopressin-induced water permeability (22, 34). Insel and co-workers (18) have shown expression and signaling of P2Y2 receptors as well as other P2Y isoforms in the distal nephron kidney epithelial cell model, MDCK. Deetjen et al. (9) showed the functional presence of a luminal P2Y2 receptor in isolated perfused mouse cortical collecting duct. Bailey et al. (2) have shown basolateral expression of functional P2Y1 receptor as well as UTP-stimulated P2Y2 receptor that may be P2Y2 and/or P2Y4. Filipovic et al. (13) documented molecular and functional expression of a P2X1-like purinergic receptor channel in LLC-PK1 cells. P2X7 expression and its possible relationship to apoptosis have also been defined in cultured mesangial cells (35). P2X1 receptor channel expression in renal vascular smooth muscle has also been studied thoroughly by Burnstock and co-workers (6).

At the present time, the existence of P2 receptors in PKD renal cyst epithelial cells remains unknown. Our hypothesis is that these cells do express purinergic receptors and that autocrine ATP signaling via these receptors stimulates Cl⁻ secretion in PKD. Our results provide evidence that 1) PKD epithelial monolayers release ATP in a dynamic and regulated manner; 2) PKD epithelial ATP release is potentiated by hypotonicity; 3) PKD epithelia express multiple purinergic receptor subtypes to receive that autocrine signal; 4) PKD epithelia respond to nucleotides, via purinergic receptors, with increases in [Ca²⁺]; and (5) P2Y- and P2X-selective agonists stimulate Cl⁻ secretion across PKD epithelial monolayers. As such, all limbs of an autocrine extracellular purinergic signaling loop exist at the level of the PKD epithelium and may exacerbate fluid accumulation that may contribute to increased cyst size and the slow, constant progression of PKD.

MATERIALS AND METHODS

ADPKD and ARPKD cell primary culture. Human ADPKD epithelial primary cultures (hADPKD), human whole kidney epithelial primary cultures (hRE), and mouse collecting duct primary cultures from B6 control and cpk ARPKD mice were grown on diluted Vitrogen (collagen types I and IV diluted 1:15 in Dulbecco’s phosphate-buffered saline; Collagen, Palo Alto, CA)-coated 35-mm culture dishes (Corning) and 25- and/or 75-cm² culture flasks (Falcon) or on filter supports (Millicell or Corning Transwell) in a defined medium [renal epithelial basal medium (REBM), Clonetics] supplemented with a REBM BulletKit (Clonetics) containing 0.5% FBS, human epidural growth factor (EGF; 0.5 ml), insulin (0.5 ml), hydrocortisone (0.5 ml), epinephrine (0.5 ml), triiodothyronine (0.5 ml), transferrin (0.5 ml), gentamycin (0.5 ml), and 1× penicillin/streptomycin (diluted in 500 ml of REBM). Concentration of the supplemented hormones are proprietary information not supplied by Clonetics.

Temperature-sensitive hADPKD cell lines. A temperature-sensitive hADPKD epithelial cell line (a generous gift of Dr. Patricia Wilson, Mt. Sinai School of Medicine, New York, NY) was grown at 32–33°C (but never above 33°C) in a 5% CO₂ humidified incubator in MEM with Earle’s salts (GIBCO BRL) supplemented with 10% FBS (GIBCO BRL), 1× L-glutamine, 1× penicillin/streptomycin, and 1× fungizone. Once confluent, these cells (ADPKD 33°C) were subcultured onto filters and switched to a similar environment but at 37°C to promote differentiation into a monolayer. These monolayers were tight to fluid; however, the transepithelial resistance (Rₑ) was not sufficiently high enough to perform Ussing chamber measurements. ATP release assays, RTPCR, and biochemistry could, however, be performed on this ADPKD cell line.

Primary cultures of hADPKD grown as monolayers for Ussing chambers. hADPKD epithelial cells were harvested from surface cysts of kidneys removed from four patients with ADPKD. The procedure for establishing primary cultures of ADPKD cells has been published elsewhere (29, 47). Briefly, kidneys were removed, placed in sterile bags, and shipped on wet ice to our laboratory by overnight delivery. During dissection of the cyst wall, fibrous tissue was manually removed to reduce the amount of fibroblasts in the primary culture and to enrich the culture with epithelial cells. The dissected tissue was several times rinsed in DMEM/F-12 containing penicillin/streptomycin, minced with sterile surgical scissors, and incubated for 8 h in media containing 220 IU/ml collagenase (type IV, Cooper Biochemical, Malvern, PA) at 37°C. Small pieces of tissue and dispersed cells were then rinsed in DMEM/F-12 supplemented with 5% FBS and insulin-transferrin-selenium (ITS). Cells were allowed to attach and propagate on plastic until they were harvested by trypsinization. Transport studies were performed in cells from the original primary culture or after the first passage.

Bioluminescence detection of ATP released from epithelial cell cultures and monolayers. Monolayers were washed three times in PBS to remove any FBS present in the culture medium. The methodology for ATP release assays has been published in detail for experiments performed in epithelial monolayers (44).

Bioluminescence detection of ATP degradation by epithelial cell cultures. All details concerning this assay have been published previously (44). Briefly, however, a known quantity of ATP (10 μM) was spiked into the culture medium, and aliquots of the medium were taken before and after exposure to cells at the time points shown. Cells were grown in flasks and studied in flasks at 37°C, where ecto-ATPases would be maximally active. All aliquots were screened by the luciferase-luciferin assay to measure ATP-driven bioluminescence that decayed over time on exposure to the cells.

Preparation of total RNA for PCR. The above cell models were grown to confluence in collagen-coated flasks, and the cells were lysed by TRIzol reagent (Life Technologies, Grand Island, NY), according to the manufacturer’s instructions. Aspects of the methods below have been published elsewhere (30).

Preparation of cDNA. Total RNA was recovered by centrifugation, resuspended in diethylpyrocarbonate (DEPC)-treated water (10–30 μl depending on size of the pellet), and treated with RNase-free DNase (Life Technologies) to remove contaminating genomic DNA. Two to four microliters of total RNA, mixed with 12–14 μl of DEPC-treated water, 2 μl of
DNase buffer, and 2 μl of RNase-free DNase I enzyme, were incubated at room temperature for 15 min. The reaction was stopped by adding 2 μl of 25 mM EDTA to chelate Ca\(^{2+}\) and Mg\(^{2+}\), and the DNase-treated samples were heated at 65°C for 10 min to inactivate the DNase I enzyme. Murine Moloney leukemia virus (MMLV)-RT (Life Technologies) was used to reverse transcribe total RNA to cDNA. Each reaction contained 20–22 μl of the total RNA template (DNase-treated sample as above), 2.5 μl of a dNTP mixture (dATP, dCTP, dGTP, and dTTP, 2 μM each, Pharmacia), 2 μl oligo-(dT\(_{12-18}\)) primer (Life Technologies), 10 μl of 5× RT buffer, 2.5 μl of dithiothreitol (0.1 M), 9.8 μl of DEPC-treated water, and 2 μl of MMLV-RT enzyme (Life Technologies). Oligo(dT) primer ensures that all messenger RNA species are reverse transcribed to cDNA. The reaction was incubated for 1 h at 37°C in a thermocycler. cDNA samples were then incubated at 90°C for 5 min in the thermocycler to inactivate the RT enzyme. cDNA samples were used that day or soon after for PCR.

**PCR of epithelial cDNA.** We used degenerate and specific PCR primers designed to amplify the P2X gene family and specific members of the P2Y receptor gene family, P2Y1, P2Y2, and P2Y6. P2X degenerate primers were designed to a common conserved sequence among rat P2X1, P2X2, and P2X3 cDNAs. P2Y1, P2Y2, and P2Y6 primers were designed to the human cDNAs. Each PCR reaction contained 3–5 μl of cDNA template, 33.8–35.8 μl of ultraviolet-sterilized, double-distilled water, 1 μl of dNTP mix (2 μM, Pharmacia), 1 μl forward primer and reverse primer (A and B below; 2 μM final), 0.2 μl of Taq polymerase (Perkin-Elmer), 5 μl of 10× PCR buffer, and 3 μl of a MgCl\(_2\) solution (1.5 mM final concentration of MgCl\(_2\)). The cycling parameters were 1) a 5-min 94°C “hot start”; 2) 40 cycles of 30 s at 94°C, 1 min at appropriate annealing temperature (indicated with each primer pair below), and 1 min at 72°C; and 3) 10 min elongation at 72°C to end the reaction. PCR products were visualized on a 1.5% agarose gel run with a 100-bp DNA ladder. β-Actin PCR amplification was performed to authenticate the cDNA before amplification with P2X or P2Y primer pairs. The primer sequences are shown below.

- **β-Actin A:** 5’-TGA CGG GGT CGT CCA CAC TGT GCC CAT CTA-3’
- **β-Actin B:** 5’-CTA GAA GCA TTG CGG TGG AGC ATG GAG GG-3’
  (60°C annealing temperature)

**P2XR-degenerate A:** 5’-TTC ACC MTY YTC ATC AAR AAC AGC ATC-3’
**P2XR-degenerate B:** 5’-TGG CAA AYC TGA AGT TGW AGC C-3’
  (52°C annealing temperature)

**P2Y1 A:** 5’-AAG AGC GCC TTC CAG TTT TAC TAC-3’
**P2Y1 B:** 5’-CAG ATT TCT GGG GTC TGG AAA TTC-3’
  (60°C annealing temperature)

**P2Y2 A:** 5’-CGT CAT CCT TGT CTG TTA CGT GCT-3’
**P2Y2 B:** 5’-CAT CCG CCG AAT GTC CTT AGT G-3’
  (60°C annealing temperature)

**P2Y6 A:** 5’-TGC CAC CCA CAA CCT GTG TCT ACC C-3’
**P2Y6 B:** 5’-AGT AGA AGA GGA TGG GGT CCA GCC C-3’
  (60°C annealing temperature)

**DNA sequencing of PCR products.** PCR products were isolated from agarose gel slices using a Qiaquick gel extraction kit (Qiagen). PCR products were ligated into a pGEM-T vector system (Promega) and then transformed into JM109 high-efficiency competent cells (Promega). Transformed bacteria were then plated onto Luigi-Bertani-agar plates containing ampicillin (100 μg/ml), 5-bromo-4-chloro-3-indoly-d-galactoside (40 μg/ml), isopropyl-D-thiogalactopyranoside (100 μg/ml) for ampicillin selection of successful transformants and blue/white selection of successful PCR product ligation. White colonies were picked off the plate to inoculate a 6-ml Luigi-Bertani growth with continued ampicillin selection. The pGEM-T plasmids with insert were purified using a PerfectPrep miniprep kit (5 Prime-3 Prime). The purified DNA was denatured in 0.2 N NaOH, precipitated in 7.5 M NH\(_4\)Cl and 100% ethanol, and sequenced using the Sequenase dideoxy termination method (Amersham/US Biochemicals) using [α-\(^{32}\)P]dATP (NEN-DuPont) incorporation in our laboratory. The resulting DNA sequence was read by two independent investigators and screened with the BLAST algorithm.

**Immunoblotting with P2X receptor channel isoform-specific antibodies.** Cells were lysed in a buffer containing 10 mM Tris, 0.5 mM NaCl, 0.5% Triton X-100, 50 μg/ml aprotinin (St. Louis, MO), 10 nM leupeptin, 100 μg/ml pepstatin A (Sigma) adjusted to pH 7.2–7.4. Twenty micrograms of protein were run per lane and separated on an 8% SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (Osmonics, Westborough, MA). Immunoblotting was performed with rabbit polyclonal antibodies to P2X1, P2X2, P2X4, and P2X7 at a dilution of 1:500 (Alomone Laboratories, Jerusalem, Israel) or with anti-P2X5 at 1:1,000 dilution (a generous gift of Drs. Mark Voigt and Terry Egan, St. Louis University, St. Louis, MO). Reactivity was detected by horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:3,000 dilution, New England BioLabs, Beverly, MA). Enhanced chemiluminescence was used to visualize the secondary antibody.

**Fura 2 imaging of intracellular Ca\(^{2+}\)−.** Cytosolic Ca\(^{2+}\)− concentration ([Ca\(^{2+}\)\(_i\)]) of hADPKD epithelial cells was measured with dual-excitation wavelength fluorescence microscopy (Deltscan, Photon Technologies, Princeton, NJ) after cells were loaded with the permeant form of the fluorescent probe fura 2-acetoxyethyl ester (fura 2-AM; Teflabs, Austin, TX). Fura 2 fluorescence was measured at an emission wavelength of 510 nm in response to excitation wavelengths of 340 and 380 nm, alternated at a rate of 25 Hz by a computer-controlled chopped assembly. Autofluorescence-corrected ratios (340 nm/380 nm) were calculated at a rate of 5 points/s using PTI software. ADPKD cells were grown on collagen-coated coverslips (1:30 dilution of Vitrogen 100 in PBS) cut to fit a cuvette at a 45° angle. Cells were incubated in media containing 5 μM fura 2-AM and 1 mg/ml Pluronic F-127 dissolved in DMSO for 2 h to allow loading of the dye into the cells. After loading, coverslips were rinsed in Ringer solution to remove extracellular fura 2-AM and positioned in the cuvette at a 45° angle from the excitation light. Two glass capillary tubes were inserted into the top of the cuvette out of the path of the excitation light. One tube was extended to the bottom of the cuvette and connected by way of polyethylene tubing to an infusion pump. The other capillary tube was positioned at the top of the cuvette and served to remove fluid from the cuvette. Flow rate through the cuvette was ~5 ml/min. A Ringer solution was used containing (in mM) 148 NaCl, 5 KCl, 1 MgSO\(_4\), 1.6 NaHPO\(_4\), 0.4 NaH\(_2\)PO\(_4\), 5 d-glucose, 1.5 CaCl\(_2\), and 10 HEPES at pH 7.4. Experiments were performed at room temperature. Cells were incubated for 20 min in the control Ringer solution to ensure conversion of fura 2-AM to fura 2. Fluorescence intensities at both wavelengths were assessed, and only those preparations in which there were >50,000 counts/s for both wavelengths
were used for experiments. The fluorescence ratio (340/380 nm) was then monitored for at least 100 s to establish a stable baseline value. Purinergic agonists, antagonists, or scavengers were then added to the Ringer solution to test the effects of these agents on [Ca$^{2+}$]. The 340/380 ratios (R) were converted into [Ca$^{2+}$] values using the equations of Grynkiewicz et al. (15) as follows

$[\text{Ca}^{2+}] = K_d \times (R - R_{\text{min}})/(R_{\text{max}} - R) \times (S_{380}/S_{340})$

where $K_d$ is the dissociation constant of fura 2 for Ca$^{2+}$, $R_{\text{max}}$ and $R_{\text{min}}$ are R values under saturating and Ca$^{2+}$-free conditions, respectively, and $S_{380}$ and $S_{340}$ are the fluorescent signals (S) emitted by Ca$^{2+}$-free (f) and Ca$^{2+}$-bound (b) forms of fura 2 at a wavelength of 380 nm. In situ cell calibrations that at 380 nm under Ca$^{2+}$kiewicz et al. (15) as follows

RESULTS (1).

were measured with an epithelial voltage-clamp apparatus ADPKD cells (5

membrane of cpk

ATP release was signi

both sides with a Ringer solution containing (in mM) 147 Na$^+$, 119 Cl$^-$, 1.2 Mg$^{2+}$, 1.2 SO$_4^{2-}$, 1.2 HCO$_3^-$, 6 alanine, 5 K$^+$, 5 acetate, 5 glucose, 4 lactate, 2.5 HPO$_4^{2-}$, 1.2 Mg$^{2+}$, 1.2 SO$_4^{2-}$, 1 citrate, 0.5 butyric acid, and 14 raffinose equilibrated with 5% CO$_2$-95% O$_2$. Short-circuit current ($I_s$), transepithelial voltage, and $R_{te}$ were measured with an epithelial voltage-clamp apparatus (Warner Instrument, Hamden, CT) as previously described (1). $R_{te}$ of the ADPKD monolayers ranged from 0.1 to 1 kΩ. In electrophysiological studies, forskolin and DIDS were dissolved in ethanol, and bumetanide and diphenylamine-2-carboxylate (DPC; Fluka Chemical) were dissolved in DMSO as 1,000× stock. The maximum %DMSO or ethanol in the preparation never exceeded 0.1%.

Data analysis and statistics. In Table 1, luminescence values (in arbitrary light units), absolute concentration of ATP, and rate of appearance of ATP in the medium are shown. As a caveat, however, all three parameters are dependent on three factors: 1) release of ATP by one or more cellular mechanisms; 2) consumption of ATP by the luciferase-luciferin detection reagent; and 3) degradation of that ATP before it is consumed by endogenously expressed ecto-ATPases and ecto-apyrases. Thus absolute concentration and rate of release are merely estimates, and they probably represent underestimates of the absolute concentration of released ATP and the ATP release rate. The statistical tests used are presented below (see figure legends).

Materials. All chemicals were obtained from Sigma unless otherwise noted. The luciferase-luciferin reagent is derived from an ATP assay kit purchased from Calbiochem.

RESULTS

ATP release is potenitated in ARPKD and ADPKD epithelial monolayers in vitro. Table 1 shows that constitutive (basal) and hypotonicity (or swelling)-induced ATP release was significantly greater across the apical membrane of cpk mouse collecting duct epithelial

Table 1. Magnitude of ATP release from normal and PKD epithelial monolayers

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Luminescence, ALU</th>
<th>Absolute [ATP], nM</th>
<th>Rate of ATP Appearance, fM·mm⁻¹·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>hRE primary (6)</td>
<td>14.71 ± 2.62</td>
<td>0.1</td>
<td>0.048</td>
</tr>
<tr>
<td>Basal</td>
<td>38.20 ± 6.17</td>
<td>2.0</td>
<td>0.948</td>
</tr>
<tr>
<td>DIDS</td>
<td>7.31 ± 1.12</td>
<td>0.01</td>
<td>0.005</td>
</tr>
<tr>
<td>hADPKD primary (6)</td>
<td>46.42 ± 6.45</td>
<td>3.0</td>
<td>1.416</td>
</tr>
<tr>
<td>Basal</td>
<td>164.7 ± 21.34†</td>
<td>9.0</td>
<td>4.260</td>
</tr>
<tr>
<td>DIDS</td>
<td>56.33 ± 11.80</td>
<td>3.5</td>
<td>1.654</td>
</tr>
</tbody>
</table>

Autosomal dominant cell models and their controls

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Luminescence, ALU</th>
<th>Absolute [ATP], nM</th>
<th>Rate of ATP Appearance, fM·mm⁻¹·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>38.18 ± 3.00</td>
<td>2</td>
<td>0.079</td>
</tr>
<tr>
<td>Hypo 55%</td>
<td>115.3 ± 5.33</td>
<td>8</td>
<td>3.780</td>
</tr>
<tr>
<td>DIDS</td>
<td>17.55 ± 1.97</td>
<td>0.1</td>
<td>0.036</td>
</tr>
<tr>
<td>cpk ARPKD pooled (13)</td>
<td>51.98 ± 7.97*</td>
<td>4</td>
<td>1.896</td>
</tr>
<tr>
<td>Hypo 55%</td>
<td>152.7 ± 12.37†</td>
<td>9</td>
<td>4.260</td>
</tr>
<tr>
<td>DIDS</td>
<td>28.43 ± 2.47</td>
<td>1</td>
<td>0.468</td>
</tr>
<tr>
<td>cpk ARPKD cysts (6)</td>
<td>27.46 ± 6.78</td>
<td>1</td>
<td>0.468</td>
</tr>
<tr>
<td>Hypo 55%</td>
<td>135.8 ± 22.18</td>
<td>8</td>
<td>3.780</td>
</tr>
<tr>
<td>DIDS</td>
<td>26.06 ± 3.68</td>
<td>1</td>
<td>0.468</td>
</tr>
<tr>
<td>cpk ARPKD no cysts (7)</td>
<td>73.75 ± 6.31*</td>
<td>4</td>
<td>1.896</td>
</tr>
<tr>
<td>Hypo 55%</td>
<td>167.2 ± 11.89†</td>
<td>9</td>
<td>4.260</td>
</tr>
<tr>
<td>DIDS</td>
<td>30.46 ± 3.40</td>
<td>1.5</td>
<td>0.708</td>
</tr>
</tbody>
</table>

Values are means ± SE with no. of monolayers in parentheses. hRE, human whole kidney epithelial primary cultures; PKD, polycystic kidney disease; ADPKD and ARPKD, autosomal dominant and autosomal recessive PKD, respectively. The units for luminescence are arbitrary light units ([ALU]). The standard curves with known quantities of ATP were: No ATP, 1.459; 1.0 M ATP, 15.28; 10⁻³ M ATP, 28.06; 10⁻² M ATP, 261.9; 10⁻¹ M ATP, 3,402; 10⁻⁰ M ATP, 9,950; and 10⁰ M ATP, >9,999. Absolute ATP ([ATP]) concentration was estimated from this standard curve as a whole number (in nM). All values with Calbiochem reagent fell in the nanomolar range. The rate of appearance of ATP (function of release rate in competition with the rate of degradation) was calculated as $[\text{ATP}]$·mm diameter of monolayer (or dish)⁻¹·min⁻¹. Human ADPKD primary cells grown in dishes are a more precise measure of ATP that may be released by a large cyst, whereas cells grown on filters are done as a microassay. This preparation had a constitutive or basal ATP release of 1,773 ± 218 ALU, which corresponded to an absolute ATP concentration ([ATP]) of 45 nM and a rate of appearance of ATP in the medium of 61.9 fM·mm⁻¹·min⁻¹. With 55% hypotonicity (Hypo), volume-induced ATP release was 4,614 ± 662 ALU, which corresponded to an absolute [ATP] of 115 nM and a rate of appearance of ATP in the medium of 158.6 fM·mm⁻¹·min⁻¹. Addition of DIDS attenuated ATP release to 127.2 ± 34.45 ALU, blocking both constitutive and swelling-induced ATP release to an absolute concentration of 5 nM and a rate of 6.895 fM·mm⁻¹·min⁻¹. Statistics done on ALU values also apply to calculated absolute [ATP] from the standard curve as well as rate of appearance of ATP in the medium. All hypotonicity-stimulated luminescence values are significantly greater than controls ($P < 0.05$ or lower by paired Student’s t-test) and are DIDS-inhibited values from the hypotonicity-stimulated values (by the same $P < 0.05$ or lower and t-test). *Significantly greater bioluminescence in PKD models vs. control models ($P < 0.05$) by unpaired ANOVA. †$P < 0.01$ difference by unpaired ANOVA.
monolayers vs. those from B6 controls. The luminescence difference was even greater if the data in the cpk group are separated between monolayers where “domes” (tubule- or cystlike) structures were visibly emerging from the monolayer and monolayers that had no such structures (Table 1). It is likely that the domes observed in cystic monolayers may have trapped some of the released ATP. ATP release under basal conditions was similar across the basolateral membrane of B6 and cpk monolayers; however, hypotonicity-induced ATP release was again augmented in cpk monolayers (data not shown). ATP release is also greater in hADPKD monolayers vs. the most appropriate companion control, monolayers of hRE monolayers. These studies show that ATP release and signaling is enhanced in the mouse ARPKD and hADPKD epithelial models. This latter result agrees with previously published data from hADPKD monolayers compared with a panel of different normal control primary cultures from proximal tubule and kidney cortex (50). Because DIDS is a potent blocker of ATP release, these results suggest that a transport mechanism is responsible, at least in part, for ATP release in these renal cell models.

Degradation of ATP by ecto-ATPases on the surface of renal epithelial cells is lacking in PKD epithelial models. Once released, extracellular ATP is subject to degradation. To examine this possibility, we compared the ability of normal and ADPKD cell models to degrade 10 μM ATP added to culture medium. Figure 1 shows the bioluminescence of samples of cell culture medium taken minutes and hours after addition of the ATP-containing medium, whereas ATP-containing medium that was not exposed to cells served as the control. In normal renal epithelial cell models, 50% of the total ATP in the medium was degraded by 20 min, and <10% of the total ATP was remaining by 2 h. Interestingly, degradation of ATP in ADPKD epithelial cell models occurred at a much slower rate. Fifty percent of the total ATP was degraded by 3 h, and ~10% of the ATP was remaining by 8 h. Taken together, these results suggest that ATP released into the medium is degraded into its metabolites in both normal and PKD epithelial models; however, and importantly, ATP is degraded four- to sixfold more slowly in PKD cell models, suggesting that the autocrine signal may last longer in the PKD epithelial microenvironment.

Multiple P2X and P2Y receptors are expressed in ARPKD and ADPKD renal epithelial cell lines and primary cultures. For ATP release and autocrine extracellular ATP signaling to be relevant to the pathogenesis of PKD, purinergic receptors that bind ATP as their physiological ligand must be expressed on the PKD epithelium. Table 2 shows the results of degenerate RT-PCR analysis for the P2X purinergic receptor channel gene family and specific RT-PCR for P2Y G protein-coupled purinergic receptors (P2Y1, P2Y2, and P2Y6) in ARPKD and ADPKD epithelial cell models and their counterpart controls. Discrete PCR products of the expected size were amplified by degenerate primers for P2XR (330 bp) and specific primers for P2Y1 (750 bp), P2Y2 (610 bp), and P2Y6 (870 bp). PCR products were excised as distinct bands, subcloned, and sequenced to confirm the identity of all amplified PCR products (data not shown). Mixed human normal and ADPKD epithelial cell samples expressed P2Y2 and P2Y6, but not P2Y1. In contrast, mouse B6 and cpk ARPKD collecting duct epithelial cell models expressed P2Y2 and P2Y1, but not P2Y6. The latter data are consistent with published work from mIMCD-K2 cells, a mouse IMCD clonal epithelial cell line, that also showed expression of P2Y1 and P2Y2, but not P2Y6 (30). Because degenerate RT-PCR for the P2X purinergic receptor channel gene family amplified a PCR product...
of the expected size, Table 3 shows the results of a more rigorous sequencing analysis of the degenerate P2X PCR products. Each product was also subcloned and transformed. In this case, however, multiple white ampicillin-resistant colonies were sequenced to gauge the incidence of P2X receptor channel subtype sequences in a given cell model. In human ADPKD cell models, an equal incidence of P2X4 and P2X5 sequences was found. In the counterpart human mixed renal epithelial cultures, P2X4 and P2X5 were found in similarly high incidence along with multiple P2X7 sequences. In mouse collecting duct epithelial cell models, sequences from P2X3 and P2X4 (but not P2X5) were found. In addition, sequences for P2X1 and P2X2 were found more rarely (Table 3). The latter data are consistent with published work from mIMCD-K2 cells, a mouse IMCD clonal epithelial cell line, that also showed expression of P2X3 and P2X4, but not other subtypes (30). Taken together, these results show that multiple subtypes of the P2X purinergic receptor channel gene family and the P2Y G protein-coupled purinergic receptor gene family are likely expressed in mouse and human epithelial cell models of ARPKD and ADPKD.

RT-PCR can often amplify mRNA species that are never translated into protein. Further confirmation of P2X receptor channel expression was obtained by immunoblotting with subtype-specific antibodies in the hADPKD 33°C cell line. Unfortunately, not enough membrane protein was obtained to perform this analysis in hADPKD primary cultures. Immunoblotting of membrane proteins revealed expression of nascent (unglycosylated) P2X4 and P2X5 receptor channel protein as well as higher molecular weight glycosylated forms of P2X4 and P2X5 that ran at least 20 kDa higher than the predicted molecular mass of these proteins (40–50 kDa) (Fig. 2). Although P2X1, P2X2, and P2X7 were not present in these membrane protein lysates, these data show that P2X4 and P2X5 are expressed by hADPKD epithelia. Functional assays using agonists selective for P2X receptor channels confirm expression of P2X receptor channel protein on the cell surface (see below), in particular, the apical membrane of a polarized PKD epithelium.

**Table 3. Incidence of specific P2X subtype sequences amplified by degenerate RT-PCR**

<table>
<thead>
<tr>
<th>Cell Line or Primary Culture</th>
<th>P2X1</th>
<th>P2X2</th>
<th>P2X3</th>
<th>P2X4</th>
<th>P2X5</th>
<th>P2X6</th>
<th>P2X7</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>hADPKD primary</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>hADPKD 33°C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>hRE primary</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>5</td>
<td>0</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>Mouse B6 WT CD primary</td>
<td>4</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Mouse cpk ARPKD CD primary</td>
<td>0</td>
<td>1</td>
<td>11</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

A given amplified PCR product from a cDNA sample reverse transcribed from total RNA extracted from the epithelial cell models listed above may have as many as 4 P2XR isoforms. WT, wild-type; CD, collecting duct. Similar analysis was performed in epithelial cell lines and primary cultures from airway and gastrointestinal tract and from an mIMCD-K2 cell line (bold nos. indicate abundantly expressed subtypes). Identity of a specific P2X subtype sequence was identified via BLAST analysis using the National Center for Biological Information website and the BLAST algorithm. Total is the total number of white colonies isolated and grown to extract, purify, and sequence the pGEM-T sequencing plasmid containing the degenerate P2Xr PCR product insert.
lial primary cultures (data not shown). Interestingly, when P2X receptor agonists were added first, a sustained increase in \([\text{Ca}^{2+}]_i\) was observed (Fig. 3). However, after prior stimulation with P2Y receptor agonists, P2XR-selective agonists only triggered a transient spike in \([\text{Ca}^{2+}]_i\) (Fig. 3). In all cases, both transient and sustained components of the \([\text{Ca}^{2+}]_i\) increase were observed in response to P2Y agonists (Fig. 3). Reversibility with washout of the agonist was only tested when a sustained response was observed (Fig. 3). More interesting still, a lack of desensitization in P2Y and P2X receptor-mediated \([\text{Ca}^{2+}]_i\) responses was noticed. It is well known that P2Y receptors as well as a subset of P2X receptors (that includes P2X4 and P2X5) are slow to desensitize compared with other receptor families (28). Taken together, these results show that both P2Y and P2X receptors increase \([\text{Ca}^{2+}]_i\) in human PKD epithelial cells. These data also confirm RT-PCR and immunoblot analysis showing that multiple P2X and P2Y receptor subtypes are expressed by PKD epithelial cell models.

**P2XR- and P2Y-selective agonists stimulate secretory anion transport by hADPKD epithelia.** The effect of purinergic receptor agonists on anion secretion across confluent hADPKD cell monolayers was assayed by measuring \(I_{SC}\) in an Ussing chamber system. Benzamil, an amiloride derivative, was added to the apical media to inhibit \(\text{Na}^+\) absorption through epithelial \(\text{Na}^+\) channels. Figure 4A shows a typical response to apical application of ATP, Bz-ATP, and UTP. Each purinergic agonist caused \(I_{SC}\) to increase and peak \(\sim 1-2\) min after addition. Table 4 lists the average peak-stimulated responses to apical addition of ATP, Bz-ATP and UTP to cell monolayers from various kidney preparations. Because of the heterogeneity of origin of ADPKD cysts along the nephron, it was critical to show the data in this manner. ATP caused a transient increase in \(I_{SC}\) in all monolayers \((n = 20)\) cultured from four hADPKD kidneys. In comparison, the response to apical addition of UTP was less consistent. In hADPKD \(_{K156}\) cells, apical addition of UTP transiently increased \(I_{SC}\) in all monolayers \((n = 4)\), whereas only four of seven monolayers from hADPKD \(_{K140}\) showed an increase in positive \(I_{SC}\). The responses to UTP suggest the presence of P2Y2 receptors on the PKD epithelium; however, we cannot rule out the fact that UDP, a metabolite of UTP, may also stimulate P2Y4 or P2Y6 receptors expressed on the PKD epithelium.

To examine the sidedness of functional expression of P2X receptors, we tested the effect of applying the P2X receptor-selective agonist Bz-ATP on the apical or basolateral side of hADPKD monolayers. The apical application of Bz-ATP stimulated a transient increase in \(I_{SC}\) by 1.0 ± 0.1 μA/cm² (Fig. 4B and Table 4). In

![Fig. 3. Agonists to both P2X receptor channels and P2Y G protein-coupled receptors increase cytosolic calcium concentration (\([\text{Ca}^{2+}]_i\)) in hADPKD cells. See text for summarized data. Typical time courses are shown where P2X and P2Y agonist cocktails were added in different order and in different protocols. Before time 0, the fluorescence was allowed to stabilize for at least 10 min before the runs shown. Curiously, when P2X agonists were added first, a small inhibition followed by a sustained increase in \([\text{Ca}^{2+}]_i\), was observed. However, when P2Y agonists were added, a transient spike and a sustained plateau in \([\text{Ca}^{2+}]_i\), were routinely observed. After P2Y agonist stimulation, subsequent addition of P2X agonists caused only a transient increase in \([\text{Ca}^{2+}]_i\).](http://ajprenal.physiology.org)
comparison, Bz-ATP applied to the basolateral side of the monolayer failed to increase $I_{SC}$. These data suggest that P2X receptor channels are expressed primarily on the apical membrane of the PKD epithelium.

To define whether increases in $I_{SC}$ were due primarily to secretory Cl$^-$/H$^+$ transport, we determined whether apical ATP-induced $I_{SC}$ could be inhibited with Cl$^-$/H$^+$ transport blockers. In this study, paired hADPKD monolayers were used. Benzamil was included in the apical media of both monolayers to eliminate possible effects of ATP on Na$^+$/H$^+$ absorption through the epithelial Na channel. The effect of ATP (10 μM) was tested on monolayers bathed in either control medium or media containing Cl$^-$/H$^+$ transport inhibitors. DPC (1 mM) and DIDS (50 μM), inhibitors of Cl$^-$/H$^+$ channels, were added to the apical medium, and bumetanide (100 μM), an inhibitor of Na-K-2Cl cotransport, was added to the basolateral medium 25 min before the addition of ATP (Fig. 5). In four pairs of monolayers, the addition of ATP increased $I_{SC}$ by 2.7 $\pm$ 0.6 A/cm$^2$ in the control group and only 0.9 $\pm$ 0.1 A/cm$^2$ ($P$ < 0.05) in the presence of the Cl$^-$/H$^+$ transport inhibitors. DIDS also inhibited residual sustained $I_{SC}$ stimulated by ATP (Fig. 5B). These data suggest that the increase in $I_{SC}$ with apical application of ATP is due primarily to Cl$^-$ secretion.

cAMP-dependent Cl$^-$ secretion through apical cystic fibrosis transmembrane conductance regulator (CFTR) Cl$^-$ channels has previously been shown to be a major process involved in fluid secretion by ADPKD renal cyst epithelia (16, 29, 43). In the present study, we confirmed that ATP is working on a process that is independent of the cAMP pathway. First, the addition of ATP further increased $I_{SC}$ above a current stimulated initially by forskolin (data not shown). Second, the increase in $I_{SC}$ with apical ATP was insensitive to protein kinase A (PKA) inhibition. Pretreatment for 15

Table 4. Purinergic agonist stimulation of short-circuit current in different hADPKD primary cultures

<table>
<thead>
<tr>
<th>hADPKD Code No. for Primary Culture</th>
<th>No. of Monolayers</th>
<th>$\Delta I_{SC}$, μA/cm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP, 10 μM</td>
<td>98, 99, 140, 156</td>
<td>1.6 $\pm$ 0.3*, 0.8 $\pm$ 0.2*, 1.4, 2.7 $\pm$ 0.4*</td>
</tr>
<tr>
<td>Bz-ATP, 10 μM</td>
<td>156</td>
<td>1.0 $\pm$ 0.1*</td>
</tr>
<tr>
<td>UTP, 10–100 μM</td>
<td>140, 156</td>
<td>0.2 $\pm$ 0.1, 1.7 $\pm$ 0.5*</td>
</tr>
</tbody>
</table>

Values are means $\pm$ SE. Summary of the effect of ATP, Bz-ATP and UTP on short-circuit current ($I_{SC}$) across cultured ADPKD cell monolayers. Monolayers of cells cultured from kidney 156 generated the largest increase ($\Delta I_{SC}$) in response to ATP and UTP, compared with monolayers from other kidney preparations. The response of hADPKD140 cell monolayers to apical UTP was variable (see text).

* $P < 0.05$ compared with 0, Student’s $t$-test.
min with H-89 (10 μM), a PKA inhibitor, had little effect on a 10 μM apical ATP-induced increase in $I_{SC}$ of 2.6 μA/cm², a response comparable with the response of ATP by untreated monolayers (data not shown). In the presence of H-89, forskolin failed to increase $I_{SC}$ (data not shown); however, in an untreated monolayer from the same kidney preparation, forskolin increased $I_{SC}$ by 3.3 μA/cm². Third, pretreatment with 1,2-bis(2-aminophenoxy)ethane-$N,N,N',N'$-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) reduced the baseline current and eliminated the peak ATP response (Fig. 6).

Taken together, these data confirm that ATP is activating a Cl⁻ secretory process that is independent of a cAMP/PKA regulation.

**DISCUSSION**

These results are the first to show all limbs of an extracellular autocrine nucleotide signaling pathway that exists at the level of the PKD epithelial cell layer in vitro (Fig. 7). In vivo, this monolayer of PKD epithelial cells would encapsulate an ADPKD cyst or line a dilated ARPKD tubule. ATP release is greater under constitutive or activated conditions in PKD epithelial cell models from ARPKD and ADPKD. Once released, ATP as an agonist is free to bind to multiple P2Y G protein-coupled receptor and P2X receptor channel subtypes expressed on the ADPKD or the ARPKD epithelium. In a polarized ADPKD epithelium, P2X receptors were expressed primarily on the apical plasma membrane. In ADPKD cells, both types of purinergic receptors increase $[Ca^{2+}]_i$. Moreover, they trigger sustained increases in $[Ca^{2+}]_i$, a process that may trigger downstream Ca²⁺-dependent signaling pathways. The same is true in normal primary cultures of renal epithelia (Schwiebert EM and Taylor AL, unpublished observations). To close the autocrine purinergic loop, purinergic agonists stimulate Cl⁻ secretion across human ADPKD epithelial monolayers. Similar results were found in a normal immortalized mIMCD-K2 cell line by authors of this study (30). It is unclear whether Cl⁻ secretion, fluid secretion, or transepithelial ion transport per se plays a role in ARPKD. This issue is under investigation in our laboratory. Because these studies are a novel description of extracellular purinergic signaling in ARPKD and ADPKD, one can only draw analogies to purinergic signaling in normal and diseased epithelia from the
stimulating increases in \[Ca^{2+}\] signal and transduce it into stimulation of \[Cl^-\] secretion. Both receptor subtypes are poised on the luminal membrane to receive the extracellular autocrine ATP ultrasound in vivo (50). In a subset of those cysts in vitro and in human ADPKD cysts tapped via fluids from microdissected ADPKD samples, ATP was found in high-nanomolar-to-micro-molar amounts and, in one sample, at 10 \(\mu\)M. This reflects marked purinergic signaling within this abnormal microenvironment. In this study, we show additional evidence for enhanced ATP release in vitro ARPKD and ADPKD models vs. relevant controls. It is also important to note that it is not absolutely necessary for ATP release and signaling to be augmented in PKD. Its mere presence in an altered microenvironment where this signaling is trapped and/or prolonged may be detrimental.

At least three possible mechanisms explain this enhanced ATP release. First, enhanced and dysregulated ATP release may be a secondary phenotype caused by a primary defect in one or both of the polycystin gene products found in ADPKD. The PKD-2 gene product, polycystin-2, has a topology not unlike a \(Ca^{2+}\)-permeable cation channel and has been hypothesized to be a unique type of ion channel that interacts with and is regulated by polycystin-1 (5, 32). Second, PKD epithelial cells have an undifferentiated and proliferative phenotype. Enhanced cellular metabolism and growth may account for the enhanced ATP release (43, 49). This would be an explanation for the phenotype found in both ARPKD and ADPKD. A third and alternative interpretation of enhanced ATP release across the apical and basolateral membrane that cannot be ruled out is an abnormally potentiated ATP release mechanism. This may also occur in both forms of the disease.

Detailed analysis of ATP release mechanisms and their regulation in carefully genotyped PKD cell models is warranted to address their possibilities and will be addressed in future studies.

This unique pathophysiological architecture, wherein the cylindrical tubule is transformed into the spherical and encapsulated ADPKD cyst, creates an ideal environment for autocrine signaling (43). It is also likely, but not proven, that higher concentrations of ATP metabolites may be present within the cyst. ADP and adenosine are also potent \(Cl^-\) and fluid secretagogues. In addition, the decreased capacity of ADPKD cells to degrade ATP suggests that ATP itself may be present for prolonged periods within the cyst lumen. It is tempting to speculate that ecto-ATPases, ecto-apyrases, and ecto-nucleotidases, normally expressed on the luminal membrane of some renal epithelia, may be lacking and/or mislocalized in PKD. In ARPKD, the tubules never close off completely; however, turbulent flow in this dilated tubule may create unstirred layers that form a microenvironment for autocrine signaling.

Knowing that purinergic agonists are present in the cyst lumen is not enough. Our molecular and functional studies show that P2Y and P2X receptors are also expressed in ARPKD and ADPKD epithelial cell models. Using chamber measurements of hADPKD epithelial monolayers showed expression of P2X and P2Y receptors on the luminal or apical membrane of PKD epithelia, while P2Y receptors were also expressed functionally on the serosal or basolateral membrane. Thus these receptors are poised to receive the autocrine ATP signal at both membranes, and they need not be mislocalized. Because they are expressed naturally in this modified nephron architecture, purinergic

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**Polarized ADPKD Epithelium**

Fig. 7. Working model illustrating the extracellular autocrine purinergic signaling system present in the PKD epithelial microenvironment that may lead to increased cyst volume, cyst size, and enhanced disease progression in PKD. The figure depicts two neighboring ADPKD epithelial cells that would encapsulate an ADPKD cyst. Similar signaling, but perhaps stimulation of different endpoints, may occur in ARPKD epithelial cells. All cells would release ATP, bind ATP, and secrete \[Cl^-\]. ATP release from the cell on the left and ATP binding to receptors, stimulating increases in \([Ca^{2+}]\), and \[Cl^-\] secretion in the cell on the right, are shown for simplicity and clarity of the working model. This extracellular autocrine signaling system likely affects \(Ca^{2+}\)-dependent \[Cl^-\] channels that are independent of the cAMP-regulated cystic fibrosis transmembrane conductance regulator (CFTR) \[Cl^-\] secretory pathway. Both receptor subtypes are poised on the luminal membrane to receive the extracellular autocrine ATP signal and transduce it into stimulation of \[Cl^-\] secretion. ATP release may happen constitutively as well as become augmented by \(Ca^{2+}\) agonists or changes in extracellular osmolality.
nergic receptors and signaling may be unintentionally detrimental in PKD. These results agree with several other recent studies that show concomitant expression of P2X and P2Y receptors in the same renal epithelial cell model. In a study performed in part by one author of this study, P2X3, P2X4, P2Y1, and P2Y2 receptors were found in an mLIMCD-K2 cell line derived from inner medullary collecting duct (30). Interestingly, this agrees with purinergic receptor expression in the mouse B6 and cpk collecting duct models. With regard to in vitro cell lines and primary cultures derived from defined nephron segments, collecting duct models are the point along the nephron where P2Y and P2X expression changes. For example, up to the collecting duct, P2X4 and P2X5 are abundant. In the collecting duct, this pattern changes to P2X3 and P2X4. This is borne out in our data in Table 3 that shows P2X4 and P2X5 in heterogeneous hRE and hADPKD models, whereas the mouse B6 and cpk models reflect the collecting duct phenotype.

Concomitant expression of P2Y and P2X receptors occurs in epithelial cells derived from other tissues. In a recent study by Luo and co-workers (27), P2Y and P2X receptors were found on the luminal and serosal membranes of isolated and perfused rat pancreatic ducts. These receptors stimulated Cl− channel activity as well as triggered increases in [Ca2+]i in isolated ducts or duct cells. In non-cystic fibrosis (CF) and CF airway and gastrointestinal models, evidence for P2Y receptor expression and P2X receptor expression in epithelial models is well documented (26, 45, 48). In the kidney, P2Y2 receptor expression has been found by numerous laboratories to exist in all regions of the kidney (cortex, outer medulla, and inner medulla) and in isolated segments (9, 23). In isolated perfused mouse cortical collecting duct, Leipziger and co-workers (9) have found that P2Y2 receptors are expressed on the luminal membrane of mouse cortical collecting duct where they increase [Ca2+]i (9). P2X receptor channels may mediate [Ca2+]i influx directly. Importantly, an alternative mechanism for P2XR-mediated increases in [Ca2+]i, which we cannot rule out, is that P2XRs themselves do not mediate Ca2+ influx but depolarize the membrane potential sufficiently to allow Ca2+ influx via voltage-gated Ca2+ influx channels (45). Taken together, expression of purinergic receptors as well as purinergic signaling via [Ca2+]i, and other pathways in other epithelial cell models are in profound agreement with this study.

In vitro studies showed that growth factors, like nucleotide agonists and their metabolites, are also found in hADPKD cyst fluid (31, 49). In a subset of cells that line the cyst, growth factor receptors (the EGF receptor, for one) are mislocalized to the apical or luminal membrane of the cyst (49). Transgenic studies, in which EGF receptor-deficient mice were crossed with the orpk ARPKD mice, showed that progression of cyst development and increased cyst size were slowed significantly (33). This autocrine signaling has been implicated in both ARPKD and ADPKD. Again, it is important to emphasize that mislocalization does not need to occur for purinergic receptors and signaling; they are already expressed in the proper functional location and, therefore, may be detrimental. It should be noted that the growth factor studies, first by Wilson and co-workers (49) and later by other laboratories, were the first description of autocrine signaling by an agonist and its possible contribution to PKD (31, 33). Similar work in mouse models of ADPKD and ARPKD must be performed in these animals to solidify a role for autocrine ATP signaling in PKD cyst progression.

Purinergic agonist stimulation of Cl− secretion has also been well documented in normal epithelial models from kidney and other tissues. Purinergic agonists also stimulate Cl− secretion from epithelia derived from CF tissues, underscoring the possible therapeutic manipulation of purinergic regulation for the benefit of CF (24, 25, 42, 45). However, in PKD cysts, this normal physiological stimulation of Cl− secretion becomes detrimental. Once Cl− secretion becomes directed into a “closed-off” cyst, the cyst slowly expands due to Cl− secretion (and the fluid secretion that follows osmotically).

Indeed, expression of CFTR and cyclic AMP stimulation of CFTR in a subset of epithelial cells that line ADPKD cysts also becomes detrimental in the PKD disease paradigm (11, 16, 29, 43). Purinergic agonists, however, appear to stimulate other types of Cl− channels that may be expressed in the PKD epithelium. Indeed, purinergic agonist stimulation of Cl− ion transport has been studied since the early 1980s in MDCK cells by Simmons and co-workers (40, 41) and involves multiple types of Cl− channels. Very recently, Simmons and co-workers revisited these studies and showed that both Ca2+ agonists and cAMP agonists stimulate Cl− secretion across mLIMCD-K2 cell monolayers (3).

Because inhibitors of cAMP signaling do not affect nucleotide-stimulated Cl− secretion, Ca2+-dependent Cl− channels are more likely targets of purinergic signaling. These may include the CaCC (CLCA) Cl− channels (8) and/or a subset of Ca2+-sensitive and voltage-sensitive CLC Cl− channel family, CLC-3 and CLC-5 (46). Indeed, BAPTA-AM pretreatment of monolayers attenuated ATP- and UTP-dependent stimulation of Cl− transport. The fact that luminal purinergic agonists were more potent than when applied serosally suggests that an apical membrane-delimited regulation of Cl− channels by purinergic receptors may be more direct and robust. Moreover, the lack of an increase in ISc with the basolateral addition of purinergic agonists is consistent with a previous report that ADPKD epithelial cells may have a low abundance of a Ca2+-dependent K+ channel on the basolateral side of the cell (Grantham JJ, unpublished observations). Indeed, in that study, charybdotoxin, a specific inhibitor of the Ca2+-dependent K+ channel and 1-EBIO, a direct activator of the channel, did not affect ISc or ADPKD microcyst formation in hydrated collagen gel (Grantham JJ, unpublished observations). Studies in airway and intestinal cell models of Cl− secretion show
DETREMITRAL EXTRACELLULAR PURINERGIC SIGNALING IN PKD

that P2X receptors stimulate Cl− secretion in the apical and basolateral membranes (45).

In summary, this study suggests strongly that extracellular purinergic signaling, via sustained increases in [Ca2+]i and stimulation of Cl− secretion, may contribute detrimentally to the progression of ADPKD by trapping a normally functioning extracellular autocrine signaling cascade, especially, a Cl− and fluid secretagogue, in an abnormal nephron architecture. The importance of transepithelial ion transport to the progression of ARPKD still needs to be investigated further. Moreover, there may be a role for extracellular nucleotides and nucleosides in the abnormal growth, proliferation, and altered development of ARPKD and/or ADPKD epithelial cells. Extracellular purinergic agonists are mitogens or comitogens with growth factors for renal epithelial and mesangial cells in vitro (19, 20, 36). This additional detrimental role of purinergic signaling in PKD must also be examined.

Possible cell biological maneuvers should be explored that interfere with purinergic signaling (ATP or adenosine scavengers, P2 and P1 purinergic receptor antagonists, inhibitors of ATP release); these agents may have therapeutic benefit by slowing the progression of PKD once cyst formation has begun. Because it is difficult to deliver any therapeutic agent to the lumen of an ADPKD cyst or a dilated ARPKD tubule, delivery before these structures have formed may be prudent. Small-molecule screening for inhibitors of ATP release is also a viable option. Finally, genetic cross of purinergic receptor knockout mice with PKD may have therapeutic benefit by slowing the progression of PKD once cyst formation has begun. Because it may have therapeutic bene

REFERENCES


