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Altered ATP-Sensitive P2 Receptor Subtype Expression in the Han:SPRD cy/+ Rat, a Model of Autosomal Dominant Polycystic Kidney Disease

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Key Words

ATP · P2 receptors · Polycystic kidney disease · Han:SPRD cy/+ · Rat

Abstract

The effects of extracellular ATP on fluid secretion and reabsorption by renal epithelial cells, as well as its known effects on cell proliferation and death, are potentially important contributory factors in the development and growth of renal cysts. In this study, we have investigated the protein and mRNA expression of several P2Y receptor subtypes ($P2Y_{1,2,4,6}$), as well as the $P2X_5$ and $P2X_7$ receptors, in kidney tissue from the Han:SPRD (cy/+) rat model of polycystic kidney disease. All of the P2Y receptors tested for, and the $P2X_5$ and $P2X_7$ subtypes, were located on the cyst-lining cells of Han:SPRD (cy/+) rat polycystic kidneys; most immunostaining was cytosolic and we could not confidently localize it to one or other membrane. However, the staining pattern for $P2Y_6$ was uniquely granular when compared with the other P2 receptors. $P2Y_2$ and $P2Y_6$ receptor mRNA was increased in both homozygote (cy/cy) and heterozygote (cy/+) rat kidneys when compared with unaffected littermates. The protein levels of $P2Y_2$ and $P2Y_6$ receptors were also increased, being undetectable or at a low level, respectively, in control tissue. Finally, $P2X_7$ receptor mRNA was increased in cy/+, but not in cy/cy rat kidneys. Our results show that a number of P2Y receptor subtypes, as well as the $P2X_5$ and $P2X_7$ receptors, are clearly expressed in cyst-lining cells in the Han:SPRD (cy/+) rat model of renal cystic disease. Furthermore, $P2Y_2$ and $P2Y_6$ receptor mRNA and protein levels are markedly increased in cystic rat kidneys compared with normal rats of the same genetic background. Thus, the most consistent findings were an increase in the expression of $P2Y_2$, $P2Y_6$ and $P2X_7$ receptors in cystic tissue. Given the widely reported effects of stimulating these P2 receptor subtypes in epithelial and other renal cells, they could contribute to the development and growth of renal cysts: extracellular ATP and its products 'trapped' in cyst fluid may activate P2 receptors expressed by cyst-lining cells, causing cyst expansion from increased fluid secretion and/or reduced reabsorption, as well as an increase in cell turnover (re-modeling).

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Abbreviations used in this paper

ADPKD	autosomal dominant polycystic kidney disease
HPRT	hypoxanthine phosphoribosyl transferase
PBS	phosphate-buffered saline
TUNEL	terminal deoxynucleotidyl transferase-mediated nick end-labeling

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Introduction

Formation and progressive enlargement of renal cysts is a well-characterized feature of most renal cystic diseases, especially autosomal dominant polycystic kidney disease (ADPKD), leading to destruction of normal renal tissue and impaired renal function. ADPKD is caused by loss-of-function mutations of PKD1 (encoding polycystin 1, a membrane receptor) and PKD2 (encoding polycystin 2, a calcium-permeable ion channel) genes, although the mechanisms underlying cyst formation remain unclear [Sutters and Germino, 2003]. Once formed, the cysts can expand by enhanced proliferation of lining epithelial cells and increased luminal fluid transport driven by transepithelial Cl^- secretion [Grantham et al., 1989; Mangoo-Karim et al., 1995], as the normal and predominantly reabsorptive role of these epithelial cells is changed to a secretory one. Recently, both increased ATP release from primary cultures of human polycystic kidney epithelia and elevated levels of ATP in cyst lumen fluid have been reported [Wilson et al., 1999; Schwiebert et al., 2002]. Moreover, extracellular ATP is a potent stimulus to transepithelial fluid secretion by acting on G-protein-coupled P2Y receptors [Schwiebert and Zsembery, 2003]. Activation of either P2X or P2Y receptors can lead to a rise in intracellular calcium concentration that in turn could trigger activation of calcium-sensitive chloride channels, increasing Cl^- secretion, and/or inhibit the epithelial sodium channel (ENaC), decreasing sodium reabsorption [Cuffe et al., 2000; Wildman et al., 2003]. Furthermore, P2Y receptor signalling via adenylate cyclase will increase cAMP levels and also stimulate renal epithelial cell Cl^- secretion and proliferation [Mangoo-Karim et al., 1989; Hanaoka and Guggino, 2000; Yamaguchi et al., 2000].

Metabotropic G-protein-coupled P2Y receptors and ionotropic P2X receptors have been found in the kidney in all nephron segments examined [Bailey et al., 2000a, 2000b; Schwiebert and Kishore, 2001; Turner et al., 2003]. Current data suggest that $P2Y_1$ and $P2Y_2$ receptors are involved in cell growth and proliferation [Greig et al., 2003b], and that the $P2X_5$ and $P2X_7$ receptors can influence cell turnover by affecting cell proliferation, differentiation and death (by necrosis or apoptosis) [Schulze-Lohoff et al., 1998; Groschel-Stewart et al., 1999a, 1999b; Harada et al., 2000; Greig et al., 2003b; Verhoef et al., 2003]. Recently, multiple P2X and P2Y receptor mRNA transcripts have been detected in human ADPKD and in *cpk* mouse renal epithelial cell primary cultures [Schwiebert et al., 2002].

The Han:SPRD (cy/+) rat model of ADPKD produces cystic changes along the nephron that are very similar to those occurring in human ADPKD [Kaspareit-Rittinghausen et al., 1990; Cowley et al., 1993; Schafer et al., 1994]. In both rats and humans the cystic epithelium is poorly differentiated, with basement membrane thickening and increased cell proliferation [Cowley et al., 1993; Schafer et al., 1994; Ramasubbu et al., 1998]. In the early stages of the rat model, cysts develop from proximal tubules, but in the later stages all nephron segments are involved [Kaspareit-Rittinghausen et al., 1990], although the majority remain proximal in origin [Schafer et al., 1994]. Similar to the disease in humans, males are more severely affected than females [Schafer et al., 1994] and the severity of the disease in homozygotes, regardless of gender, is significantly more pronounced than in heterozygotes, suggesting that differences in cystic phenotype are related to gene dosage [Cowley et al., 1993]. Furthermore, heterozygous animals exhibit a more slowly progressive form, which is closer to human ADPKD [Schafer et al., 1994].

In the present study our aim was limited and primarily descriptive: to examine for any change in the pattern of P2 receptor expression in this renal cystic disease model, which might provide a clue as to a potential functional role for one or more of these ATP-sensitive receptors. Using immunohistochemistry and currently available antibodies, we have examined the pattern of expression of $P2Y_{1,2,4,6}$ and $P2X_5$ and $P2X_7$ receptor subtypes in the Han:SPRD (cy/+) rat at essentially one adult time point. (Previously, we have also published the pattern of renal P2 receptor expression in the Sprague-Dawley rat, SPRD [Turner et al., 2003; Bailey et al., 2004].) In addition, we have used real-time PCR to determine the relative abundance of P2 receptor subtype mRNA in both cy/cy homozygote and cy/+ heterozygote rats compared with unaffected (wild-type) control animals.

Methods

Immunohistochemistry

Kidney tissue from 36-day-old heterozygote Han:SPRD (cy/+) rats was a generous gift from Prof. Gretz (University of Heidelberg, Germany). It was embedded in Tissue-Tek (Sakura Finetek, Mijdrecht, The Netherlands) for cryosectioning. Tissue was sectioned at 8 μm using a cryostat (Reichert Jung CM1800), collected on gelatin-coated slides and air-dried at room temperature. The slides were stored at −20 °C and allowed to return to room temperature for at least 10 min prior to further use. The slide-mounted sections were fixed in 4% formaldehyde and 0.2% of a saturated picric acid solution in phosphate-buffered saline (PBS) for 2 min. To inactivate

Table 1. Forward (F) and reverse (R) sequences of the oligonucleotide primers used in this study

Gene	Accession	No.	5′ to 3′ Sequence	Expected product size, bp
$P2Y_1$	NM_012800	F	ACGTCAGATGAGTACCTGCG	289
		R	CCCTGTCGTTGAAATCACAC	
$P2Y_2$	XM_346560	F	ACTTTGTCACCACCAGCGTGAG	279
		R	TGACGTGGAAAGGCAGGAAG	
$P2Y_4$	Y11433	F	TGTTCCACCTGGCATTGTCAG	294
		R	AAAGATTGGGCACGAGGCAG	
$P2Y_6$	NM_057124	F	TGCTTGGGTGGTATGTGGAGTC	339
		R	TGGAAAGGCAGGAAGCTGATAAC	
$P2X_5$	X97328	F	TGTCATTCCATCTCAGGGGG	286
		R	TTCGGCATCCTTTAGAAGGG	
$P2X_7$	X95882	F	GTGCCATTCTGACCAGGGTTGTATAAA	353
		R	GCCACCTCTGTAAAGTTCTCTCCGATT	
HPRT	XM343829.1	F	GCTGACCTGCTGGATTACATTA	410
		R	CCACTTTCGCTGATGACACAA	

endogenous peroxidase, the sections were then treated with 50% methanol containing 0.4% hydrogen peroxide for 10 min. Non-specific protein binding sites were blocked by 20-min incubation with 10% normal horse serum in PBS containing 0.05% thimerosal (Sigma-Aldrich, Poole, UK). The P2 polyclonal antibodies were diluted to 1.25–5 μg/ml (determined as optimal by prior titration) with 10% normal horse serum, and the sections were incubated with the primary antibodies overnight at room temperature. $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor antibodies were obtained from Alomone Laboratories (Jerusalem, Israel), $P2Y_6$ receptor antibody was a gift from Prof. Leipziger (University of Aarhus, Aarhus, Denmark), and $P2X_5$ and $P2X_7$ receptor antibodies were from Roche Bioscience (Palo Alto, Calif., USA). The secondary antibody was a biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch, Luton, UK) used at a dilution of 1:500 for 30 min, followed by the ExtrAvidin peroxidase conjugate (Sigma-Aldrich) at 1:1,000 for 30 min.

The nickel-intensified 3,3′-diaminobenzidine reaction was performed for 5 min according to the protocol developed by Llewellyn-Smith et al. [Llewellyn-Smith et al., 1992]. Sections were dehydrated in isopropyl alcohol and mounted in Eukitt (BDH/Merck, Leicester, UK). In the case of fluorescent microscopy, the secondary antibody was cy3-labeled anti-rabbit IgG (Abcam, Cambridge, UK). Controls were performed with pre-immune IgG and with the P2X antibodies pre-absorbed with the homologous peptides; no staining was observed. The results were documented using the Zeiss Axioplan light/fluorescent microscope (Jena, Germany) and images captured using a Leica DC200 digital camera (Leica, Wetzlar, Germany).

Identification of Apoptotic Cells

The terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) assay was performed using the in situ cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany). TUNEL identifies fragmented DNA as a marker of apoptosis. Briefly, Han:SPRD cy/+ kidney sections were fixed with 4% formaldehyde in PBS for 20 min and then washed three times with PBS. Cells were permeabilized with 0.1% Triton X-100, 0.1% sodium citrate solution in PBS for 2 min at 4°C, rinsed three times with PBS and incubated for 1 h in the TUNEL reaction mixture (terminal deoxynucleotidyl transferase with FITC-conjugated dUTP). After a further washing in PBS, the slides were mounted in Citifluor and examined with a Zeiss Axioplan light/fluorescent microscope and images were captured using a Leica DC200 digital camera (Leica).

RNA and Protein Extraction

Heterozygote and homozygote Han:SPRD (cy/+) kidney tissue from 28-day-old rats was provided by Prof. Woo (University of California, USA) for the extraction of RNA and proteins. Fresh whole kidney was snap-frozen in liquid nitrogen and ground to a powder using a pestle and mortar. One hundred micrograms of powdered tissue were resuspended in 1 ml of TRIzol® reagent (Gibco BRL) and passed through a pipette several times to ensure a homogeneous suspension. The remaining powdered tissue was resuspended in ice-cold Ripa buffer (50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 m*M* EDTA) containing 10% protease inhibitors (Sigma, UK) for immunoblotting.

RNA was extracted using TRIzol/chloroform extraction and isopropyl alcohol precipitation. The final pellet was air dried and resuspended in RNAse-free distilled water. RNA concentration and purity were determined by spectrophotometry. Messenger RNA was isolated using oligo(-dt)-coated magnetic beads according to the manufacturer's protocol (PolyATract; Promega, Southampton, UK). An amount equivalent to 1 μg of total RNA was reverse transcribed with 0.5 μg oligo(-dt) 12–18 primer and a first-strand cDNA synthesis kit (Superscript II RNase H^- reverse transcriptase, Gibco BRL).

Real-Time PCR

The resulting cDNA transcripts of whole kidney mRNA were used for PCR amplification using the Roche Lightcycler (Roche Diagnostics) and QuantiTect SYBR® Green PCR kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. Specific primers were designed from the rat sequences for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2X_5$ and $P2X_7$ and the constitutively expressed gene hypoxanthine

phosphoribosyl transferase (HPRT; see table 1 for primer sequences). To quantify P2 receptor mRNA expression, standard curves were generated with known amounts of each gene product. A ratio of relative abundance of the gene of interest (P2) to the 'house-keeping' gene HPRT was calculated by the Lightcycler Relative Quantification software version 1.0 (Roche Diagnostics). Melting curve analysis was carried out to ensure primer specificity. PCR products were also analyzed by gel electrophoresis and visualized using a Bio-Rad multi-imager (Bio-Rad, Hemel Hempstead, UK).

Immunoblotting

Cell debris was removed from kidney homogenates by centrifugation, and protein concentration was determined by spectrophotometry. Ten micrograms of protein were electrophoresed on 12% SDS-PAGE gels and then transferred to Hybond ECL-nitrocellulose membrane (Amersham Biosciences, Amersham, UK) using Bio-Rad semi-dry transfer apparatus. Membrane blots were then washed in distilled water (3 × 1 min) and incubated in 10% glutaraldehyde overnight. Membranes were blocked with 3% milk in PBS containing 0.05% Tween-20 for 1 h, and probed overnight with either $P2Y_2$ or $P2Y_6$ receptor antibody diluted in PBS. A peroxidase-linked donkey anti-rabbit IgG and ECL-positive chemiluminescence (Amersham Biosciences) was used for detection and visualized using a Bio-Rad Multi-imager (Bio-Rad).

Statistics

Real-time PCR results are expressed as means ± SD of *n* observations, and percentage changes are compared with control. To compare sets of data, we used 'Graphpad Instat' version 3.06 and a one-way ANOVA with the Tukey-Kramer multiple comparisons post hoc test, as appropriate. Differences were considered statistically significant when $p < 0.05$.

Results

Immunohistochemistry

Using the polyclonal antibodies to P2 receptor subtypes, we were able to localize the distribution of $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in 36-day-old Han:SPRD (cy/+) rat kidney. However, no suitable antibodies for $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ were available to us, nor was sufficient tissue available for us to use nephron segment co-localizing antibodies.

All of the P2 receptor subtypes tested were detected on epithelial cells lining renal cysts; however, not every cyst was immunopositive for each receptor subtype. Cysts that were immunopositive for $P2Y_1$ were predominantly located in the renal cortex (fig. 1a) and this receptor appeared to be mainly cytoplasmic in cyst-lining cells (fig. 2a). Glomerular mesangial cells were also positive for $P2Y_1$ receptors, and the staining pattern was the same as that found in normal Sprague-Dawley rats [Turner et al., 2003; Bailey et al., 2004]. $P2Y_2$ receptor expression was also cytoplasmic in the lining cells of both large and small cysts, but again, not all cysts were immunopositive for this receptor subtype (fig. 1b, 2b). Glomerular podocytes (not shown) were immunopositive for $P2Y_2$ receptors, as reported previously in normal Sprague-Dawley rat kidneys [Turner et al., 2003; Bailey et al., 2004].

Many cysts located in the cortex were immunopositive for $P2Y_4$ and $P2Y_6$ receptors (fig. 1c, d), perhaps indicative of the tubule segment from which the cyst was derived. In a previous study of normal rat kidney, $P2Y_4$ receptors were detected on basolateral membranes of proximal convoluted tubule cells [Turner et al., 2003], and $P2Y_6$ receptors have been detected in S1 and S2 proximal convoluted tubule cells [Leipziger, pers. commun.; Bailey et al., 2004). Dense $P2Y_4$ receptor expression was detected in the cytoplasm of epithelial cells of small cysts, but the staining pattern was more scattered in cells lining larger cysts (fig. 1c). This was not seen with $P2Y_6$ receptor expression, which had a granular and cytoplasmic staining pattern in both large and small cysts (fig. 1d, 2c).

$P2X_5$ receptor immunoreactivity was detected in the cytoplasm of cells lining mainly small, though some large, cysts in the cortex (fig. 1e). Many small and large cysts were immunopositive for $P2X_7$ receptors; the staining pattern for this receptor was particularly intense in cells lining smaller cysts (fig. 1f).

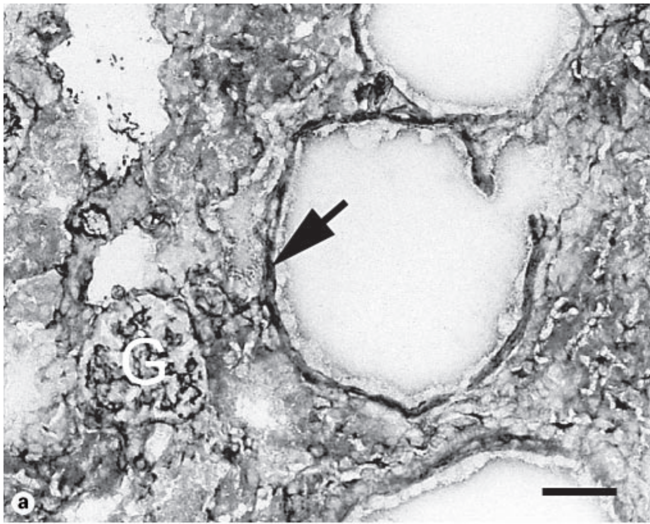
TUNEL Assay

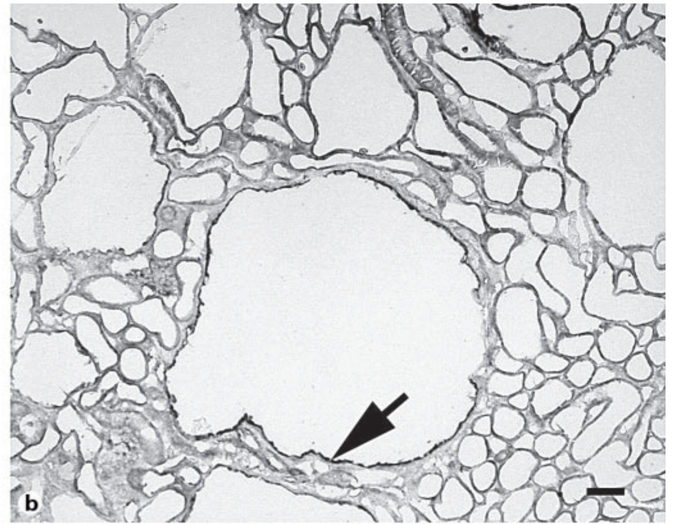
TUNEL-positive nuclei, a marker of cell apoptosis, were detected on epithelial cells lining renal cysts (arrows), the interstitium and some glomerular cells (fig. 3a). Apoptotic cells were found to be mainly associated with small cysts. However, TUNEL-positive nuclei were not detected in normal rat kidney tissue (fig. 3b).

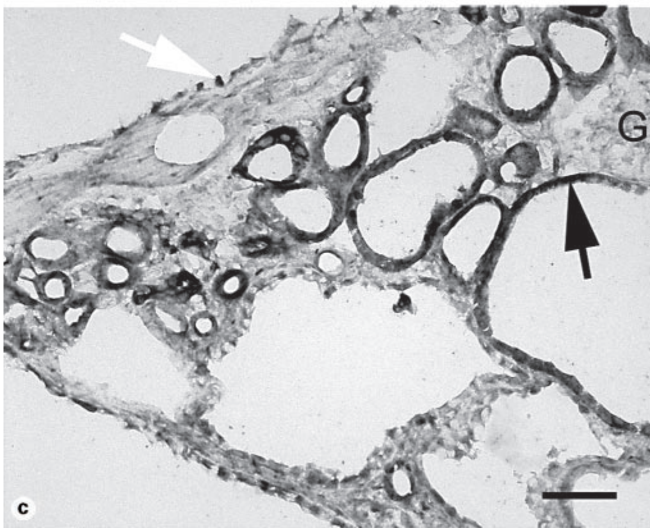
Real-Time PCR

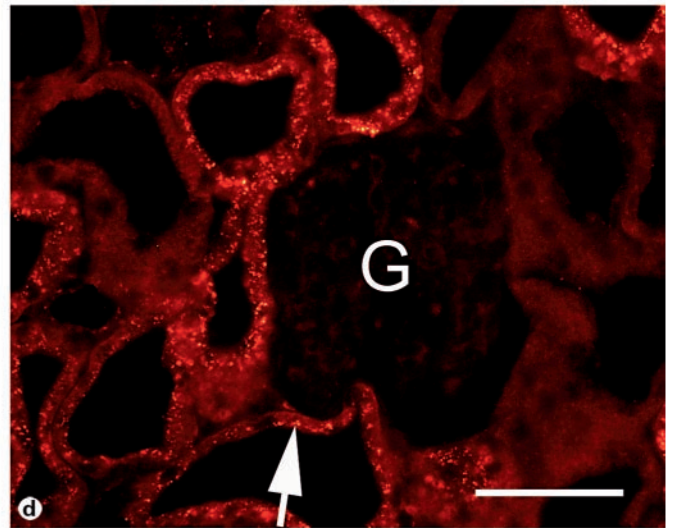
To determine the relative abundance of P2 receptor mRNA in 28-day-old Han:SPRD rat kidney tissue, a ratio was calculated of the gene of interest (P2 receptor) to a constitutively expressed 'house-keeping' gene (HPRT). Experiments were repeated in duplicate or triplicate on 5–8 animals in each group.

$P2Y_2$ receptor mRNA abundance was increased in both cy/+ (78%, not significant) and cy/cy (224%, $p < 0.001$) animals when compared with wild-type littermates (fig. 4a). Levels of $P2Y_6$ receptor mRNA were increased by 125% ($p \le 0.001$) in the cy/+ renal tissue and 120% ($p < 0.001$) in cy/cy animals when compared with control (fig. 4b). Levels of $P2Y_1$ receptor mRNA remained un-









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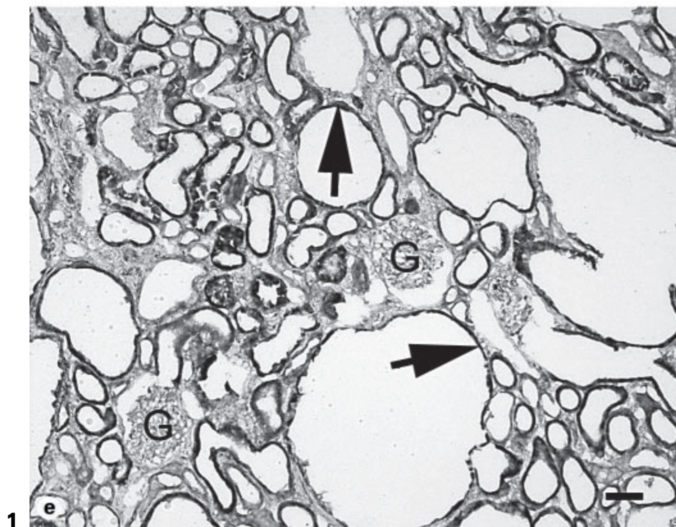

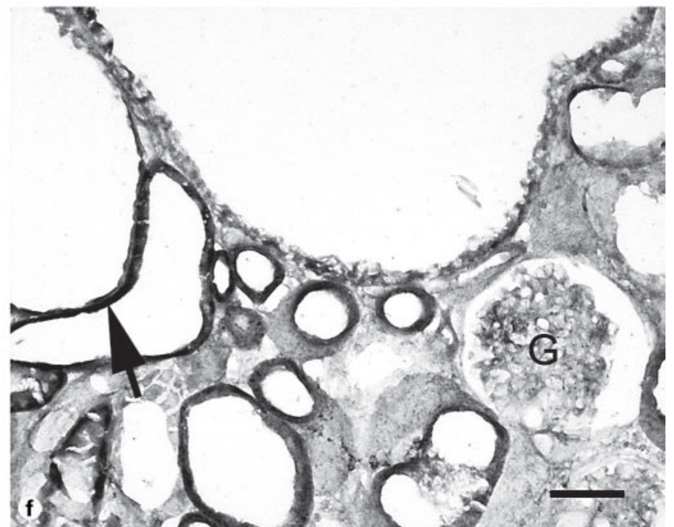

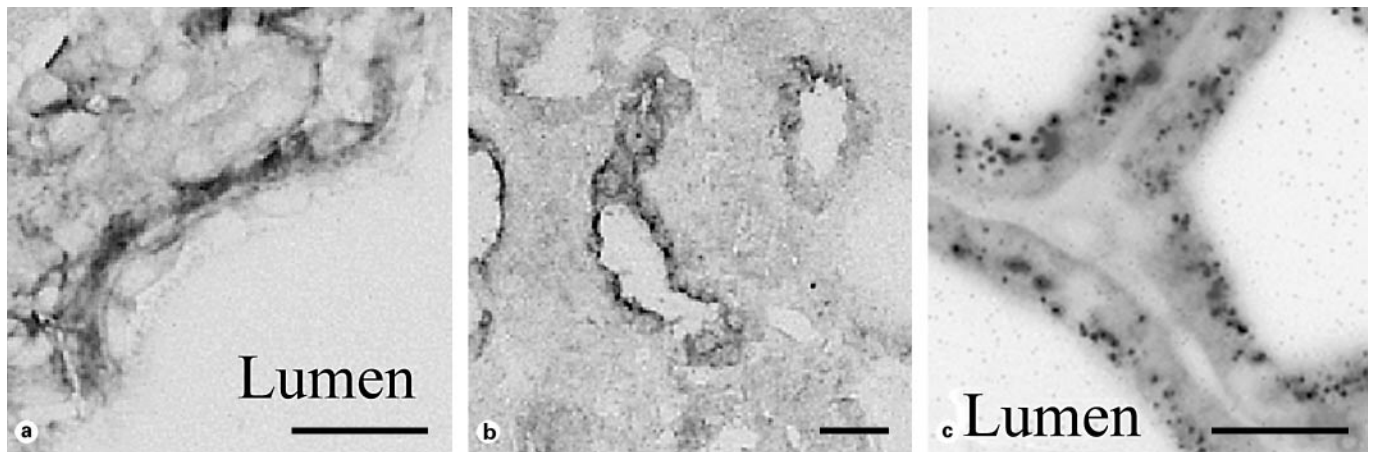

Fig. 2. Higher magnification photomicrographs of the lining of renal cysts showing $P2Y_1$ receptor immunoreactivity predominantly and diffusely cytoplasmic (**a**); $P2Y_2$ receptor immunoreactivity (**b**), and $P2Y_6$ receptor immunoreactivity, cytoplasmic with a granular appearance (**c**; see also fig. 1d). Scale bar = 30 µm.

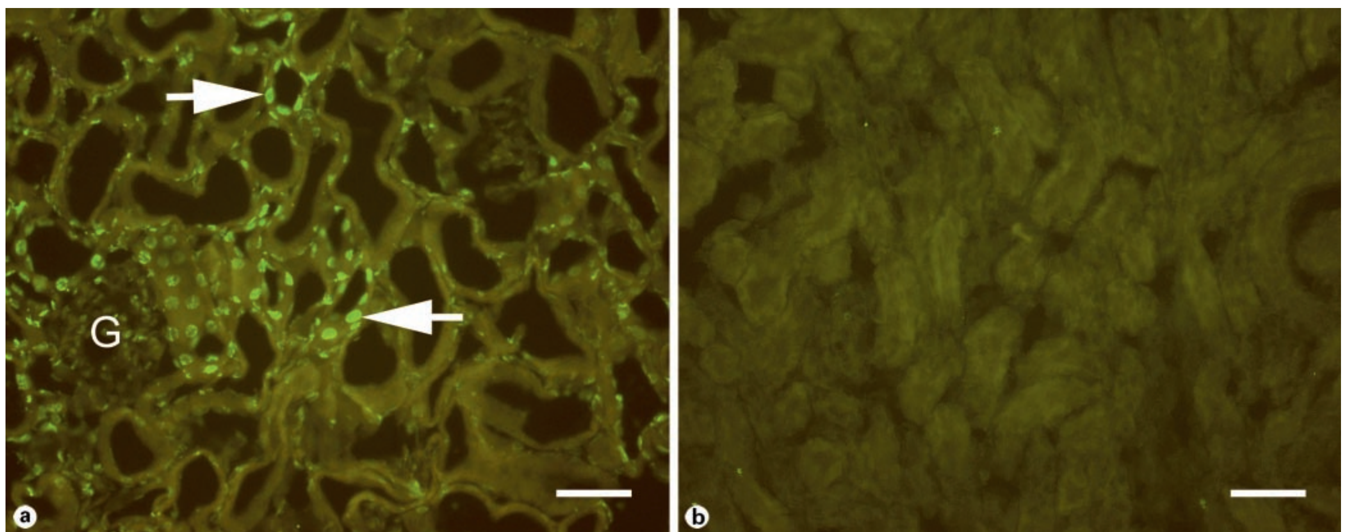

Fig. 3. a TUNEL-positive nuclei were detected in many epithelial cells lining small cysts (arrows), in the renal interstitium and in glomeruli (G). **b** TUNEL-positive nuclei could not be detected in normal rat kidney (fig. 2b). Scale bar = 50 µm.

Fig. 1. Representative photomicrographs of the renal cortex showing P2 receptor expression on cyst cells. Scale bar = 50 µm. G = Glomerulus. **a** $P2Y_1$ receptor immunoreactivity in the cytoplasm of a subpopulation of cyst-lining cells (arrow) and in glomerular (G) mesangial cells (shown previously in normal rats [Turner et al., 2003; Bailey et al., 2004]). **b** $P2Y_2$ receptor immunoreactivity in the cytoplasm of cyst-lining cells (arrow); smaller cysts show little or no receptor expression. **c** Dense $P2Y_4$ receptor immunoreactivity on cells lining small cysts (black arrow) and several immunopositive cells in larger cysts (white arrow). **d** $P2Y_6$ immunoreactivity in the cytoplasm of many cyst-lining cells of both large and small cysts (arrow). Note the granular pattern of staining. **e** $P2X_5$ receptor immunoreactivity seen mainly on lining cells of small cysts and some larger cysts (arrows). **f** $P2X_7$ receptor immunoreactivity on cyst-lining cells (arrow).

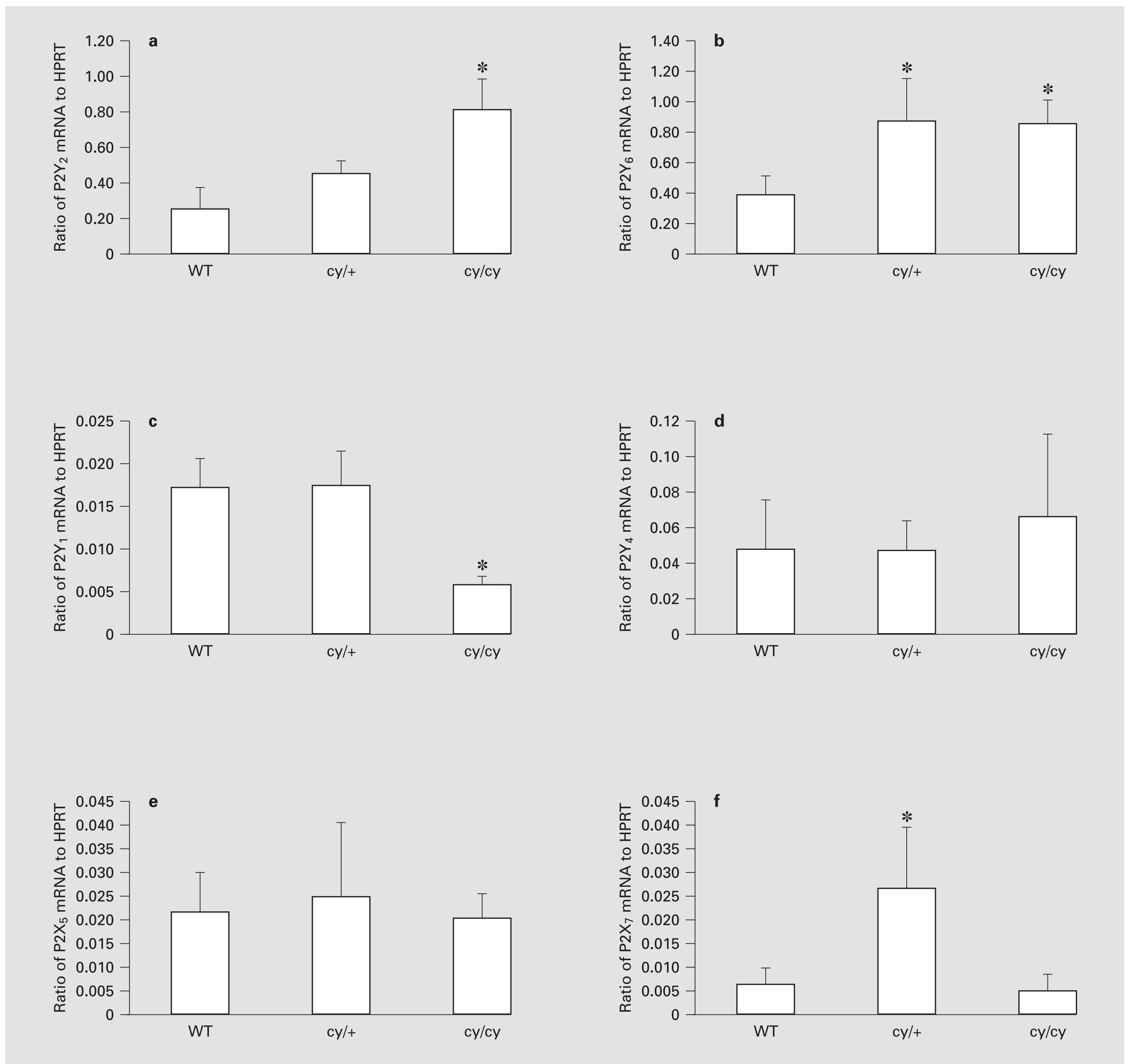

Fig. 4. Relative abundance of P2 receptor mRNA expressed in arbitrary units as a ratio to that of the 'house-keeping' gene HPRT. WT = Wild type; cy/+ = heterozygote for cysts; cy/cy = homozygote for cysts (* $p < 0.001$). Bars represent means ± SD. **a** $P2Y_2$. **b** $P2Y_6$. **c** $P2Y_1$. **d** $P2Y_4$. **e** $P2X_5$. **f** $P2X_7$.

changed in the cy/+ heterozygotes, although there was a significant decrease of 67% ($p < 0.001$) in the cy/cy homozygote renal tissue (fig. 4c). However, there was no significant increase in either $P2Y_4$ (fig. 4d) or $P2X_5$ (fig. 4e) receptor mRNAs when compared with control, although there was a small and non-significant increase of 39% in $P2Y_4$ receptor mRNA in the cy/cy group. $P2X_7$ receptor mRNA was increased by 330% ($p < 0.001$) in the cy/+ genotype, but there was no significant change in cy/cy kidney tissue (fig. 4f).

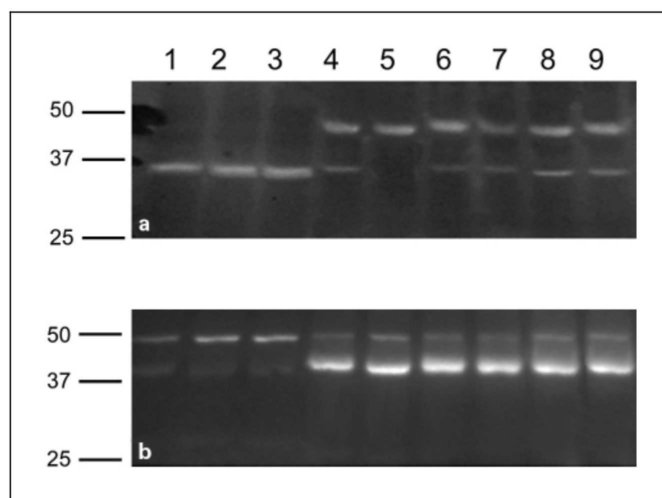

Fig. 5. Sample immunoblots using polyclonal antibody to $P2Y_2$ and $P2Y_6$ receptor protein: lanes 1–3 are whole-kidney lysates extracted from wild-type littermates, lanes 4–6 are whole-kidney lysates extracted from heterozygote cy/+ Han:SPRD rat kidney, and lanes 7–9 are whole-kidney lysates extracted from homozygote cy/cy Han:SPRD rat kidney. Sample immunoblot probed with $P2Y_2$ receptor antibody showing bands of the expected size at ~47 kDa with both cy/+ and cy/cy rat kidney protein, but not in control. In control tissue, only a smaller, ~36-kDa band was detected (also weakly in cy/+ and cy/cy samples). Sample immunoblot probed with $P2Y_6$ receptor antibody showing an increase in signal for the expected ~40-kDa band in both cy/+ and cy/cy rat kidney protein extracts compared with wild-type littermates. A larger ~50-kDa band was also detected in control samples, but less strongly in cy/+ and cy/cy samples.

Immunoblotting

In view of the large increases in $P2Y_2$ and $P2Y_6$ receptor mRNA in cystic kidney tissue and since increases in protein could not be quantified by immunohistochemistry, we carried out immunoblots with receptor antibodies on 28-day-old cy/+ and cy/cy Han:SPRD rat kidney. $P2Y_2$ receptor antibody produced clear bands of the expected size (47 kDa) in both cy/+ and cy/cy rats, but could not be detected in wild-type controls (fig. 5a). A second band of approximately 36 kDa was detected in control rats, but was only very weakly detectable in cystic rats. $P2Y_6$ receptor antibody produced a clear band at the expected size of 40 kDa. An increase in $P2Y_6$ receptor protein (40 kDa) could be detected in both cy/+ and cy/cy kidney tissue compared with control (fig. 5b), which matches the RT-PCR data. A second band of approximately 50 kDa was also detected in control rats, but was seen only very faintly in cystic rat whole kidney extract.

Discussion

Emerging data suggest a complex pattern of P2 receptor expression in many types of epithelia, including that of the kidney, in which evidence for both basolateral and apical cell membrane expression of P2 receptors has been reported [Bailey et al., 2000b; Deetjen et al., 2000; Kishore et al., 2000; Bailey et al., 2001; Turner et al., 2003]. ATP in tubular fluid, acting via P2 receptors, is postulated to play a role in controlling renal epithelial cell function [McCoy et al., 1999; Cuffe et al., 2000; Schwiebert and Kishore, 2001], and disturbances in ATP signaling might contribute to some forms of renal tubular dysfunction. In a previous study by Schwiebert et al. [2002], P2 receptor mRNA transcripts were identified in monolayer cultures of human ADPKD and *cpk* mouse kidney cells. In the present study, we have used immunohistochemistry and real-time PCR to explore P2 receptor subtype expression and distribution in cystic tissue from the Han:SPRD (cy/+) rat model of ADPKD.

The Han:SPRD rat is a well-characterized model of ADPKD in which initially most cysts derive from the proximal tubule [Kaspareit-Rittinghausen et al., 1990]. However, unlike ADPKD, a reversal of tubular cell polarity does not seem to be a feature [Schafer et al., 1994; Obermuller et al., 1997]. Several P2X and P2Y receptor proteins were detected on epithelial cells that line renal cysts in the Han:SPRD rat model; we also found elevated mRNA levels for the $P2Y_2$ and $P2Y_6$ receptors compared with controls, as well as increased amounts of whole-kidney receptor protein. We might expect $P2Y_6$ receptor expression in proximal-tubule-derived cysts, since previous studies provide functional and molecular evidence for $P2Y_6$ receptor expression in the proximal convoluted tubule [Bailey et al., 2001], and its protein has also been detected in the cytoplasm [Bailey et al., 2004] and basolateral membrane [Leipziger, pers. commun.] of this nephron segment. However, although proximal tubule cells express $P2Y_2$ receptor mRNA [Bailey et al., 2000b], the protein has not been detected immunohistologically in this segment [Turner et al., 2003], or only at a very low level [Bailey et al., 2004]. Functionally, the ATP-sensitive $P2Y_2$ receptor stimulates calcium-activated Cl^- secretion and inhibits Na^+ absorption in the mouse cortical collect-

ing duct [Cuffe et al., 2000; Deetjen et al., 2000; Lehrmann et al., 2002], and rabbit distal convoluted tubule [Rubera et al., 2000]. Moreover, the UDP-sensitive $P2Y_6$ receptor can stimulate Cl^- secretion via both calcium-sensitive Cl^- channels and cAMP-regulated CFTR (cystic fibrosis transmembrane conductance regulator) [Kottgen et al., 2003]. Activation of either $P2Y_2$ and/or $P2Y_6$ receptors could therefore elevate solute concentrations in cyst fluid by increasing Na^+ and Cl^- content, which would then osmotically drive fluid secretion into cysts and so increase their size. In fact, stimulation of human ADPKD primary cultures with a cocktail of P2Y receptor agonists (ATP, UTP and UDP) results in elevated levels of intracellular calcium and Cl^- secretion [Schwiebert et al., 2002]. Since a cyst is an enclosed environment, ATP and its metabolites could be present for prolonged periods, potentially over-stimulating apical P2 receptors and/or disturbing any physiological balance in coordinated activity. Moreover, increased fluid secretion might also cause additional stretch-induced release of ATP, also activating basolateral receptors, and thus a positive feedback loop.

In the present study, $P2Y_1$ receptors were detected mainly in the cytoplasm of cells lining rat polycystic kidney cysts, although in our previous report in the normal SPRD rat this receptor was detected on the apical membrane of the rat proximal straight tubule [Turner et al., 2003]. Though the staining pattern appeared to be altered from that of normal kidneys (more cytoplasmic), $P2Y_1$ receptor mRNA levels in cy/+ animals remained unchanged, and levels were actually decreased in the more severely affected cy/cy animals. In a recent report, the $P2Y_1$ receptor has been identified in the basal layer of both adult and foetal epidermis in association with proliferating cells [Greig et al., 2003a, 2003b]. Of perhaps more relevance to renal cells, an ATP/ADP-sensitive P2Y receptor has been reported to stimulate renal mesangial cell proliferation [Vonend et al., 2003], although other studies of mesangial cells suggest a UTP-triggered response is more compatible with either $P2Y_2$ or $P2Y_4$ receptor stimulation [Schulze-Lohoff et al., 1992; Harada et al., 2000]. In the present study, $P2Y_4$ receptor protein was clearly evident in cells lining small cysts, but larger cysts revealed a more scattered pattern of expression. Despite the altered tissue architecture, levels of $P2Y_4$ receptor mRNA did not change with the cystic genotype. In normal rat kidney, $P2Y_4$ receptor protein was found on the basolateral membrane in cells of the proximal convoluted tubule [Turner et al., 2003]. Therefore, expression on small and predominantly proximal tubule-derived cysts may not indicate a pattern greatly different from that in normal rat kidney. Histological examination of the Han:SPRD (cy/+) renal tissue revealed that although P2 receptors were expressed on the cystic epithelium, not every cyst examined was immunopositive for a given receptor.

$P2X_5$ receptors were also detected in rat cystic epithelium, although this receptor was found on epithelia of predominantly smaller cysts, and levels of $P2X_5$ receptor mRNA remained unchanged, regardless of the cystic genotype. Normally, this receptor is expressed apically on cells of the proximal straight tubule, and principal cells of the collecting duct [Turner et al., 2003]. $P2X_5$ receptors have been linked to differentiation of skeletal muscle cells and cultured keratinocytes [Ryten et al., 2002; Greig et al., 2003b]; although cystic epithelia often appear not to be fully differentiated, lacking the apical microvilli normally seen in mature renal epithelium.

$P2X_7$ receptor expression is barely detectable in normal renal epithelium [Turner et al., 2003]; however, we found dense $P2X_7$ receptor expression in both small and large cysts, and increased mRNA expression in the cy/+ genotype. Increased $P2X_7$ receptor protein and mRNA have also been found in the *cpk* mouse model of PKD [Hillman et al., 2002], and a recent study of diabetic and hypertensive glomerular injury in the rat has demonstrated enhanced $P2X_7$ receptor expression [Vonend et al., 2004]. Normally, activation of P2X receptors by nucleotides leads to formation of a non-selective cation-permeable channel and an increase in intracellular calcium. In addition to this effect, there is growing evidence to suggest that $P2X_7$ receptor activation is part of the normal inflammatory response via its interactions with various cytokines and as a mediator of cell apoptosis [Harada et al., 2000; Verhoef et al., 2003]. The ability of extracellular ATP to trigger apoptosis via the $P2X_7$ receptor has been reported in a number of different cell types, including thymocytes [Zheng et al., 1991], dendritic cells [Coutinho-Silva et al., 1999] and mesangial cells [Harada et al., 2000]. In HEK-293 cells, an embryonic kidney cell line, striking membrane blebbing and micro-vesiculation have been observed within seconds to minutes of receptor activation, a phenomenon in which large membrane-bound vesicles are extruded rapidly from the cell surface as they undergo apoptosis [Wilson et al., 2002]. Increased caspase activity and elevated Bcl-2 expression (B-cell lymphoma-2), both of which are involved in the apoptotic pathway as pro- and anti-apoptotic proteins, respectively, have been found in the Han:SPRD rat kidney [Ecder et al., 2002] and in a mouse model of PKD [Ali et al., 2000]; moreover, over-expression of Bcl-2 prevents cyst formation by Madin-Darby canine kidney cells in collagen cul-

Table 2. Summary of results of P2 receptor expression patterns in the Han:SPRD cy/+ rat compared with the findings in our earlier studies of P2 receptor expression in the kidney of normal Sprague-Dawley (SPRD) rats [Turner et al., 2003; Bailey et al., 2004]

P2 subtype	P2 nephron location in normal SPRD rats	P2 location in the Han:SPRD cy/+ rat cystic epithelia	P2 *mRNA* and *protein* expression in the Han:SPRD rat
$P2Y_1$	Vascular SMC; glomerular MC; peritubular fibroblasts, PT S3 segment apical membrane	Cytoplasmic and membrane staining	Levels of *mRNA* remained unchanged with cy/+ and decreased by 67% with cy/cy animals
$P2Y_2$	Glomerular PD; tAL, TAL, CD (intercalated cells)	Cytoplasmic and membrane staining	Levels of *mRNA* increased by 78% with cy/+ and 224% with cy/cy animals; *protein* (47 kDa) was low or undetectable in control and elevated with cy/+ and cy/cy animals
$P2Y_4$	PCT basolateral membrane	Mainly cytoplasmic staining	Levels of *mRNA* remained unchanged
$P2Y_6$	PT	Cytoplasmic staining with a granular pattern	Levels of *mRNA* increased by 125% with cy/+ and 120% in cy/cy animals; *protein* (40 kDa) was increased with cy/+ and cy/cy animals
$P2X_5$	PT S3 segment (apical), medullary CD (principal cells), cortical CD (minimal)	Mainly cytoplasmic staining	Levels of *mRNA* remained unchanged
$P2X_7$	Low level expression in some glomeruli	Mainly cytoplasmic staining	Levels of *mRNA* increased by 330% with cy/+ but were unchanged with cy/cy animals

CD = Collecting duct; MC = mesangial cells; PCT = proximal convoluted tubule; PD = podocytes; PT = proximal tubule; SMC = smooth muscle cells; tAL = thin ascending limb; TAL = thick ascending limb.

ture [Lin et al., 1999]. Thus, an imbalance between such factors may determine the level of apoptosis, which might in turn be related to cyst formation. Increased numbers of apoptotic cells have also been reported in human polycystic kidney when compared with normal tissue [Woo, 1995], although more usually in the interstitium between the cysts [Hillman et al., 2002]. However, recent studies in the Han:SPRD rat have reported epithelial cyst wall [Ecder et al., 2002; Hocher et al., 2003] and glomerular [Hocher et al., 2003] apoptosis, as we observed. These various studies suggest that apoptosis, normally a tightly regulated mechanism for maintaining normal cell turnover, tissue repair and re-modeling [Savill et al., 1996], is increased in some renal cystic diseases, perhaps as a mechanism for deleting damaged cells and promoting increased cell turnover. Interestingly, the distribution of apoptotic cells we found in epithelial cells lining the smaller cysts in cy/+ animals was not dissimilar to the pattern of expression we observed for the $P2X_7$ receptor. Also in this context, as well as increased expression of the putative pro-apoptotic $P2X_7$ receptor, expression of the $P2Y_2$ and $P2Y_6$ receptors was increased in cystic tissue. In addition to their already discussed potential effects on fluid and electrolyte transport, these receptors can stimulate epithelial cell proliferation, which in the case of the $P2Y_6$ receptor appears to be inflammatory cytokine-dependent [Schafer et al., 2003] and anti-apoptotic [Kim et al., 2003].

In summary, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2X_5$ and $P2X_7$ receptors were detected on the epithelial cells lining renal cysts in the Han:SPRD cy/+ rat model of ADPKD. Compared with our earlier study in normal SPRD rats (table 2), cell distribution of all the P2 receptors examined appeared to be more diffuse (except for $P2Y_6$, which was strikingly granular) and cytoplasmic, possibly reflecting a more primitive and less-differentiated state of cyst-lining cells. $P2Y_2$ receptor mRNA was significantly increased with the cy/cy genotype, and $P2Y_6$ receptor mRNA was elevated with both cy/+ and cy/cy genotypes. Furthermore, $P2Y_2$ and $P2Y_6$ receptor proteins were readily detectable in cystic rat kidneys, whereas the former could not be detected in control kidneys and the latter was expressed only at a low level.

Finally, nucleotides present in cyst lumen fluid could activate G-protein-coupled P2Y receptors, potentially causing detrimental cyst expansion due to increased and osmotically driven fluid secretion. From evidence in other tissues, $P2Y_1$, $P2Y_2$, $P2Y_6$ and $P2X_5$ receptors might also be involved in cell proliferation and differentiation (although only the expression levels of $P2Y_2$ and $P2Y_6$ increased significantly), and the $P2X_7$ receptor (the expression of which also increased) in renal cell death by apoptosis, all mechanisms potentially relevant to cyst growth. Further studies will be necessary to determine if these changes precede or follow the development of renal cysts, and thus a pathophysiological role for P2 receptors

in ADPKD and other renal cystic diseases. If a functional link can be established, therapies based on inhibiting P2 receptor actions might eventually have therapeutic use in ameliorating cyst progression.

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