THE POSSIBLE ROLE OF P2 RECEPTORS IN THE PATHOGENESIS OF RENAL CYSTIC DISEASE AND OTHER FORMS OF RENAL CELL DYSFUNCTION

A thesis submitted by

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Abstract

Extracellular nucleotides, such as ATP and UTP, are now widely accepted as regulators of diverse cellular functions including regulation of ion transport in epithelial tissues. They signal via multiple P2 receptor subtypes. Current evidence suggests that P2 receptors are expressed in kidney epithelia and that they may influence transport of ions and fluid. Consequently, alterations in the physiology of P2 receptor signalling may be involved in the development of renal diseases such as autosomal dominant polycystic kidney disease (ADPKD), an inherited kidney disease in which abnormal cell physiology is thought to contribute to disease progression. In this thesis, the expression and role of P2 receptors in the kidney and their possible influence on ADPKD cyst growth have been investigated.

Several P2 receptor subtypes were identified on specific cells of the normal rat kidney. Mostly P2Y receptors were detected in the glomerulus and tubule epithelium, but also $P2X_5$ receptors were found on collecting duct cells and $P2X_4$ and $P2X_6$ receptors were expressed at a low level throughout the nephron. The $P2Y_{1,2,4,6}$ and the $P2X_5$ and $P2X_7$ subtypes were detected on the cyst lining cells of (cy/+) rat polycystic kidneys, and $P2Y_{2,6}$ and $P2X_7$ receptor mRNA was increased when compared to normal rat kidneys. $P2X_7$ receptor expression was also increased in rodent models of glomerular injury. *In vitro*, inhibition of mostly P2Y receptor subtypes with antagonists or removal of ATP from the growth medium significantly reduced the growth of MDCK microcysts.

This work has extended the current knowledge of P2 receptor expression in healthy and diseased kidney tissue. The pattern of P2 receptor expression in renal cysts indicate that these receptors may play a role in cyst formation and progression. Their influence is most likely via ATP-stimulated chloride secretion and accelerated proliferation of epithelial cells; both of which are key factors that affect ADPKD cyst growth. These findings support the hypothesis for a role of P2 receptors in renal cyst growth and enlargement. However, these initial observations require more study, since there are many P2 receptor subtypes, one of which might prove to be a therapeutic target to limit cyst growth and preserve renal function.

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List of abbreviations used in this thesis

α,β-MeATP, α,β-methylene adenosine triphosphate

AC, adenylate cyclase

ADP, adenosine 5' –diphosphate

ADPβS, adenosine 5'-O-[2-thiodiphosphate]

ADPKD, autosomal dominant polycystic kidney disease

AQP, aquaporin

ARPKD, autosomal recessive polycystic kidney disease

ATP, adenosine 5' –triphosphate

ATPγS, adenosine 5'-O-[3-thiotriphosphate]

BBG, Coomassie brilliant blue G

Bcl-2, B-cell lymphoma-2

BzATP, 2',3'-O-(4-benzoyl)benzoyl -adenosine-triphosphate

cAMP, 3',5'-cyclic adenosine monophosphate

CCD, cortical collecting duct

CFTR, cystic fibrosis transmembrane conductance regulator

DAG, diacylglycerol

DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid

GBM, glomerular basement membrane

GFR, glomerular filtration rate

IFN-γ, interferon-γ

IL-1β, interleukin-1β

IP_3, inositol-1,4,5-triphosphate

I_{sc}, short circuit current

IMCD, inner medullary collecting duct

LPS, lipopolysaccharide

MAP, mitogen-activated protein kinase

MDCK, Madin Darby canine kidney

2-MeSATP, 2-Methylthio ATP

MRS 2179, 2'-deoxy-N^6-methyladenosine-3',5'-bisphosphate

OMCD, outer medullary collecting duct

PAS, periodic acid Schiff

PC-1, polycystin-1

PC-2, polycystin-2

PCT, proximal convoluted tubule

PIT, 2'2' pyridilisatogen tosylate

PLC, phospholipase C

PKA, protein kinase A

PPADS, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonate

PVDF, poly(vinylidenedifluoride)

RB2, reactive blue 2

RT-PCR, reverse transcription polymerase chain reaction

SAPK, stress activated protein kinase

SDS-PAGE, Sodium dodecylsulphate -Polyacrylamide gel electrophoresis

STZ, streptozotocin

tAL, thin ascending limb (of Henlē)

TAL, thick ascending limb (of Henlē)

TGF, tubulo-glomerular feedback

TNF-α, tumour necrosis factor-α

TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl)- adenosine-triphosphate

UDP, uridine diphosphate

UTP, uridine triphosphate

Chapter 1

Introduction

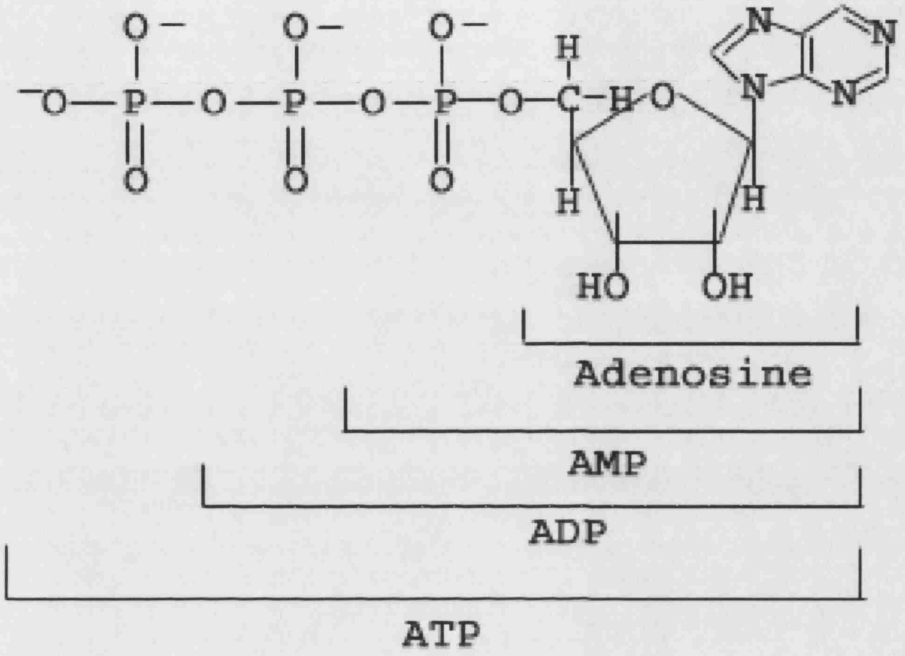
1.1 The discovery of ATP

In 1929 the German chemist Karl Lohmann, whilst studying the properties of frog muscle extract, discovered a compound with a high-energy phosphate bond. That compound was adenosine triphosphate (ATP) (Lohmann, 1929). ATP belongs to the purine family and is made up of a heterocyclic nitrogenous base (adenine), a 5' carbon sugar (ribose) and a chain of three ionised groups (phosphates) bound to the ribose (Figure 1.1). In 1937, Kalckar demonstrated, using cell-free extracts of kidney cortex, that ATP was generated during the breakdown of glucose to lactic acid (anaerobic glycolysis), within the citric acid cycle (Kalckar, 1937). Not long after this, Lipmann suggested that ATP is the main carrier of chemical energy in the cell and coined the phrase "energy-rich phosphate bonds"(Lipmann, 1941). He proposed the ATP cycle that describes the process of energy release from the hydrolysis of ATP to adenosine 5'-diphosphate (ADP) and inorganic phosphate (Pi) and the subsequent conversion back to ATP by oxidative phosphorylation. ATP is essential at many levels of cellular metabolism, providing the energy required for biosynthesis and anabolic pathways. As a result of these studies, ATP was soon thought of as the most fundamental of all biomolecules and the universal source of readily available chemical energy for all living cells.

1.2 The discovery of nucleotide receptors

The widespread abundance, multifunctional and intracellular nature of ATP made it difficult for many researchers to except it has an extracellular role. It was Drury and Szent-Györgyi who first recognised the potent extracellular

Figure 1. 1 **Molecular structure of ATP, ADP, adenosine 5' monophosphate (AMP) and adenosine**



Denoted (from left to right) three phosphate groups, a ribose sugar moiety, and an adenine structure showing how ATP can be cleaved to form ADP, AMP or adenosine.

actions of purine nucleotides, namely adenosine and ATP, on mammalian heart (Drury & Szent-Györgyi, 1929). These investigators measured the heart rate of guinea pigs during intravenous injections of adenosine and adenosine 5'-monophosphate (AMP) and found they have pronounced biological effects including slowing of heart rate, arterial dilatation, and inhibition of intestinal smooth muscle contraction. In 1934, Gillespie described the ability of adenine compounds to cause vasodilatation and hypotension, whilst ATP caused an increase in blood pressure in the rabbit and the cat that was never observed with adenosine or AMP (Gillespie, 1934). This was the first indication of different actions of adenosine and ATP and therefore the first indication of the possible existence of different purine receptors. Most of the early investigations into the effects of adenosine and ATP, concentrated on the heart and vasculature (Emmelin & Feldburg, 1948;Moir & Downs, 1972). In addition to the cardiovascular system, diverse biological responses to extracellular purines and pyrimidines have been documented in many tissues, for example in kidney (Harvey, 1964), urinary bladder (Burnstock *et al.*, 1972), guinea pig trachea and taenia coli (Axelsson *et al.*, 1965;Coleman, 1976;Farmer & Farrar, 1976), and human skin (Coutts *et al.*, 1981)

A major line of research is now concerned with purines as neurotransmitters. Holton and Holton first documented ATP release from sensory nerves in 1953, when they suggested ATP and its breakdown products produced vasodilatation via stimulation of sensory nerves supplying the rabbit ear (Holton & Holton, 1953). It was nearly twenty years later that Burnstock published a review referring to a third component of the autonomic nervous system that was neither adrenergic (releasing noradrenaline) nor cholinergic

(releasing acetylcholine) (Burnstock, 1972). He proposed that the principle substance released by these nerves was a purine nucleotide and thus called these nerves "purinergic". It is now recognised that ATP acts as a neurotransmitter, cotransmitter and neuromodulator in many systems (Burnstock, 1999).

1.3 The proposed mode of action of nucleotide receptors

Purines and pyrimidines mediate their effects by interactions with distinct cell-surface receptors. Early purinoceptor nomenclature, before cloning methods were established, was based on the pharmacological profiles of isolated tissue preparations and by receptor binding studies (using agonists and antagonists). In 1978, Burnstock proposed a basis for distinguishing two types of purinergic receptors and designated them P1 and P2 receptors (Burnstock, 1978). P1 receptors had the highest affinity for adenosine followed by AMP, ADP and ATP, and P2 receptors had the highest affinity for ATP, then ADP, AMP and adenosine (Farmer & Farrar, 1976). Methylxanthines were reported to be potent antagonists of P1 receptors (Curnish *et al.*, 1972), whereas quinidine compounds (Burnstock *et al.*, 1972), high concentrations of 2-substituted imidazoline compounds (Rikimaru *et al.*, 1971), and 2'2' pyridilisatogen tosylate (PIT) (Spedding *et al.*, 1975) antagonised P2 receptors. Tissue distribution studies revealed some broad trends, P1 receptors predominated in the cardiovascular system, in the trachea and in brain while P2 receptors were predominantly found in the gastrointestinal and urogenital system (Burnstock, 1978).

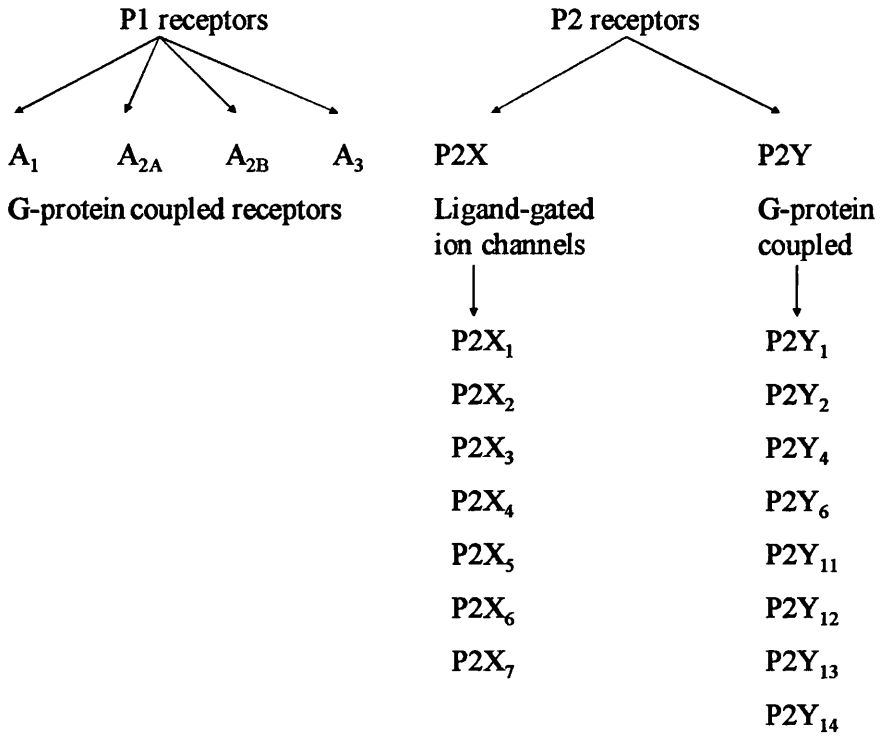
It was soon apparent that this classification of purinergic receptors could be extended. Firstly, two subclasses of P1 receptor were identified in cultured brain cells (van Calker *et al.*, 1979) and later termed "A1" and "A2" in the

nomenclature. Secondly, P2 receptors showed different sensitivities to the blocking effects of apamin, from honey-bee venom, (Shuba & Vladimirova, 1980) and the purinoceptor on platelets was sensitive to ADP (Colman *et al.*, 1980) whilst mast cell receptors were sensitive to ATP but not ADP (Dahlquist & Diamant, 1970). In 1985, Burnstock and Kennedy suggested the P2 class of receptor could be further subdivided based on pharmacological studies of vascular tissues. They suggested that the population mediating contraction with a high affinity for α,β-methylene ATP should be named P_{2X}, and the population mediating relaxation with a high affinity for 2-methylthio-ATP should be named P_{2Y} (Burnstock & Kennedy, 1985). In 1990, Kennedy suggested that both P1 and P2 purinoceptor classes could be further subdivided based on biochemical, ligand binding and pharmacological studies. P1 receptors could be divided into A1, A2 and there was evidence to support an A3 subtype. In addition to the P_{2X} and P_{2Y} receptors, the use of ATP analogues and the activity of antagonists at P2 receptors led to the definition of P_{2X}, P_{2Y}, P_{2T}, P_{2U}, and P_{2Z} receptors (Cusack & Hourani, 1990;Kennedy, 1990). Kennedy also suggested purinoceptor classification on the basis of transduction, following electrophysiological experimentation on smooth muscle cells and neurones. He proposed that the P_{2X} receptor is an ion channel and the P_{2Y} receptor a metabotropic receptor (Kennedy, 1990).

The first P_{2Y} receptor was cloned from chick brain in 1993 and based on sequence similarities it was classified as a G-protein coupled receptor (Webb *et al.*, 1993). When expressed in *Xenopus* oocytes this receptor produced a slowly developing ATP-stimulated calcium-activated inward current. This receptor was subsequently designated P_{2Y1}. A second P_{2Y} receptor, P_{2Y2}, was cloned from a

murine neuroblastoma cell line and it was activated by adenine and uridine nucleotides (Lustig *et al.*, 1993). P_{2Y1} and P_{2Y2} receptors closely corresponded to the P_{2Y} receptor and P_{2U} receptor previously characterised by pharmacological criteria (Communi *et al.*, 2000). The structural similarities between chick P_{2Y1} and murine P_{2Y2} receptor proteins provided the requisite tools for a wave of homology screening. The emergence of genetic sequence data allowed detailed studies of the properties of cloned receptors expressed in cell lines. The determination of receptor sequences also enabled the development of selective antibodies against specific receptor subtypes. As structural information became available, it was apparent that receptor sequences of P2 receptor families were related but distinct. The previously described nomenclature system was confusing and contradictory. Therefore, following an International Union of Pharmacology (IUPHAR) committee on receptor nomenclature, it was recommended that the new system of classification of purinoceptors proposed by Abbracchio and Burnstock (1994) should be adopted, namely that a P_{2X} family should encompass ATP-activated, ligand-gated ion channels, a P_{2Y} family should comprise of metabotropic ATP receptors and also a P_{2Z} family for the non-selective pore forming receptor (now known to be $P2X_7$) (Figure 1.2). The subcommittee also recommended the previously widely used term "P2 purinoceptor" would be changed to "P2 receptor", and that "$P2X_1$" replaces '$P_{2X}1$". For the continuation of this thesis, the currently accepted version of nomenclature for P2 receptors will be used. At this time there are seven homomeric P2X receptors (ligand-gated ion channels) and eight mammalian G-protein coupled P2Y receptors (Ralevic & Burnstock, 1998;Khakh *et al.*, 2001;King & Burnstock, 2002;Abbracchio *et al.*, 2003).

Figure 1. 2 **Current nomenclature and classification of P2 receptors according to the IUPHAR subcommittee. For completeness, P1 receptor classification has been included in this diagram**

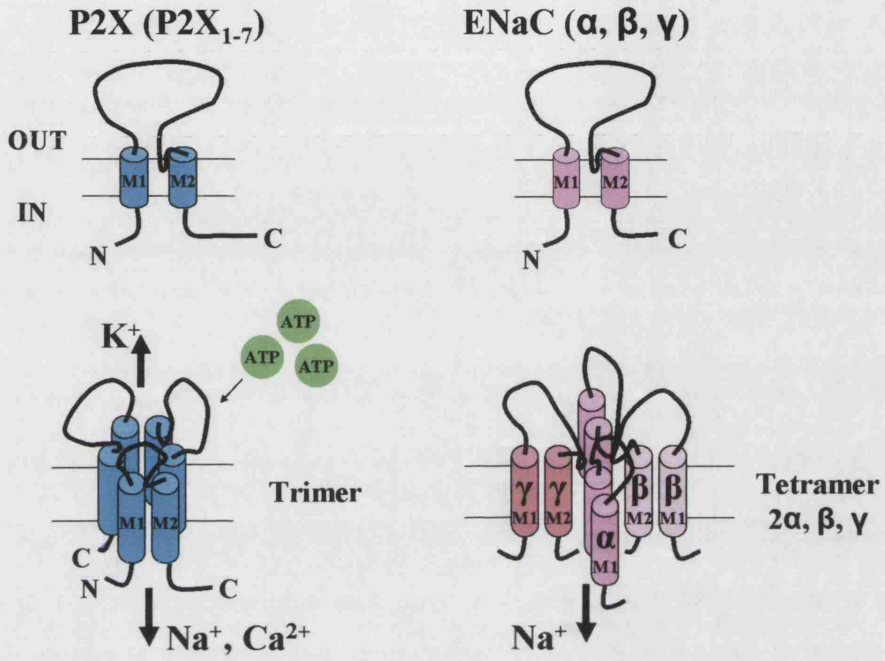


1.3.1 The structure and function of P2X receptors

P2X receptor channels are highly permeable to Ca^{2+}, but also to other cations such as Na^+ and K^+ (Liu & Adams, 2001). This Ca^{2+} permeability is important because activity-dependent Ca^{2+} signals mediated by these receptors are likely to affect downstream intracellular signalling (Rogers *et al.*, 1997). The $P2X_{1-7}$ proteins have 384 ($P2X_4$) to 595 ($P2X_7$) amino acid residues in each subunit and share 39.2 to 55.4% sequence identity at the amino acid level. Comparison of the amino acid sequences of $P2X_{1-7}$ receptors reveals 75 conserved residues (see Appendix 1), with most sequence variation occurring in the COOH terminal regions (Newbolt *et al.*, 1998;North, 2002). Each receptor subunit is characterised by two membrane-spanning domains, a large extracellular loop with ten conserved cysteines, and short intracellular NH_2- and COOH termini (Brake *et al.*, 1994;Kennedy *et al.*, 1997;Newbolt *et al.*, 1998). The overall structure resembles that of the inwardly rectifying K^+ channel and epithelial Na^+ channels (Figure 1.3) (Valera *et al.*, 1994;Surprenant *et al.*, 1995). The $P2X_7$ subunit has a much longer COOH terminus and contains an additional hydrophobic domain that is sufficiently long to traverse the plasma membrane (Surprenant *et al.*, 1996). Membrane topology algorithms suggest an intracellular location, but there is no definitive evidence that places the $P2X_7$ COOH terminus inside or outside of the cell (North, 2002).

Functional expression studies have provided evidence for heteromultimerisation where at least two different subunits can contribute to the ion channel (Koshimizu *et al.*, 2002). Coexpression of P2X subunits in defolliculated *Xenopus* oocytes has revealed several functional heteromultimers, $P2X_{4/6}$ (Le *et al.*, 1998), $P2X_{1/5}$ (Le *et al.*, 1999), $P2X_{2/6}$ (King *et al.*, 2000),

Figure 1. 3 **Proposed membrane topology of a P2X receptor compared with the epithelial sodium channel (ENaC)**



Structurally, both P2X receptor and ENaC subunits have two membrane spanning domains (M1 and M2), a large extracellular loop and short COOH- and NH_2-termini. Expression analysis whereby a cDNA encoding 2 to 6 linked, radiolabelled P2X monomers were expressed in Xenopus oocytes revealed the existence of functional trimers (Nicke et al., 2003) requiring three molecules of ATP to activate the channel (Bean, 1990). However, ENaC exists as a heterotetramer composed of two α subunits separated by β and γ subunits (Firsov et al., 1998).

$P2X_{2/3}$ (Liu *et al.*, 2001) and $P2X_{1/2}$ (Brown *et al.*, 2002), often with a novel pharmacological phenotype comprising aspects of both subunits. Torres and co-workers have determined which pairs of subunits are potentially able to co-assemble by co-immunoprecipitation of epitope-tagged subunits after expression in human embryonic kidney (HEK 293) cells (Torres *et al.*, 1999)(Table 1.1). With the exception of $P2X_7$, all of the subunits were able to co-assemble, but $P2X_3$ and $P2X_4$ presented a more restricted pattern of co-assembly. As previously stated, all P2X receptors are cation selective channels permeable to Na^+, K^+ and Ca^{2+} (Liu & Adams, 2001). Although much valuable information can be derived from studies of populations of cells in culture, there are potential pitfalls associated with *in vitro* analysis, for example, interference from endogenously expressed P2 receptors, and the possibility that receptor expression may be altered by culture conditions. A more useful technique for studying P2 receptor function is the *Xenopus* oocytes expression system first demonstrated by Gurdon *et al* (Gurdon *et al.*, 1971). The large size (1 mm), high efficiency for expressing foreign membrane proteins and lack of endogenous P2 receptors make oocytes ideal for pharmacological studies with minimal interference from extraneous factors. This system is now widely used and has proved an important tool for studying structure and function of many recombinant receptors and ion channels. Expression of P2X receptors in oocytes and electrophysiological recordings after application of agonists and antagonists allows channel activity to be recorded. Exposure of oocytes expressing heterologous P2X receptors to a brief application of an agonist results in an increase in intracellular Ca^{2+} and Na^+ and an efflux of K^+. Considerable effort has been devoted to defining the ligand recognition characteristics of each P2X receptor subtype using agonists and

Table 1. 1 **Heteromultimerisation capabilities of P2X receptor subunits**

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$
$P2X_1$	+	+	+	-	+	+	-
$P2X_2$		+	+	-	+	+	-
$P2X_3$			+	-	+	-	-
$P2X_4$				+	+	+	-
$P2X_5$					+	+	-
$P2X_6$					+	-	-
$P2X_7$							+

Pairs of P2X receptor subunits, carrying either one of two epitope tags were expressed in HEK293 cells. If the receptor subunits formed multimers, then those that precipitated with antibody to one epitope could be detected with an antibody to the second epitope. Potential multimers are indicated by '+' (Torres et al., 1999).

antagonists (Table 1.2). ATP activates all of the known P2X assemblies, but the potency of ATP can vary enormously depending on subunit composition (North, 2002). There are also differences in the desensitisation properties of P2X receptors. Desensitisation refers to the decline in current elicited by ATP in the continual presence of ATP. $P2X_1$ and $P2X_3$ are referred to as rapidly desensitising (approximately 300ms for $P2X_1$), whereas, $P2X_2$, $P2X_4$, and $P2X_5$ desensitise 100 -1000 times more slowly (Werner *et al.*, 1996;North, 2002). Several P2X subtype selective antagonists have been developed and these are largely based on analogues of either suramin or Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS). There is no pharmacological profile for homomeric $P2X_6$, since no recordable currents are evoked by ATP when this receptor is expressed in oocytes (North, 2002). P2X receptors are known as ionotropic receptors since they function with multiple subunits and form an ion channel that opens on binding of an extracellular ligand.

1.3.2 The structure and function of P2Y receptors

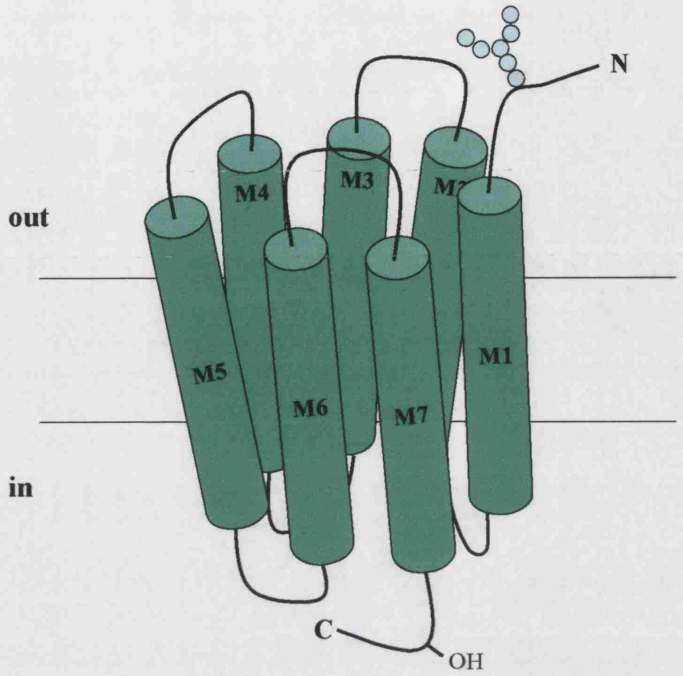
Eleven different P2Y receptors have been cloned so far and shown to act as receptors for nucleotides by expression in functional systems. The receptor proteins contain the typical features of G-protein coupled receptors including seven predicted hydrophobic transmembrane domains, with short extracellular NH_2- and intracellular COOH- terminals (Figure 1.4) (Abbracchio & Burnstock, 1998). There are eight mammalian subtypes $P2Y_{1,2,4,6,11,12,13,}$ and $_{14}$ (Lazarowski *et al.*, 2003), while $P2Y_3$, tp2y and $p2y_8$ are exclusively found in non-mammalian tissues (Webb *et al.*, 1996;Bogdanov *et al.*, 1997;Boyer *et al.*, 2000), however these may be species orthologues of mammalian P2Y receptors. The avian $P2Y_3$ receptor resembles the mammalian $P2Y_6$ receptor in amino acid sequence and

Table 1. 2 **Principal putative agonists and antagonists and desensitisation (Ds) rate of P2X receptors**

Receptor subtype	Agonists	Antagonists	Ds. rate	Reference
$P2X_1$	α,β-MeATP ATP 2-MeSATP BzATP	TNP-ATP Suramin PPADS RB2	Rapid	(Valera *et al.*, 1994;Wildman *et al.*, 2002)
$P2X_2$	2-MeSATP ATP ATPγS	RB2 PPADS	Very slow	(Brake *et al.*, 1994)
$P2X_3$	α,β-MeATP 2-MeSATP ATP	TNP-ATP PPADS RB2	Rapid	(Chen *et al.*, 1995;Seguela *et al.*, 1996)
$P2X_4$	BzATP ATP 2-MeSATP	TNP-ATP BBG	Slow	(Bo *et al.*, 1995)
$P2X_5$	ATP 2-MeSATP ATPγS	PPADS Suramin RB2	Very slow/ none	(Collo *et al.*, 1996;Wildman *et al.*, 2002)
$P2X_6$	ATP	No data	No data	(Collo *et al.*, 1996)
$P2X_7$	BzATP ATP 2-MeSATP	BBG PPADS TNP-ATP	none	(Surprenant *et al.*, 1996)

Abbreviations: α,β-MeATP, α,β-methylene adenosine triphosphate; ATPγS, adenosine 5'-O-[3-thiotriphosphate]; BBG, Coomassie brilliant blue G; BzATP, 2',3'-O-(4-benzoyl)benzoyl -adenosine-triphosphate; 2-MeSATP, 2-Methylthio ATP; RB2, reactive blue 2; TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl)-adenosine-triphosphate. (Table adapted from (Burnstock, 2004)

Figure 1. 4 **Proposed membrane topology of a P2Y receptor**



In common with other G-protein coupled receptors, P2Y receptors have seven putative membrane spanning domains (M1-7), each believed to constitute an α-helix, which are connected by three extracellular and three intracellular hydrophilic loops. At the short, extracellular NH_2 terminus is a putative glycosylation site (indicated by the 'Y' shaped structure in the diagram) which is conserved among the P2Y receptor family. The hydroxyl group on the intracellular COOH terminus represents a potential phosphorylation site. (Kennedy et al., 1997).

pharmacological properties (Webb *et al.*, 1996). There is also similarity between the rat $P2Y_4$ receptor and the turkey tp2y receptor as well as the p2y8 receptor expressed in the neural plate of *Xenopus* laevis (Boyer *et al.*, 2000). The non-sequential numbering of the P2Y receptor family is caused by the recognition that certain receptors had been wrongly identified as P2Y receptors. Until receptors are confirmed as P2Y receptors they are designated with lower case letters. The p2y7 receptor has subsequently been identified as a leukotriene B_4 receptor (Akbar *et al.*, 1996), and the p2y5, p2y9 and p2y10 receptors were found to be unresponsive to nucleotides (Li *et al.*, 1997;Rao *et al.*, 1999;Noguchi *et al.*, 2003). More recently, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ receptors were identified by homology cloning and confirmed as nucleotide receptors (Communi *et al.*, 1997;Hollopeter *et al.*, 2001;Zhang *et al.*, 2002). Recently, the UDP-glucose receptor has been included in the P2Y receptor family as $P2Y_{14}$ due to structural similarity to other known P2Y receptors (Abbracchio *et al.*, 2003).

The P2Y receptor family differs from other known G-protein coupled receptor families by having a high diversity in the amino acid sequences among its members. The human $P2Y_1$ receptor and the human $P2Y_{12}$ receptor share only 19% sequence identity, although similarities between species homologues of the same subtype are much higher (Sak & Webb, 2002). The human $P2Y_1$ receptor and the bovine $P2Y_1$ receptor share 96% amino acid sequence identity calculated using the ClustalW service at the EMBL European Bioinformatics Institute (Sak & Webb, 2002). A more detailed examination of the sequence similarity between subtypes reveals that there are several highly conserved motifs within the transmembrane regions (see Appendix 2) (Abbracchio & Burnstock, 1998;Von Kugelgen & Wetter, 2000). There is currently little evidence to

suggest that P2Y receptors form functional dimers or oligomers, although there is evidence for a $P2Y_1$/adenosine A_1 dimer. The resulting $A_1R/P2Y_1$ heteromeric complex portrayed a reduced A_1 receptor pharmacological profile with an enhanced response to the $P2Y_1$ agonist ADPβS (Yoshioka *et al.*, 2001).

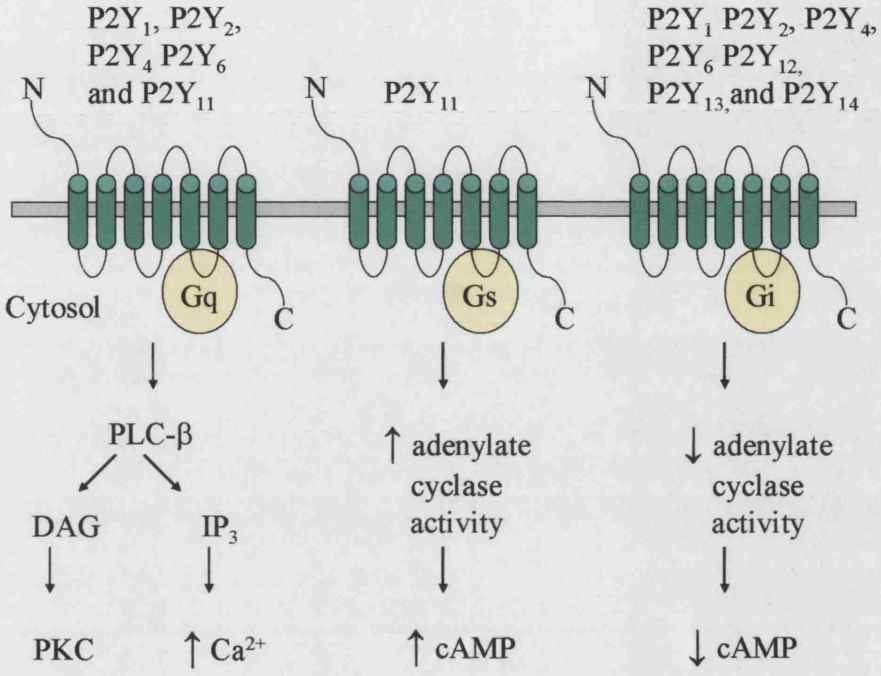
$P2Y_1$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ receptors are principally activated by adenine nucleotides, whereas $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors are activated by uracil nucleotides (Ralevic & Burnstock, 1998). The recently cloned $P2Y_{14}$ receptor specifically responds to UDP-glucose and related sugar nucleotides (Abbracchio *et al.*, 2003). In many cases, receptor subtypes that recognise the same physiologic agonist preferentially activate different signalling pathways (Table 1.3). Most of the work to date has emphasised linkage to different classes of G-proteins as the explanation for differences in intracellular signalling (Figure 1.5). In heterologous expression systems, recombinant $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors share a common signal transduction pathway involving coupling to heterotrimeric G_q protein which activates intracellular signalling cascades mainly the phospholipase C_β (PLC_β) isoform which, via 1,4,5-inositol triphosphate (IP_3), triggers a rise in intracellular Ca^{2+} from stores in the endoplasmic reticulum (Dubyak, 1991;Communi *et al.*, 2000). G- proteins consist of three subunits, α, β, and γ, and are located on the inner surface of the plasma membrane in association with G-protein coupled transmembrane receptors. Upon activation of the receptor, Gα exchanges GDP for GTP and dissociates from Gβ and Gγ to activate an effector molecule for example adenylate cyclase (AC) (Hollenberg, 1987). There are several different types of G protein but, in terms of P2Y receptor coupling, G_q activates phospholipase C (Filtz *et al.*, 1994) which generates the second messengers IP_3 and 1,2 -

Table 1. 3 **Properties of P2Y receptors**

Receptor Subtype	Principal natural ligand	G protein	Signal transduction	Reference
$P2Y_1$	ADP	G_q G_i	↑ PLC ↓ AC	(Filtz *et al.*, 1994;Boyer *et al.*, 1993)
$P2Y_2$	UTP=ATP	G_q or $G_{i/o}$	↑ PLC	(Nicholas *et al.*, 1996)
$P2Y_4$	UTP	G_q or $G_{i/o}$	↑ PLC	(Communi *et al.*, 1996a;Nicholas *et al.*, 1996)
$P2Y_6$	UDP	G_q or $G_{i/o}$	↑ PLC	(Communi *et al.*, 1996b;Nicholas *et al.*, 1996)
$P2Y_{11}$	ATP	G_q or G_s	↑ PLC ↑ AC	(Torres *et al.*, 2002;Qi *et al.*, 2001a)
$P2Y_{12}$	ADP	G_i	↓ AC	(Hollopeter *et al.*, 2001)
$P2Y_{13}$	ADP	G_i	↓ AC	(Communi *et al.*, 2001)
$P2Y_{14}$	UDP-glucose	$G_{i/o}$	↓ AC	(Abbracchio *et al.*, 2003)

Up arrows indicate an increase in phospholipase C (PLC) or adenylate cyclase (AC) activity, whereas down arrows signify a decrease in activity.

Figure 1. 5 **G-protein coupling and signalling pathways of P2Y receptors**



P2Y receptor coupling to Gq activates phospholipase C isoform β (PLC-β) which generates the second messengers 1,2-diacylglycerol (DAG) and Inositol 1,4,5- triphosphate (IP_3). DAG activates protein kinase C (PKC) which phosphorylates a variety of target proteins which, for example, control cell growth and differentiation (Toker, 1998). IP_3 releases Ca^{2+} from stores in the endoplasmic reticulum which can affect Ca^{2+} sensitive ion channels. P2Y receptor coupling to Gs activates adenylate cyclase, whereas Gi inhibits adenylate cyclase. Adenylate cyclase converts ATP to cAMP and pyrophosphate.

diacylglycerol (DAG), the latter activates protein kinase C which is involved in a multitude of physiological processes (Toker, 1998). G_s stimulates adenylate cyclase activity which catalyses the conversion of ATP to cAMP, and G_i inhibits adenylyl cyclase activity (Figure 1.5)(Von Kugelgen & Wetter, 2000). G_i and G_o are sensitive to pertussis toxin which ADP-ribosylates the α subunit preventing the G-protein/receptor interaction (Moss, 1987). $P2Y_1$ receptor activation can also directly modulate ion channel function, for example in rat cerebellar neurons, $P2Y_1$ activation leads to opening of an outwardly rectifying K^+ current via coupling of the β, and γ-subunits of the G protein to a K^+ channel (O'Grady *et al.*, 1996). $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors may also couple to pertussis toxin sensitive $G_{i/o}$ to liberate regulatory β and γ-subunits and modulate other ion channels (Communi *et al.*, 1996a). $P2Y_{11}$ receptors couple to G_s to raise intracellular cAMP levels (Torres *et al.*, 2002) and $P2Y_6$ receptors may also raise cAMP levels although it is unclear whether this is through direct coupling to G_s (Kottgen *et al.*, 2003).

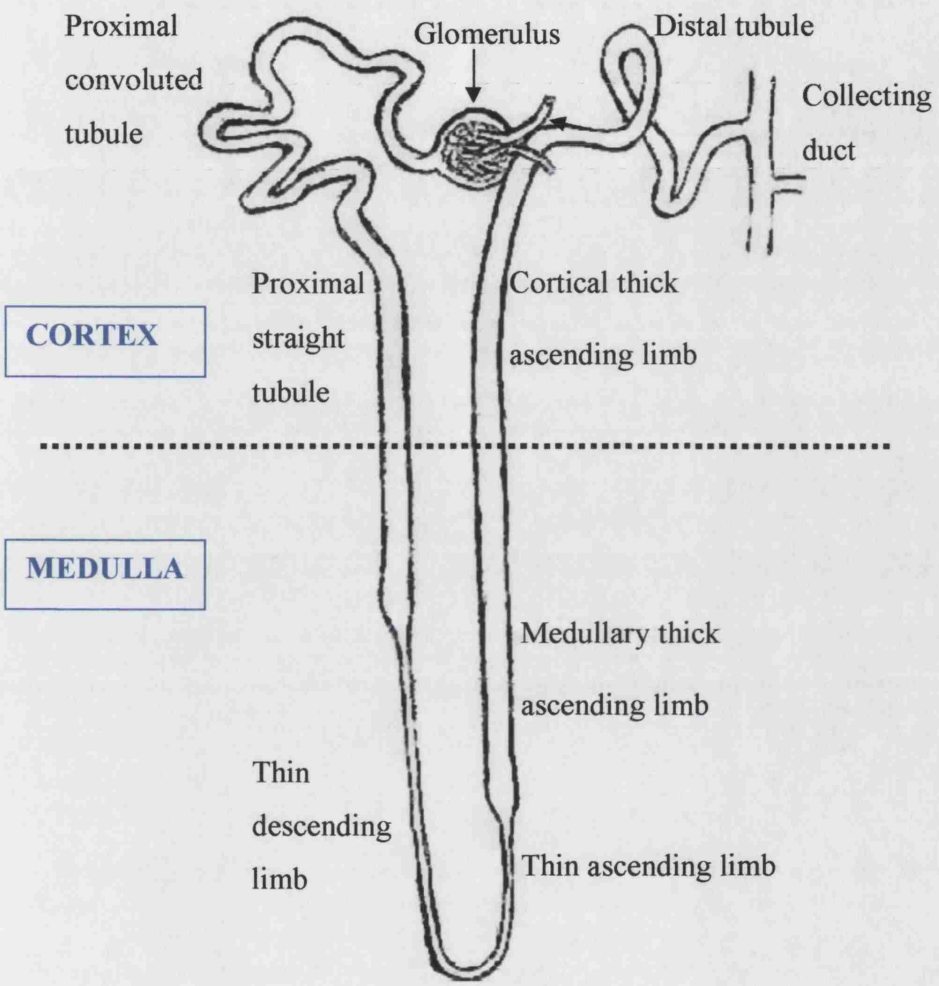
Nucleotides, via the activity of P2 receptors, are now emerging as physiological regulators of a number of cellular functions in many different cell types. Expression of P2 receptors has been described in virtually every major system of the body including the respiratory system, immune system and cardiovascular system (reviewed in (Ralevic & Burnstock, 1998)). There is much interest in P2 receptor modulation of cell function and the role of P2 receptors in several cell types including immune cells and epithelial cells. This thesis focuses on expression of P2 receptors in the kidney. The kidney is important in the regulation of body fluids and electrolytes and ultimately assists in the maintenance of homeostasis. This is achieved by glomerular ultrafiltration

and selective reabsorption or secretion of ions and water through the epithelial cells of the nephron. Figure 1.6 shows a schematic diagram of the structures of the anatomical nephron. The nephron consists of a single layer of polarised epithelial cells, and consequently there may be distinct P2 receptor subtypes in each membrane domain.

1.4 Expression pattern and function of P2 receptors in the kidney

The earliest studies of the effects of ATP in the kidney report that direct renal artery infusion of ATP increased renal blood flow and reduced glomerular filtration rate (GFR) (Harvey, 1964;Tagawa & Vander, 1970). Many of the subsequent studies report a vasoconstriction of the renal arteries upon infusion with ATP (Baylin *et al.*, 1966;Murphy *et al.*, 1969;Hrdina *et al.*, 1970). These studies suggest that the renal effects of adenine nucleotides are different from the vasodilatation elicited by adenosine (see section 1.2). Later investigations of the potential role of ATP and P2 receptors in the control of renal function were concerned with ATP release from sympathetic nerve endings acting as a cotransmitter at postsynaptic sites since both the juxtaglomerular apparatus and proximal tubules are densely innervated (Schwartz & Malik, 1989). The primary role of the juxtaglomerular apparatus is to maintain the glomerular filtration rate at a high and nearly constant level in the face of large variations in both systemic blood pressure and daily intake of water and electrolytes (Vallon, 2003). Mitchell and Navar (1993) hypothesised a role for extracellular ATP in the control of tubuloglomerular feedback (TGF) by modulation of preglomerular vascular resistance (Mitchell & Navar, 1993). Autoregulation of renal blood

Figure 1. 6 **Schematic diagram of the nephron**



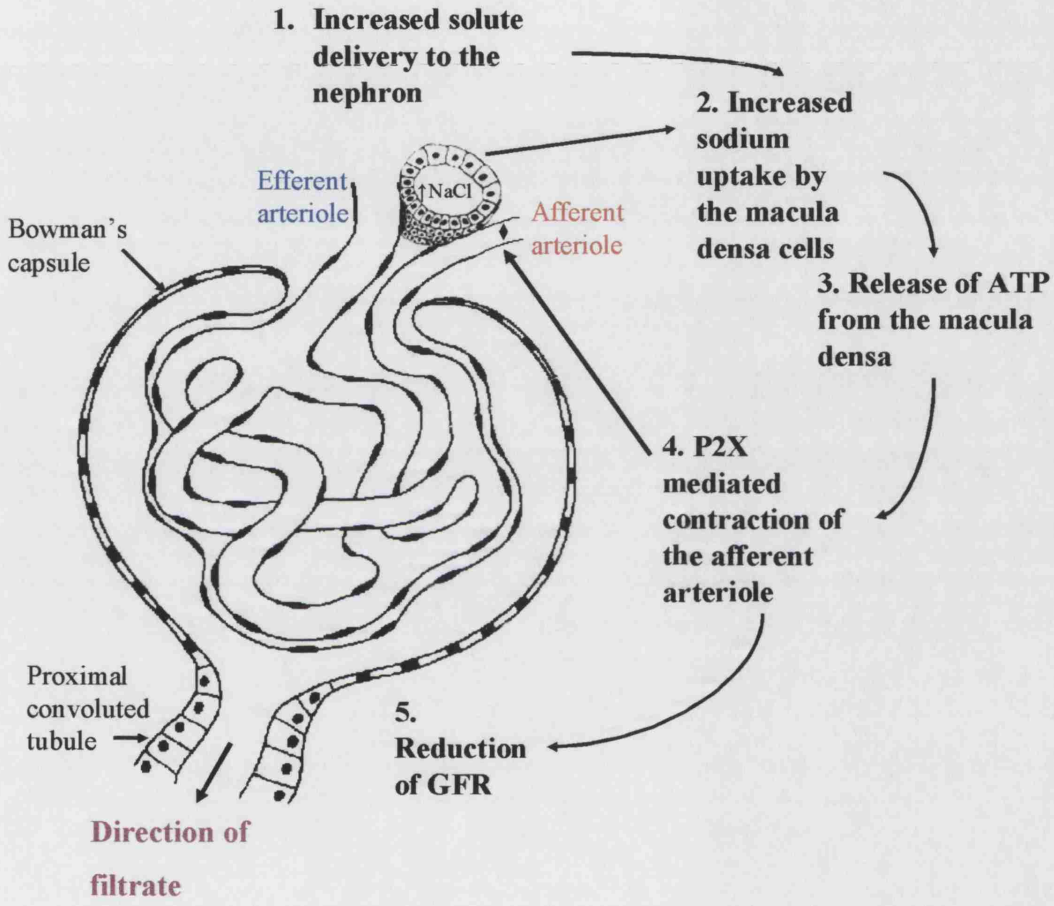
The glomerulus is the filtration apparatus of the kidney, it consists of a capillary tuft surrounded by Bowman's capsule, a single layer of glomerular epithelial cells, and within the capsule are podocytes and the mesangium. The proximal convoluted tubule extends from Bowman's capsule. The loop of Henle consists of the straight portion of the proximal tubule, the thin descending and ascending limbs and the thick ascending limbs (TAL). The site on the TAL where the glomerulus makes contact is the macula densa (MD). The distal tubule extends from the macula densa to the collecting duct.

flow involves alterations in preglomerular resistance through TGF-dependent alterations in afferent arteriolar resistance in response to signals transmitted from the macula densa, a group of specialised cells situated where the thick ascending limb makes contact with the glomerulus (Figure 1.6). The macula densa detects tubular fluid sodium chloride (NaCl) and sends a signal that regulates afferent arteriolar resistance (either by vasoconstriction or vasodilatation) thus altering glomerular filtration.

More recent studies, largely carried out by Inscho and co-workers, indicate that extracellular ATP can affect the renal microcirculation directly through binding to P2X and P2Y receptors on renal vascular smooth muscle cells. Studies using isolated perfused juxtamedullary nephron and continual monitoring of arteriolar diameter have shown that the renal microvasculature exhibits a marked and segmental responsiveness to the effects of extracellular ATP (Inscho *et al.*, 1992;Inscho *et al.*, 1998). These investigators demonstrate that the afferent arteriole is far more responsive to ATP than either the arcuate or cortical radial artery, whilst the efferent arteriole is unresponsive. However the effect of infused P2 receptor agonists on renal blood flow and perfusion pressure are dependent on a number of different factors including the species being studied and the resting vascular tone. For example, infusion of ATP directly into the rat kidney evokes vasoconstriction, but vasodilatation when vascular tone is elevated (Eltze & Ullrich, 1996). Infusion of ATP into the dog kidney produced vasodilatation by stimulating the synthesis and release of nitric oxide (Majid & Navar, 1992). Pre-treatment with the nitric oxide inhibitor nitro-L-arginine abolished vasodilatation (Eltze & Ullrich, 1996;Majid *et al.*, 1999). Broadly however, P2X receptor activation leads to a marked vasoconstriction of renal

blood vessels whereas P2Y receptor activation can cause vasoconstriction or trigger vasodilatation via release of endothelium-derived relaxing factors predominantly nitric oxide (Churchill & Ellis, 1993a;Von Kugelgen *et al.*, 1995;Eltze & Ullrich, 1996;Majid *et al.*, 1999;Wangensteen *et al.*, 2000). The control of TGF by ATP has been investigated by Inscho and co-workers over a number of years and supports the hypothesis that extracellular ATP activates afferent arteriolar P2 receptors to stimulate autoregulatory adjustments in preglomerular resistance probably via multiple P2 receptors (Figure 1.7) (Inscho *et al.*, 1992;Inscho *et al.*, 1998;Inscho & Cook, 2001;White *et al.*, 2001;Inscho & Cook, 2002). However, the exact mechanism by which TGF signals from the macula densa are transmitted to P2 receptors on the afferent arteriole to regulate glomerular filtration is yet to be determined.

Figure 1. 7 **Schematic diagram of a glomerulus showing a possible role for extracellular ATP in P2X-mediated vasoconstriction and control of tubuloglomerular feedback**



(1) Increased delivery of NaCl in the nephron (2) increases sodium uptake across the apical membrane of macula densa cells and (3) stimulates local release of ATP. (4) ATP activates P2X receptors leading to contraction of the afferent arteriole thus (5) reducing the filtered load on the glomerulus and reducing glomerular filtration rate (GFR).

1.5 Potential sources of extracellular ATP in the kidney

Several studies have shown that ATP is co-released with acetylcholine or with noradrenaline from nerve termini via synaptic vesicles (Dowdall *et al.*, 1974;Burnstock, 1995). In a recent report, Vonend and co-workers have shown that electrical field stimulation of the sympathetic nerves in the renal cortex induces release of ATP, but this only accounted for 25% of the total cortical ATP content, with the remainder coming from non-neuronal sources (Vonend *et al.*, 2002). There are multiple segments along the nephron that could be sources of extracellular ATP, for example proximal tubule epithelial cells have a large number of mitochondria, which are necessary to support a high metabolic rate, and consequently this cell type are a rich source of ATP. The work of Schwiebert and co-workers has demonstrated that under basal conditions, high nanomolar concentrations of ATP are released from proximal tubule cell primary cultures and cell lines (Schwiebert, 2001). They have also shown lower nanomolar concentrations of ATP are released from thick ascending limb and collecting duct cell models (Schwiebert, 2001). Apart from epithelial cells, other potential sources of ATP in the kidney include the cells present in the renal vascular system such as endothelial and smooth muscle cells (Pearson & Gordon, 1979;Bodin & Burnstock, 1996), platelets (Detwiler & Feinman, 1973), mononuclear cells (Maugeri *et al.*, 1990), and erythrocytes (Bergfeld & Forrester, 1992).

Due to its charge and size, ATP cannot cross the cell membrane, therefore either vesicular exocytosis or other transport mechanisms are required to facilitate its movement. The ATP-binding cassette (ABC) family of proteins

have been identified as membrane transporters of ATP, in particular multidrug resistance glycoproteins have been directly implicated in ATP transport (Cantiello *et al.*, 1998;Schwiebert, 1999;Roman *et al.*, 2001). These proteins are active transmembrane pumps which can transport various substrates including inorganic ions, sugars, peptides and amino acids (Holland *et al.*, 2003). Cellular release of ATP can be induced by a range of stimuli including hypoxia (Bergfeld & Forrester, 1992), acute inflammation (Bodin & Burnstock, 1998), fluid shear stress (Bodin & Burnstock, 2001a), osmotic shock (Jans *et al.*, 2002), and mechanical deformation such as distension of the ureter (Knight *et al.*, 2002). Once released from cells, ATP may directly influence either the cell it is released from (autocrine) or an adjacent cell (paracrine) or cells at a more distant site. However in the kidney, as in other tissues, extracellular ATP and other nucleotides are rapidly hydrolysed by membrane-bound ecto-enzymes such as ecto-5'-nucleotidase (Dawson *et al.*, 1989). Therefore, P2 receptor signalling in the kidney must involve a finite balance between ATP release and ATP breakdown.

1.6 Expression of P2 receptors along the nephron

The effect of extracellular ATP on renal epithelial cells was first reported in 1972, in which ATP was shown to increase calcium release from a suspension of cortical tubules (Rorive & Kleinzeller, 1972). Since this time, measurements of intracellular Ca^{2+}, using the fluorescent probe fura-2, have provided functional evidence for both P2X and P2Y receptor activation since both types of receptor can trigger a rise in intracellular Ca^{2+} (He *et al.*, 2003). The difference being that P2X receptors induce Ca^{2+} influx, while activation of P2Y receptors results in

intracellular Ca^{2+} mobilisation. Identification of the particular receptor(s) involved is based on agonist and antagonist profiles.

1.6.1 The expression and function of P2 receptors in the glomerulus

Nucleotide-induced rises in intracellular Ca^{2+} have been used to identify P2Y receptors in cultured glomerular endothelial cells (Pavenstadt *et al.*, 1992;Briner & Kern, 1994) and rat mesangial cells (Pfeilschifter, 1990). Most of the current research in the glomerulus has focussed on mesangial cells, with few reports of podocyte or endothelial cell P2 receptor expression. Fischer and co-workers identified mRNA transcripts for $P2Y_1$, $P2Y_2$, $P2Y_6$ and $P2X_7$ receptors and, using patch-clamp techniques, determined that P2 receptor agonists depolarise cultured podocytes and increase intracellular Ca^{2+} (Fischer *et al.*, 2001). Pfeilschifter identified several second messengers of P2Y receptor signalling (see Figure 1.5), including activation of PLC, and concomitant production of IP_3 and diacylglycerol (DAG) in mesangial cells (Pfeilschifter, 1990). Schulze-Lohoff reported inhibition of cAMP formation in mesangial cells by P2Y coupling to Gi (Schulze-Lohoff *et al.*, 1995). Furthermore, P2Y receptor activation in rat mesangial cells results in activation of phospholipase D and the mitogen-activated protein (MAP) kinase cascade (Pfeilschifter & Merriweather, 1993). This suggests the presence of several signalling pathways utilised by P2Y receptors in cultured mesangial cells.

There are a number of reports that extracellular nucleotides can regulate mesangial cell proliferation *in vitro* via P2Y receptor activation (Schulze-Lohoff *et al.*, 1992;Huwiler & Pfeilschifter, 1994;Ishikawa *et al.*, 1994;Harada *et al.*, 2000). These studies measured the incorporation of ^{3}H-thymidine into the cellular DNA of mesangial cells after agonist stimulation of P2 receptors. More

recently, ATP-induced proliferation of mesangial cells was shown to be through P2Y receptor activation of the $MAPK^{42/44}$ signal transduction pathway (Vonend *et al.*, 2003). Extracellular nucleotides may also activate the stress-activated protein kinase (SAPK) and the p38-stress-activated protein kinase ($MAPK^{38}$) cascades in mesangial cells via P2Y receptors (Huwiler *et al.*, 1997;Huwiler *et al.*, 2000). These cascades are most often activated by cellular stresses such as chemicals, heat, osmotic shock and UV irradiation (reviewed in Paul *et al.*, 1997). The balance between nucleotide-stimulation of the $MAPK^{42/44}$ pathway (Vonend *et al.*, 2003), and the SAPK and $MAPK^{38}$ pathways determines whether cells survive or undergo programmed cell death (Huwiler *et al.*, 1997;Huwiler *et al.*, 2000). The signal that determines which pathway is activated is currently unknown. Activation of the $P2X_7$ receptor, in cultured rat mesangial cells, induced apoptosis and an increase in p53, a component of the apoptotic pathway. This effect could be inhibited by the $P2X_7$ antagonist, ox-ATP (Schulze-Lohoff *et al.*, 1998). Harada and co-workers reported reduced cell numbers, DNA cleavage and upregulation of $P2X_7$ receptor mRNA in mesangial cells stimulated with 3'-O-(4-benzoyl)benzoyl-ATP (BzATP) (Harada *et al.*, 2000). Studies of mesangial cell proliferation and apoptosis are of particular relevance to renal diseases such as diabetic nephropathy, glomerulonephritis and IgA nephropathy where these processes are often simultaneously increased (Griffin *et al.*, 2003;Kurogi, 2003).

1.6.2 The expression and function of P2 receptors in the proximal tubule

Several studies describe the effect of nucleotides on intact, micro-dissected tubules, most notably Bailey and co-workers provide functional and molecular evidence for P2 receptors along the rat renal tubule and report

segment-specific P2Y receptor expression. In the proximal convoluted tubule there is evidence for $P2Y_1$, $P2Y_6$ and either $P2Y_2$ or $P2Y_4$ receptors (Bailey *et al.*, 2000b;Bailey *et al.*, 2001). Expression of P2 receptors in the porcine proximal tubule cell line (LLC-PK_1) has also been described in which P2Y receptor activation increased intracellular Ca^{2+} (Anderson *et al.*, 1991) and, coupled to a G protein, inhibited adenylate cyclase activity (Harada *et al.*, 1991). P2X receptors have also been demonstrated in LLC-PK_1 cells by measuring whole cell conductance in response to agonists and some antagonists (Filipovic *et al.*, 1998). ATP-induced rises in intracellular Ca^{2+} were observed in isolated tubules of the marine teleost (Renfro *et al.*, 1982), in micro-dissected *Necturus* proximal tubule (Bouyer *et al.*, 1998) and in cultured mouse proximal tubule cells in which mRNA transcripts for $P2X_4$, $P2Y_1$ and $P2Y_2$ receptors were also found (Takeda *et al.*, 1998). In cultured rat proximal tubule cells, $P2Y_1$ and $P2Y_2$ receptors, coupled to IP_3, mediated release of Ca^{2+} from intracellular stores (Dockrell *et al.*, 2001).

Functionally, P2 receptors in the proximal tubule may influence cell metabolism and the hormonal regulation of this segment. Agonist stimulation of $P2Y_1$ and $P2Y_2$ or $P2Y_4$ receptors has been shown to stimulate gluconeogenesis in isolated rat proximal tubules (Cha *et al.*, 1995;Mo & Fisher, 2002) and to stimulate renin secretion in rat cortical slices (Churchill & Ellis, 1993b). Renin plays an important role in renal haemodynamics, since it is released from juxtaglomerular cells in response to stimuli from the macula densa. Renin is part of the pathway that, along with angiotensin converting enzyme, results in angiotensin II production that affects many aspects of cardiovascular control. The renin-angiotensin system influences systemic blood pressure, blood volume

and intake and excretion of salt and water. Effects of ATP on other hormones or hormone receptors has also been described, P2X receptor agonists enhanced the inhibition of sodium-dependent phosphate uptake by modulating the activity of parathyroid hormone in proximal tubule cells derived from opossum kidney (Lederer & McLeish, 1995). In LLC-PK_1 cells, modulation of the binding affinity of epidermal growth factor receptor by extracellular ATP involved stimulation of PLC and activation of PKC (Harada *et al.*, 1993) suggesting involvement of P2Y receptors. It is likely that extracellular ATP exerts autocrine or paracrine control of renal epithelial cell function, since tightly regulated physiological release of ATP has been shown to occur in renal epithelial cells (Roman *et al.*, 2001;Vonend *et al.*, 2002). ATP released as a cotransmitter during renal sympathetic nerve stimulation or from renal vascular or tubule cells, may directly affect tubular electrolyte and water transport.

1.6.3 The expression and function of P2 receptors in the loop of Henle

In the thin limbs of Henle from the rat kidney, basolaterally applied ATP and UTP triggered a rise in intracellular Ca^{2+} (Bailey *et al.*, 2000b). In the mouse, P2Y agonists induced Ca^{2+} transients in thick ascending limb, and ATP and UTP were equipotent suggesting $P2Y_2$- or $P2Y_4$-like receptors (Paulais *et al.*, 1995). There are few studies addressing functional consequences of P2 receptors in the loop of Henle, however there is considerable interest in P2 mediated control of TGF (see Figure 1.7). The thick ascending limb makes contact with the glomerulus via the macula densa from where signals are sent to the afferent arteriole to mediate GFR (Vallon, 2003). The mechanisms and nature of these signals are currently unknown. However, ATP and UTP basolaterally applied to microdissected glomeruli with attached thick ascending limb and macula densa,

induced a rise in intracellular Ca^{2+} dependent on PLC (Liu *et al.*, 2002). There was no effect on macula densa Ca^{2+} when ATP was microperfused through the lumen of the thick ascending limb, suggesting, in agreement with the antagonist profile, that macula densa cells express basolateral $P2Y_2$ receptors (Liu *et al.*, 2002). Furthermore, Bell reported NaCl concentration-dependent ATP release from the basolateral membrane of macula densa cells through a large-conductance anion channel (Bell *et al.*, 2003). These reports, together with those that functionally identify P2 receptors in cells of the afferent arteriole and glomerulus, provide increasing evidence that P2 receptors are modulators of TGF (see Figure 1.7).

1.6.4 The expression and function of P2 receptors in the distal tubule

The distal tubule extends from the macula densa to the collecting duct. Functional studies of P2 receptor activity in this segment are limited and to date have been confined to cultured cells. In A6 cells, a distal tubule-like cell line derived from *Xenopus laevis*, extracellular ATP stimulated Cl^- secretion and the Na^+-K^+-$2Cl^-$ cotransporter (Middleton *et al.*, 1993;Banderali *et al.*, 1999). Patch-clamp analysis of A6 cells, and the use of specific channel-blockers, established that ATP activates both Cl^- and K^+ channels by a Ca^{2+}-dependent mechanism (Nilius *et al.*, 1995). Apical $P2Y_2$ receptors in cultured rabbit distal tubule cells generated an increase in intracellular Ca^{2+} and an increase in Cl^- secretion via calcium-sensitive Cl^- channels (Bidet *et al.*, 2000;Rubera *et al.*, 2000). In cultured mouse distal convoluted tubule cells, ATP inhibits both basal and hormone-stimulated magnesium uptake via P2X receptors (Dai *et al.*, 2001). $P2X_{1\text{-}5}$ and $P2Y_2$ receptor mRNA was detected in these cells by RT-PCR analysis (Dai *et al.*, 2001). The distal tubule is a site of significant Mg^{2+} reabsorption and

plays an important role in determining the final urinary concentration rate (Quamme & de Rouffignac, 2000).

1.6.5 The expression and function of P2 receptors in the collecting duct

One of the proposed roles for P2Y receptors in the kidney is with the modulation of ion channels, and therefore transport of ions either in to or out of the cell (see section 1.3.2). In 1979 Simmons demonstrated, using Madin Darby canine kidney (MDCK) cells grown on a semi permeable filter and mounted in Ussing chambers, that extracellular ATP stimulates Cl^- ion transport (Simmons, 1979). Further investigations revealed that the Cl^- conductance was controlled by P2 receptors activated by UTP and ATP and similar responses could be detected on both the basolateral and apical membranes (Simmons, 1981a). MDCK cells are believed to have a distal tubule/collecting duct phenotype (Arthur, 2000), and therefore are included in this section detailing P2 receptor expression in the collecting duct. Extracellular nucleotides (ATP, ADP and UTP) were also shown to hyperpolarize MDCK cells by activating inwardly rectifying potassium channels dependent on intracellular Ca^{2+} (Lang *et al.*, 1988;Friedrich *et al.*, 1989;Lang & Paulmichl, 1989).

The effects elicited by P2 receptor activation are dependent on the species and receptor subtype, the cell type and the signal transduction pathway. In MDCK cells alone, P2 receptor activation involves a vast complexity of signal transduction cascades for regulation of ion transport including, transient increases in intracellular Ca^{2+} (Paulmichl & Lang, 1988), PLC and phopholipase A_2 (Firestein *et al.*, 1996), PKC (Xing *et al.*, 1997), activation of cAMP (Post *et al.*, 1998), MAPK (Orlov *et al.*, 1999) arachidonic acid release (Xing *et al.*, 1999) and activation of adenylate cyclase (Woo *et al.*, 1998) (Ostrom *et al.*,

2001). Measurements of short circuit current (I_{sc}) and intracellular Ca^{2+} provide evidence for $P2Y_2$ receptors in isolated cortical collecting duct from mouse (Deetjen *et al.*, 2000;Lehrmann *et al.*, 2002), and rabbit (Woda *et al.*, 2002) and Kishore provided direct evidence for $P2Y_2$ receptor expression in isolated rat inner medullary collecting duct by detecting both mRNA and protein (Kishore *et al.*, 2000). Extracellular nucleotides, via $P2Y_2$, have been shown to stimulate Cl^- secretion and inhibit Na^+ absorption in mouse inner medullary collecting duct cells (mIMCD-K2) (McCoy *et al.*, 1999) and mouse M1 cells (Cuffe *et al.*, 2000) and to inhibit Na^+ and Ca^{2+} absorption in rabbit cortical collecting duct primary culture (Koster *et al.*, 1996). Stimulation of $P2Y_2$ receptors also inhibits K^+ secretion from mouse cortical collecting duct principal cells by inhibiting the apically expressed small conductance K^+ channel (SK) (Lu *et al.*, 2000). Activation of $P2Y_2$ receptors in the rat inner medullary collecting duct has been shown to enhance release of prostaglandin E2 (Welch *et al.*, 2003), and to inhibit the action of vasopressin on tubule cell water permeability (Kishore *et al.*, 1995;Edwards, 2002) providing direct evidence for a role in modulation of water transport. Finally, increased proliferation of cultured inner medullary collecting duct cells in response to ATP and UTP suggests a mitogenic role for $P2Y_2$ receptors via phospholipase C (Ishikawa *et al.*, 1997).

Most of the studies described rely upon the relative potency of receptor agonists and antagonists for identification and characterisation of the P2 receptors expressed on renal epithelial cells. The findings are summarised in Tables 1.4 and 1.5. The evidence suggests that there are multiple P2 receptors expressed on both apical and basolateral domains of tubule epithelial cells, the renal vasculature and cells of the glomerulus providing an elaborate system for

paracrine and autocrine control of renal function. The complexity of the system is magnified since multiple P2X and P2Y receptors may be expressed on the same cell and there are many different cell types along the nephron. P2 receptors and subsequent intracellular signals provide a potential coupling between metabolic demand and solute reabsorption by the kidney. Although it is possible to measure the effects of ATP on kidney cells, the role of P2 receptors in renal function *in vivo* is still under investigation and not fully elucidated. However, it is becoming evident that alterations in the physiology of P2 receptor signalling may be involved in the development of a variety of pathologies including autosomal dominant polycystic kidney disease (ADPKD), an inherited kidney disease in which abnormal cell physiology is thought to contribute to disease progression.

Table 1. 4 **Current evidence for segment specific localisation and function of P2 receptors in the proximal tubule and loop of Henle.**

Nephron segment	Receptor	Function	Reference
Proximal tubule	P2Y	↓AC, ↑PKC	(Anderson *et al.*, 1991)
	P2Y	renin secretion	(Churchill & Ellis, 1993b)
	P2Y	regulate EGFR	(Harada *et al.*, 1993)
	P2	↑PTH activity	(Lederer & McLeish, 1995)
	Apical P2Y	$\downarrow Na^+$-K^+-ATPase	(Jin & Hopfer, 1997)
	P2Y	$\uparrow[Ca]_i$	(Bouyer *et al.*, 1998)
	$P2X_1$	$\uparrow[Ca]_i$, $\uparrow Na^+$, $\uparrow K^+$ absorption	(Filipovic *et al.*, 1998)
	$P2Y_1$, $P2Y_2$, $P2X_4$	$\uparrow[Ca]_i$	(Takeda *et al.*, 1998)
	Basolateral $P2Y_1$, $P2Y_{2/4}$, $P2Y_6$	$\uparrow IP_3$ and $\uparrow[Ca]_i$	(Bailey *et al.*, 2000b;Bailey *et al.*, 2001)
	$P2Y_1$, $P2Y_2$	$\uparrow IP_3$ and $\uparrow[Ca]_i$	(Dockrell *et al.*, 2001)
	$P2Y_1$, $P2Y_{2/4}$	gluconeogenesis	(Cha *et al.*, 1995;Mo & Fisher, 2002)
Loop of Henle	$P2Y_2$ (dTL) $P2Y_2$ or $_4$ (ATL)	$\uparrow[Ca]_i$	(Bailey *et al.*, 2000b)
	$P2Y_2$ (TAL)	$\uparrow[Ca]_i$	(Liu *et al.*, 2002)

Table 1. 5 **Current evidence for segment specific localisation and function of P2 receptors in the distal tubule and collecting duct.**

Nephron segment	Receptor	Function	Reference
Distal tubule	P2	↑ Na^+-K^+-$2Cl^-$ cotransport	(Middleton *et al.*, 1993)
	P2	↑$[Ca]_i$, ↑K^+, ↑Cl^- currents	(Nilius *et al.*, 1995)
	Apical $P2Y_2$	↑Cl^- secretion	(Banderali *et al.*, 1999;Bidet *et al.*, 2000;Rubera *et al.*, 2000)
	P2X	↓Mg^{2+} uptake	(Dai *et al.*, 2001)
MDCK[1]	P2Y	↑Cl^- secretion	(Simmons, 1981b)
	$P2Y_1$, $P2Y_2$, $P2Y_{11}$	↑$[Ca]_i$, ↑cAMP, ↑PKC	(Insel *et al.*, 1996;Zambon *et al.*, 2000)
	$P2Y_{11}$	↑cAMP	(Torres *et al.*, 2002;Zambon *et al.*, 2001)
	P2Y (UTP)	↓ Na^+-K^+-Cl^-	(Brindikova *et al.*, 2003)

1. Note that MDCK cells are a heterogeneous population of cells with characteristics of both distal tubule and collecting duct (Arthur, 2000).

Continuation of table 1.5

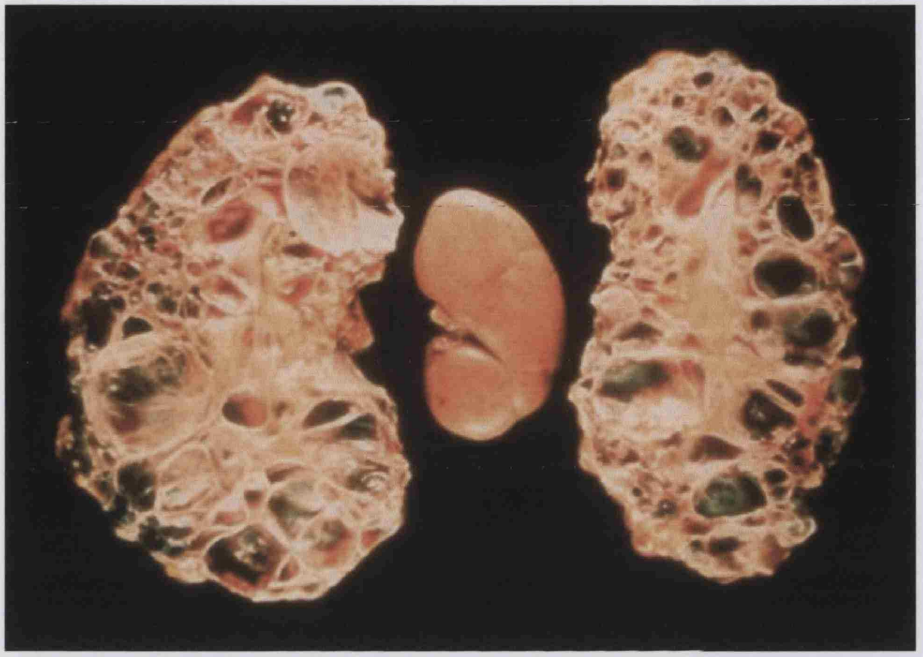
CCD	P_{2U} ($P2Y_2$)	$\downarrow Na^+$, $\downarrow Ca^{2+}$ absorption	(Koster *et al.*, 1996)
	Apical $P2Y_2$	$\downarrow K^+$ secretion	(Lu *et al.*, 2000)
	$P2Y_2$	$\uparrow Cl^-$ secretion, $\downarrow Na^+$ absorption	(Cuffe *et al.*, 2000;Deetjen *et al.*, 2000;Bouyer *et al.*, 1998;Lehrmann *et al.*, 2002)
	Apical $P2Y_2$	$\uparrow [Ca]_i$	(Deetjen *et al.*, 2000;Woda *et al.*, 2002)
IMCD	P2Y	$\uparrow [Ca]_i$	(Ecelbarger *et al.*, 1994)
	$P2Y_2$	$\uparrow IP_3$, activate MAPK, $\uparrow$ cell proliferation	(Ishikawa *et al.*, 1997)
	Apical P2X, P2Y	$\downarrow Na^+$ absorption, $\uparrow Cl^-$ secretion	(McCoy *et al.*, 1999) (Boese *et al.*, 2000)
	Basolateral $P2Y_2$	$\downarrow$vasopressin activity	(Kishore *et al.*, 1995;Edwards, 2002)
	$P2Y_2$	$\uparrow$release of prostaglandin E_2	(Welch *et al.*, 2003)

1.7 Autosomal dominant polycystic kidney disease (ADPKD)

ADPKD occurs with a frequency of 1 in 1000 births worldwide and is transmitted as an autosomal dominant trait with complete penetrance (Dalgaard, 1957). In addition there is also a recessive form of the disease (ARPKD) that is usually lethal within the first year of life. For the purpose of this thesis, only the autosomal dominant form will be discussed.

In Western Europe, ADPKD accounts for 6% of all patients requiring renal replacement therapy (Zeier *et al.*, 1996). The disease was first described by the 18th century Italian anatomist, Domenico Gusmano Galeazzi who treated three patients with abdominal symptoms that were thought to derive from renal stone disease in one case, a liver tumour in the second and an intestinal tumour in the third case. Upon post-mortem examination, the kidneys of all three patients were enlarged and completely overwhelmed by numerous vesicles of different sizes ((Galeazzi, 1757) as cited by (Fogazzi, 1998)). In 1888 the term 'polycystic kidneys' was introduced by Felix Lejars in his doctoral thesis. He described the clinical features and symptoms of the disease in order to allow for clinical diagnoses during life rather than at post-mortem and emphasised the bilateral nature of the condition (Lejars, 1888). Cysts grossly enlarge the kidney (Figure 1.8) and interfere with kidney function, resulting in chronic high blood pressure, anaemia and kidney infection. Renal function typically deteriorates through early to mid-adult life, although the rate of progression can vary significantly (Osathanondh & Potter, 1964). Cysts can arise from the glomeruli and all tubular structures although Heggo noted that cysts were most numerous in the glomeruli, in the angle of the loop of Henle and especially in the collecting

Figure 1. 8 **Size comparison of normal and ADPKD kidneys**



ADPKD kidneys (left and right) are grossly enlarged compared to a normal kidney (centre), and cysts and fibrotic tissue replace most of the normal renal parenchyma.

From : http://info.med.yale.edu/pharm/hermen/polycystinprojects.html.

tubules (Heggo, 1966). Blood vessels and functioning nephrons are compressed by the expanding cysts, and remodelling of the extracellular matrix occurs. Loss of kidney function is due to cystic degeneration of functional nephrons and progressive fibrosis of renal tissue. In the end-stage kidney, cysts and fibrotic tissue have replaced most of the normal renal parenchyma. Several extrarenal manifestations, including hepatic cysts, cardiac valve abnormalities and arterial aneurysms contribute to the morbidity and mortality of the disease (Milutinovic *et al.*, 1980;Fick *et al.*, 1995).

1.7.1 The role of polycystins in ADPKD

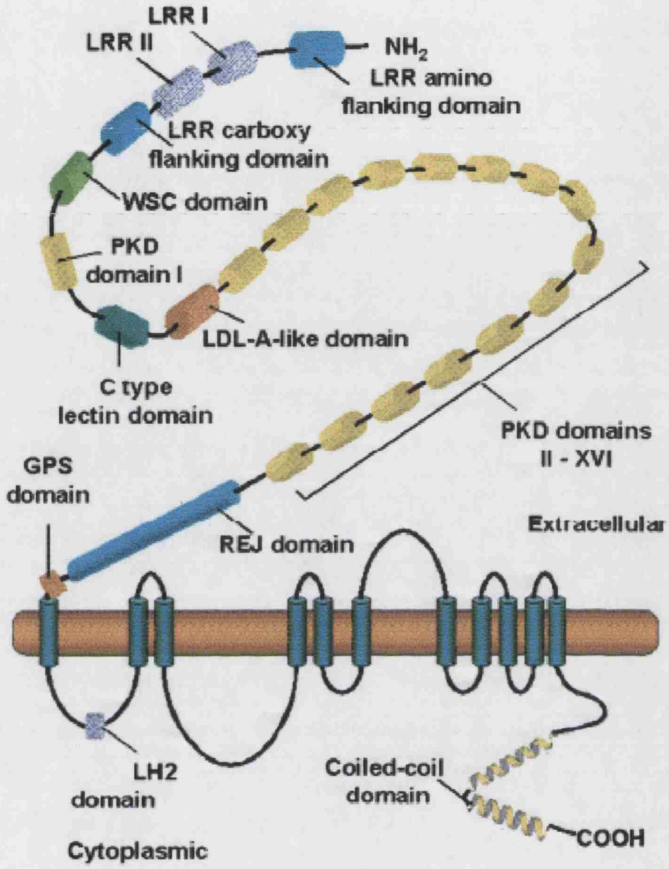
More than 85% of patients affected with ADPKD have mutations in the *PKD1* gene, while most other cases are due to mutations in *PKD2* and in rare cases, in a third undescribed locus (Ariza *et al.*, 1997;Gallagher *et al.*, 2000). Most of the mutations in the *PKD1* and *PKD2* genes lead to a loss of gene function due to premature termination of translation of the protein product (Calvet & Grantham, 2001). It is possible to subdivide ADPKD into two types depending on which gene is mutated. Disease progression tends to be more rapid with ADPKD type 1 but in all other respects, type 1 and type 2 share identical disease phenotypes (Hateboer *et al.*, 1999). The protein products coded by these genes are polycystin-1 and polycystin-2 respectively.

1.7.2 The *PKD1* gene and ADPKD

Genetic linkage analysis of affected families enabled identification of the *PKD1* gene which was located to the short arm of chromosome 16 (16p13.3) in 1985 (Reeders *et al.*, 1985). It was not until 1994 that the European Polycystic Kidney Disease Consortium cloned and sequenced the *PKD1* gene (Wunderle *et*

al., 1994;The European Polycystic Kidney Disease Consortium, 1994). *PKD1* spans approximately 52 kb and consists of 46 exons, encoding a large 4302 amino acid protein called polycystin-1 with a molecular mass of 462kDa (Hughes *et al.*, 1995;Sandford *et al.*, 1997). The presence of leucine rich repeats (LRR) and a carbohydrate binding domain in the long extracellular NH_2-terminus suggests possible protein-protein and protein-carbohydrate interactions (Figure 1.9) (Sandford *et al.*, 1997;Malhas *et al.*, 2002). Many proteins containing LRRs are thought to be involved in protein-protein interactions and at least half of such proteins participate in signal transduction pathways (Kobe & Kajava, 2001). There is significant sequence homology between the extracellular domain of polycystin-1 and a sea urchin sperm glycoprotein receptor for egg jelly (suREJ) involved in fertilisation (Moy *et al.*, 1996). The suREJ protein binds the glycoprotein coat of the egg jelly, triggers an influx of extracellular Ca^{2+} and regulates ion transport in the sperm acrosome reaction. This may support the theory that polycystin-1, like the suREJ protein, is involved with Ca^{2+} signalling (Moy *et al.*, 1996). There is also evidence to suggest that polycystin-1 is involved with cell-cell (Streets *et al.*, 2003) and cell-matrix interactions (Malhas *et al.*, 2002). Current evidence suggests the transmembrane portion consists of eleven hydrophobic domains leading to a short cytoplasmic COOH-terminus (Bycroft *et al.*, 1999;Nims *et al.*, 2003). The COOH- terminal region is characterised by a series of motifs including the potential for protein-kinase A phosphorylation (Parnell *et al.*, 1999), coupling to heterotrimeric G proteins (Parnell *et al.*, 1998), a regulator of G protein signalling (RGS7) (Kim *et al.*, 1999), and a stimulator of activation protein-1 (AP-1) and PKC (Arnould *et al.*, 1998;Parnell *et al.*, 2002).

Figure 1. 9 **Schematic diagram of polycystin-1**



The predicted membrane topology for polycystin-1 suggests eleven transmembrane domains, a long NH_2- terminus and a short COOH-terminus (Nims et al., 2003). Starting at the NH_2-terminus, the LRR (leucine-rich repeat) motifs are indicative of protein-protein interaction or binding to the extracellular matrix (Sandford et al., 1997). The function of the WSC motif, and the 16 PKD domains is currently unknown. The C-type lectin domain typically binds carbohydrates in the presence of Ca^{2+}, interacts with glycoproteins involved in the extracellular matrix and is possibly involved with cell adhesion and recognition (Weston et al., 2001). The LDL-A-like motif was originally described in the LDL (low density lipoprotein) receptor, and because of their

hydrophobic nature they have been implicated in ligand binding regions (Hughes et al., 1995). The REJ (receptor for egg jelly) domain which has homology with the REJ protein, a membrane glycoprotein which is involved in changes in Ca^{2+} *and* Na^{+} *ion channel function (Moy et al., 1996). The LH-2 (lipoxygenase homology 2), motif may be involved in protein-protein or protein-lipid interactions. The coiled-coil domain can heterodimerise with polycystin-2 and is thought to comprise part of a voltage-gated channel (Qian et al., 1997;Newby et al., 2002;Delmas et al., 2004). The COOH-terminus also contains a tyrosine phosphorylation site, a G-protein activation site, and AP-1 and PKC sites but for clarity these were not included on the schematic diagram. (Figure 1.9 was adapted from* http://www.cimr.cam.ac.uk/medgen/pkd/pkd1/pkd1pic.htm *and Ikeda & Guggino, 2002).*

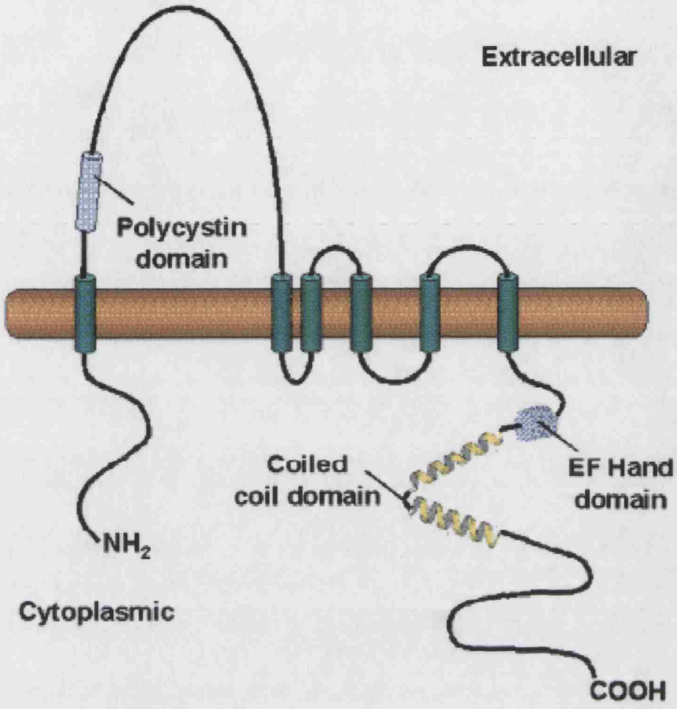
1.7.3 The *PKD2* gene and ADPKD

The *PKD2* gene is located on the long arm of chromosome 4 (4q21-23) and was identified by linkage analysis of affected families and positional cloning (Mochizuki *et al.*, 1996a). The gene is comprised of 15 exons and spans approximately 68 kb (Hayashi *et al.*, 1997). Polycystin-2 protein consists of 968 amino acids and has intracellular NH_2- and COOH-termini with a calculated molecular mass of 110 kDa (Mochizuki *et al.*, 1996a). Hydropathy analysis of the protein predicts six transmembrane domains (Figure 1.10) (Mochizuki *et al.*, 1996a). Polycystin-2 has significant amino acid homology to the transient receptor potential (*trp)* family of store-operated calcium channels (Koulen *et al.*, 2002). The COOH-terminus of *PKD2* contains an EF-hand domain, a protein structure that typically binds calcium and is common with voltage–gated calcium channels (de Leon *et al.*, 1995;Mochizuki *et al.*, 1996a).

1.7.4 Function of polycystin-1 and polycystin-2

Several investigators have proposed that polycystin-1 and polycystin-2 interact and are components of a signalling cascade, which modulates intracellular Ca^{2+} and other signal transduction pathways (Tsiokas *et al.*, 1997). Studies have shown that the COOH-termini of polycystin-1 interacts with and regulates polycystin-2 via their coiled-coiled domains (Qian *et al.*, 1997;Newby *et al.*, 2002) and form a Ca^{2+} channel (Delmas *et al.*, 2004). Mutations in the cytoplasmic tail of either protein abolishes the ability for the receptors to interact (Xu *et al.*, 2003). Over expression of the polycystin-1 COOH-terminal tail upregulates a Ca^{2+}-permeable non-specific Ca^{2+} channel in *Xenopus* oocytes that elevates intracellular Ca^{2+} (Vandorpe *et al.*, 2001;Vandorpe *et al.*, 2002).

Figure 1. 10 **Schematic diagram of polycystin 2**



The predicted membrane topology for polycystin-2 has six transmembrane domains and intracellular NH_2- and COOH- termini (Mochizuki et al., 1996b). The polycystin domain is a conserved amino acid motif with as yet no ascribed function. The EF Hand domain is a structure that may bind calcium and is common to voltage-gated calcium channels (Mochizuki et al., 1996b). The coiled-coil domain can heterodimerise with polycystin-1 (Qian et al., 1997;Newby et al., 2002). (Figure 1.10 was adapted from http://www.cimr.cam.ac.uk/medgen/pkd/pkd2l/pkd2lpic.htm and Ikeda & Guggino, 2002).

Evidence suggests that polycystin-2 is translocated to the plasma membrane in the presence of polycystin-1 and together they form a functional cation-permeable channel (Hanaoka *et al.*, 2000;Delmas *et al.*, 2004).

Immunohistochemical studies demonstrate that polycystin-1 and polycystin-2 are highly expressed in foetal tissues and the expression patterns are similar with prominent expression in maturing proximal and distal tubules (Geng *et al.*, 1997;Ibraghimov-Beskrovnaya *et al.*, 1997;Ong *et al.*, 1999a;Ong *et al.*, 1999b). Several lines of evidence suggest a connection between the polycystins and primary cilia, a highly conserved organelle thought to be an important sensor of environmental signals (Praetorius & Spring, 2003). The genes mutated in three mouse models of PKD, polaris (*orpk*), cystin (*cpk*) and inversin (*inv*) have been localised along with polycystin-1 and polycystin-2 to the primary cilia (Yoder *et al.*, 2002). One of the earliest roles for the primary cilia in the mammalian embryo is the development of left-right asymmetry. The *orpk* mouse has shortened cilia and shows left-right asymmetry defects (Murcia *et al.*, 2000). There is functional evidence that polycstin-1 and polycystin-2 can mediate a fluid-sensitive mechanotransduction pathway in primary cilia. The ciliary membrane is rich in receptors, ion channels, and signalling proteins which may be activated by mechanical or chemical stimuli (Rosenbaum & Witman, 2002). In cultured MDCK cells, the renal primary cilium acts as a flow sensor mediating a large increase in intracellular Ca^{2+} in response to fluid flow (Praetorius & Spring, 2003). Nauli and co-workers demonstrated that cultured polycystin-1 *null* cells formed normal cilia but were unable to increase Ca^{2+} influx in response to fluid flow. In wild-type cells, shear stress elicited a rapid rise in intracellular Ca^{2+} that was dependent on Ca^{2+} influx rather than release from stores (Nauli *et*

al., 2003). Furthermore, the polycystin 1/2 complex may be involved in cell cycle regulation and therefore mutations in either gene can result in dysregulated growth (Bhunia *et al.*, 2002). However, the exact mechanisms by which mutations in *PKD1* and *PKD2* lead to cyst formation still remain unclear.

1.8 Possible mechanisms of cystogenesis

Since there are about a million nephrons per kidney and some nephrons give rise to more than one cyst one would expect there to be more than a million cysts in an ADPKD kidney. However the number of cysts is considerably less than a million (Heggo, 1966), which suggests that not all nephrons become cystic. If all cells carry the mutation, there must be other regulatory factors that influence cystogenesis.

1.8.1 The two-hit hypothesis of cystogenesis

The analysis of individual kidney cysts revealed that for both *PKD1* and *PKD2* the germline mutation is present in all cells, but cyst formation is not triggered until the second allele is rendered inactive by somatic mutation (Koptides *et al.*, 1998;Koptides *et al.*, 1999). A mutation in the second allele, possibly in a small population of cells, would result in the complete loss of function of polycystin-1 or polycystin-2 respectively. A study carried out by Wu and co-workers in *Pkd1* and *Pkd2* knockout mice supports this two-hit hypothesis. Mice in which only one *Pkd2* allele has been inactivated do not develop polycystic kidneys, whereas cysts accumulate rapidly in *Pkd2* (-/-) mice (Wu *et al.*, 2002). A similar two-hit model applies to patients with ADPKD, analysis of cysts from patients with type 2 ADPKD with known germline mutations in *PKD2* demonstrated somatic mutations in the copy of *PKD2*

inherited from the unaffected parent (Pei *et al.*, 1999). There may also be a transheterozygous state with mutations in both PKD genes. One mutation, for example in *PKD1* is a germline mutation, and the second-hit mutation in *PKD2* is of a somatic nature (Koptides *et al.*, 2000;Watnick *et al.*, 2000). This condition also provides additional evidence that polycystin-1 and polycystin-2 interact and share a common pathway.

1.9 The enlargement of ADPKD cysts

Following initiation of a cyst, growth is the result of proliferation of incompletely differentiated epithelial cells and the accumulation of fluid within the lumen (Wilson, 1991;Ye & Grantham, 1993). A detailed scanning electron microscopic study of the morphology of 387 cysts from 10 patients with ADPKD showed that only 11.1% were lined by epithelia that are typical for various sections of the nephron, in 84% the epithelia were phenotypically undefined and in 4.9% cells were markedly hyperplastic (Grantham *et al.*, 1987). As cysts enlarge, they lose their tubular connection and become isolated from the glomerulus thus requiring transepithelial transport of solutes and fluid for further expansion (Grantham *et al.*, 1987). The alteration of normally absorptive epithelium into cystic secretory epithelium is a key factor in cyst pathophysiology. Initial investigations suggest that the increased cell proliferation and fluid accumulation is a result of abnormal polarity of multiple ion transporters and growth factor receptors, including the normally basolateral Na^+-K^+-ATPase pump and epidermal growth factor receptor (Wilson *et al.*, 1991;Du & Wilson, 1995). It has been suggested that the resulting apical sodium secretion could participate in driving fluid transport into the cyst lumen.

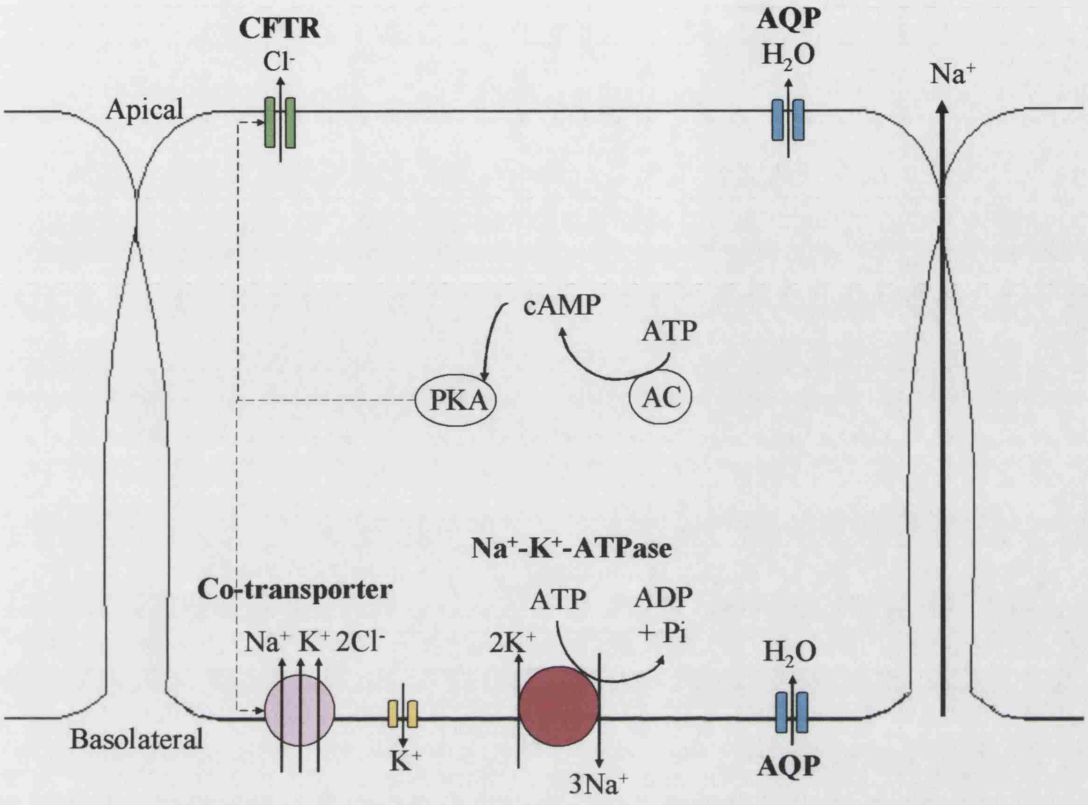
However, several other researchers found little or no apical expression of Na^+-K^+-ATPase in human ADPKD cyst epithelium (Carone *et al.*, 1994;Brill *et al.*, 1996) in renal cysts of the DBA/2FG-*pcy* mouse model (Kawa *et al.*, 1994) or in the Han:SPRD cy/+ rat model of ADPKD (Schafer *et al.*, 1994). In these studies, expression of Na^+-K^+-ATPase was exclusive and normally located to the basolateral membrane and it has been suggested that the discrepancy may be due to methodological differences. Carone and co-workers investigated the effect of ischemia on the distribution of Na^+-K^+-ATPase by incubating small samples of human kidney at 20°C for varying lengths of time. Na^+-K^+-ATPase was found in the apical membrane of tubule cells after ischemic periods of 30 minutes or longer, suggesting redistribution of Na^+-K^+-ATPase could be an artefact induced by ischemia during tissue processing (Carone *et al.*, 1994).

Analysis of intact isolated cysts from patients with end-stage ADPKD and monolayer cultures of human and canine kidney tubule epithelial cells demonstrated that fluid secretion can be induced by native cyst fluid or by activation of the adenylate cyclase signal transduction pathway using forskolin (Ye & Grantham, 1993;Grantham *et al.*, 1995;Yamaguchi *et al.*, 1995). In the absence of stimulation, ADPKD epithelia absorbed fluid in a manner similar to wild-type tubules. This suggests the presence of a secretagogue in the cyst fluid and that the cystic epithelium is not terminally committed to secretion. Cyst fluid also stimulated the proliferation rate of MDCK cells grown on plastic dishes as measured by ^{3}H-thymidine incorporation. Cyst fluid was a more potent mitogen than forskolin and was approximately equal in strength to epidermal growth factor (Yamaguchi *et al.*, 1995). It was later shown that cAMP and cAMP agonists also stimulate fluid secretion and proliferation of ADPKD

epithelial cells, but not normal human kidney cells, by activating the MAPK pathway (Hanaoka & Guggino, 2000;Yamaguchi *et al.*, 2000).

In most secretory epithelia such as the trachea or intestine, fluid secretion depends on a transepithelial, cAMP-stimulated Cl^- secretion (Sullivan *et al.*, 1998). In the standard model for Cl^- secretion (Figure 1.11), Na^+ and K^+ are carried into the cell by the basolaterally located Na^+-K^+-$2Cl^-$ co-transporter, and cycle back out across the basolateral membrane via the Na^+-K^+-ATPase and a K^+ channel. The Na^+-K^+-ATPase establishes and maintains the chemical gradient for Na^+ that drives the electrically neutral co-transport. An apical Cl^- channel provides the pathway for Cl^- efflux. The force driving Cl^- efflux is the electrochemical gradient for Cl^- established by the continued activity of the co-transport (Liedtke, 1989). Normally, Cl^- is the predominant anion in the glomerular ultrafiltrate and is reabsorbed along the nephron either by transcellular or paracellular pathways (Schild *et al.*, 1988). By monitoring, in parallel, currents and fluid secretion in intact cyst walls and monolayers of cultured ADPKD cyst cells, Mangoo-Karim and co-workers demonstrated that cAMP agonists induce fluid secretion and increase luminal electronegativity (Mangoo-Karim *et al.*, 1995). Fluid secretion however was abolished by replacement of Cl^- in the basolateral medium and could be inhibited by bumetanide (an inhibitor of the Na^+-K^+- Cl^- cotransport) applied basolaterally or by the addition of chloride channel inhibitors to the apical side (Mangoo-Karim *et al.*, 1995). In MDCK microcysts, fluid secretion was enhanced by cAMP agonists and inhibited by pre-treatment with 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and basolaterally-applied ouabain, furosemide and amiloride (Tanner *et al.*, 1992). DIDS is an inhibitor of Cl^- channels including

Figure 1. 11 **Model of fluid secretion driven by a Cl^- conductance**



Na^+, K^+ and Cl^- are carried into the cell by the basolateral Na^+-K^+-$2Cl^-$ cotransporter. Na^+ and K^+ are carried back out of the basolateral membrane via the Na^+-K^+-ATPase and the K^+ channel. Cl^- exits the cell via an apical Cl^- channel (CFTR) along the electrochemical gradient. Continued activity of the Na^+-K^+-ATPase and Na^+- K^+-Cl^- cotransporter maintain the electrochemical gradient. The activity of the two transporters is regulated by protein kinase A (PKA) under the control of cAMP. Cl- secretion drives movement of Na^+, via a paracellular route, and water, either transcellularly via aquaporins (AQP) or paracellularly, through electrical and osmotic coupling, respectively.

the Ca^{2+} activated Cl^- channels and outwardly rectifying Cl^- channels, furosemide blocks Na-K-2Cl^- cotransport, ouabain and amiloride are inhibitors of the Na^+-K^+-ATPase. CFTR has been implicated in the pathway of fluid secretion in ADPKD cystic epithelia. This receptor is a cAMP-dependent Cl^- channel required for Cl^- secretion and fluid production in airway epithelia (Jiang *et al.*, 1993). CFTR mRNA has been detected in all nephron segments and CFTR protein has been detected in the proximal tubule, thin limbs of Henle's loop, and on the apical membrane of distal tubule, cortical collecting duct and inner medullary collecting duct by immunohistochemistry (Crawford *et al.*, 1991;Morales *et al.*, 1996). CFTR has been detected on the apical surface of epithelial cell cultures derived from ADPKD cysts (Davidow *et al.*, 1996;Hanaoka *et al.*, 1996), and most cells that were positive for CFTR were also positive for the Na-K-2Cl^- cotransporter (Lebeau *et al.*, 2002). These studies show that key components for Cl^- secretion are present in ADPKD epithelial cells including apical Cl^- channels, and basolaterally located Na^+-K^+-ATPase and Na-K-2Cl^- cotransport. Driven by the osmotic gradient, water crosses the epithelium and accumulates in the cyst lumen either by a transcellular or by a paracellular pathway.

1.10 A hypothetical link between P2 receptors and ADPKD cyst enlargement

The work of Schwiebert and co-workers provides several lines of evidence for autocrine ATP-induced stimulation of Cl^- secretion in ADPKD cells. ATP release is enhanced from *cpk* mouse collecting duct monolayers and human ADPKD cells compared to controls; degradation of released ATP by

ecto-ATPases in ADPKD cells occurs at a much slower rate than in controls; multiple P2X and P2Y receptor mRNAs were detected and P2X and P2Y receptor agonists stimulated Cl^- secretion and an increase in intracellular Ca^{2+} in ADPKD cells (Schwiebert *et al.*, 2002). Furthermore, ATP has been shown in the micromolar range in the apical medium of cultured human ADPKD cyst cells (Wilson *et al.*, 1999;Schwiebert *et al.*, 2002). There are several reports that chloride secretion in ADPKD cyst cells is regulated by cAMP, which stimulates PKA activation to regulate activity of both CFTR and Na^+-K^+-$2Cl^-$ cotransport (see Figure 1.11) (Gregory *et al.*, 1990;Slotki *et al.*, 1993). Further evidence shows increased production of cAMP in a mouse model of ADPKD (Yamaguchi *et al.*, 1997) and cAMP stimulated fluid secretion and proliferation of primary cultures of human ADPKD cells (Hanaoka & Guggino, 2000). Production of cAMP can be regulated in renal cells by activation of P2Y receptors and subsequent stimulation or inhibition of adenylate cyclase activity (Anderson *et al.*, 1991). Several other lines of evidence suggest that other membrane ion channels are also regulated by extracellular nucleotides via P2 receptors including Na^+, Ca^{2+} (Koster *et al.*, 1996;McCoy *et al.*, 1999) and K^+ channels (Lu *et al.*, 2000). In distal tubule and collecting duct cell lines, P2Y receptors regulate chloride secretion by activation of PLC, increased intracellular Ca^{2+} and activation of calcium-activated Cl^- channels (Simmons, 1981b;Banderali *et al.*, 1999;Bidet *et al.*, 2000;Boese *et al.*, 2000;Cuffe *et al.*, 2000). Therefore stimulation of P2Y receptors can alter ion transport properties of renal epithelial cells potentially causing enhanced secretion and reduced absorption of ions from the tubule lumen. There may also be an association between the polycystin pathway and ATP-stimulated Cl^- secretion. Expression of the polycystin-1

COOH-terminal tail has been shown to enhance ATP-induced Ca^{2+} release in human kidney cells (Aguiari *et al.*, 2003) and to promote ATP-stimulated Cl^- secretion in a mouse collecting duct cell line (Wildman *et al.*, 2003b). Ion and fluid transport become encapsulated within the cyst lumen such that any released secretagogue or mitogen may create an autocrine/paracrine cycle of growth and enlargement. Thus apical release of ATP or its metabolites in to ADPKD renal cysts might, via stimulation of P2 receptors, enhance fluid secretion and therefore cyst expansion.

1.11 Aims of this Thesis

Reports dating back over 20 years document the effects of extracellular ATP on renal tissue, and much of the current knowledge comes from the use of kidney-derived cell lines. However, there is a distinct lack of evidence documenting P2 receptor protein expression in the native mammalian kidney. Several studies show functional expression of P2 receptors, using agonists and antagonists, or the presence of P2 receptor mRNA in isolated tubules and in cultured kidney cells. These studies have shown that there are many P2 receptor subtypes in the kidney and both P2X and P2Y receptors may be expressed in the same epithelial cell type and often in the same membrane domain (see section 1.6). From these studies it seems that P2Y receptor subtypes are the most dominant types of P2 receptor in the kidney epithelia and that P2X receptors dominate in the renal vasculature. 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 & Kishore, 2001), and disturbances in ATP signalling might contribute to some forms of renal tubular dysfunction. Extracellular ATP has been implicated in the enlargement of ADPKD cysts and significant levels of ATP in cyst fluids and enhanced ATP release from ADPKD epithelia have been detected (Wilson *et al.*, 1999;Schwiebert *et al.*, 2002).

The aim of the experiments undertaken in this thesis was to extend the investigation of P2 receptor expression and function in normal and ADPKD kidney. Initially, P2 receptor protein and mRNA expression and localisation in normal rat kidney were investigated. Secondly, P2 receptor protein localisation was examined in a rat model of ADPKD, the Han:SPRD cy/+ rat. Levels of P2

receptor mRNA in this rat model were compared with control rats. To further address the potential role for P2 receptors in ADPKD, a cell culture model of renal cyst formation was used to study the process and mechanism(s) of fluid accumulation and cyst growth in relation to P2 receptor activity. P2 receptor agonists and antagonists were used and cyst growth was monitored over a period of time. Finally, since the $P2X_7$ receptor has been associated with mesangial cell death (see section 1.6.1), and with apoptosis and recruitment of inflammatory cytokines (Verhoef *et al.*, 2003), a final study investigates P2 receptor expression in three rodent models of renal glomerular cell damage.

Chapter 2

General Methods

2.1 Localisation of P2 receptor proteins by immunohistochemistry

2.1.1 Preparation of tissue samples

Generally, tissue was embedded in OCT compound (BDH/Merck, Leicester, UK), mounted onto cork discs and frozen in iso-pentane (Sigma-Aldrich Co. Ltd., Poole, UK) pre-cooled in liquid nitrogen. 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 -80°C and allowed to return to room temperature for at least 10 min prior to further use.

2.1.2 Primary antibodies

The P2X immunogens used were peptides corresponding to 15 receptor subtype-specific amino acids in the C-terminal region of the receptor. See Table 2.1 for peptide sequences.

The synthetic peptides corresponding to P2X receptor subtypes were covalently linked to keyhole limpet haemocyanin and the conjugate was administered to rabbits at monthly intervals (performed by Research Genetics, Hunstsville, Ala., USA). Immunoglobulin G (IgG) fractions were isolated from the immune and pre-immune sera ($P2X_{1-7}$), following the method of Harboe and Ingild (Harboe & Ingild, 1973). The protein concentration was determined at 280 nm using an extinction factor of 1.43 for 1 mg/ml. The specificity of the antibodies was verified by immunoblotting with membrane preparations from CHO-K1 cells expressing the cloned $P2X_{1-7}$ receptors. Immunoblotting studies

have shown that anti-$P2X_{1-7}$ antibodies specifically recognize the recombinant receptors expressed in CHO-K1 cells (Oglesby *et al.*, 1999).

Rabbit polyclonal antibodies to $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptor subtypes were obtained from Alomone Laboratories Ltd. (Jerusalem, Israel). $P2Y_6$ receptor antibody was a generous gift from Prof. Jens Leipziger (Aarhus University, Denmark). See Table 2.1 for peptide sequences. The specificity of P2 subtype specific antibodies was immunohistochemically determined by replacement of primary antibody with non-immune rabbit serum. Pre-absorption of both P2X and P2Y subtype antibodies with excess of the appropriate synthetic peptide used for generation of the antibodies eliminated immunoreactivity.

2.1.3 Avidin-biotin technique

Slide-mounted sections were fixed for 2 minutes in 4% formaldehyde (VWR International Ltd, Poole, UK) and 0.2% of a saturated picric acid solution (Sigma-Aldrich Co. Ltd., Poole, UK) in phosphate-buffered saline (PBS). Slides were then washed three times 5 minutes with excess PBS. To inactivate endogenous peroxidase, the sections were then treated with 50% methanol (VWR International Ltd, Poole, UK) containing 0.4% hydrogen peroxide (Sigma-Aldrich Co. Ltd., Poole, UK) for 10 min. Non-specific protein binding sites were blocked by 20 min incubation with 10% normal horse serum (NHS) (Invitrogen Ltd., Renfrew, UK) in PBS containing 0.05% thimerosal (Sigma-Aldrich Co. Ltd., Poole, UK). The rabbit polyclonal P2 receptor antibodies (Roche bioscience, Palo Alto, Ca, USA) were diluted to 1.25–5 μg/ml (determined as optimal from prior titration) with 10% NHS, and the sections were incubated with the primary antibodies overnight at room temperature. The

Table 2. 1 **Peptide sequences for P2 receptor subtype specific antibodies**

P2 Receptor subtype	Corresponding amino acids	Sequence
$P2X_1$	385–399	ATSSTLGLQENMRTS
$P2X_2$	458–472	QQDSTSTDPKGLAQL
$P2X_3$	383–397	VEKQSTDSGAYSIGH
$P2X_4$	374–388	YVEDYEQGLSG-EMNQ
$P2X_5$	437–451	RENAIVNVKQSQILH
$P2X_6$	357–371	EAGFYWRTKYEEARA
$P2X_7$	555–569	TWRFVSQDMADFAIL
$P2Y_1$	242-258	VRALIYKDLDNSPLRRKS
$P2Y_2$	227-244	KPAYGTTGLPRAKRKSVR
$P2Y_4$	337-350	HEESISRWADTHQD
$P2Y_6$	283-298	YKGTRPFASANSVLDP

secondary antibody was a biotinylated donkey anti-rabbit immunoglobulin G (IgG) serum (Jackson Immunoresearch, Luton, UK) used at a dilution of 1:500 for 30 minutes, followed by the ExtrAvidin peroxidase conjugate (Sigma-Aldrich Co., Ltd., Poole, UK) at 1:1000 for 30 min.

Sections bound with ExtrAvidin peroxidase were incubated with nickel-intensified 3,3'-diaminobenzidine (DAB) for 5 minutes. The solution contained 0.05% DAB, 0.04% nickel ammonium sulphate, 0.2% β-D-glucose, 0.004% ammonium nitrate, and 1.2U/ml glucose oxidase (all from Sigma-Aldrich Co., Ltd., Poole, UK). Sections were then washed in excess PBS, dehydrated in 2 washes of isopropyl alcohol and mounted in Eukitt (VWR International Ltd, Poole, UK).

2.1.4 Immunofluorescent technique

Slide-mounted sections were fixed for 2 minutes in 4% formaldehyde diluted with phosphate-buffered saline (PBS). Slides were then washed three times 5 minutes with excess PBS. Non-specific protein binding sites were blocked by 20 min incubation with 10% normal horse serum (NHS) (Invitrogen Ltd., Renfrew, UK) in PBS containing 0.05% thimerosal (Sigma-Aldrich Co., Ltd., Poole, UK). Sections were then incubated with the P2 receptor primary antibodies overnight at room temperature. The secondary antibody was either streptavidin-conjugated fluorescein-isothiocyanate (FITC) (ICN, Biomed, CA), or a cy3 labelled anti-rabbit IgG (Abcam Ltd., Cambridge, UK). The former gives green fluorescence and the latter red. Slides were then mounted in citifluor (Citifluor Ltd, Leicester, UK) and examined immediately.

2.1.5 Light and fluorescence Microscopy

Slides were examined using the Zeiss Axioplan light/fluorescent microscope (Carl Zeiss International, Göttingen Germany) and the images were captured using a Leica DC200 digital camera (Leica Microsystems, Wetzlar, Germany).

2.2 Detection of P2 receptor mRNA by reverse-transcription polymerase chain reaction (RT-PCR)

The reverse-transcription (RT) polymerase chain reaction (PCR) is a sensitive method for detection of mRNAs in cells and tissues.

2.2.1 Extraction of RNA

Precautions against contaminating samples with RNAses were observed. The bench working area was wiped with RNAse Zap (Ambion Ltd., UK), disposable gloves were worn at all times, glassware and the pestle and mortar were baked at 150°C for 4 hours prior to use and sterile disposable plasticwear and pipettes reserved for RNA work only were used. Fresh whole kidney was snap frozen in liquid nitrogen and ground to powder using a cold pestle and mortar. One hundred mg of powdered tissue was resuspended in 1ml of TRIzol® reagent (Invitrogen Ltd., Renfrew, UK) and passed through a pipette several times to ensure a homogeneous suspension. RNA was extracted using TRIzol®/chloroform extraction and isopropyl alcohol precipitation. Tissue samples were incubated in TRIzol reagent for five minutes at room temperature to allow complete dissociation of nucleoprotein complexes. Two hundred µl of chloroform were added and the samples were mixed thoroughly, incubated for 3 minutes and then centrifuged at 12000 x g for 15 minutes at 4°C. After

centrifugation, the upper aqueous phase containing the RNA was removed to a separate clean tube and the lower organic phase was discarded.

RNA was precipitated from the aqueous phase by addition of 500µl of isopropyl alcohol (Sigma-Aldrich Co., Ltd., Poole, UK), incubated at room temperature for 10 minutes and then centrifuged at 12000 x g for 10 minutes (4°C). The resulting RNA pellet was washed with 75% ethanol and centrifuged at 7500 x g for 5 minutes (4°C). The final pellet was air dried and resuspended in RNAse free distilled water (Promega UK Ltd., Southampton, UK). RNA concentration and purity were determined by measuring the absorbance of RNA in water at 260 nm using a spectrophotometer (Beckman DU 650 Spectrophotometer, High Wickam, Bucks. UK). RNA concentration was calculated using the formula A_{260} * 40 = µg RNA/ml. In distilled water, the RNA had an $A_{260/280}$ ratio of 1.9 – 2.1 indicating RNA free of contamination. RNA samples were stored at -80°C until further use.

2.2.2 Synthesis of copy DNA (cDNA)

One 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 (Invitrogen Ltd., Renfrew, UK). The reaction buffer contained 20mM Tris-HCL (pH 8.4), 50mM KCl, 500µM each of dATP, dCTP, dGTP, dTTP, 5mM Dithiothreitol, 40 units of RNaseOUT™ Recombinant Ribonuclease Inhibitor and 50 units of superscript™ II reverse transcriptase in a 20µl reaction volume. The reaction was incubated for 50 minutes at 42°C in a Hybaid sprint thermocycler (Thermo Electron Molecular Biology, USA). To inactivate the RT enzyme samples were heated to 70°C for 10 minutes, storage was at -20°C.

2.2.3 Polymerase chain reaction (PCR)

Copy DNA transcripts were used as a template with the PCR Core System I (Promega UK Ltd., Southampton, UK). Each PCR reaction contained 5.0pmol of forward primer, 5.0pmol of reverse primer 1.5mM $MgCl_2$, 500µM each of dATP, dCTP, dGTP, dTTP, 0.5 units of *Taq* polymerase and 1xPCR buffer in a 20µl reaction. The cycling parameters were initial denaturing at 95°C for 3 minutes, unless stated 30 cycles of denaturing at 95°C for 30 seconds, annealing for 1 minute and extension at 72°C for 1 minute, followed by a final extension step at 72°C for 5 minutes using a Hybaid PCR Sprint thermal cycler (Hybaid, Middlesex, UK). See Table 2.2 for primer sequences, annealing temperatures and expected product sizes. The primers were synthesised by Sigma-Genosys Ltd. (Poole, Dorset, UK) according to the specified sequences. The house keeping genes β-Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed to incorporate areas of sequence that were highly conserved between species of rat, mouse and human. Primer specificity was determined by performing a BLAST search where a primer sequence can be checked against a database of all published genomic sequences (web address: http://www.ncbi.nlm.nih.gov/blast/).

2.2.4 Agarose gel electrophoresis

Resulting PCR products were resolved on a 2% (w/v) agarose gel in TAE buffer (Tris-Acetate EDTA) containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich Co., Ltd., Poole, UK). PCR bands were observed under ultraviolet illumination and images were captured using Fluor-s Multiimager (Bio-Rad, Hemel Hempstead, Herts, UK) imager. PCR products produced a single clear

Table 2. 2 **Primer sequences, annealing temperature (Tm) and expected product sizes for gene specific primers used in this study**

Gene	Accession number		5' to 3' Sequence	Tm (°C)	Size (base pairs)
Rat $P2Y_1$	NM_012800	F	ACGTCAGATGAGTACCTGCG	58	289
		R	CCCTGTCGTTGAAATCACAC		
Rat $P2Y_2$	XM_346560	F	ACTTTGTCACCACCAGCGTGAG	58	279
		R	TGACGTGGAAAGGCAGGAAG		
Rat $P2Y_4$	Y11433	F	TGTTCCACCTGGCATTGTCAG	58	294
		R	AAAGATTGGGCACGAGGCAG		
Rat $P2Y_6$	NM_057124	F	TGCTTGGGTGGTATGTGGAGTC	56	339
		R	TGGAAAGGCAGGAAGCTGATAAC		
Rat $P2X_5$	X97328	F	TGTCATTCCATCTCAGGGGG	58	286
		R	TTCGGCATCCTTTAGAAGGG		
Rat $P2X_7$	X95882	F	GTGCCATTCTGACCAGGGTTGTATAAA	58	353
		R	GCCACCTCTGTAAAGTTCTCTCCGATT		
β-Actin	BC002409.2	F	ACCTTCAACACCCCAGCCATGTACG	65	698
		R	CTGATCCACATCTGCTGGAAGGTGG		
GAPDH	BC014085	F	GCCATCAATGACCCCTTCAT	54	281
		R	GAGGGGGCAGAGATGATGAC		
HPRT	XM343829.1	F	GCTGACCTGCTGGATTACATTA	60	410
		R	CCACTTTCGCTGATGACACAA		

band of the expected size and this was confirmed by sequencing performed by Oswel DNA Sequencing Laboratories (Southampton, UK). In all experiments, the presence of possible contaminants was investigated using control RT-PCR assays of samples in which either RNA had been excluded (blank) or reverse transcriptase had been omitted from the RT mixture.

2.3 Quantitative analysis of P2 receptor mRNA using real-time PCR

Real time PCR allows continuous monitoring of PCR product information. Fluorescent double-stranded DNA dyes such as SYBR® Green I can be used to monitor PCR product accumulation after each cycle of amplification (Wittwer *et al.*, 1997). Real time PCR can be conducted either as a one- or a two step reaction. Two step RT-PCR, in which cDNA is produced in a separate reaction tube to the PCR, has the advantage over one step RT-PCR, in which the reverse transcriptase and the PCR are conducted in the same reaction tube. The cDNA template is more stable than RNA and there is often enough cDNA to perform a number of subsequent PCR reactions. Moreover, since the cDNA reaction product used as a starting template for the PCR is the same for both the control gene and for the gene of interest this eliminates variations due to fluctuations in efficiency of the reverse transcriptase step. For these reasons, the two step protocol for real time PCR was used.

2.3.1 Extraction of messenger RNA for real-time PCR

RNA was extracted by TRIzol®/chloroform extraction and isopropyl alcohol precipitation as described in section 2.2.1. Messenger RNA was isolated using oligo(dT) coated magnetic beads (PolyATract, Promega UK Ltd.,

Southampton, UK). The system uses a biotinylated oligo(dT) primer to hybridize at high efficiency in solution to the 3′ poly(A) region present in most mature eukaryotic mRNA species. The oligo(dT)-mRNA hybrids were captured using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The particles were captured against the magnetic stand against the wall of the eppendorf tube, and washed at high stringency using sodium chloride-sodium citrate (SSC) buffer (consisting of 0.6 M NaCl and 60 mM Na_3 citrate). The mRNA was eluted from the solid phase by the addition of ribonuclease-free deionized water.

2.3.2 Synthesis of cDNA for real-time PCR

An amount equivalent to 1μg of total RNA was reverse transcribed with 0.5μg oligo(-dt) 12-18 primer and a first-strand cDNA synthesis kit, (Superscript II RNase H^- reverse transcriptase, Invitrogen Ltd., Renfrew, UK) as described in section 2.2.2.

2.3.3 Real-time PCR amplification

The resulting cDNA transcripts of whole kidney mRNA were used for PCR amplification using the Roche Lightcycler (Roche diagnostics, Penzberg, Germany) and QuantiTect SYBR® Green PCR kit (Qiagen, Crawley, UK). QuantiTect SYBR® Green I PCR master mix contains SYBR® Green I, HotStarTaq DNA Polymerase, and a dNTP mix. SYBR® green I, present in the PCR mix, only emits light when bound to double-stranded DNA, once bound it is excited at 494 nm and emits light at 521nm. The lightcycler fluorimeter monitors emissions at 521nm, and values are recorded by a computer (Dell Computers, Bracknell, UK). Each PCR reaction mix contained 1.0 μl cDNA template, 5.0

pmol forward and 5.0 pmol reverse primers, 10 µl 2 x QuantiTect SYBR® Green I PCR master mix and distilled water to a final volume of 20µl. For primer sequences see Table 2.2.

2.3.4 Real-time PCR cycling parameters

The hot start Taq polymerase present in the SYBR® Green I PCR master mix significantly reduces non-specific priming and the formation of primer dimers. The PCR cycling conditions were initial denaturation of 95°C for 15 minutes, denaturation at 94°C for 15 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 30 seconds. The temperature of fluorescence acquisition was set at 78°C for 5 seconds, 20°C below the product melting temperature. The product melting temperature was determined in a test run by examining the melting curve (described in section 2.3.7). All experiments were performed in duplicate and for each sample the gene of interest and the control gene were run in parallel. A ratio of relative abundance of the gene of interest to the constitutively expressed gene hypoxanthine phosphoribosyl transferase (HPRT) was calculated by the Lightcycler Relative Quantification software version 1.0 (RelQuant) (Roche Diagnostics, Penzberg, Germany). HPRT was chosen as a control gene because its expression level is similar to the genes of interest (P2 subtypes). The house keeping genes β-Actin and GAPDH were both considered but rejected since they had higher expression levels than the P2 receptor genes which could lead to false negative results for low abundance mRNAs (Serazin-Leroy *et al.*, 1998).

2.3.5 The second derivative maximal method

The lightcycler software version 3.5 (Roche diagnostics, Penzberg, Germany) calculates the PCR cycle at which the maximal increase in fluorescence occurs in the log/linear phase of cycling. This is known as the second derivative maximum method, and the cycle at which this occurs can be, and is usually different for each sample. The cycle number at which maximal increase in fluorescence occurs is compared to that of standards with known concentration of PCR product using RelQuant software (Roche Diagnostics, Penzberg, Germany). A standard curve for each gene was prepared with PCR products in which the concentration has been determined by spectroscopy.

2.3.6 Preparation of standard curves

PCR products (dsDNA) for each gene were separated on a 2% agarose-TAE gel and purified using a Geneclean kit (Qbiogene, Cambridge, UK). 300mg of gel containing the PCR band of interest was excised from the agarose gel and placed in 400 µl glassmilk (silica beads in high salt solution). DNA binds to silica in high salt solutions although the mechanism for this has not been fully described (Vogelstein & Gillespie, 1979). The glassmilk-DNA solution was heated at 55°C for five minutes to melt the agarose gel. After cooling, the mixture was placed in a GENECLEAN spin filter column and centrifuged at 13,000 x g for 1 minute. DNA was retained by the membrane-filter and the supernatant was discarded. The membrane-filter was washed twice with wash buffer and dried by centrifuging for a further 2 minutes. DNA was eluted with 10-25µl distilled water and centrifuged at 13,000 x g for 1 minute. The concentration of each PCR product DNA was determined using a

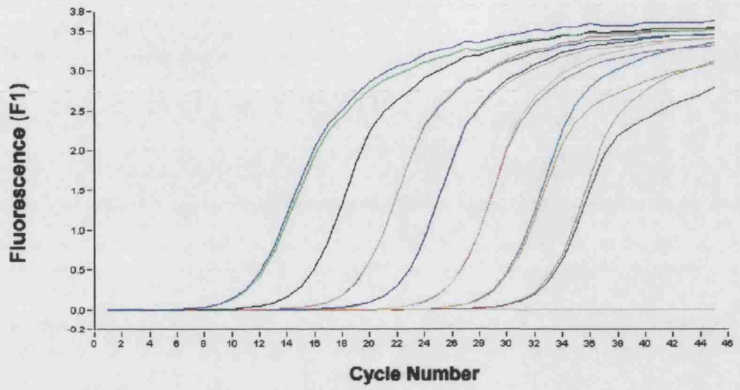
spectrophotometer (Beckman DU 650 Spectrophotometer, High Wickam, Bucks. UK), and calculated using the formula A_{260} * 50 = μg DNA/ml. Purified DNA was serially diluted 10- fold covering a dynamic range of 7 logarithmic orders. 1.0 μl of each standard dilution was amplified by PCR using the lightcycler and specific primers. One set of gene of interest standards were run in duplicate with the HPRT standards to generate two standard curves.

2.3.7 Analysis of the melting curve

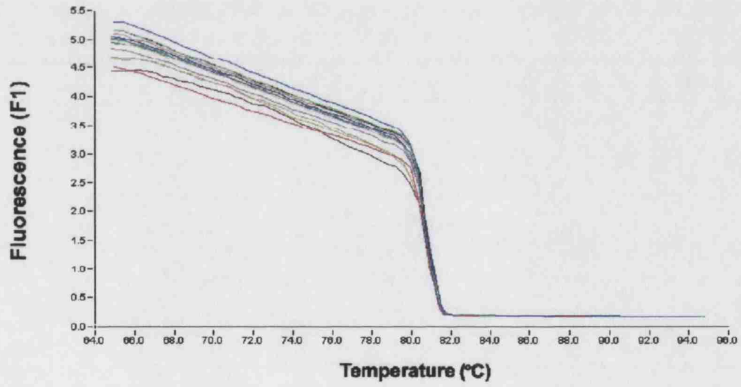
After completion of the PCR amplification cycles, a melting curve was determined for each PCR product. Each dsDNA has its own melting temperature (Tm) based on the strand length and the G-C content. Therefore melting curve analysis can be used to identify unwanted by-products of the PCR reaction such as primer dimer. The PCR products were heated to 65°C and then the temperature was increased slowly (0.5°C per second) whilst fluorescence was continually monitored. At low temperatures all DNA is double stranded, SYBR green I binding and fluorescence is maximal. As the temperature is increased, DNA products are denatured, and the fluorescence decreases. A melting curve is produced by plotting fluorescence against temperature. Primer dimer and other short non-specific products can be distinguished using this method, since they usually melt at lower temperatures than the desired product. Increasing the fluorescence acquisition temperature used during PCR to above the melting temperature of primer-dimer and non specific products eliminates any fluorescence due to the presence of these products. PCR products were also analyzed by gel electrophoresis as described in section 2.2.4 and visualized using a Bio-Rad multi-imager (Bio-Rad, Hemel Hempstead, Herts, UK).

Figure 2. 1 **Represenative graphs showing light cycler fluorescence emission data for a standard curve and a melting curve**

A



B



A standard curve can be generated by performing real-time PCR amplification of serially diluted purified PCR product DNA (A). The log concentration of purified DNA was plotted against the cycle number at which the greatest increase in fluorescence was detected. PCR product melting curves (B) were generated by gradual heating to 95°C with continued monitoring of fluorescence. At the melting temperature of the PCR product, there is a steep decrease in fluorescence emission since the dsDNA is denatured.

2.4 Detection of P2 receptor protein by immunoblotting

2.4.1 Preparation of protein samples

Fresh whole kidney was snap frozen in liquid nitrogen and ground to powder using a cold pestle and mortar. One hundred mg of powdered tissue was resuspended in ice-cold ripa buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1mM EDTA) containing 10% protease inhibitors (Sigma-Aldrich Co. Ltd., Poole, UK). Cell debris was removed from kidney homogenates by centrifugation at 12000 x g for 5 minutes and protein concentration was determined using the BCA™ (bicinchoninic acid) protein Assay (Perbio Science UK Ltd., Cheshire, UK). A standard curve was prepared by diluting bovine serum albumin (2.0 mg/ml) to achieve final concentrations of 0, 25, 125, 250, 500, 750 and 1000 µg/ml using ripa buffer as a diluent. To prepare the BCA™ protein assay working reagent, 50 parts of reagent A were combined with 1 part of reagent B. 50 µl of sample or standards were added to 1.0ml of BCA™ working reagent and after vortexing, incubated at 37°C for 30 minutes. The standards or the samples were placed in plastic cuvettes, and the absorbance at 562nm was determined using a spectrophotometer (Beckman DU 650 Spectrophotometer, High Wickam, Bucks. UK). The standard curve was prepared and the protein concentration of the samples was determined by extrapolating from the graph.

2.4.2 SDS-PAGE

Sodium dodecylsulphate -Polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmili (Laemmli, 1970) using 1 mm thick 10% slab gels. All reagents were of electrophoresis grade from Bio-Rad

laboratories, Hemel Hempstead, Herts, UK. The SDS-PAGE gel consisted of a stacking gel overlaid onto a separating gel. The stacking gel comprised of 5% of 30% Acrylamide/Bis solution (ratio 37.5:1) in a buffer of 0.125 M Tris-HCl, pH 6.8, containing 0.1% (w/v) SDS and polymerisation was initiated by addition of 0.1% (w/v) ammonium persulphate and 0.05% (v/v) tetramethylethylenediamine (TEMED). The separating gel comprised of 10% of a 30% acrylamide/Bis solution (ratio 37.5:1) in a buffer of 0.375M Tris-HCl, pH 8.8 with 0.1% SDS. The gel was polymerised by addition of 0.1% (w/v) ammonium persulphate and 0.016% (v/v) TEMED.

After determination of protein concentration, 10μg of protein homogenate was diluted with an equal volume of sample buffer (0.125M Tris-HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, 0.6M β-mercaptoethanol and 0.02% Bromophenol blue) and were heated at 40°C for 30 minutes. Ten μl aliquots of the protein samples containing 10 μg protein were loaded into each lane of the 1.0 cm stacking gel, and then proteins were resolved by the 6.0 cm separating gel. The electrode buffer comprised 0.025M Tris, 0.192 glycine and 0.1% (w/v) SDS, pH 8.3. Electrophoresis was conducted at 40mA (constant current) for 1 hour using a Bio-Rad mini-gel system.

2.4.3 Electro-blotting

Semi-dry electroblotting was performed on a Bio-Rad Trans-Blot SD semi-dry transfer cell in which the electrode plates are in direct contact with filter paper as buffer reservoirs. Filter paper and membranes were trimmed to match the dimensions of the gel and equilibrated in transfer buffer (Towbin buffer consisting of 25mM Tris, 192 mM glycine, 20% methanol, pH 8.1-8.5). The

blotting assembly from anode to cathode contained a filter paper sheet (Bio-Rad, Hemel Hempstead, Herts, UK), the gel, poly(vinylidene difluoride) (PVDF) membrane and a second filter paper. Small-pore PVDF membrane (0.2 μm; from Bio-Rad Hemel Hempstead, Herts, UK) was used throughout for this study. The electroblotting was carried out at constant current (100mA for 2 gels) for 1.5 hour using (ECPS 3000/150 Pharmacia Power supply).

After transfer, the membrane blots were washed in distilled water (3x1 minute) and incubated in 10% glutaraldehyde (VWR International Ltd, Poole, UK) overnight. The membranes were washed in distilled water (3x5 minute) and, to block non-specific protein binding sites, incubated with 5% (w/v) non-fat dry milk (Marvel, Cadbury's, Birmingham, UK) in PBS/Tween 20 (0.1%) overnight at 4°C with gentle agitation. The PVDF membrane was incubated overnight at room temperature with the rabbit affinity-purified antibody raised against P2 receptor subtypes (Table 2.1). The membrane was washed 1x15 minutes and then a further 3x5 minutes with PBS/TWEEN 20. After the primary antibody incubation, membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (Amersham Biosciences, Bucks, UK) for 1 hour before employing the Enhanced Chemiluminescent (ECL) western blotting detection system (Amersham Biosciences, Bucks, UK). The bands were analysed using Fluor-s Multi-imager (Bio-Rad, Hemel Hempstead, Herts, UK).

All immunohistochemistry and molecular biology data presented in this thesis is representative of at least three replicates.

Chapter 3

The pattern of distribution of selected ATP-sensitive P2 receptor subtypes in normal rat kidney

3.1 Introduction

There is increasing interest in the potential role of P2 receptors in normal and abnormal renal function, and several studies have attempted to identify the P2X and P2Y receptor subtypes expressed in mammalian kidney (Paulais *et al.*, 1995;Bailey *et al.*, 2000b;Deetjen *et al.*, 2000;Bailey *et al.*, 2001;White *et al.*, 2001). However, until now, such studies have relied largely on measurements of altered intracellular calcium concentration in response to ATP (as a functional index of P2Y receptor activation), as well as analysis of P2Y receptor mRNA expression (Bailey *et al.*, 2000b;Bailey *et al.*, 2001), with only limited immunohistochemical studies of P2 receptor protein localisation, such as $P2X_1$ (Chan *et al.*, 1998a;Hansen *et al.*, 1999).

The present study was to investigate the P2 receptor distribution in normal rat kidney, using immunohistochemistry and the P2 receptor antibodies currently available in conjunction with RT-PCR using gene specific primers for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors. Taken together with previous functional studies (Bailey *et al.*, 2000b;Bailey *et al.*, 2001), this was intended to provide a more complete picture of the distribution of purinergic P2 receptors in specific structures of the normal rat kidney.

3.2 Methods

3.2.1 Preparation of samples

Procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986. Briefly, 250 g body weight Sprague-Dawley rats were killed by intra-peritoneal injection of 90 mg/kg Sagatal and the left

kidneys removed. Tissue was embedded in OCT compound (BDH/Merck, Leicester, UK) sectioned at 8 μm as described in Chapter 2 section 2.1.1.

3.2.2 Localisation of P2 receptor subtypes by immunohistochemistry

The avidin-biotin technique was used as described previously in section 2.1.3 with P2 receptor antibodies listed in table 2.1. In the case of fluorescent microscopy, the secondary antibody was either streptavidin-conjugated fluorescein-isothiocyanate (FITC) (ICN, Biomed, CA), or a cy3 labelled anti-rabbit IgG (Abcam Ltd., Cambridge, UK). The former gives green fluorescence and the latter red.

In the case of double-labelling experiments, marker antibodies (listed in table 3.1) were detected with FITC labelled anti-mouse IgG. For double-labelling experiments in which both antibodies were raised in the same species, further steps were required to ensure prevention of cross-reactivity. P2 receptor antibodies were detected by tyramide signal amplification (Renaissance, TSA indirect, NEN, USA), using antibody concentrations below the detection limit of a fluorophore-coupled secondary antibody. After incubation overnight with the first antibody, the layers of secondary antibody were: biotinylated donkey anti-rabbit IgG (Jackson immunoresearch, Luton, UK) and ExtrAvidin peroxidase (Sigma Aldrich Co. Ltd., Poole, UK). The tyramide signal amplification solution was applied for 8 minutes and the final layer was streptavidin fluorescein (Amersham Biosciences, Bucks, UK), which emits green fluorescence. Finally, sections were incubated overnight with the second antibody and then detected with donkey anti-rabbit Cy3 (red fluorescence).

Table 3. 1 **Specific antibodies used as markers for identification of renal cell types**

Antibody used	**Immunopositive cell type**	**Source**
Anti-aquaporin-1 (AQP1)	Thin descending limb	Abcam Ltd., Cambridge, UK
Anti-aquaporin-2 (AQP2)	Principal cells of the collecting duct	Gift from Dr. D. Marples
Anti-CLC-K1	Thin ascending limb	Alomone Laboratories Ltd., Jerusalem, Israel
Anti-ecto-5'-nucleotidase	Peritubular fibroblasts	Gift from Dr. J. Sévigny
Anti-smooth muscle actin	Vascular smooth muscle	Sigma Aldrich Co. Ltd., Poole, UK
Anti-Tamm Horsfall protein	Thick ascending limb	Biogenesis Ltd., Poole, UK
Anti-Thymocyte-1 (Thy-1)	Mesangial cells	Abcam Ltd., Cambridge, UK
Anti-Wilms-Tumour-1 (WT-1)	Podocytes	Santa Cruz Biotechnlogy, CA, USA

3.2.3 Identification of P2Y receptor mRNA transcripts by reverse-transcriptase polymerase chain reaction

RNA was extracted from whole kidney using TRIzol/chloroform extraction and isopropyl alcohol precipitation as described in section 2.2.1. The final pellet was air dried and resuspended in RNAse free distilled water. RNA concentration and purity was determined by spectrophotometry. RT-PCR was carried out on a Hybaid PCR Sprint thermal cycler (Hybaid, Middlesex, UK). Messenger RNA samples extracted from microdissected tubules were a gift from Dr Martine Imbert-Teboul (CEA Saclay, Gif sur Yvette, France). 100 pg of mRNA was reverse transcribed for 50 minutes at 42°C with 0.5 µl of oligo$(dT)_{12-18}$ using a first-strand cDNA synthesis kit for RT-PCR (Superscript II RNase H^- reverse transcriptase, Invitrogen Ltd., Renfrew, UK). Negative controls were carried out in the absence of reverse transcriptase. 50% of the resulting cDNA template or negative control was used for PCR amplification with PCR Core System I (Promega, Southampton, UK) using gene specific primers (sequences are listed in Table 2.2).

Whole kidney and microdissected tubule PCR products were resolved on a 2% (w/v) agarose gel containing 10µg/ml ethidium bromide and visualised under ultraviolet illumination. Images were captured using a Fluor-S Multi-Imager (Bio-Rad, California). The amount of mRNA was standardised between samples using the housekeeping genes GAPDH and β-Actin.

3.3 Results

The present study reports the distribution of $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$, $P2Y_1$, $P2Y_2$ $P2Y_4$, and $P2Y_6$ receptor proteins in normal rat

kidney. However, suitable commercial antibodies for $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ were not available. RT-PCR was used to detect mRNA with primers for the rat $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors in microdissected tubules and glomeruli.

3.3.1 Identification of P2 receptors in the renal vasculature

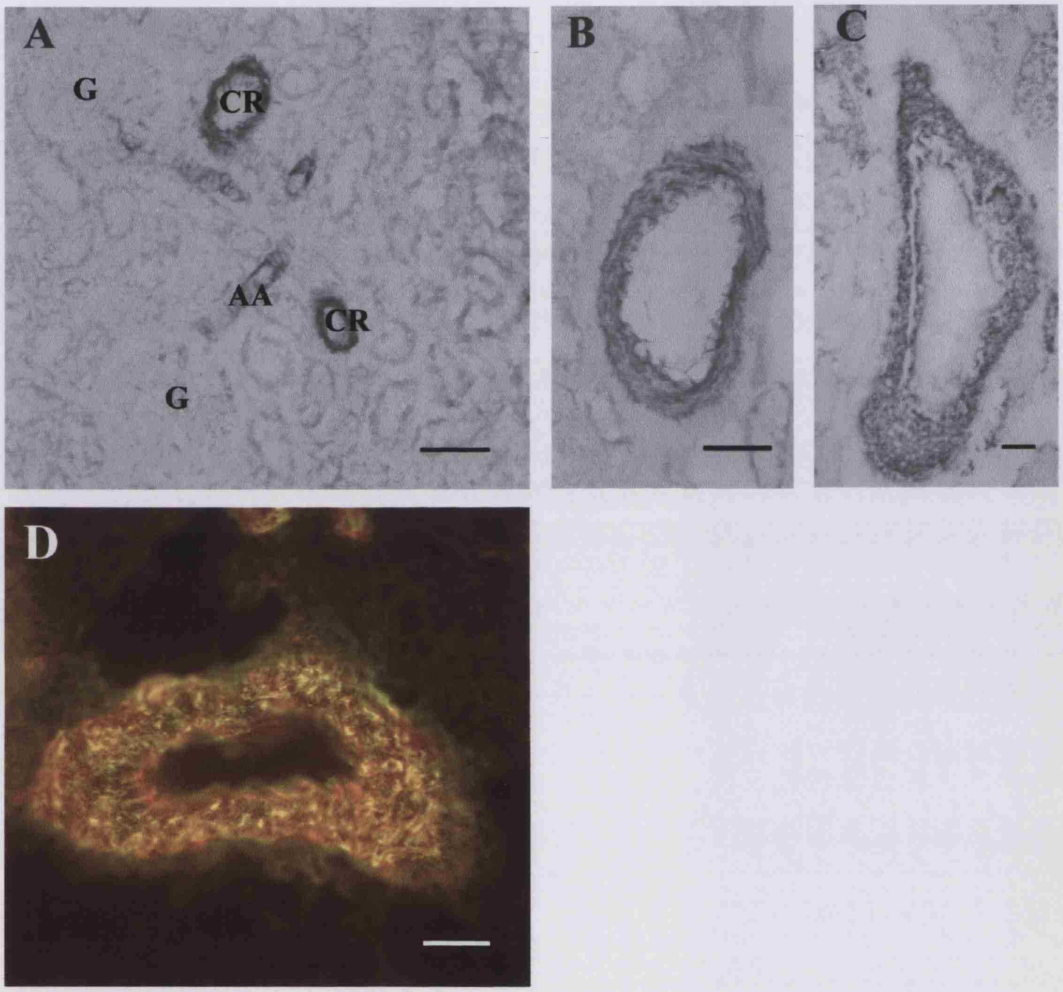
$P2X_1$ receptor immunoreactivity was found to have a widespread distribution in the smooth muscle cells of the renal vasculature, including the renal artery, arcuate and cortical radial arteries and afferent arteriole (Figure 3.1A). The localisation of $P2X_1$ receptors on the renal vasculature has been reported previously (Chan *et al.*, 1998a). Similarly, $P2X_2$ receptor immunoreactivity was also seen on the smooth muscle cells of the renal vasculature, but was predominantly found in larger intrarenal arteries and veins (Figure 3.1B). No other P2X subtype tested immunolocalised to the vasculature. $P2Y_1$ receptors were extensively distributed throughout the smooth muscle layer of intrarenal arteries and veins, including both the afferent and efferent arterioles (not shown) and the interlobar artery (Figure 3.1 C). The smooth muscle layer distribution of $P2X_1$, $P2X_2$ and $P2Y_1$ receptors was confirmed by double labelling with anti-smooth muscle actin (Figure 3.1 D).

3.3.2 Identification of P2 receptors in the glomerulus

Immunoreactivity for $P2Y_1$ and $P2Y_2$ receptors was localised to the glomeruli. A typical mesangium-like staining pattern was seen with many mesangial cells immunopositive for $P2Y_1$ (Figure 3.2 A and Figure 3.2 C), which was confirmed by double labelling with anti-Thymocyte-1 (Thy-1), a well-characterized mesangial cell marker (Figure 3.2 F). $P2Y_1$ receptor antibodies did

not co-localize with anti-endothelium (Ox43) or anti-Wilm's Tumour 1 (WT-1, for podocytes). $P2Y_1$ receptor immunoreactivity was also seen on the peritubular and periglomerular fibroblasts and was confirmed by double labelling with anti-ecto 5'- nucleotidase (Figures 3.2 C-E). $P2Y_1$ positive fibroblasts were distinguished from peritubular dendritic cells using anti-MHCII. Immunoreactivity for $P2Y_2$ receptors was detected on podocytes, as determined by double labelling with an antibody to the podocyte nuclear protein, WT-1 (Figure 3.2 G). $P2Y_2$ receptor antibodies did not co-localise with either anti-endothelium (Ox43) or anti-Thy-1 antibody staining. In some instances, a very low level of $P2X_7$ receptor immunoreactivity was detectable in a few glomeruli. $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptor mRNA was detected in microdissected whole glomeruli (Figure 3.3).

Figure 3. 1 **Photomicrographs showing P2 receptor expression on cells of the renal vasculature**



A. $P2X_1$ immunoreactivity on smooth muscle cells of the afferent arteriole (AA) and cortical radial artery (CR). G = glomeruli. (Scale bar = 50μm).

B. $P2X_2$ positive smooth muscle cells of the arcuate artery. (Scale bar = 50μm).

C. $P2Y_1$ immunoreactivity in the smooth muscle layer of an interlobar artery. (Scale bar = 50μm).

D. $P2Y_1$ immunoreactivity (red) co-localised with smooth muscle actin (green) in which areas of co-staining appear yellow. (Scale bar = 50μm).

Figure 3. 2 **Photomicrographs showing P2 receptor expression on cells of the glomerulus**

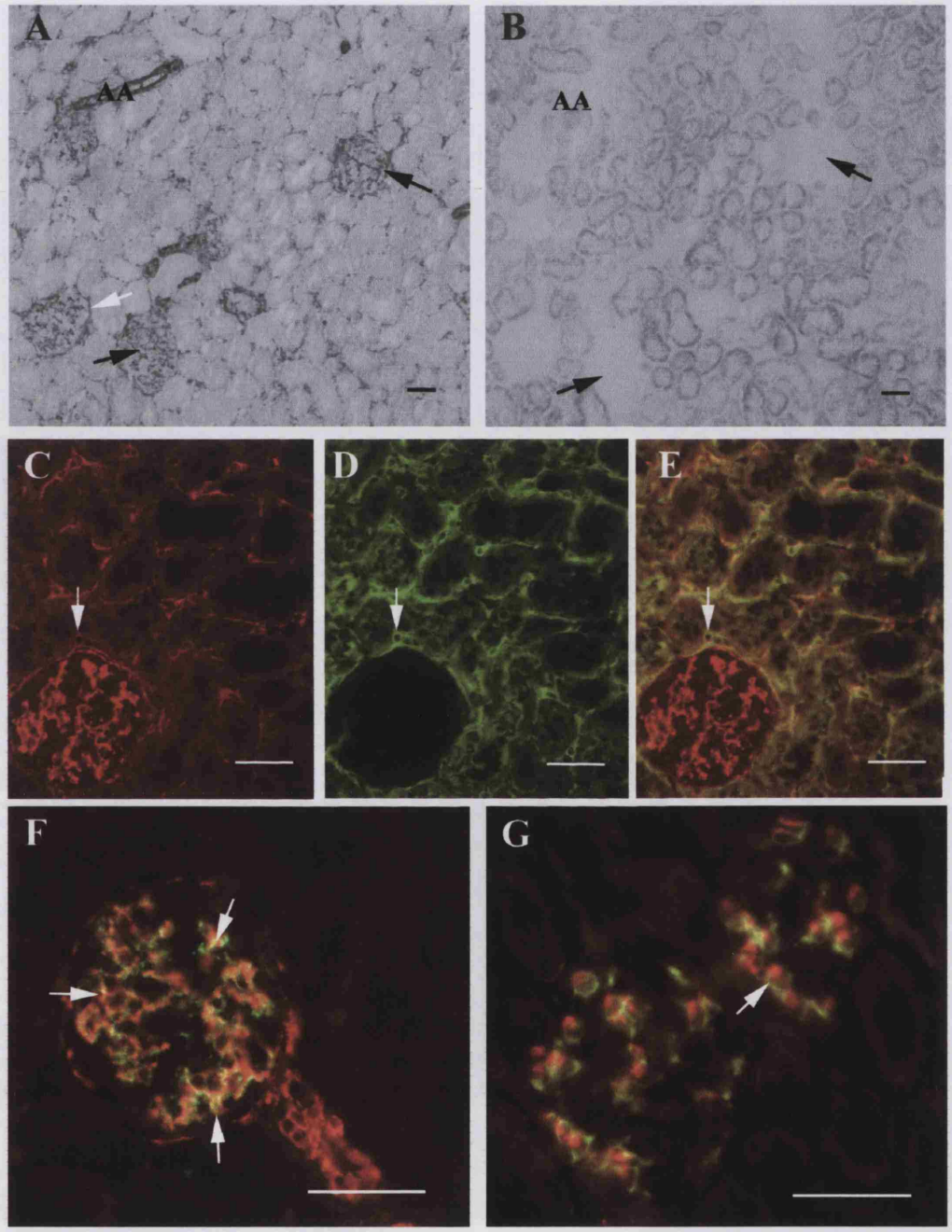


Figure 3.2 legend

A. $P2Y_1$ immunoreactivity in the glomerulus (black arrows), afferent arteriole (AA) and periglomerular fibroblasts (white arrow). (Scale bar = 50μm).

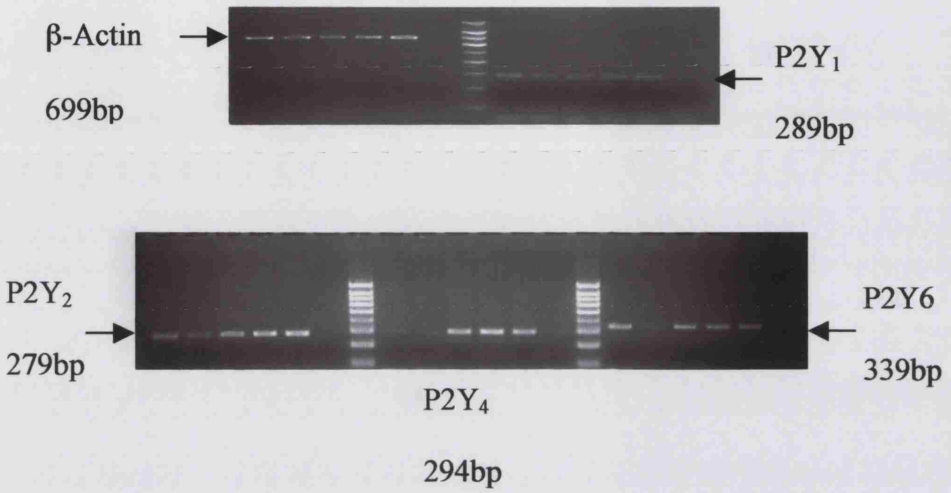
B. Corresponding section to Figure 2A pre-absorbed with excess $P2Y_1$ synthetic peptide eliminated immunoreactivity. (Scale bar = 50μm).

C, D, E. Peritubular and periglomerular fibroblasts immunopositive for $P2Y_1$ (red) and ecto-5'-nucleotidase (green), plate E is the overlay. Arrow indicates the same cell in plates C, D, E. Absence of immunostaining in the glomerulus on plate D confirms that there was no cross-reactivity between the same species antibodies. (Scale bar = 50μm).

F. A subpopulation of mesangial cells immunopositive for $P2Y_1$ (red) double labelling with anti-thy-1, a mesangial cell marker (green). (Scale bar = 50μm).

G. $P2Y_2$ immunoreactivity (green) in the cytoplasm of podocyte cells, identified by the podocyte nuclear protein WT-1 (red) (Scale bar = 50μm).

Figure 3. 3 **P2Y receptor subtype mRNA in microdissected glomeruli of five control rats**



The concentration of mRNA was standardized using the housekeeping gene β-Actin. $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ mRNA was present in the glomeruli of all the animals tested. The size of the expected PCR products are shown in base pairs (bp).

3.3.3 Identification of P2 receptors in the proximal tubule

$P2Y_4$ receptors immunolocalised to proximal convoluted tubule (PCT) cells and the positive staining was distributed in basolateral membranes (Figures 3.4 A and 3.4 B). This P2 receptor subtype was not seen in any other segment of the nephron. $P2Y_6$ receptor immunoreactivity was detected in the apical and basolateral membrane of proximal convoluted tubules (Figure 3.4 E). Apical membranes of the S3 segment in the outer medulla were immunopositive for $P2Y_1$ and $P2X_5$ receptors (Figure 3.4C and 3.8D-F respectively), which was confirmed by counter-staining with periodic acid Schiff (PAS) (Yabuki *et al.*, 1999). This reaction detects glycogenic mucopolysaccharides in the brush border and positivity is seen with a magenta colour reaction. $P2Y_1$ and $P2X_5$ immunoreactivity was not detected in the proximal convoluted tubule. The only other P2 subtypes that localized to the proximal tubule were $P2X_4$ and $P2X_6$ (Figure 3.4D); however, these receptors were expressed at a low level throughout the renal tubule. Messenger RNAs for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors were detected in the proximal tubule (Figure 3.5 and 3.6).

Figure 3. 4 **Photomicrographs showing P2 receptor expression in the renal cortex and outer medulla**

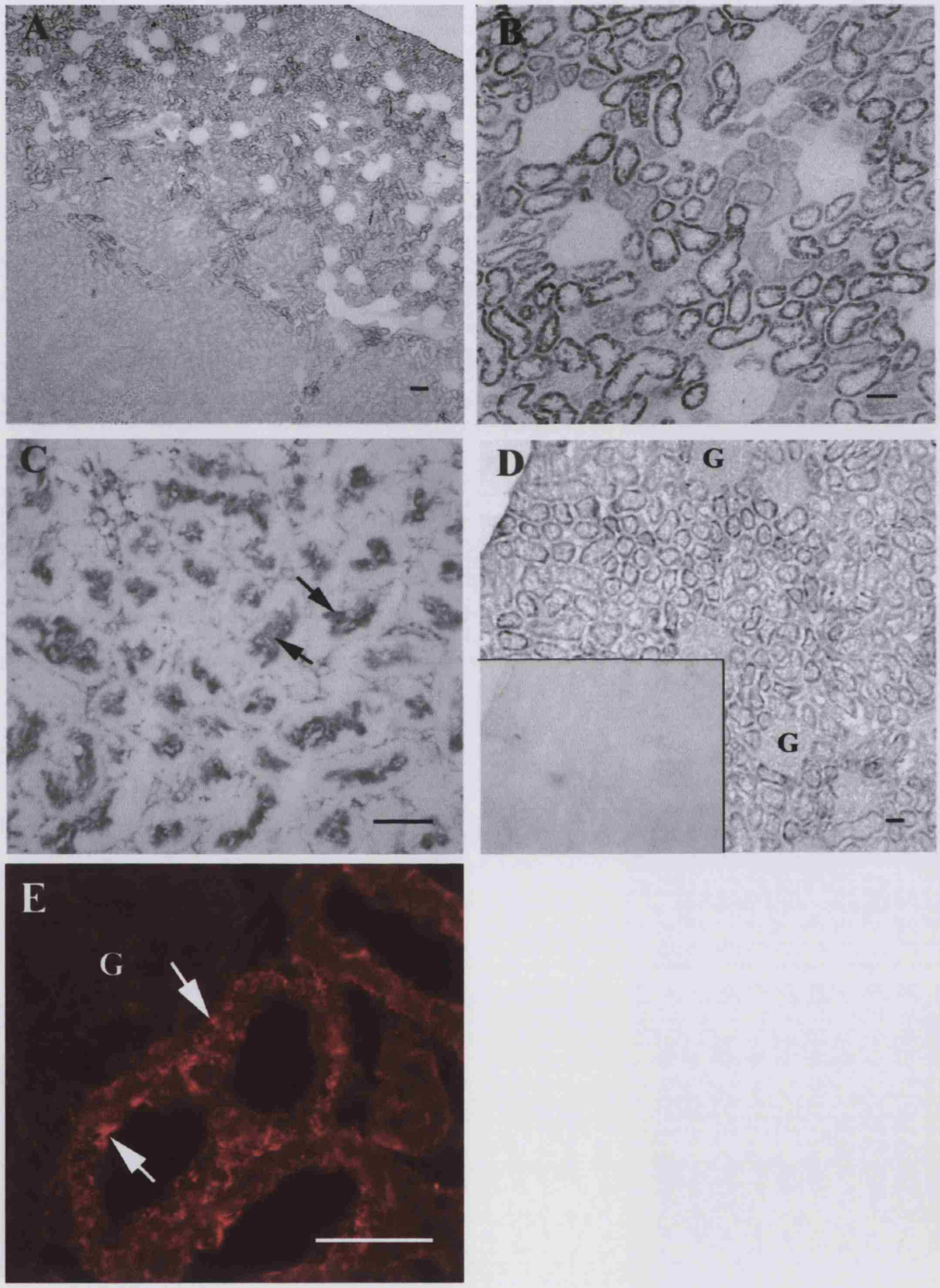


Figure 3.4 legend

A. Low magnification photomicrograph of the cortex showing immunoreactivity for $P2Y_4$ receptors in the basolateral membranes of proximal convoluted tubules. (Scale bar = 100μm)

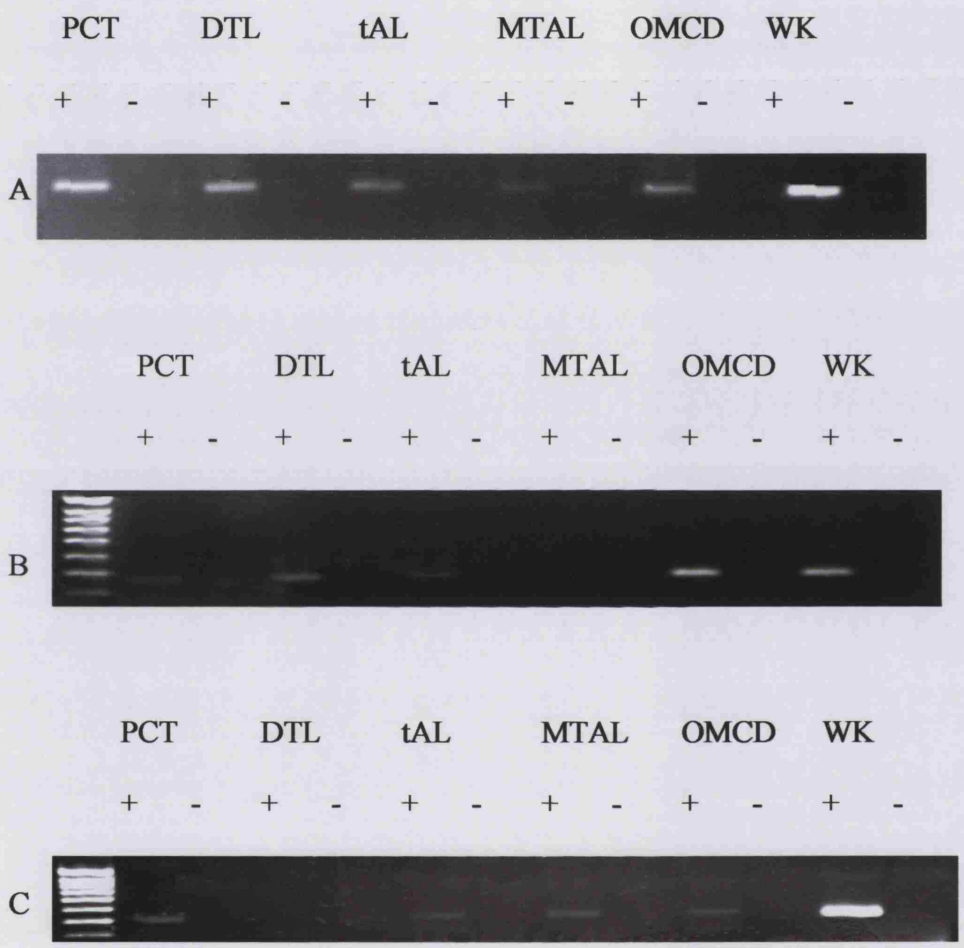
B. Higher magnification of the cortex showing $P2Y_4$ receptor immunoreactivity in the basolateral membranes of proximal convoluted tubules. (Scale bar = 50μm)

C. High magnification showing $P2Y_1$ immunoreactivity at the brush border of S3 segments in the outer stripe of the outer medulla. (Scale bars = 50μm).

D. Low-level expression of $P2X_6$ receptors in the basolateral membranes of renal tubules (Scale bar = 100μm). Insert – similar region of the renal cortex of the corresponding section pre-absorbed with excess $P2X_6$ synthetic peptide. G - glomerulus

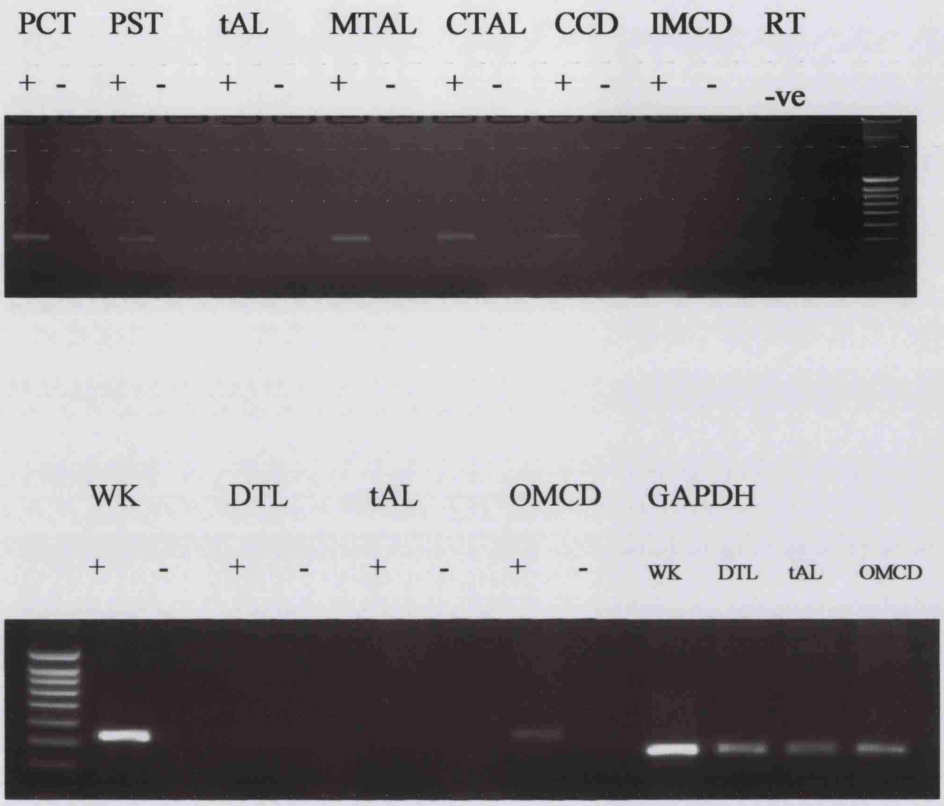
E. Apical and basolateral expression (arrows) of $P2Y_6$ receptors in the proximal convoluted tubule. G – glomerulus, (Scale bar = 50μm).

Figure 3. 5 **Example gels showing A - $P2Y_1$, B - $P2Y_2$ and C - $P2Y_4$ receptor mRNA transcripts in microdissected tubule segments of normal rat kidney**



RT-PCR experiments were carried out in the presence '+' or absence '-' of reverse transcriptase. (WK- whole kidney; PCT – proximal convoluted tubule; DTL – descending thin limb (of Henle); tAL – thin ascending limb; MTAL – medullary thick ascending limb; OMCD – outer medullary collecting duct).

Figure 3. 6 **Example gels depicting $P2Y_6$ receptor mRNA transcripts in microdissected tubule segments of normal rat kidney**



RT-PCR experiments were carried out in the presence '+' or absence '-' of reverse transcriptase. (WK- whole kidney; PCT – proximal convoluted tubule; PST – proximal straight tubule; DTL – descending thin limb (of Henle); tAL – thin ascending limb; CTAL – cortical thick ascending limb; MTAL – medullary thick ascending limb; CCD – cortical collecting duct; OMCD – outer medullary collecting duct; IMCD – inner medullary collecting duct).

3.3.4 Identification of P2 receptors in the loop of Henle

$P2Y_2$ receptors immunolocalised intracellularly to the thin ascending limb of Henle, this was confirmed by double labelling with anti-CLC-K1 (Figure 3.7 A-C). $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor mRNA transcripts were detected in this segment (Figure 3.5). $P2Y_2$ receptors were located intracellularly in cells of the thick ascending limb (Figure 3.7 D-F), where mRNA transcripts for $P2Y_2$ and $P2Y_6$ were detected in the cortical portion and $P2Y_1$, $P2Y_2$ $P2Y_4$ and $P2Y_6$ were detected in the medullary portion (Figure 3.5 and 3.6). The thin descending limb of Henle, identified using aquaporin-1, was negative for $P2Y_2$ receptors however, mRNA transcripts for $P2Y_1$ and $P2Y_2$ receptor subtypes was detected in this segment (Figure 3.5).

3.3.5 Identification of P2 receptors in the collecting duct

$P2Y_2$ receptors were located on the intercalated cells of the medullary (Figure 3.7 G), but not cortical collecting duct. Messenger mRNA transcripts for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors were detected in the outer medullary-collecting duct (Figures 3.5 and 3.6). $P2X_5$ receptors were most densely expressed on inner medullary collecting duct (IMCD) principal cells (Figure 3.8 A) with a gradual diminution of expression (Figure 3.8 B) towards the cortex (cortical collecting ducts, CCD), where there was minimal or no immunoreactivity (Figure 3.8 C). This was the most frequent staining pattern observed, but an alternative pattern was also observed, characterized by a lack of $P2X_5$ receptor expression on any part of the collecting duct (Figures 3.8 D-F) and $P2X_5$ expression only detected on the apical membrane of S3 segments. Of note

Figure 3. 7 **Photomicrographs showing P2 receptor expression in the loop of Henle and collecting duct**

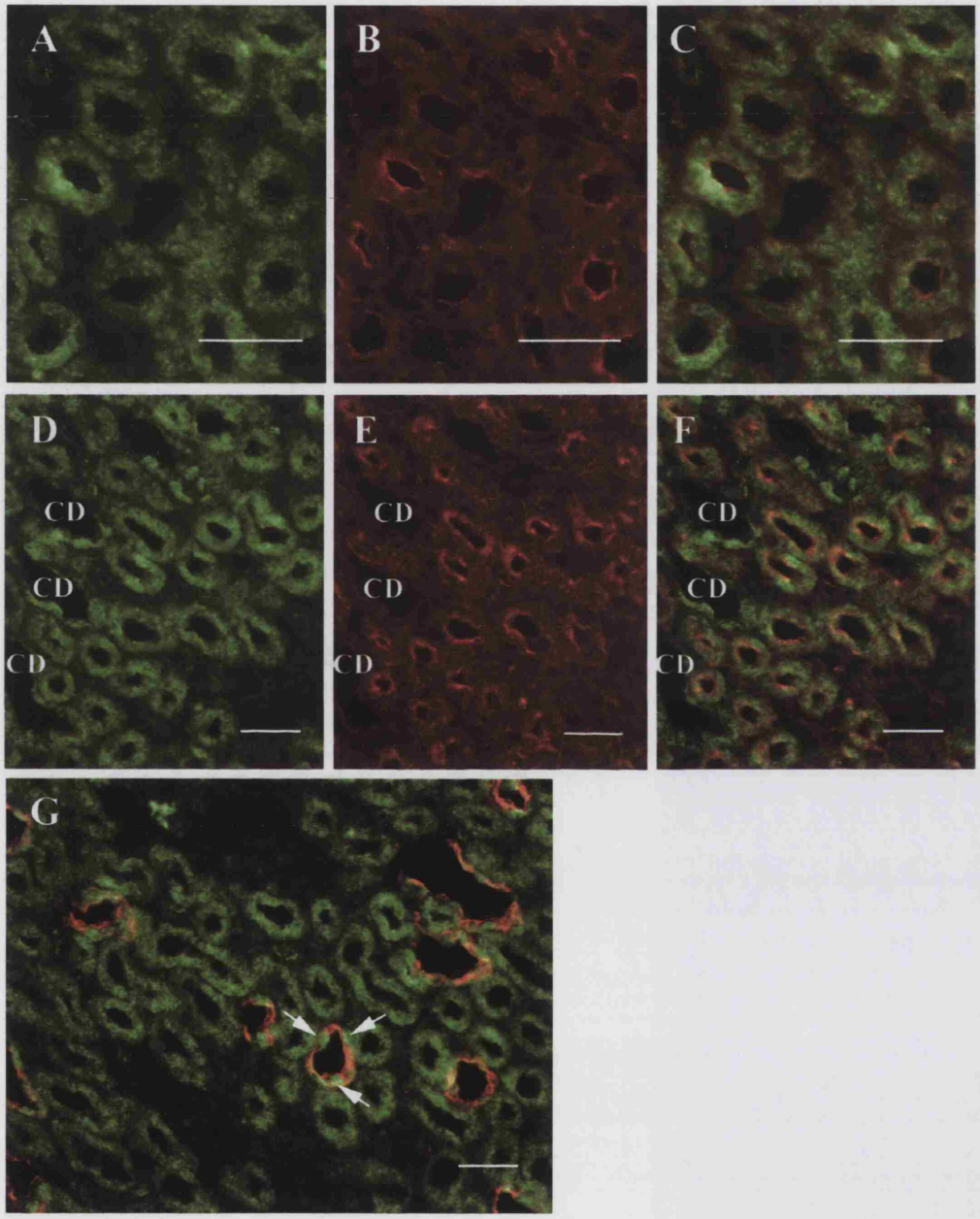


Figure 3.7 legend

A. Intracellular location of $P2Y_2$ receptor in cells of the thin ascending limb (tAL) of Henles Loop identified by anti-CLC-K1 (B) immunoreactivity only in the tAL on both apical and basolateral plasma membranes (Scale bar = 50μm).

C. Overlay of plate A and B depicting anti-$P2Y_2$ and anti-CLC-K1 on the same cells of the tAL (Scale bar = 50μm).

D. Intracellular location of $P2Y_2$ receptor (green) in cells of the thick ascending limb (TAL) of Henle identified by anti-Tamm Horsfall protein (red) expressed on the apical membrane of TAL cells (E). CD = collecting duct (Scale bar = 50μm).

F. Overlay of plate D and E depicting $P2Y_2$ receptor (green) and Tamm Horsfall Protein (red) on the same cell of the TAL. CD = collecting duct (Scale bar = 50μm).

G. $P2Y_2$ receptor expression (green) on intercalated cells (arrows) of the collecting duct. Principal cells of the collecting duct identified by anti-aquaporin 2 (red) (Scale bar = 50μm).

Figure 3. 8 **Photomicrographs showing $P2X_5$ receptor expression in the collecting duct and S3 segment**

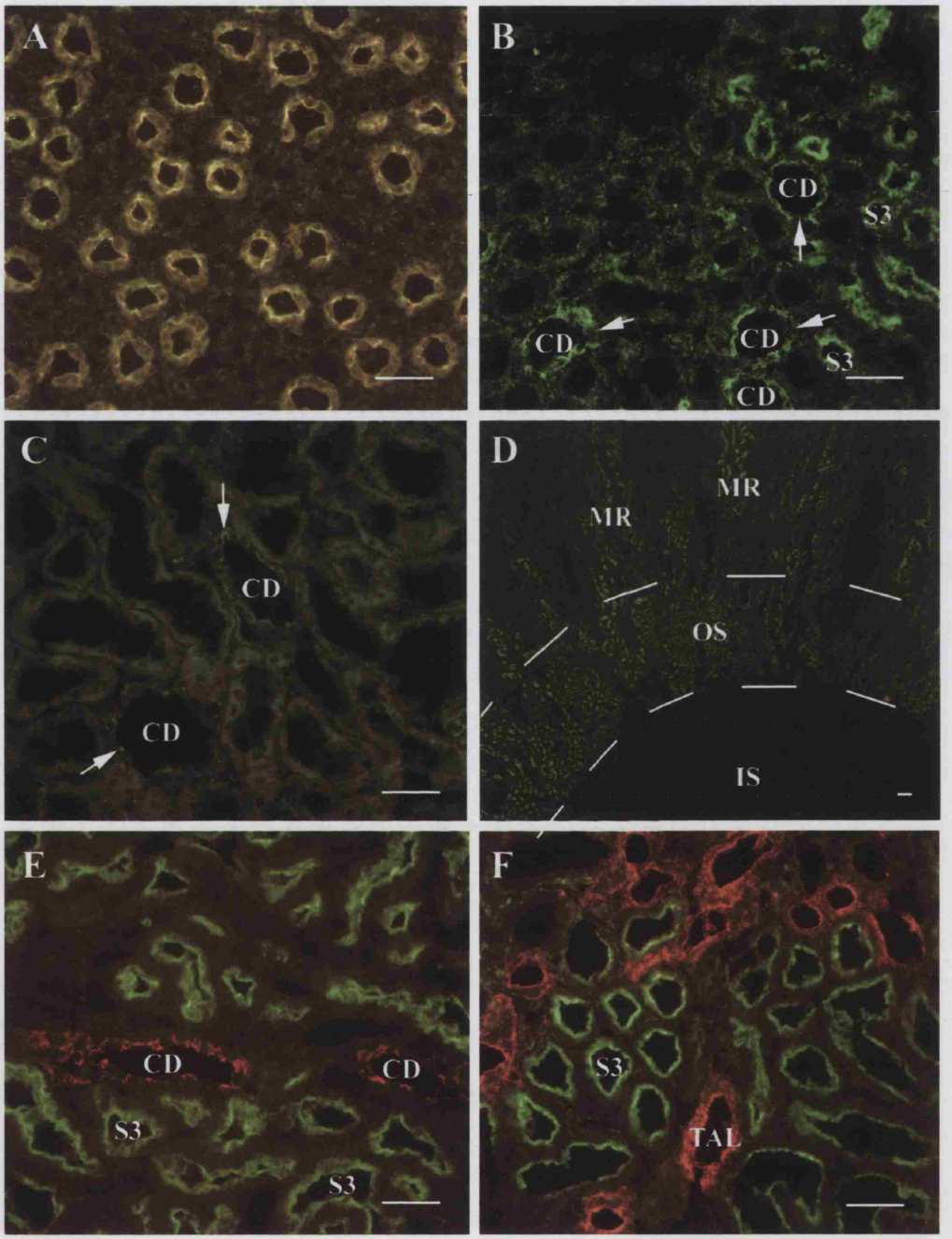


Figure 3.8 legend

A. Photomicrograph showing collecting ducts in the inner stripe of the inner medulla immunopositive for $P2X_5$ (green) and aquaporin 2 (red). Areas of co-localisation appear yellow (Scale bar = 50μm).

B. $P2X_5$ receptor expression on collecting duct (CD) principal cells and S3 cells. Arrows indicate intercalated cells. (Scale bar = 50μm).

C. Low level of expression of $P2X_5$ receptor (arrows) on cortical collecting duct cells in the outer cortex.

D. Low magnification photomicrograph showing $P2X_5$ immunoreactivity in the S3 segment. MR= medullary rays, OS= outer stripe, IS= inner stripe (Scale bar = 100μm). Note lack of immunostaining in the inner stripe (alternate pattern).

E. Apical expression of $P2X_5$ receptor on S3 segment (green) with lack of expression on collecting duct cells (red) identified by anti-aquaporin 2 (Scale bar = 50μm).

F. Apical expression of $P2X_5$ receptor on S3 segment (green) with lack of expression on TAL cells (red) identified by anti-Tamm Horsfall protein. (Scale bar = 50μm).

is the inner stripe (IS) of inner medulla in Figure 3.8 D compared with Figure 3.8 A (the more common staining pattern), which shows the same region at a higher magnification.

3.4 Discussion

The present study shows that all of the P2 receptor subtypes examined are distributed throughout the nephron and renal vasculature; the findings are summarized in Figure 3.9 and table 3.2. Previous studies have shown that renal cells express P2 receptors, but this work has largely been carried out on various renal cell types in culture;(Pfeilschifter, 1990;Filipovic *et al.*, 1998;Banderali *et al.*, 1999;McCoy *et al.*, 1999;Dockrell *et al.*, 2001;Fischer *et al.*, 2001;White *et al.*, 2001;Zambon *et al.*, 2001) rather than studies of native tissue. Sources of extracellular ATP to act on these receptors could include perivascular and peritubular nerve terminals, circulating erythrocytes, aggregating platelets, infiltrating mast cells, and renal endothelial and epithelial cells (Chan *et al.*, 1998b;Bodin & Burnstock, 2001b). Even though the gradient for ATP efflux is large, the amount of locally released ATP is probably in the high nanomolar to low micromolar range, but still in excess of the concentration required for receptor stimulation (Schwiebert, 2001).

3.4.1 P2 receptors and the renal vasculature

$P2X_1$ receptors immunolocalised to the intrarenal vasculature, including the afferent arteriole, which confirms previous reports and is consistent with functional studies (Chan *et al.*, 1998a;Hansen *et al.*, 1999). $P2X_1$ receptors were expressed by the renal artery, arcuate and cortical radial artery, but not by the renal tubules or glomeruli. $P2X_2$ receptors were also present in the smooth

Figure 3. 9 **A stylised nephron indicating the location of P2 receptor subtypes detected by immunohistochemistry (IC denotes intercalated cells)**

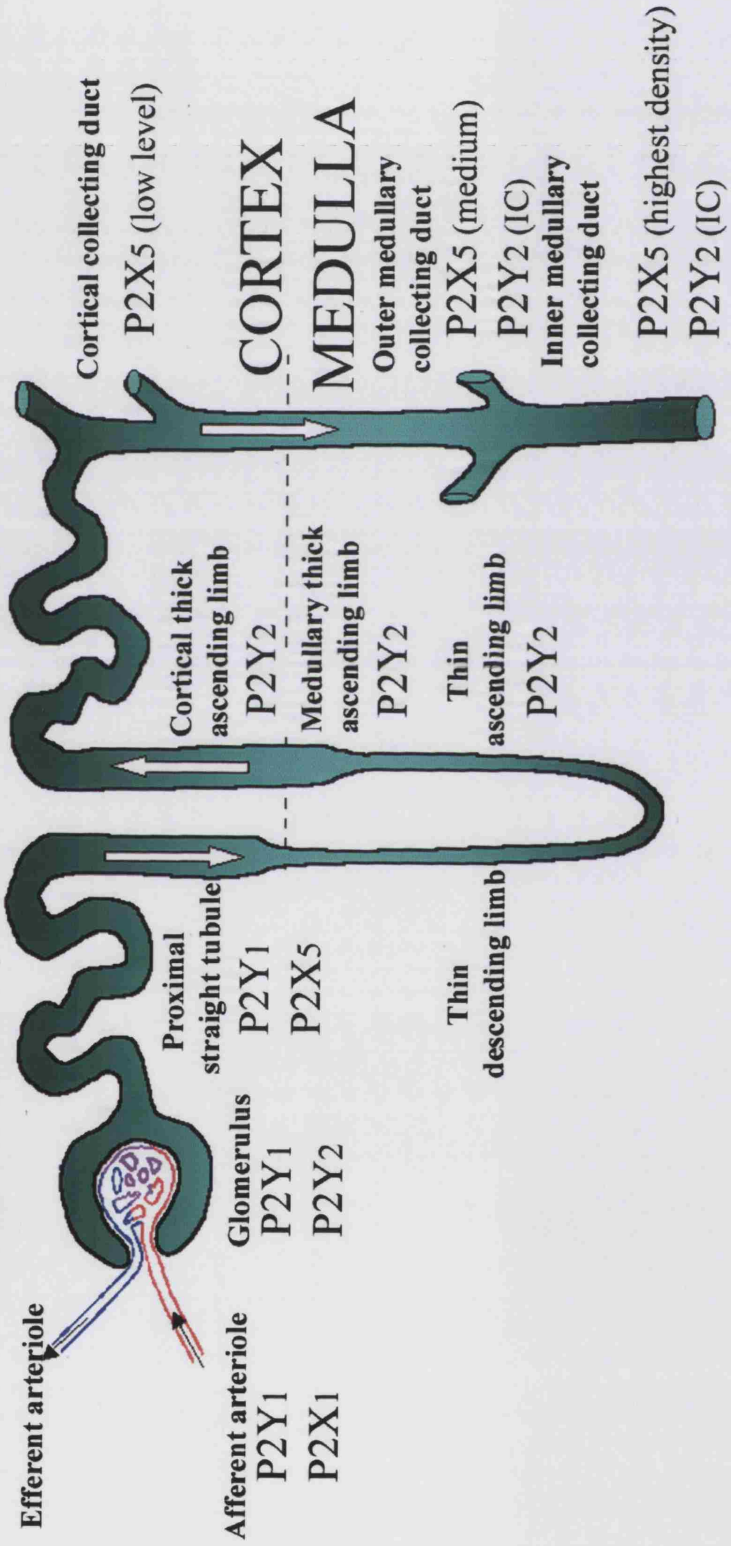


Table 3. 2 **Summary of the immuno-positive nephron segments for P2 receptors in the normal rat kidney**

Subtype	Location along the nephron
$P2Y_1$	Vascular SMC; glomerular MC; peritubular fibroblasts, PT S3 segment apical membrane.
$P2Y_2$	Glomerular PD; tAL, TAL, CD (intercalated cells)
$P2Y_4$	PCT basolateral membrane
$P2X_1$	Vascular SMC
$P2X_2$	Vascular SMC (larger intrarenal vessels)
$P2X_3$	Not detected.
$P2X_4$	Low level expression throughout nephron
$P2X_5$	PT S3 segment (apical), medullary CD (principal cells), cortical CD (minimal)
$P2X_6$	Low level expression throughout nephron
$P2X_7$	Low level expression in some glomeruli

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

muscle cells of the larger renal arteries, but not the afferent or efferent arterioles or tubule epithelium. Sustained vasoconstriction of the afferent arteriole in response to ATP has been demonstrated, whereas the efferent arteriole shows no such response (Inscho *et al.*, 1992). A later study by the same group (Inscho et al, 1998) showed that rat juxtamedullary afferent arterioles are highly sensitive to the $P2X_1$ and $P2X_3$ agonists α,β-methylene-ATP and β,γ-methylene ATP, respectively. In this study, pharmacological evidence for a P2Y receptor that responded to UTP was also found, suggesting that it was the $P2Y_2$ or $P2Y_4$ receptor subtype. However, the present study reports expression of the $P2Y_1$ receptor (which is ADP responsive) in the vascular smooth muscle of both afferent and efferent arterioles, and not expression of $P2Y_2$ or $P2Y_4$ receptors. It is possible that these receptors are normally expressed at very low levels, which could not be detected by immunohistochemistry.

3.4.2 Expression of P2 receptors in the glomerulus

Recently, Jankowski et al (2001) reported that isolated rat glomeruli, when pre-contracted with angiotensin II, could be relaxed by ATP, ADP and UTP. They confirmed the presence of P2Y receptors in glomeruli using various ATP analogues: relaxation was seen with both 2-methylthio-ATP (2-MeSATP), a potent agonist at the $P2Y_1$ receptor, and UTP, which stimulates both $P2Y_2$ and $P2Y_4$ receptors (Jankowski *et al.*, 2001). In keeping with this functional study, both $P2Y_1$ and $P2Y_2$ receptors immunolocalised to rat glomeruli: $P2Y_1$ receptors labelled mesangial cells, identified by co-localisation with anti-Thy-1, a mesangial cell marker, and $P2Y_2$ receptors labelled podocyte cells, identified by co-localisation with anti-WT-1, a podocyte nuclear protein. Furthermore, mRNA transcripts for $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors were detected in whole

glomeruli. There are recent reports of P2Y receptor expression in intact and isolated glomeruli by measuring inositol triphosphate (IP_3) production in response to ATP, UTP and other P2Y receptor agonists (Bailey *et al.*, 1999;Bailey *et al.*, 2004). Fischer *et al* (2001) detected $P2Y_2$ and $P2Y_6$ receptors in cultured podocytes using patch clamp recordings and measurements of intracellular free calcium concentration. Evidence for a P2Y receptor in cultured mesangial cells was also found by measuring the production of IP_3 in response to P2Y receptor agonists (Pfeilschifter, 1990).

Mesangial cells contain contractile proteins and are probably involved in glomerular contraction and relaxation. Their contractile elements alter cell shape and/or volume, and, via their contact with glomerular capillaries, they can alter the surface area available for filtration (Pfeilschifter, 1990). In endothelium and smooth muscle, $P2Y_1$ receptors are thought to mediate responses that include smooth muscle relaxation and release of endothelium-derived relaxing factor (Von Kugelgen & Wetter, 2000). The action of extracellular ATP on glomerular mesangial cells via P2Y receptors might alter glomerular perfusion pressure and thus glomerular filtration. Recently, Nishiyama et al (2001) reported a study in which they found a positive correlation between tubulo-glomerular feedback (TGF)-induced adjustments of renal pre-glomerular resistance and extracellular ATP concentrations. Furthermore, Bell and co-workers provided direct evidence that ATP is released from the basolateral membrane of macula densa cells via a maxi anion channel in response to increased luminal NaCl concentration. The released ATP can thereby transmit signals to mesangial cells adjacent to the macula densa via P2 receptors (Bell *et al.*, 2003). ATP release from the macula densa was demonstrated at NaCl concentrations in the physiological range for

TGF signalling (0-60 mmol/l) and the concentration of released ATP induced a P2 receptor-stimulated rise in intracellular Ca^{2+} in cultured mesangial cells (Komlosi *et al.*, 2004). It is also possible that ATP released from macula densa cells may directly trigger TGF signalling at the afferent arteriole smooth muscle cells via $P2X_1$ and $P2Y_1$ receptors. Taken together, these findings suggest that extracellular ATP could have a paracrine or autocrine role in controlling not only the renal microvasculature (Figure 1.7) but also affecting glomerular function.

The present study reports expression of $P2Y_1$ receptors on renal interstitial fibroblasts, a cell known to be the source of erythropoietin (Maxwell *et al.*, 1993) and to also express ecto-5'-nucleotidase activity. This enzyme catalyses dephosphorylation of AMP to adenosine and, under hypoxic conditions, adenosine may stimulate erythropoietin production from these cells (Kaissling *et al.*, 1993). There is also evidence that anaemia itself increases ecto-5'-nucleotidase activity in rat kidney (Le Hir *et al.*, 1989) and that hypoxia induces release of adenosine via the extracellular catabolism of ATP (Conde & Monteiro, 2004). At present the role of $P2Y_1$ receptors on renal fibroblasts is not clear, but it is interesting to note that this receptor might link ATP release to erythropoietin synthesis and release (via the breakdown product adenosine) *in vivo*.

3.4.3 Expression of P2 receptors in proximal tubule

Recently, based on agonist potencies, Dockrell et al (2001) demonstrated the presence of either a $P2Y_2$ or a $P2Y_4$ receptor in primary cultures of proximal tubule cells. Pharmacologically, the agonist profile indicates a $P2Y_1$-like receptor, because ATP, ADP and 2MeSATP were equipotent; in addition, the response to both UTP and ATPγS indicates either $P2Y_2$ or $P2Y_4$ receptors (Bailey

et al., 2000b). Furthermore, an increase in intracellular calcium was reported in response to UDP, indicative of $P2Y_6$ receptor expression (Bailey *et al.*, 2001). In the present study RNA transcripts for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors were detected in microdissected proximal tubule segments. Immunohistochemistry confirms the presence of $P2Y_4$ receptors in the basolateral membrane and $P2Y_6$ receptors in both apical and basolateral membranes of PCT. The apical membrane of the later and straight portion (S3) of the proximal tubule was immuno-positive for $P2Y_1$ and $P2X_5$ receptors. However, mRNA for $P2Y_2$ receptors was also detected in the S3 segment, and suggests that under certain conditions these receptors might be expressed, or that their expression level is normally too low to be detected by immunohistochemistry.

The physiological significance of P2X and P2Y receptors in the proximal tubule is still unclear. P2Y receptor activation can inhibit the basolaterally located Na^+-K^+-ATPase (Jin & Hopfer, 1997), a key enzyme in maintaining vectorial transepithelial Na^+ transport throughout the renal tubule. In the proximal tubule, ATP can stimulate gluconeogenesis, probably via the $P2Y_1$ receptor (Cha *et al.*, 1995), and can also enhance facilitated, GLUT-mediated, glucose transport across the brush border membrane (Marks *et al.*, 2000). Hence, P2 receptors in the proximal tubule may have multiple roles affecting both membrane transport and metabolic function (section 1.6.2).

Interestingly, $P2X_4$ and $P2X_6$ receptor immunoreactivity was found throughout the nephron, suggesting that both receptor subtypes could have a more general regulatory role in cell function. These P2X subtypes are capable of forming heteromeric receptors with other members of the P2X family (Torres *et*

al., 1999). $P2X_6$ cannot exist alone, but can form receptor complexes with $P2X_1$, $P2X_2$, $P2X_4$ and $P2X_5$ (see Table 1.1).

3.4.4 P2 receptors and the thin limbs of Henle

In addition to a potential effect on proximal tubule function, ATP filtered at the glomerulus, or released from proximal tubule cells, might travel down the nephron to act at more distant sites on apically expressed P2 receptors. The present study identified $P2Y_1$ and $P2Y_2$ receptor mRNA transcripts in the descending and ascending thin limbs, and $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ mRNA transcripts in thick ascending limb (TAL). $P2Y_2$ receptors were detected by immunohistochemistry in the thin ascending limb of Henle (tAL), and the TAL, although the expression appears to be predominantly intracellular. Consistent with these findings, a previous study measuring intracellular calcium in the thin limbs found that basolaterally applied ATP and UTP were equipotent, suggesting the presence of a $P2Y_2$-like receptor (Bailey *et al.*, 2000b). Autoradiographical studies have also found dense binding sites for [^{35}S] ATPγS in the TAL, again indicative of a $P2Y_2$-like receptor, and in agreement with current findings (Bailey *et al.*, 2000a).

3.4.5 P2 receptors and the distal tubule

Most P2 receptor studies of the distal tubule have been performed on cell lines. The A6 cell line, derived from *Xenopus laevis*, exhibits transport properties similar to the mammalian distal tubule. Using the patch clamp technique and measurements of short-circuit current (I_{sc}) Banderali et al (1999) showed that apical application of nucleotides in A6 cells invoked a transient increase in Cl^- secretion, mediated by a $P2Y_2$–like receptor that activates an

apical Cl^- channel. However in the present study, only $P2X_4$ and $P2X_6$ receptors were detected on the basolateral membrane of the rat distal tubule but this segment was also shown previously to be unresponsive to basolateral application of ATP (Bailey *et al.*, 2000b).

3.4.6 P2 receptors and the collecting duct

Much of the previous work on collecting duct cells has focused on the presence of a P2Y receptor. Indeed, $P2Y_2$ receptors have been identified in isolated mouse cortical collecting duct (Deetjen *et al.*, 2000), in isolated rat inner medullary collecting duct (Kishore *et al.*, 2000), and in Madin Darby Canine Kidney Cells (MDCK) in culture (Zambon *et al.*, 2001). In keeping with these results, $P2Y_2$ receptors were identified in rat inner and outer medullary collecting duct by immunohistochemistry, and mRNA transcripts for $P2Y_{1,2,4}$ and $P2Y_6$ receptors. However, $P2Y_2$ receptor immunoreactivity was not detected in the cortical collecting duct; although *in vitro* perfusion experiments have identified $P2Y_1$ and $P2Y_2$ receptors in the outer medullary collecting duct (Bailey *et al.*, 2000b). Interestingly, the highest density of $P2Y_2$ receptor expression was on intercalated cells, identified by their lack of staining for aquaporin 2 receptors. By contrast, in a mouse cortical collecting duct cell line, Cuffe *et al* (2000) have described inhibition of Na^+ absorption and stimulation of Cl^- secretion by ATP, which they attributed to apical and basolateral $P2Y_2$ receptor stimulation. Moreover, ATP acting basolaterally has been shown to inhibit the effect of vasopressin on increasing the water permeability of the rat inner medullary collecting duct and is again thought to be $P2Y_2$-mediated (Kishore *et al.*, 1995;Edwards, 2002), which is perhaps surprising given the finding that $P2Y_2$ immunostaining was most pronounced on intercalated type cells. McCoy *et al*

(1999) also presented evidence for multiple P2X and P2Y receptors in a mouse inner medulla collecting duct cell line (mIMCD-K2) and suggested that these receptors could be involved in modulating Na^+ and Cl^- transport.

$P2X_5$ receptor expression on principal collecting duct cells presented two interesting patterns: the first showed very little expression on collecting duct cells in the outer cortex, with increasing density of receptor expression further along the collecting duct into the inner cortex and outer medulla, with highest expression in the inner stripe of the inner medulla. This pattern was in addition to the apical S3 $P2X_5$ receptor expression already mentioned. The second pattern, which was less frequently observed, showed no collecting duct expression of $P2X_5$ receptors, but just apical S3 expression. As yet, this apparent change in pattern of $P2X_5$ receptor immunostaining cannot be explained, since the rats studied were all male and kept under identical conditions.

The results presented in this Chapter clearly show that purinergic P2 receptors are widely expressed throughout the rat kidney, on the vascular and epithelial cells of all nephron segments, as well as cells of the glomerulus. $P2X_5$, $P2X_6$ $P2Y_4$ and $P2Y_6$ receptors are located exclusively on renal tubule cells. In other tissues, there is a wide range of biological functions elicited by nucleotide P2 receptors, including altered ion transport and vascular tone (Abbracchio & Burnstock, 1998). However, the function of these receptors in the kidney remains largely unknown and is still highly speculative, but from their distribution at least, they are likely to influence both glomerular and tubular cell function.

Chapter 4

Altered P2 receptor expression in the Han:SPRD cy/+ rat: a model of autosomal dominant polycystic kidney disease (ADPKD)

4.1 Introduction

Formation and progressive enlargement of renal cysts is a well-characterised feature of autosomal dominant polycystic kidney disease (ADPKD), leading to destruction of normal renal tissue and impaired renal function. The disease 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 are still unclear (see section 1.8). 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, it has been shown that fluid extracted from the lumen of ADPKD cysts contained as much as 0.5 to 10 μM ATP, and ADPKD primary cultures released significantly more ATP than a normal PCT cell primary culture (Wilson *et al.*, 1999;Schwiebert *et al.*, 2002). Typically, in studies of recombinant P2 receptors expressed in *Xenopus* oocytes, EC_{50} values (concentration of agonist eliciting 50% of the maximal response) for P2X and P2Y receptors are in the low micromolar range (1 to 10 μM) for ATP (North & Surprenant, 2000;Sak & Webb, 2002); therefore it is possible that the concentration of ATP that Wilson and co-workers measured in cyst fluids could activate P2 receptors. Moreover, extracellular ATP is a potent stimulus to transepithelial fluid secretion by acting on G-protein coupled P2Y receptors (Schwiebert & 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.*, 2003a). Furthermore, P2Y receptor signalling via adenylate cyclase will increase cAMP levels and also stimulate renal epithelial cell Cl^- secretion and proliferation (Hanaoka & Guggino, 2000;Mangoo-Karim *et al.*, 1989;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 (see Chapter 3), (Bailey *et al.*, 2000a;Bailey *et al.*, 2000b;Schwiebert & Kishore, 2001). Current data suggest that $P2Y_1$ and $P2Y_2$ receptors are involved in cell growth and proliferation (Schulze-Lohoff *et al.*, 1992;Harada *et al.*, 2000;Greig *et al.*, 2003b;Vonend *et al.*, 2003), and that the $P2X_5$ and $P2X_7$ receptors can influence cell turnover by affecting cell proliferation, differentiation and death (Schulze-Lohoff *et al.*, 1998;Groschel-Stewart *et al.*, 1999b;Groschel-Stewart *et al.*, 1999a;Harada *et al.*, 2000;Greig *et al.*, 2003b;Verhoef *et al.*, 2003). Recently, $P2Y_{1,2}$ and $P2Y_6$ receptor mRNA transcripts and $P2X_4$ and $P2X_5$ receptor proteins have been detected in human ADPKD and in *cpk* mouse renal epithelial cell primary cultures (Schwiebert *et al.*, 2002). In addition, expression of a truncated polycystin-1 fusion protein in a renal collecting duct cell line enhances ATP-stimulated transepithelial Cl^- secretion via P2Y receptor activation, which suggests a possible interaction between the polycystin signalling pathway (in ADPKD) and P2Y receptor signal transduction (Hooper *et al.*, 2003;Wildman *et al.*, 2003b).

The Han:SPRD (cy/+) rat model of ADPKD was chosen for the purpose of this study due to its similarity to human ADPKD (Table 4.1). The model was discovered by Kaspareit-Rittinghausen who noted a spontaneous mutation

Table 4. 1 **Comparison of human ADPKD and the Han:SPRD rat model**

Feature	**Han:SPRD cy/+**	**Human ADPKD**
Inheritance	Autosomal dominant	Autosomal dominant
Gene Locus	?	16 and 4
Gender difference	+	+
Uraemia	+	+
Hypertension	+	+
Proteinuria	+	+
Hyperlipidemia	+	+
Matrix alterations	+	+
Extrarenal manifestations	+	+

The heterozygous rat model is characterised by a slow progression of uraemia, proteinuria and hyperlipidemia, and histological changes that resemble those seen in human ADPKD, including alterations of the basement membrane and extracellular matrix. However, in rats there is a lack of extra renal manifestations, which have only been seen as the development of liver cysts in older females (Schafer et al., 1994).

Table adapted from Gretz et al., (1996)

resulting in PKD had occurred in a colony of Sprague-Dawley rats (Kaspareit-Rittinghausen *et al.*, 1990). Genetic analysis suggested that there was an autosomal dominant pattern of inheritance but to date the mutated genes have not been determined. In both rats and humans the cystic epithelium is poorly differentiated, with basement membrane thickening and increased cell proliferation (Cowley, Jr. *et al.*, 1993;Schafer *et al.*, 1994). 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). 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, which suggests that differences in cystic phenotype are related to gene dosage (Cowley, Jr. *et al.*, 1993). Furthermore, heterozygous animals develop a more slowly progressive form, which is closer to human ADPKD, whilst homozygotes develop an aggressive form of the disease leading to early death at 3 to 4 weeks of age (Schafer *et al.*, 1994).

In this Chapter, using immunohistochemistry with currently available antibodies, the pattern of expression of $P2Y_{1,2,4,6}$ and $P2X_5$ and $P2X_7$ receptor subtypes in the Han:SPRD (cy/+) rat model of polycystic kidney disease was examined. Quantitative real-time PCR was used 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.

4.2 Methods

4.2.1 Localisation of P2 receptor subtypes by immunohistochemistry

Kidney tissue from 36-day-old heterozygote Han:SPRD (cy/+) rats (n=5) was a generous gift from Prof. Gretz (University of Heidelberg, Germany). It was embedded in OCT compound (BDH/Merck, Leicester, UK) and sectioned at 8 μm as described in section 2.1.1. The avidin-biotin technique for immunohistochemistry was used as described in section 2.1.3. $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor antibodies were obtained from Alomone Laboratories Ltd. (Jerusalem, Israel), $P2Y_6$ receptor antibody was a gift from Prof. Leipziger (Aarhus University, Denmark), and $P2X_5$ and $P2X_7$ receptor antibodies were from Roche Bioscience (Palo Alto, Ca, USA). The secondary antibody was a biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch, Luton, UK) used at a dilution of 1:500 for 30 minutes, followed by the ExtrAvidin peroxidase conjugate (Sigma-Aldrich Co. Ltd., Poole, UK) at 1:1000 for 30 minutes.

In the case of fluorescent microscopy (described in detail in section 2.1.4), the secondary antibody was cy3 labeled anti-rabbit IgG (Abcam Ltd., Cambridge, UK). Controls were performed with pre-immune IgG and with the P2 antibodies pre-absorbed with the homologous peptides; no staining was observed. The results were documented using the Zeiss Axioplan light/fluorescent microscope (Carl Zeiss International, Göttingen Germany) and images captured using a Leica DC200 digital camera (Leica Microsystems, Wetzlar, Germany).

4.2.2 Identification of apoptotic cells by TUNEL assay

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). During apoptosis, DNAse activity not only generates double-stranded, low-molecular-weight DNA fragments (mono- and oligonucleosomes), but also introduces strand breaks ("nicks") into the high-molecular-weight DNA. These processes can be identified by labelling the free 3'-OH termini with terminal deoxynucleotidyl transferase (TdT), which attaches labelled nucleotides to all 3'OH-ends. Han:SPRD cy/+ kidney sections were fixed with 4% formaldehyde in PBS for 20 minutes and then washed three times with PBS. Cells were permeabilised with 0.1% triton-X-100 (Sigma-Aldrich Co. Ltd., Poole, UK), 0.1% sodium citrate solution in PBS for 2 minutes at 4°C, rinsed three times with PBS and incubated for 1 hour in the TUNEL reaction mixture (contains 0.2 U/µl TdT enzyme, 6 nM fluorescein-16-dUTP, 6 nM dATP in TdT buffer). After a further washing in PBS to stop the reaction, the slides were mounted in citifluor and examined with a Zeiss Axioplan light/fluorescent microscope (Carl Zeiss International, Göttingen Germany) and images were captured using a Leica DC200 digital camera (Leica Microsystems, Wetzlar, Germany).

4.2.3 RNA and protein extraction

Heterozygote and homozygote Han:SPRD (cy/+) kidney tissue from 28-day-old rats was provided by Prof. D Woo (University of California, USA) for extraction of RNA and proteins. Fresh whole kidney was snap frozen in liquid nitrogen and ground to powder using a pestle and mortar. One hundred mg of

powdered tissue was resuspended in 1ml 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 containing 10% protease inhibitors (Sigma Aldrich Co. Ltd., Poole, 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 (PolyATract, Promega, Southampton, UK) as described in section 2.3.1. An amount equivalent to 1μg of total RNA was reverse transcribed with 0.5μg oligo(-dt) 12-18 primer and a first-strand cDNA synthesis kit (Superscript II RNase H⁻ reverse transcriptase, Gibco BRL, UK), described in detail in section 2.3.2.

4.2.4 Quantitative analysis of P2 receptor mRNA by real-time PCR

The resulting cDNA transcripts of whole kidney mRNA were used for PCR amplification using the Roche Lightcycler (Roche diagnostics, Germany) and QuantiTect SYBR® Green PCR kit (Qiagen, West Sussex UK) described in more detail in section 2.3. 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 2.2 for primer sequences). PCR products were also analyzed by gel electrophoresis and visualized using a Bio-Rad multi-imager (Bio-Rad, Hemel Hempstead, Herts, UK).

4.2.5 Detection of $P2Y_2$ and $P2Y_6$ receptor protein by immunoblotting

As described in section 2.4, 10 μg of protein homogenate was electrophoresed on 12% SDS-PAGE gels and then transferred to Hybond ECL-nitrocellulose membrane (Amersham Biosciences, Bucks, UK, UK) using Bio-Rad semi-dry transfer apparatus as described in section 2.4.3. Membranes were probed overnight with either $P2Y_2$ or $P2Y_6$ receptor antibody diluted in PBS/tween 20. A peroxidase-linked donkey anti-rabbit IgG and ECL+ chemiluminescence (Amersham Biosciences, Bucks, UK, UK) was used for detection and visualized using a Bio-Rad Multi-imager (Bio-Rad, Hemel Hempstead, Herts., UK).

4.2.6 Statistics

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

4.3 Results

4.3.1 Localisation of P2 receptor protein by immunohistochemistry

Using the polyclonal antibodies to P2 receptor subtypes, 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 was sought. However, no suitable commercial antibodies for

$P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ were available, nor was sufficient tissue available to use segment co-localising antibodies.

All of the P2 receptor subtypes tested were localized 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 (Figure 4.1A) and this receptor appeared to be mainly cytoplasmic in cyst lining cells. Glomerular mesangial cells were also positive for $P2Y_1$ receptors and the staining pattern was the same as that found in normal animals (Chapter 3). $P2Y_2$ receptor expression was found on the epithelial cells lining both large and small cysts, but again, not all cysts were immunopositive for this receptor subtype (Figure 4.1B). Glomerular podocytes were immunopositive for $P2Y_2$ receptors as described previously (Chapter 3). Many cysts located in the cortex were immunopositive for $P2Y_4$ and $P2Y_6$ receptors (Figures 4.1C and 4.1D), 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 (Chapter 3) and $P2Y_6$ receptors have been detected on S1 and S2 proximal convoluted tubule cells (Chapter 3). Dense $P2Y_4$ receptor expression was detected on epithelial cells of small cysts, but the staining pattern was more scattered in cells lining larger cysts (Figure 4.1C). This was not seen with $P2Y_6$ receptor expression, which had a granular and cytoplasmic staining pattern in both large and small cysts (Figure 4.1D). $P2X_5$ receptor immunoreactivity was detected on both apical and basolateral membranes of cells lining mainly small, but also some large, cysts in the cortex (Figure 4.1E). Many small and

Figure 4. 1 **Representative micrographs of the renal cortex showing P2 receptor expression on cyst lining cells**

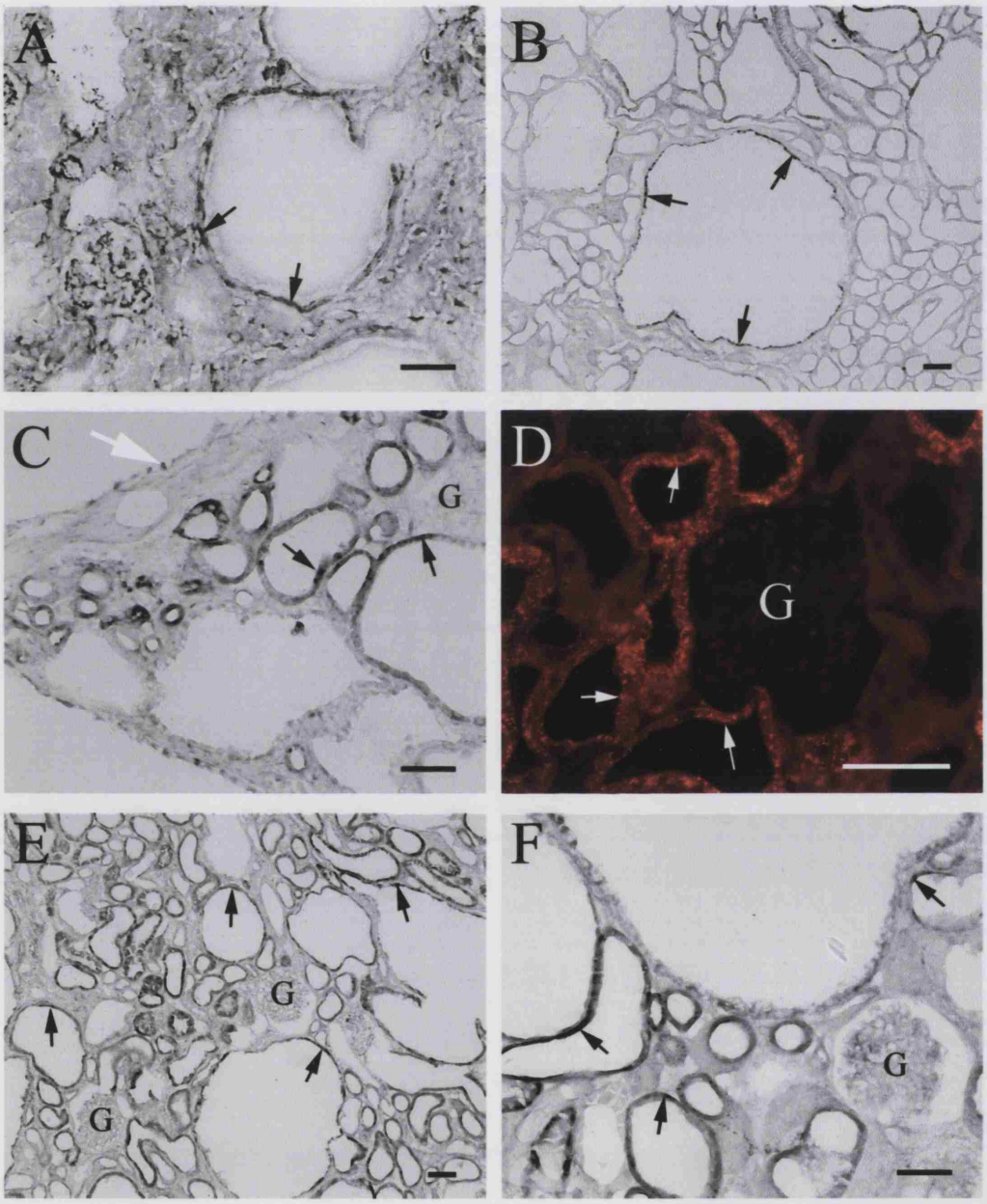


Figure 4.1 legend

A. $P2Y_1$ receptor immunoreactivity on the basolateral membrane of cyst-lining cells (arrow) and glomerular mesangial cells (G). (Scale bar = 50μm).

B. $P2Y_2$ receptor immunoreactivity on cyst lining cells (arrow); smaller cysts have minimal receptor expression. (Scale bar = 50μm).

C. Dense $P2Y_4$ receptor immunoreactivity on cells lining small cysts (black arrow), and several immunopositive cells of larger cysts (white arrow). (Scale bar = 50μm).

D. $P2Y_6$ immunoreactivity predominantly at the apical membrane of many cyst lining cells of both large and small cysts (arrow), G= glomeruli. (Scale bar = 50μm).

E. Micrograph showing dense $P2X_5$ receptor immunoreactivity mainly on small cysts but also on some large cysts (arrows). (Scale bar = 50μm).

F. $P2X_7$ receptor immunoreactivity on cyst cells (arrow) and glomerular podocyte cells (G). (Scale bar = 50μm).

large cysts were immunopositive for $P2X_7$ receptors; the staining pattern was particularly intense in cells lining smaller cysts. $P2X_7$ immunoreactivity was also detected on glomerular podocytes (Figure 4.1F).

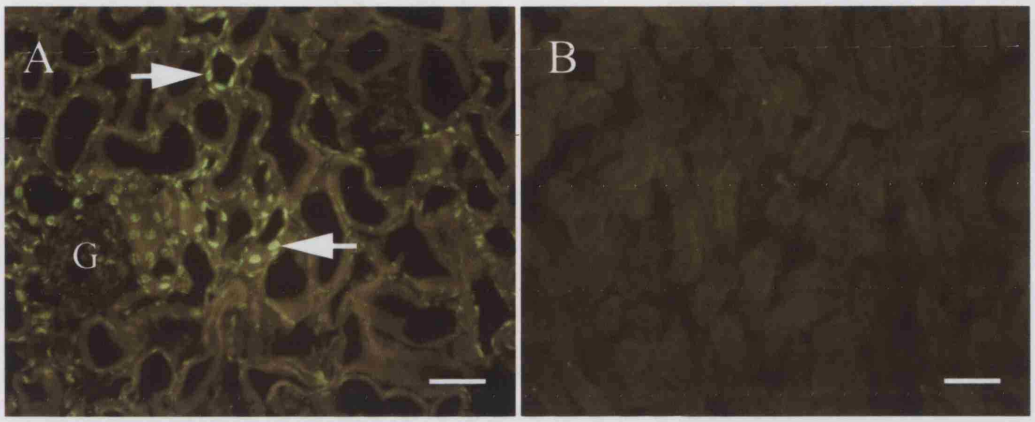
4.3.2 TUNEL Assay

TUNEL-positive cells, as a marker of apoptosis, were detected on epithelial cells lining renal cysts (arrows), the interstitium and some glomerular cells (Figure 4.2A). Apoptotic cells were found to be mainly associated with small cysts. TUNEL positive cells were not detected in normal rat kidney tissue (Figure 4.2B).

4.3.3 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 housekeeping gene HPRT. Experiments were repeated in duplicate or triplicate on 5 to 8 animals in each group. $P2Y_2$ receptor mRNA was increased in both cy/+ (78%, p=0.09) and cy/cy (224%, p<0.001) animals when compared with wild-type littermates (Figure 4.3A). Levels of $P2Y_6$ receptor mRNA were increased by 125% (p=<0.001) in the cy/+ renal tissue and 120% (p<0.001) in cy/cy animals when compared with control (Figure 4.3B). Levels of $P2Y_1$ receptor mRNA remained unchanged in the cy/+ heterozygotes, although there was a significant decrease of 67% (p<0.001) in the cy/cy homozygote renal tissue (Figure 4.3C). However, there was no significant increase in either $P2Y_4$ (Figure 4.3D) or $P2X_5$ (Figure 4.3E) receptor mRNAs when compared with control; although there was a small and non-significant

Figure 4. 2 **Identification of apoptotic cells in the Han:SPRD cy/+ rat model of ADPKD**



A. TUNEL-positive cells were detected in many epithelial cells lining small cysts (arrows) in the renal interstitium and in some glomeruli (G).

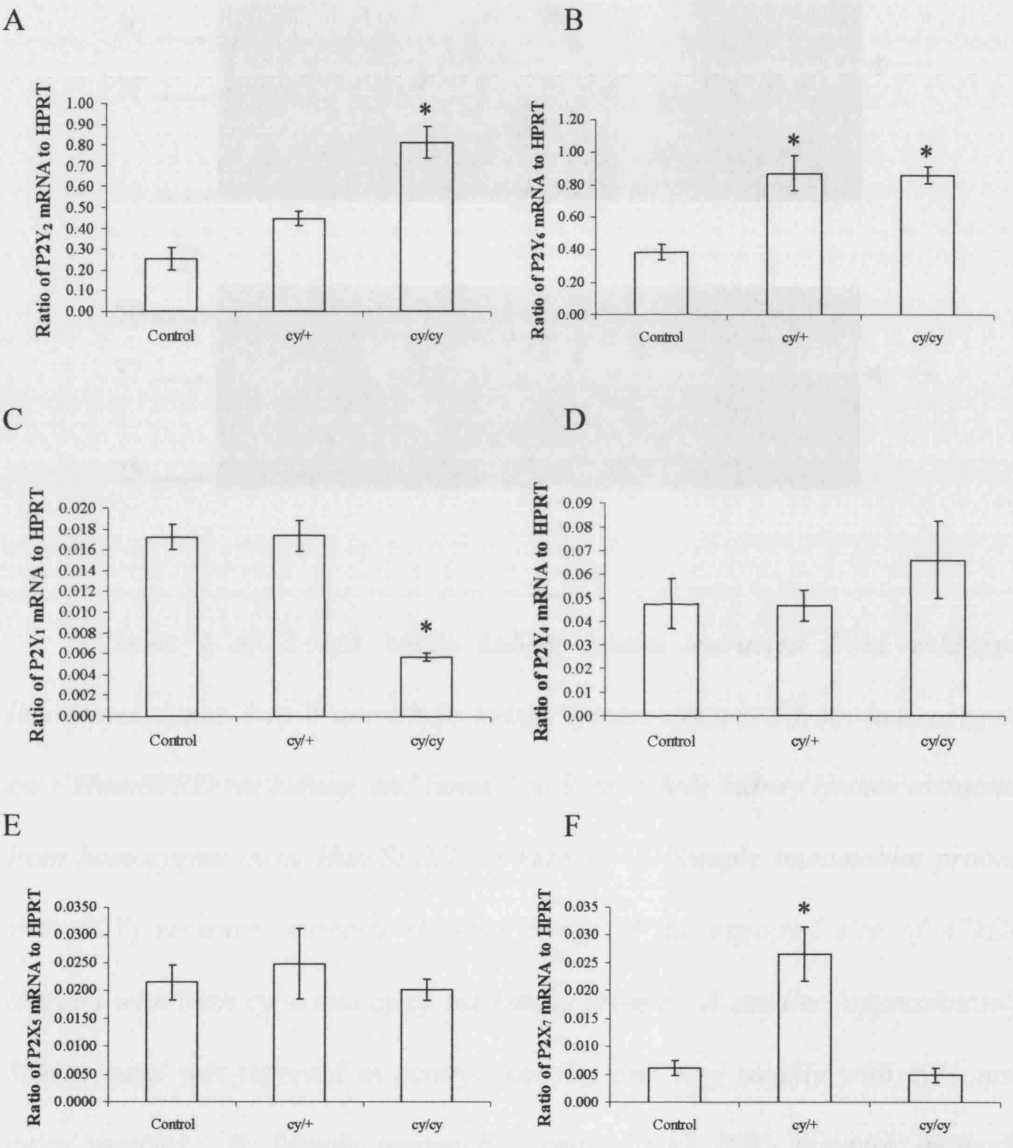
B. Apoptosis could not be detected in normal rat kidney. (Scale bar = 50μm).

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 (Figure 4.3F).

4.3.4 Immunoblotting for $P2Y_2$ and $P2Y_6$ receptor protein

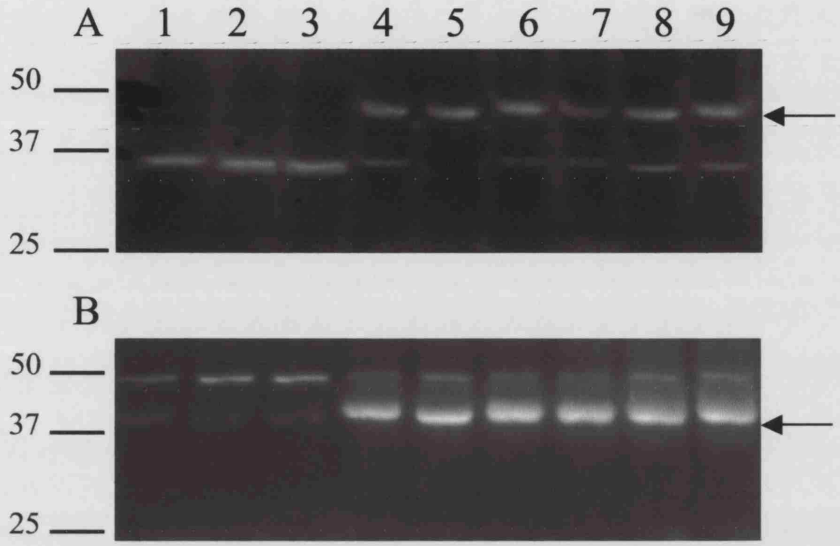
In view of the large increases in $P2Y_2$ and $P2Y_6$ receptor mRNA in 28-day-old Han:SPRD cystic kidney tissue and since increases in protein could not be quantified by immunohistochemistry, immunoblots were carried out with receptor antibodies. $P2Y_2$ receptor antibody produced clear bands of the expected size (47kDa) in both cy/+ and cy/cy rats, but could not be detected in wild-type controls (Figure 4.4A). A second band of approximately 36kDa was also detected in control rats, but was only very weakly detectable in cystic rats and possibly indicates differential post-translational modification. $P2Y_6$ receptor antibody produced a clear band at the expected size of 40kDa. An increase in $P2Y_6$ receptor protein (40kDa) could be detected in both cy/+ and cy/cy kidney tissue compared with control (Figure 4.4B), which matches the RT-PCR data. A second band of approximately 50kDa was also be detected in control rats, but was seen only faintly in cystic rat whole kidney extract.

Figure 4. 3 **Relative abundance of P2 receptor mRNA expressed as a ratio to that of the housekeeping gene HPRT. A: $P2Y_2$; B: $P2Y_6$; C: $P2Y_1$; D: $P2Y_4$; E: $P2X_5$; F: $P2X_7$**



*Control, cy/+ heterozygote for cysts; cy/cy, homozygote for cysts (n=7, *p<0.001). Bars represent means +/- SEM.*

Figure 4. 4 **Sample immunoblots using a polyclonal antibody to $P2Y_2$ or $P2Y_6$ receptor protein**



Lanes 1 to 3 are whole kidney lysates extracted from wild-type littermates, lanes 4 to 6 are whole kidney lysates extracted from heterozygote cy/+ Han:SPRD rat kidney, and lanes 7 to 9 are whole kidney lysates extracted from homozygote cy/cy Han:SPRD rat kidney. A. Sample immunoblot probed with $P2Y_2$ receptor antibody showing bands of the expected size of 47kDa (arrow) with both cy/+ and cy/cy rat kidney protein. A smaller, approximately 36kDa band was detected in control samples and very weakly with cy/+ and cy/cy samples. B. Sample immunoblot probed with $P2Y_6$ receptor antibody showing upregulation of the expected 40kDa band (arrow) with both cy/+ and cy/cy rat kidney protein compared to wild-type littermates. A larger 50kDa band could also be detected in control samples and weakly with cy/+ and cy/cy samples.

4.4 Discussion

Emerging data suggest a complex pattern of P2 receptor expression in many types of epithelia, including that of the kidney, in which both basolateral and apical cell membrane expression of P2 receptors has been reported (Chapter 3)(Bailey *et al.*, 2000b;Deetjen *et al.*, 2000;Kishore *et al.*, 2000;Bailey *et al.*, 2001). 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 & Kishore, 2001), and disturbances in ATP signalling might contribute to some forms of renal tubular dysfunction. In a previous study by Schwiebert and co-workers, P2 receptor mRNA transcripts were identified in monolayer cultures of human ADPKD and *cpk* mouse kidney cells (Schwiebert *et al.*, 2002). In the present study, immunohistochemistry and real-time PCR were used 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 characterised model of ADPKD in which initially most cysts derive from the proximal tubule (Kaspareit-Rittinghausen *et al.*, 1990). Several P2X and P2Y receptor proteins were detected on epithelial cells that line renal cysts with elevated mRNA levels and expression on cyst epithelial cells for both $P2Y_2$ and $P2Y_6$ receptor subtypes. Furthermore, upregulation of $P2Y_2$ (47 KDa) and $P2Y_6$ (40 KDa) receptor proteins could be detected in cy/+ and cy/cy rat kidneys. $P2Y_6$ receptor expression in proximal tubule-derived cysts may be expected, 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 apical membrane of this segment (see Chapter 3). However, although proximal tubule

cells express $P2Y_2$ receptor mRNA (Bailey *et al.*, 2000b), the protein has not been detected immunohistologically in this segment (Chapter 3). Functionally, the ATP-sensitive $P2Y_2$ receptor stimulates calcium activated Cl^- secretion and inhibits Na^+ absorption in mouse cortical collecting 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 (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 then osmotically drives fluid transport and increases cyst size. Indeed, 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). Furthermore, stimulation of basolateral $P2Y_2$ receptors has been shown to inhibit the activity of vasopressin thus inhibiting water reabsorption in rat *in vitro* perfused IMCD (Kishore *et al.*, 1995;Edwards, 2002). Since a cyst is an enclosed environment, ATP and its metabolites could be present for prolonged periods, potentially over-stimulating P2 receptors and/or disturbing any physiological balance in their coordinated activation. Moreover, increased fluid secretion and reduced water reabsorption might also cause additional stretch-induced release of ATP, and thus a positive feedback loop.

In the present study, $P2Y_1$ receptors were detected mainly in the cytoplasm of rat polycystic kidney cysts, though previously this receptor was detected on the apical membrane of the rat proximal straight tubule (Chapter 3). Although 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. Increased epithelial and interstitial cell proliferation in the Han:SPRD rat model has been reported previously by Ramasubbu and co-workers (Ramasubbu *et al.*, 1998). 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.*, 2003b;Greig *et al.*, 2003a). 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 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 alter 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 (Chapter 3). Therefore, expression on small and predominantly proximal tubule-derived cysts may not indicate a pattern greatly different from that in normal healthy 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 detected 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 (Chapter 3). $P2X_5$ receptors have been linked by immunohistochemistry to differentiation of squamous epithelium and gut epithelial cells (Groschel-Stewart *et al.*, 1999b;Groschel-Stewart *et al.*, 1999a), cultured skeletal muscle satellite cells (Ryten *et al.*, 2002) and to cultured adult and foetal human keratinocytes (Greig *et al.*, 2003b;Greig *et al.*, 2003a). Greig and co-workers demonstrated that the $P2X_5$ receptor agonist ATPγS halted proliferation of cultured keratinocytes indicative of cells withdrawing from the cell cycle and differentiating (Greig *et al.*, 2003b). Moreover, extracellular ATP has been shown to halt proliferation and promote markers of muscle cell differentiation such as myogenin and increase the rate of myotubule formation in cultured smooth muscle cells (Ryten *et al.*, 2002). However, both the Han:SPRD rat and human ADPKD cystic epithelia often appear de-differentiated, lacking the apical microvilli normally seen in mature renal epithelium (Grantham *et al.*, 1987;Vogel *et al.*, 2000). Currently, there is no evidence that $P2X_5$ receptor expression on cystic epithelia promotes cell differentiation.

$P2X_7$ receptor expression is barely detectable in normal renal epithelium (Chapter 3); however, in the current study dense $P2X_7$ receptor expression in both small and large cysts, and increased mRNA expression in the cy/+ genotype was demonstrated. Increased $P2X_7$ receptor protein and mRNA have also been found in the *cpk* mouse model of polycystic kidney disease (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, several lines of evidence suggest that $P2X_7$ receptor activation is part of the inflammatory response through its interactions with proinflammatory cytokines and as a mediator of cell apoptosis (Harada *et al.*, 2000;Verhoef *et al.*, 2003). $P2X_7$ receptor activation has been shown to promote the release of interleukin-1β (IL-1β) from activated macrophages (Ferrari *et al.*, 1997a;Grahames *et al.*, 1999;Verhoef *et al.*, 2003), and this proinflammatory cytokine has been demonstrated in cyst fluid from patients with ADPKD (Gardner, Jr. *et al.*, 1991;Merta *et al.*, 1997). However, to date there is no direct correlation between $P2X_7$ receptor expression and synthesis and release of proinflammatory cytokines in ADPKD.

Extracellular ATP has been shown to induce apoptosis via activation of the $P2X_7$ receptor in several cell types including thymocytes (Zheng *et al.*, 1991), cultured mesangial cells (Schulze-Lohoff *et al.*, 1998;Harada *et al.*, 2000), and dendritic cells (Coutinho-Silva *et al.*, 1999). Indeed, in the present study apoptotic cells were detected on epithelial cells lining small cysts in cy/+ animals and the pattern of expression was similar to that of the $P2X_7$ receptor staining. Previous reports demonstrate increased numbers of apoptotic cells in human PKD kidney compared with normal kidney (Woo, 1995), in the *cpk* mouse model of polycystic kidney disease (Ali *et al.*, 2000) and in the Han:SPRD (cy/+) and (cy/cy) rats (Ecder *et al.*, 2002). In these studies, apoptotic cells were detected in the same vicinity as proliferating epithelial cells involved with cyst development (Woo, 1995;Ali *et al.*, 2000;Ecder *et al.*, 2002). In addition, increased caspase activity and elevated B-cell lymphoma-2 expression (bcl-2), both of which are involved in the apoptotic pathway, were detected in the cpk mouse (Ali *et al.*,

2000) and in the Han:SPRD rat model of polycystic disease (Ecder *et al.*, 2002). Caspases are a family of cysteine proteases that are synthesised as proenzymes in normal cells and are activated when the cell receives apoptotic stimuli (Salvesen & Dixit, 1999). Interestingly, over-expression of bcl-2 in Madin-Darby canine kidney cells prevented cyst formation in collagen culture (Lin *et al.*, 1999) perhaps because bcl-2 regulates apoptosis by inhibiting caspase activation (Kluck *et al.*, 1997) Furthermore, bcl-2 knockout mice develop multicystic kidney disease with cysts arising from proximal tubule, Henle's loop, distal tubule and collecting duct (Sorenson *et al.*, 1996). These studies provide increasing evidence that apoptosis, normally a tightly regulated mechanism for maintaining normal cell turnover, tissue repair and remodelling (Savill *et al.*, 1996), is increased in some renal cystic diseases perhaps as a mechanism for deleting damaged cells and promoting increased cell turnover.

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 the P2 receptor immunohistology in normal SPRD rats (Chapter 3) the cell distribution of all the P2 receptors examined appeared to be more diffuse and cytoplasmic perhaps 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 at a low level.

Nucleotides present in cyst lumen fluid may activate P2Y receptors, potentially causing detrimental cyst expansion due to increased and osmotically

driven fluid secretion. From evidence in other tissues, $P2Y_1$ and $P2X_5$ receptors could be involved in cell proliferation and differentiation, and the $P2X_7$ receptor in renal cell death by apoptosis; all mechanisms potentially relevant to cyst growth. Further studies will be needed to determine the pathophysiological role of P2 receptors in ADPKD, and maybe other renal cystic diseases, as therapies based on inhibiting their action may have therapeutic potential in reducing cyst progression.

Chapter 5

Selective P2 receptor inhibition reduces growth rate of MDCK-derived cysts cultured *in vitro*

5.1 Introduction

The previous studies have shown expression of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ subtype mRNAs, and P2X and P2Y receptor proteins along the normal renal tubule (Chapter 3). Furthermore, there is also immunohistological evidence for expression of both P2X and P2Y receptor subtypes on cyst lining cells of the Han:SPRD cy/+ rat model of polycystic kidney disease (see Chapter 4). The present study was to investigate the role of P2 receptors on a Madin-Darby Canine kidney (MDCK) cell culture model of renal cyst formation. MDCK cells were originally derived from dog kidney distal tubule/collecting duct epithelium (Rindler *et al.*, 1979). In monolayer cultures these cells are known to exhibit ATP- stimulated Cl^- secretion (Simmons, 1981b) and to express $P2Y_1$, $P2Y_2$ $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors (Post *et al.*, 1998;Brindikova *et al.*, 2003). Stimulation of MDCK cells by P2Y receptor agonists results in the hydrolysis of phosphoinositides (Yang *et al.*, 1997), activation of mitogen-activated protein (MAP) kinase and phospholipases (Orlov *et al.*, 1999), alterations in the uptake and release of arachidonic acid (Zambon *et al.*, 2000), and the stimulation of cAMP formation by adenylyl cyclase (Torres *et al.*, 2002). In these cells, cAMP regulates Cl^- ion secretion and Na^+, K^+ ATPase activity (Simmons, 1991;Taub *et al.*, 1992). Individual MDCK cells suspended in collagen gel in the presence of the cAMP agonist forskolin proliferate and form epithelial microcysts (McAteer *et al.*, 1987;Grantham *et al.*, 1989;Mangoo-Karim *et al.*, 1989). Each cyst enlarges progressively over many days and is filled with a clear fluid (Grantham *et al.*, 1989). The cells are polarized, with the apical cell surface facing the lumen and the basolateral surface in contact with the collagen matrix (Grantham *et al.*, 1989). The transparent nature of the collagen gel allows for repeated light

microscopic examination of individual cysts so that the rate of cyst enlargement can be calculated. Using this model it was possible to add various drugs to the medium bathing the cysts and monitor the effects on cyst formation and enlargement. This Chapter reports the effects of several P2 receptor agonists and antagonists on type 1 MDCK cyst growth.

5.2 Methods

5.2.1 Cells and cell culture

Type 1 MDCK cells were a generous gift from Professor D. Sheppard (University of Bristol, UK). These cells generate cAMP-stimulated apical membrane Cl^- channels (Mohamed *et al.*, 1997). Type 1 MDCK cells were cultured in MDCK medium (a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 nutrient medium supplemented with 10% FBS, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin; all from Life Technologies Ltd., Paisley, UK) at 37 °C in a humidified atmosphere of 5% CO_2.

5.2.2 Cyst growth

To grow cysts, MDCK cells were cultured in collagen gels in the presence of cAMP agonists using a modification of the method of Grantham *et al.* (Grantham *et al.*, 1989). Cells were trypsinised with 0.25% (w/v) trypsin for 30 min at 37 °C, diluted with MDCK medium to form a suspension of 0.2 – 0.4 x 10^4 cells ml^{-1}, and aliquoted into individual wells of a 24-well plate (0.1 ml per well). Each well contained 0.4 ml of ice-cold Vitrogen (~3.0 mg ml^{-1} collagen; Cohesion Technologies Inc., Palo Alto, Ca, USA) supplemented with 10% (v/v) 10X minimum essential medium, 10 mM Hepes, 27 mM $NaHCO_3$, 100 U ml^{-1}

penicillin, and 100 μg ml^{-1} streptomycin, and adjusted to pH 7.4 with NaOH. The 24-well plate was gently agitated to distribute cells throughout the Vitrogen and incubated in a water bath at 37 °C for 90 min to promote gelation of the Vitrogen.

After gelation, 1.5 ml MDCK medium was added to each well of the 24-well plate. To promote cyst growth, the cAMP agonist forskolin (10 μM) was added to the MDCK medium. Plates were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and the MDCK medium containing forskolin was changed every two days.

Six days after seeding collagen gels with MDCK cells, cysts were readily detected at x100 magnification using an Olympus CK40 inverted microscope with phase contrast optics (Olympus Microscopes, Southall, Middlesex, UK). To test the effect of P2 receptor modulators on cyst growth, P2 receptor agonists or antagonists were added to MDCK medium in the continued presence of forskolin. MDCK medium containing forskolin and P2 receptor modulators were changed every 2 days. Photographs of individual cysts were taken before the addition of P2 receptor modulators and at 3-day intervals for the duration of the experiment. Experiments were carried out in triplicate, and for each experiment 4 wells were used per reagent. Images were captured using a Nikon Coolpix 995 digital camera (Nikon, Kingston Upon Thames, Surrey, UK). To identify individual cysts, each cyst was assigned a unique reference number using a grid placed below the 24-well plate.

5.2.3 Cyst volume measurement

The diameter of cysts was measured directly from photographs of cysts using images that had been magnified by identical amounts. Only cysts that were

near spherical in shape were measured and an average diameter was obtained from a horizontal and a vertical measurement. Cyst volume was then estimated using the formula for the volume of a sphere, $4/3(\pi)r^3$.

5.2.4 Reagents

Forskolin, PPADS (pyridoxal-phosphate-6-azophenyl-2',4'-disulphonate), RB2 (reactive blue 2), Coomassie brilliant blue G, suramin, BzATP (3'-O-(4-benzoyl)benzoyl –adenosine-triphosphate), ATPγS (adenosine 5'-O-[3-thiotriphosphate]), ADPβS (adenosine 5'-O-[2-thiodiphosphate]), MRS 2179 (2'-deoxy-N^6-methyladenosine-3',5'-bisphosphate), zinc chloride and apyrase were purchased from the Sigma-Aldrich Co. Ltd. (Poole, Poole, UK).

Stock solutions were stored at -20 °C and diluted in MDCK medium to achieve final concentrations immediately before use. Precautions against light-sensitive reactions were observed when using PPADS by wrapping experimental plates in tin foil.

5.2.5 Statistics

Results are expressed as means ± SEM of *n* observations for a total of three experiments per treatment. To compare sets of data, Kruskal-Wallis test with Dunns multiple comparisons *post hoc* test was used, calculated using 'Graphpad Instat' version 3.06. Differences were considered statistically significant when $P < 0.05$.

5.3 Results

In all experiments, unless stated otherwise, established cysts at day 6 were exposed to either agonist or antagonist for a continuous period from day 6

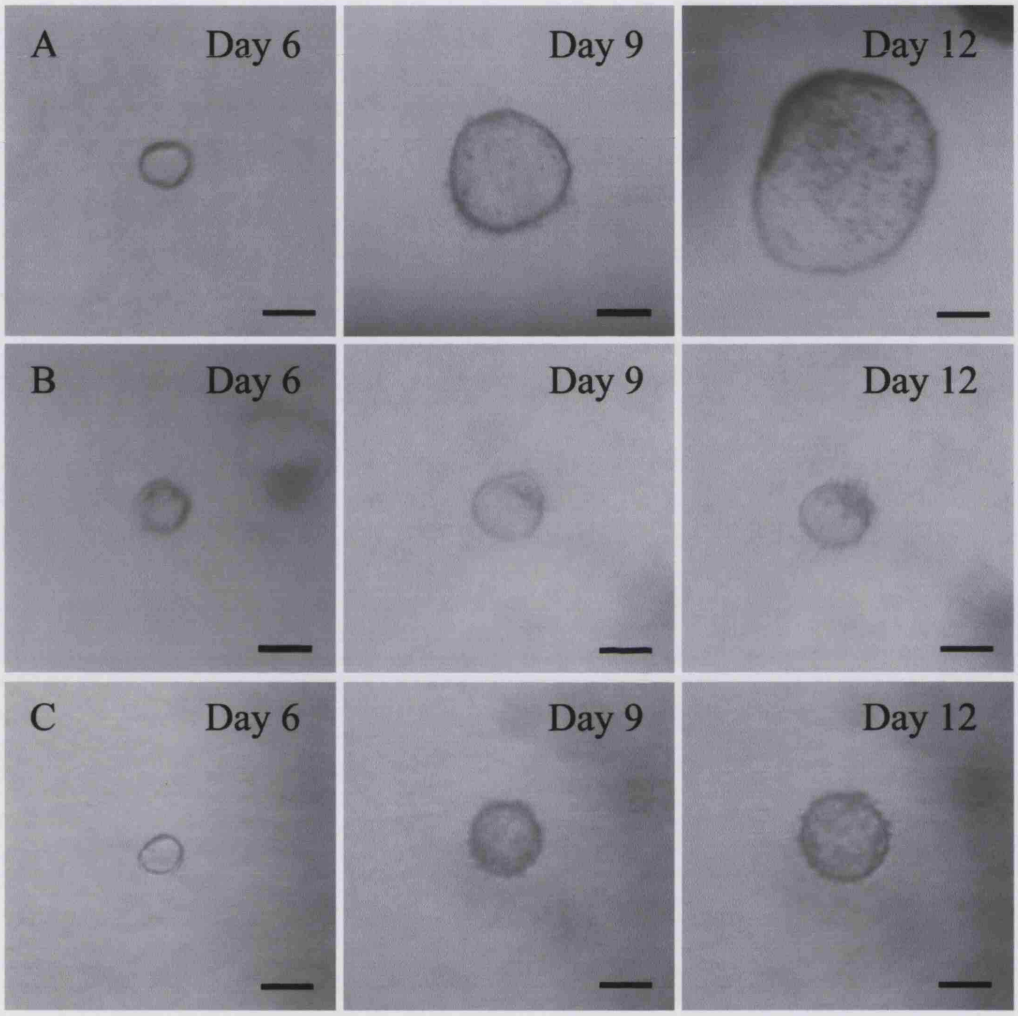
until day 12, media was changed every two days. Photographs (Figure 5.1) and measurements of cyst size were taken at three-day intervals until day 12, when the experiment was terminated.

5.3.1 Effects of P2 receptor Antagonists

As a first step in investigating the role of P2 receptors on renal cyst growth the ability of different P2 receptor antagonists to influence cyst size was determined. Most antagonists at a concentration of 100μM reduced cyst growth rate, although it was not possible to completely prevent cyst enlargement (Table 5.1 and Figure 5.2A). The rank order of potency was RB2> suramin> PPADS> MRS2179> Coomassie brilliant blue G. In non-treated, forskolin-only cysts, a growth rate of 1.06 (+/- 0.07) nl/day was recorded (Figure 5.2A). Incubation of MDCK cysts with 100μM RB2 consistently diminished the rate of cyst growth by 68% to 0.35 (+/- 0.08,) nl/day ($p<0.001$). Suramin (100μM) also reduced cyst growth rate by 51% to 0.53 (+/- 0.07) nl/day ($p<0.001$). 100μM PPADS reduced cyst growth rate to 0.74 (+/- 0.11) nl/day ($p<0.05$), and the $P2Y_1$ selective antagonist MRS 2179 reduced cyst growth rate to 0.76 (+/- 0.12) nl/day ($p=0.05$). One hundred μM coomassie brilliant blue G had no significant effect on cyst growth rate.

The most potent antagonists were RB2 and suramin. Therefore established cysts were exposed to either an increased concentration of these antagonists (1mM) or to a combination of 100μM RB2 and 100μM suramin. For this set of experiments untreated (forskolin only) cysts had a mean growth rate of 0.84 (+/- 0.12, n=27) nl/day (Table 5.2 and Figure 5.2B). The combination of RB2 and suramin reduced cyst growth rate by 88% to 0.10 (+/- 0.01) nl/day

Figure 5. 1 **Example photomicrographs showing the progressive enlargement of single MDCK cysts**



Cysts were cultured in; A, control media (10 μM forskolin only), or in the presence of B, 100μM RB2, or C, 100μM suramin **(***Scale bars = 100μm)*

Table 5. 1 **Mean cyst volume data for P2 receptor agonists and antagonists (100μM), from day 6 to day 12**

Treatment	n	Mean cyst volume (nl)		
		Day 6	Day 9	Day 12
Control	77	0.49 (0.05)	2.89 (0.20)	6.88 (0.44)
RB2	40	0.56 (0.09)	1.30 (0.11)	2.64 (0.51)
Suramin	54	0.58 (0.10)	1.95 (0.30)	3.74 (0.47)
PPADS	40	0.61 (0.12)	2.45 (0.37)	5.10 (0.76)
MRS2179	18	0.47 (0.09)	2.09 (0.32)	5.04 (0.76)
Brilliant blue G	24	0.53 (0.07)	2.56 (0.28)	6.58 (1.40)
ATPγS	24	0.52 (0.05)	3.11 (0.53)	7.21 (1.12)
ADPβS	29	0.43 (0.06)	2.65 (0.39)	7.36 (1.13)
BzATP	27	0.45 (0.06)	1.60 (0.20)	3.37 (0.30)

Numbers in brackets are standard error of the mean (SEM).

($p<0.001$). However, the greatest effect on cyst size was with 1mM RB2, when cyst growth rate was reduced by 91% to 0.07 (+/- 0.02) nl/day ($p<0.001$). Suramin (1mM), reduced cyst growth rate by 84% to 0.13 (+/-0.02) nl/day ($p<0.001$).

Emerging data also suggest an inhibitory action of Zn^{2+} ions on $P2X_1$, $P2X_4$, $P2X_5$, $P2X_7$ and $P2Y_4$ receptors; therefore we incubated cysts from day 6 to day 12 in 1mM Zn^{2+}. This reduced cyst growth rate by 95% to 0.04 (+/- 0.01) nl/day ($p<0.001$) (Table 5.2 and Figure 5.2B). Cyst size on day 12 was reduced from an untreated control cyst size of 5.59 (+/- 0.73) nl to 0.86 (+/- 0.12) nl (Figure 5.2B).

BzATP is considered an agonist at $P2X_7$ receptors, but recent data suggest that it is also a potent antagonist at $P2Y_1$ and $P2Y_4$ receptors (Wildman *et al.*, 2003c;Vigne *et al.*, 1999). BzATP (1mM) reduced cyst growth rate by 84% to 0.13 (+/- 0.02) nl/day ($p<0.001$) compared with untreated cysts (Table 5.2 and Figure 5.2B).

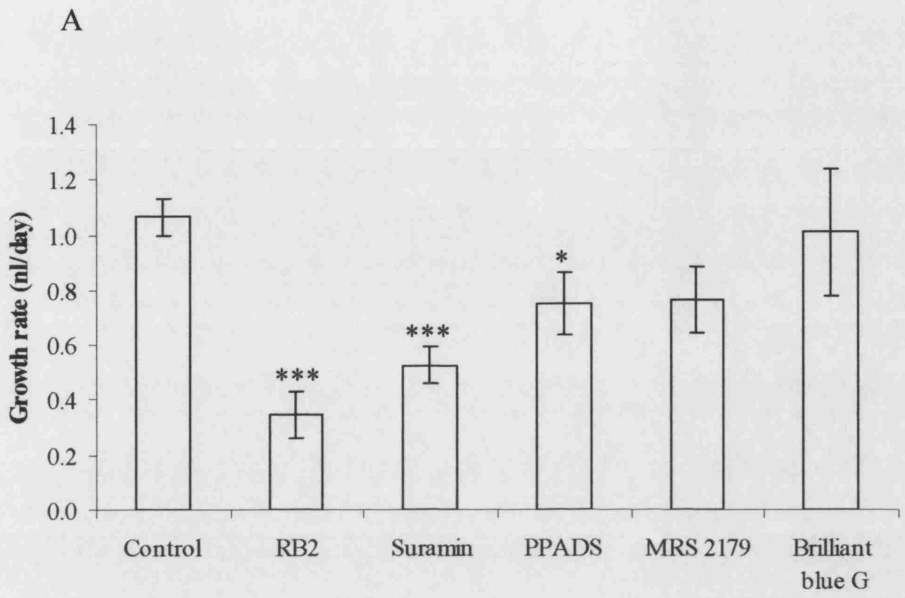
5.3.2 Effects of P2 receptor agonists

Since ATP and its derivatives may be broken down before any effects on cyst growth can be monitored, non-hydrolysable analogues of ATP and ADP were used. When treated with 100μM ATPγS or 100μM ADPβS from day 6, there was no significant change in the growth rate of MDCK cysts (Table 5.1 and Figure 5.3). Non-treated cysts had a growth rate of 1.06 (+/- 0.07) nl/day compared with 1.12 (+/- 0.18) nl/day with ATPγS and 1.15 (+/- 0.18) nl/day with ADPβS (Figure 5.3). There was no cyst formation if MDCK cells were incubated with 100μM ATPγS or 100μM ADPβS from day 0 to day 12 without

Table 5. 2 **Mean cyst volume data for P2 receptor antagonists, from day 6 to day 12. Numbers in brackets are standard error of the mean (SEM).**

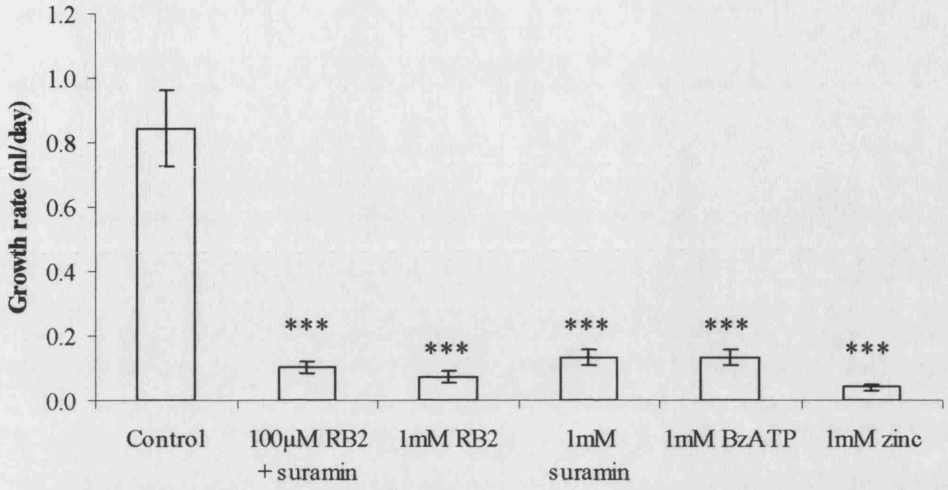
Treatment	n	Mean cyst volume (nl)		
		Day 6	Day 9	Day 12
Control	27	0.53 (0.09)	2.07 (0.25)	5.59 (0.73)
100µM RB2 + 100µM suramin	29	0.35 (0.04)	0.71 (0.08)	0.97 (0.13)
1mM RB2	21	0.50 (0.06)	0.74 (0.10)	0.94 (0.15)
1mM suramin	21	0.57 (0.06)	0.95 (0.11)	1.36 (0.17)
1mM BzATP	23	0.53 (0.07)	0.97 (0.11)	1.32 (0.16)
1mM zinc	27	0.61 (0.10)	0.75 (0.11)	0.86 (0.12)

Figure 5. 2 **A and B; Mean growth rate of MDCK cysts when cultured in the presence of P2 receptor antagonists**



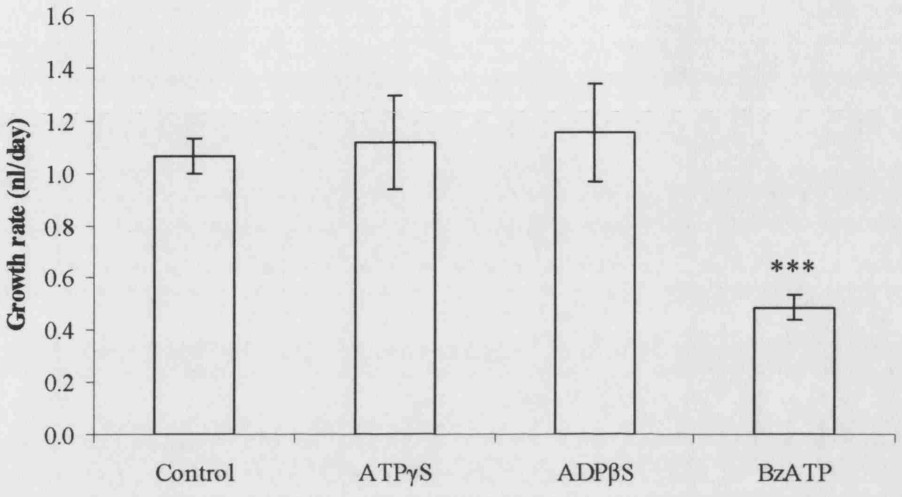
*(A) 100μM P2 receptor antagonists from day 6 to day 12. RB2, reactive blue 2; PPADS, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonate; MRS 2179, 2'-deoxy-N6-methyladenosine-3',5'-bisphosphate. (n= 18-77, * $p<0.05$, *** $p<0.001$)*

B



*(B) co-application of 100µM RB2 and 100µM suramin, 1mM RB2, 1mM Suramin, 1mM BzATP (3'-O-(4-benzoyl)benzoyl –adenosine-triphosphate) and 1mM zinc chloride (n= 21-29, ***p<0.001).*

Figure 5. 3 **Mean growth rate of MDCK cysts when cultured in the presence of P2 receptor agonists**



*BzATP, ATPγS, adenosine 5'-O-[3-thiotriphosphate]; ADPβS, adenosine 5'-O-[2-thiodiphosphate] (n= 24-77, ***p<0.001).*

forskolin (data not shown).

5.3.3 Effect of removal of ATP from growth medium with apyrase

To determine whether cyst growth was due to ATP, the adenosine 5'-triphosphatase and adenosine 5'-diphosphatase, apyrase was used. In this set of experiments, non-treated cysts had a mean growth rate of 0.67 (+/- 0.01) nl/day (Table 5.3 and Figure 5.4). Established cysts were treated with 10 units of apyrase per well from day 6 until day 12, and the media containing 10 units of apyrase was replaced every 2 days. This caused no significant reduction in MDCK cyst growth. However, treatment of established cysts with 20 units of apyrase from day 6 until day 12 reduced cyst growth rate by 46% ($p<0.01$), with a mean growth rate of 0.36 (+/- 0.01) nl/day (Table 5.3 and Figure 5.4). Furthermore, 10 units of apyrase per well from day zero for a total of 12 days, replacing the medium and enzyme every 2 days reduced cyst size by 51% to 0.33 (+/- 0.01) nl/day ($p<0.05$).

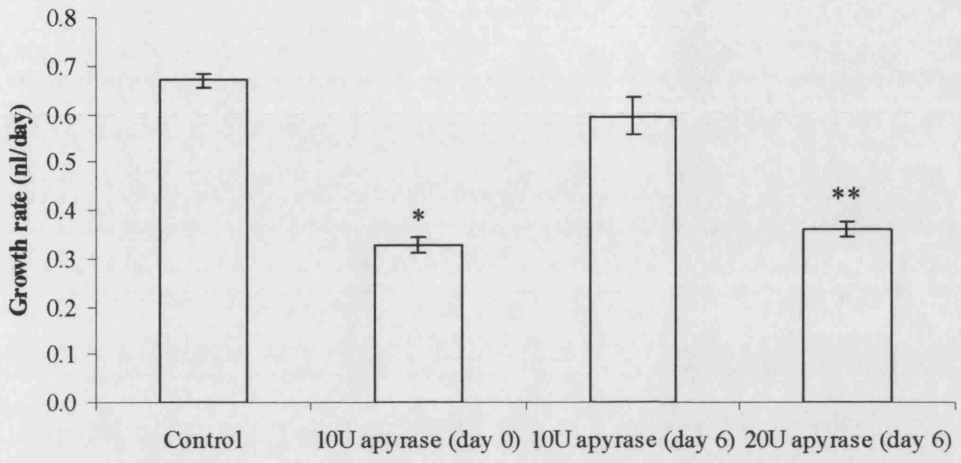
5.4 Discussion

P2 receptors are expressed by normal renal epithelial cells (See Chapter 3)(Bailey *et al.*, 2000b;Bailey *et al.*, 2001) and the cyst lining cells of the Han:SPRD cy/+ rat model of polycystic kidney disease (Chapter 4). Thus, the present study was designed to determine whether P2 receptors could be involved in renal cyst enlargement. MDCK cells, when stimulated with forskolin and grown in a collagen gel, can readily form epithelial microcysts with their apical cell membranes facing the lumen (Mangoo-Karim *et al.*, 1989). Forskolin

Table 5. 3 **Mean cyst volume data for cysts incubated in ATP deplete media from day zero through to day 12, or from day 6 to day 12. Numbers in brackets are standard error of the mean (SEM).**

Treatment	n	Mean cyst volume (nl)		
		Day 6	Day 9	Day 12
Control	36	0.38 (0.04)	1.60 (0.17)	4.40 (0.51)
10U apyrase day 0 - 12	14	0.27 (0.07)	0.88 (0.17)	2.23 (0.38)
10U apyrase day 6 - 12	13	0.41 (0.09)	1.13 (0.25)	3.98 (0.88)
20U apyrase day 6 - 12	20	0.35 (0.05)	1.18 (0.17)	2.50 (0.43)

Figure 5. 4 **Mean growth rate of MDCK cysts when cultured in ATP depleted media.**



*10U apyrase (day 0): 10 units of apyrase from day 0 onwards, 10U apyrase (day 6): 10 units of apyrase from day 6 onwards, 20U apyrase (day 6): 20 units of apyrase from day 6 onwards (n=13-36, *p<0.05, ** p<0.01).*

stimulates cAMP production, arachidonic acid release and Cl^- secretion in MDCK cell monolayers (Ostrom *et al.*, 2000), and an elevated Cl^- concentration has been recorded in MDCK cyst fluid (Mangoo-Karim *et al.*, 1989). Monolayer cultures of MDCK cells express the transporters required for Cl^- secretion, the Na^+-K^+-ATPase (Abaza *et al.*, 1974), the Na^+-K^+-$2Cl^-$ (Giesen-Crouse & McRoberts, 1987) and Cl^- and K^+ channels (Kolb *et al.*, 1985;Kolb *et al.*, 1987). In MDCK type I cells that express CFTR, stimulation by forskolin activates the CFTR Cl^- channel and results in MDCK cyst formation as does stimulation of both Ca^{2+}-activated and volume sensitive Cl^- channels with ionomycin or a 50% hypotonic solution, respectively (Li & Sheppard, 2003). MDCK cells also express $P2Y_{1,2,4,6}$ and $P2Y_{11}$ receptor mRNA transcripts (Post *et al.*, 1998;Brindikova *et al.*, 2003), although at present there are no reports of P2X receptor expression. Observations in other renal epithelia, make it likely that MDCK cells express a combination of P2Y and P2X receptors apically and basolaterally (Chapter 3)(Schwiebert & Kishore, 2001;Leipziger, 2003). Basal or apical application of ATP to monolayers of MDCK cells induces a rise in short-circuit current (I_{sc}), whereas adenosine has no such effect, excluding any contribution from P1 receptor activation (Bourcier *et al.*, 2002). The present study describes the effects of P2 receptor agonists and antagonists on MDCK cyst size.

It is clear that RB2 and suramin were the most potent of the P2 receptor antagonists tested. One hundred μM RB2 reduced cyst growth rate by 68% and 100μM suramin reduced it by 51%. When co-applied at these concentrations, cyst growth was reduced by 88%, suggesting some additivity. However, individually at higher concentrations (1mM), RB2 and suramin reduced cyst

growth rate by 91% and 84%, respectively. Basilen blue, a component of RB2, has previously been shown to inhibit ATP-induced I_{sc} in MDCK cells (Zegarra-Moran *et al.*, 1995). Unfortunately, these antagonists can affect multiple P2 receptors, including P2X receptors; although of the P2Y receptors, RB2 is most notably an inhibitor of the $P2Y_1$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ subtypes, and suramin an inhibitor of the $P2Y_1$, $P2Y_2$, $P2Y_6$ and $P2Y_{11}$ subtypes (Von Kugelgen & Wetter, 2000). Therefore, in combination, RB2 and suramin inhibit many P2Y receptor subtypes and the significant reduction in cyst growth with these agents probably indicates a predominantly P2Y receptor component to cyst enlargement. In a recent report, suramin has been shown to block ADPβS-stimulated cAMP production from MDCK-D1 cells, a sub-clone of the parent strain, via $P2Y_{11}$ receptor inhibition (Torres *et al.*, 2002). Furthermore, cAMP stimulation has been shown to enhance Cl^- secretion from MDCK cysts and MDCK monolayers in culture (Mangoo-Karim *et al.*, 1989). The P2 receptor antagonist PPADS inhibits $P2X_1$, $P2X_3$ and $P2X_5$ receptors (North, 2002;Wildman *et al.*, 2002) and is also an antagonist of $P2Y_2$ and $P2Y_4$ receptors (Wildman *et al.*, 2003c). PPADS reduced cyst growth rate by around 30%, a relatively small effect in keeping with a predominantly P2X inhibitory effect.

It is unlikely that any given P2Y receptor subtype is solely responsible for cyst growth. For example, $P2Y_1$ receptors are sensitive to most of the antagonists used in this study that were able to reduce cyst growth rate (Von Kugelgen & Wetter, 2000), but selective inhibition of this receptor with 100μM MRS2179 reduced cyst enlargement by only 30%. It is possible that inhibition of one or several P2Y receptor subtypes results in others compensating for loss of function. Based on the relative potencies of the antagonists tested in this

study, the sensitivity of $P2Y_1$, $P2Y_6$ and $P2Y_{11}$ receptors to RB2, suramin and PPADS suggests that these receptors in combination have a role in the enlargement of MDCK cysts, since their inhibition resulted in a very significant reduction in cyst growth. These P2Y receptors can be grouped together by their coupling to phospholipase C via the pertussis toxin-insensitive $G_{q/11}$ proteins (Von Kugelgen & Wetter, 2000). Moreover, $P2Y_{11}$ receptors couple to G_s and activate adenylyl cyclase, leading to cAMP formation, which can also stimulate proliferation of renal cyst epithelial cells (Mangoo-Karim *et al.*, 1989;Torres *et al.*, 2002;Yamaguchi *et al.*, 2003). Grantham and colleagues described a pronounced thickening of the walls of MDCK cysts with solute transport inhibitors, such as ouabain and vanadate (Grantham *et al.*, 1989). No such thickening was seen in the present study, suggesting inhibition of both cell proliferation and fluid transport, both known effects of P2Y receptor stimulation (McCoy *et al.*, 1999;Vonend *et al.*, 2003).

$P2Y_1$ and $P2Y_{11}$ receptors are adenine nucleotide-selective, but in contrast to the human $P2Y_{11}$ receptor, the canine receptor is more responsive to ADPβS than to ATPγS (Qi *et al.*, 2001b). ADPβS and ATPγS have previously been shown to increase cAMP production in MDCK-D1 cells via $P2Y_{11}$ receptors (Torres *et al.*, 2002). However, there was no detectable increase in cyst growth rate when these agonists were applied to the culture medium. This may be because the ion transporters involved in cyst growth are already maximally stimulated by the forskolin-induced activation of adenylyl cyclase and subsequent cAMP production. To determine if this was the case, forskolin was omitted from the culture medium and 100μM ATPγS or 100μM ADPβS were applied from day zero, but under these conditions there was no cyst formation.

However, in the presence of forskolin, when ATP and ADP were removed from the culture medium using apyrase cyst growth rate was reduced by almost half when 10 units of apyrase were added before the cysts had formed, or when 20 units were added to 6-day-old cysts. This suggests that ATP is involved in cyst formation, but does not rule out involvement of uracil nucleotides. Ten units of apyrase had little effect on 6-day-old cysts, perhaps because ATP production continued to exceed its degradation.

At present, it is not known whether MDCK cells express P2X receptors, but RB2, suramin and PPADS are active in inhibiting $P2X_{1,2,3}$, and $P2X_5$ receptors (North, 2002), so a P2X receptor component to cyst growth cannot be excluded. Nevertheless, the P2X receptor agonist, α,β-me-ATP has been reported to have no effect on I_{sc} in these cells (Zegarra-Moran *et al.*, 1995). However, Zn^{2+} ions have been shown to inhibit an ATP-induced rise in intracellular Ca^{2+} in MDCK cells (Jan *et al.*, 1999). Studies carried out in *Xenopus* oocytes have shown that Zn^{2+} ions are antagonists at $P2X_1$, $P2X_7$ and $P2Y_4$ receptors (North, 2002;Wildman *et al.*, 2002;Wildman *et al.*, 2003c). High concentrations of Zn^{2+} (300-1000μM) can also inhibit $P2X_4$ and $P2X_5$ receptor responses (Wildman *et al.*, 1999;Wildman *et al.*, 2002). To fully block $P2X_4$ and $P2X_5$ receptors 1mM Zn^{2+} was used, a concentration that inhibited MDCK cyst growth by 95%.

Cyst growth rate was halved by application of 100μM BzATP and reduced by 84% with 1mM BzATP. BzATP is a potent agonist at the $P2X_7$ receptor (North & Surprenant, 2000), but recent data suggest that it can also antagonise $P2Y_1$ and $P2Y_4$ receptors (Vigne *et al.*, 1999;Wildman *et al.*, 2003c). The $P2X_7$ receptor has generated a lot of interest recently, because of its

apparently dual function: brief exposure to an agonist results in the opening of a cation-selective channel, similar to other P2X receptors; whereas more prolonged activation results in increased cell permeability to cations and plasma membrane blebbing, leading eventually to cell death (Virginio *et al.*, 1999;Wilson *et al.*, 2002). In the present study, cysts were exposed to BzATP for 6 days and thus the response generated should be that of prolonged exposure to the agonist. In contrast to BzATP, coomassie brilliant blue G, a putative inhibitor of the $P2X_7$ receptor (Jiang *et al.*, 2000), had little effect on cyst size. It has been suggested that $P2X_7$ receptors have a role in normal cell turnover due to increased expression in shedding epithelia, such as skin epidermis and duodenal mucosa (Groschel-Stewart *et al.*, 1999a;Groschel-Stewart *et al.*, 1999b). Furthermore, recent data showed a significant decrease in cell number when BzATP was applied to cultured keratinocytes (Greig *et al.*, 2003b). Also, $P2X_7$ receptor expression has been associated with collecting duct cysts in the cpk mouse model of polycystic kidney disease, although a non-apoptotic role was suggested (Hillman *et al.*, 2002). From the present study, it was not clear whether the reduction in cyst growth rate with BzATP is due to non-selective inhibition of P2Y receptors, and therefore a reduction in ion and fluid transport, or to stimulation of the $P2X_7$ receptor causing cell loss.

In summary, these results provide evidence that P2 receptor subtypes are involved in MDCK cyst formation but, as with other epithelia, the effect is probably not due to a single receptor, rather a mixture of receptor subtypes that cannot be easily delineated using the agonists and antagonists currently available. Given the evidence that MDCK cells express P2Y receptors, the reduced cyst size with mainly P2Y receptor inhibitors, and previous data supporting P2Y-

mediated cAMP production in these cells, suggest that cyst expansion is related to stimulation of P2Y receptors and fluid secretion. ATP released from cyst lining epithelial cells (via regulated vesicular fusion or from cell damage) could be trapped in cyst fluid, where ATPase activity might be reduced (Schwiebert *et al.*, 2002), and so activate P2 receptors in an autocrine or paracrine fashion. In turn, P2Y receptor stimulation would promote Cl^- and fluid secretion, probably involving, at least in part, increased cAMP production. If ATP signalling and P2Y receptor activation are important in maintaining and increasing cyst size, then therapies based on inhibiting this pathway should slow progression of renal cystic diseases like ADPKD.

Chapter 6

Expression of the $P2X_7$ receptor in rodent models of hypertension, diabetes and glomerulonephritis

6.1 Introduction

It is clear from the results presented in the previous Chapters that $P2X_7$ receptor expression is minimal in healthy kidney but upregulated in the Han:SPRD cy/+ rat model of ADPKD. The aim of the set of experiments presented in this Chapter was to examine the distribution and localisation of the $P2X_7$ receptor in kidneys from three rodent models of chronic glomerular injury - diabetes mellitus, hypertension and glomerulonephritis. Glomerular inflammation, excessive mesangial cell proliferation and abnormal cell turnover are common features of these diseases (Griffin *et al.*, 2003;Wolf & Shankland, 2003).

$P2X_7$ (formally P_{2Z}) receptor expression was first reported in rat brain and in cells of haemopoetic origin including monocytes, macrophages, lymphocytes and bone marrow (Collo *et al.*, 1997;Labasi *et al.*, 2002). However, a low level of $P2X_7$ receptor mRNA has been reported in normal adult kidneys, although the cell type(s) that expressed the receptors were not defined (Rassendren *et al.*, 1997). In normal rat kidney, $P2X_7$ receptor expression is barely detectable (Chapter 3) however it has been identified on cultured mesangial cells (Schulze-Lohoff *et al.*, 1998;Harada *et al.*, 2000), and cultured podocytes (Fischer *et al.*, 2001;Vonend *et al.*, 2002). Although structurally similar to the other P2X receptors, the $P2X_7$ receptor has several unique properties. Its affinity for ATP is extremely low, requiring 10-100 times higher concentrations of ATP compared with other P2X receptors (Surprenant *et al.*, 1996;Rassendren *et al.*, 1997). However, it has a high affinity for BzATP, a synthetic ATP analogue (Surprenant *et al.*, 1996). Brief activation of the $P2X_7$ receptor facilitates a rapid bidirectional flux of cations thereby triggering depolarisation, collapse of the Na^+

and K^+ gradients and an influx of Ca^{2+}, a typical P2X receptor response (Rassendren *et al.*, 1997). However, sustained activation triggers formation of a large, non-specific pore allowing permeability of molecules up to 900 Da (Cockcroft & Gomperts, 1979;Virginio *et al.*, 1999). The opening of this membrane pore is known to cause cell death by disrupting ionic gradients and/or providing pathways for an efflux of vital intracellular molecules (Surprenant *et al.*, 1996). The receptor has a significantly longer intracellular C-terminus, 240 amino acids compared with 27 – 120 amino acids in the other P2X receptors. Truncation of the $P2X_7$ receptor, by removal of the last 177 residues of the C-terminus, prevents pore formation but does not affect the function as a small cation channel (Wilson *et al.*, 2002;Surprenant *et al.*, 1996). Therefore, the long C-terminus appears to be crucial for the formation of the non-selective pore.

Glomerular disease is a common cause of progressive renal failure, eventually requiring dialysis and kidney transplantation. Glomerular hypercellularity and decreased glomerular cell number, which may be associated with glomerulosclerosis, can both be observed in most types of glomerular disease (Pusey & Peters, 1993). Much research is therefore dedicated to studying factors that regulate normal cell turnover, proliferation and apoptosis. It is now known that extracellular ATP can kill cells by necrosis and/or apoptosis (Zheng *et al.*, 1991). Necrosis is often referred to as accidental cell death and is caused through irreversible damage to the plasma membrane. Apoptosis, or programmed cell death, is distinguished from necrosis by morphological and biochemical criteria. Apoptosis is associated with nuclear and cytosolic condensation and with fragmentation of chromatin and DNA. In mouse thymocytes, extracellular ATP was seen to cause the classic signs of apoptosis;

DNA fragmentation, membrane blebbing, cell shrinkage, nuclear condensation and formation of apoptotic bodies (Zheng *et al.*, 1991). Furthermore, ATP-induced apoptosis is thought to be mediated by the $P2X_7$ receptor (Di Virgilio, 1995), activation of which in human embryonic kidney (HEK293) cells revealed extensive membrane disarray and blebbing (Virginio *et al.*, 1999). None of the other P2X receptors induce membrane blebbing or apoptosis. Another unique function of the $P2X_7$ receptor is with proinflammatory events, since receptor activation facilitates processing and release of the inflammatory cytokine, interleukin-1β (IL-1β), from monocytes and macrophages (Verhoef *et al.*, 2003). There is also a study that suggests a proliferative role in $P2X_7$ receptor-transfected lymphoid cells (Baricordi *et al.*, 1999). However, the physiological and pathological role of this receptor, its regulation and tissue distribution are still under investigation. In this Chapter, $P2X_7$ receptor protein localisation has been investigated in rodent models of diabetes, hypertension and glomerulonephritis. In addition, levels of $P2X_7$ receptor mRNA have been examined in a rat model of crescentic glomerulonephritis.

6.2 Methods

All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

6.2.1 Model of diabetes

The kidneys of streptozotocin (STZ)–induced diabetic rat kidneys were kindly provided by Dr. E. Debnam (University College London, UK). Male Sprague-Dawley rats aged 6-7 weeks and weighing 230-260 g were given a single injection of STZ into a tail vein (55 mg/kg body weight, dissolved in

freshly prepared 0.05 M citrate buffer pH 4.5) under light isofluorane anaesthesia. Animals were allowed *ad libitum* access to food (diet RM1, SDS Ltd, Witham, Essex, UK) and water up to the time of experimentation, and were glycosuric 24 hours after STZ treatment (Diastix, Bayer PLC, Berkshire, UK). The animals were terminally anaesthetised with intraperitoneal pentobarbitone sodium at 3, 6 and 9 weeks post-injection and their kidneys examined by immunohistochemistry. Kidneys from age matched control rats were also examined.

6.2.2 Model of hypertension

The kidneys of hypertensive transgenic (mRen2)27 rats (TGR), which express the mouse Ren-2 gene, were kindly provided by Professor J. Mullins (Edinburgh University, UK) and Dr. J. McEwan (University College London, UK). At the time of sacrifice these animals were aged 12 weeks. TGR hypertensive rats develop severe hypertension, which begins at 4 weeks and peaks at 9 weeks of age, blood pressure decreases thereafter. No renal damage is visible until 10 weeks of age, when glomerulosclerosis becomes evident. By 18 weeks, more than 50% of glomeruli are sclerotic and the intrarenal vessels thickened. The kidney tissues of both the diabetic and hypertensive models were embedded in paraffin wax.

6.2.3 Models of glomerulonephritis

Kidneys from the murine model of anti-GBM crescentic glomerulonephritis were kindly provided by Dr. R. Tarzi (Imperial College London, UK) (Tarzi *et al.*, 2003). Most forms of human glomerulonephritis involve an autoimmune antibody response to exogenous or endogenous antigen

with deposition of host immune complexes. The mouse model is an accelerated form of nephrotoxic nephritis in which C57BL/6 mice are given an injection of sheep anti-mouse glomerular basement membrane (GBM) globulin. This binds to the glomerulus where it acts as a planted antigen to which mouse IgG becomes bound. The animals were then sacrificed at time points 24 hours and 8 days after nephrotoxic serum injection and embedded in OCT compound (BDH/Merck, Leicester, UK) in preparation for cryosectioning. The model is characterised by leukocyte infiltration, proteinuria, glomerular capillary thrombosis, glomerular crescent formation and renal impairment.

RNA samples from the rat model of anti-GBM crescentic glomerulonephritis were kindly provided by Dr. F. Tam (Imperial College London, UK) (Tam *et al.*, 1999). Male Wistar Kyoto (WKY) rats weighing 200-250g were injected with 0.1 ml of rabbit anti-rat GBM globulin and then sacrificed at time points 2,4 and 7 days after the nephrotoxic serum injection. Kidneys were removed and RNA was extracted as described in Chapter 2, section 2.2.1.

6.2.4 Immunofluorescence using anti-$P2X_7$ receptor antibody

Sections (4μm) were cut from wax embedded tissue using a microtome, placed on gelatin-coated slides and dried overnight at 37°C. The sections were subsequently de-waxed with histoclear (National Diagnostics, Hessle, UK) and rehydrated in decreasing concentrations of ethanol. Slides were heated in 10mM citrate buffer (pH 6.0) in a microwave oven for 10 minutes and allowed to cool for 1 hour. Slides were then washed three times 5 minutes with excess PBS.

Kidneys embedded in OCT compound (BDH/Merck, Leicester, UK) were sectioned at 8 μm as described in section 2.1.1. For both wax embedded and

OCT embedded sections, an immunofluorescent technique was used (section 2.1.4). Anti thymocyte-1 (thy-1) antibody (Abcam Ltd., Cambridge, UK) was used as a marker for mesangial cells and anti wilms tumour-1 (WT-1) antibody (Santa Cruz Biotechnology, CA, USA) was used as a marker for podocytes. The secondary antibody for fluorescent microscopy was Cy3 labelled anti-rabbit IgG (Abcam Ltd., Cambridge, UK) (red colour) for $P2X_7$, or anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (ICN, Biomed, CA) (green colour) for thy-1.

Antibodies for $P2X_7$ and the podocyte marker WT-1 were raised in the same species therefore tyramide signal amplification was used as described in detail in section 3.2.2. Slides were examined using a Zeiss Axioplan immunofluorescent microscope (Carl Zeiss International, Göttingen Germany) and photographs were documented using a Leica DC200 digital camera (Leica Microsystems, Wetzlar, Germany).

6.2.5 Immuno-electron microscopy of STZ-diabetic rat kidney using anti-$P2X_7$ receptor antibody

The ultra-structural localisation of $P2X_7$ receptor immunoreactivity using electron-microscopy was carried out by Dr. Andrzej Loesch (University College London, UK). Blocks of kidney (approximately 0.5 x 0.5 cm) were immersion fixed overnight at 4°C in fixative consisting of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The following day, the specimens were rinsed in phosphate buffer for several hours at 4°C, and then transferred to 0.05 M tris-buffered saline (TBS) at pH 7.6. Sections (60-70μM) were cut on a vibrotome, collected in TBS and processed for pre-embedding electron immunocytochemistry using the avidin-biotin peroxidase conjugate

procedure. Sections were washed in TBS, and to block endogenous peroxidase activity, incubated for 30 minutes in 30% methanol containing 0.3% hydrogen peroxide. Sections were washed in TBS and non-specific protein binding sites were blocked by 1.5 hour incubation with 10% normal horse serum (NHS) diluted in TBS. The $P2X_7$ receptor antibody (Roche bioscience, Palo Alto, Ca, USA) was diluted to 3 μg/ml with 10% NHS in TBS containing 0.1% sodium azide (Sigma-Aldrich Co. Ltd., Poole, UK). Sections were incubated overnight for 20 hours with the $P2X_7$ primary antibody. The secondary antibody was a biotin-conjugated donkey anti-rabbit IgG serum (Jackson Immunoresearch, Luton, UK) diluted 1 in 500 in TBS containing 1% NHS and 0.1% sodium azide (Sigma-Aldrich Co. Ltd., Poole, UK). Sections were incubated in secondary antibody for 5 hours, followed by 18 hour incubation with ExtrAvidin-horseradish peroxidase conjugate (Sigma-Aldrich Co. Ltd., Poole, UK) diluted 1 in 1500 with TBS. The sections were exposed to nickel-intensified DAB until a colour reaction could be detected, and then osmication (1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4) was carried out. Sections were dehydrated in a graded series of ethanol, embedded in Araldite, and stained with uranyl acetate and lead citrate. The sections were examined with a JEM-1010 transmission electron microscope (JEOL, Tokyo Japan).

6.2.6 Quantification of $P2X_7$ receptor mRNA in a rat model of crescentic glomerulonephritis

One μg of total RNA was reverse transcribed with 0.5μg oligo(-dt) 12-18 primer and a first-strand cDNA synthesis kit, (Superscript II RNase H^- reverse transcriptase, Gibco BRL, UK). The resulting cDNA transcripts of whole kidney mRNA were used for PCR amplification using the Roche Lightcycler (Roche

diagnostics, Penzberg, Germany) and QuantiTect SYBR® Green PCR kit (Qiagen, West Sussex UK) described in detail in section 2.3. See table 2.2 for $P2X_7$ and HPRT primer sequences.

6.2.7 Statistics

Real-time PCR results are expressed as means ± SEM of *n* observations. To compare sets of data, one-way ANOVA and the Tukey-Kramer multiple comparisons *post-hoc* test were used, calculated using 'Graphpad Instat' version 3.06. Differences were considered statistically significant when $P < 0.05$.

6.3 Results

6.3.1 Immunohistochemistry

$P2X_7$ receptor immunoreactivity was barely detectable in either normal rat or normal mouse kidney (Figures 6.1 B and 6.2 A). In contrast, $P2X_7$ receptor immunoreactivity was clearly visible in glomeruli of kidney tissue from STZ-induced diabetic rats (Figure 6.1 A) and 12-week TGR hypertensive rats (Figure 6.1 C). Increased $P2X_7$ receptor immunoreactivity was readily detectable in 3-week diabetic rats, but there was no further increase between weeks 6 and 9. No immunoreactivity was observed in diabetic or hypertensive kidney tissue when the primary antibody was replaced with non-immune serum. Similarly, no immunoreactivity was observed in diabetic or hypertensive tissue when the antibody was pre-absorbed with homologous peptide (Figure 6.1 D). In order to identify the immunopositive cell-type(s), co-localisation studies were carried out. $P2X_7$ receptor immunostaining co- localised with the podocyte nuclear marker WT-1 (Figure 6.1 E), but not the mesangial cell marker Thy-1 (Figure 6.1 F).

Figure 6. 1 **Photomicrographs showing $P2X_7$ receptor expression on cells of the rat glomerulus**

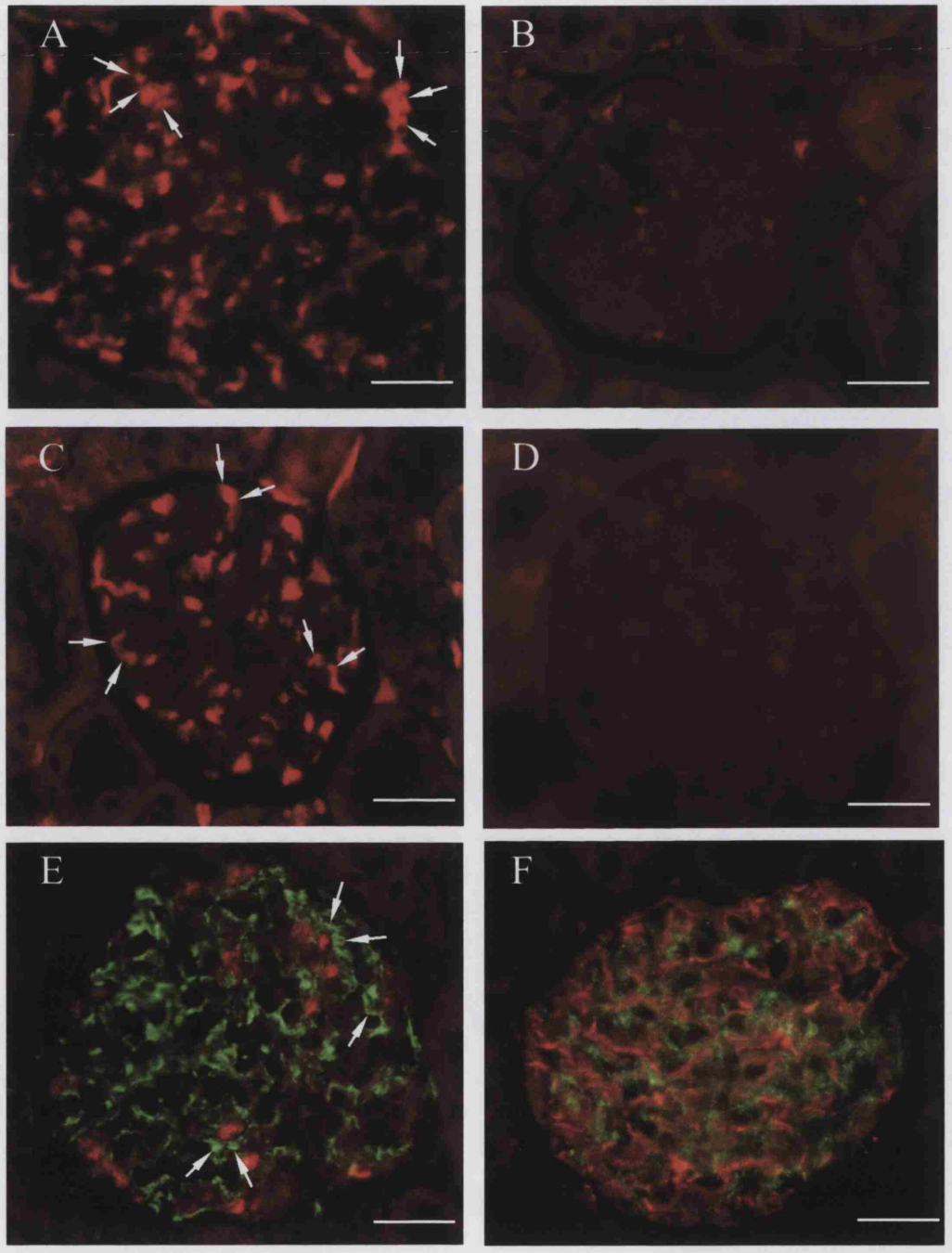


Figure 6.1 legend

A. Increased $P2X_7$ receptor immunoreactivity (arrows) in a 6-week STZ-induced diabetic rat glomerulus (Scale bar = 30μm).

B. Control rat glomerulus showing minimal $P2X_7$ receptor immunoreactivity (Scale bar = 30μm).

C. Increased $P2X_7$ receptor immunoreactivity (arrows) in a 12-week TGR hypertensive rat glomerulus (Scale bar = 30μm).

D. An immunohistochemical control showing diabetic glomerulus pre-absorbed with excess peptide showing no immunostaining (Scale bar = 30μm).

E. $P2X_7$ receptor immunoreactivity (green) on podocytes (arrows), co-localised with the podocyte nuclear protein WT-1 (red) (Scale bar = 30μm).

F. No co-localisation of $P2X_7$ immunoreactivity (red) with the mesangial cell marker Thy-1 (green) (Scale bar = 30μm).

$P2X_7$ receptor immunoreactivity was not visible in control mice glomeruli (Figure 6.2 A) but clearly visible in glomeruli of mice with accelerated nephrotoxic nephritis (Figure 6.2 B) and was readily detectable 24 hours and 8 days after injection with the nephrotoxic serum (Figure 6.2 B - D). No immunoreactivity was observed in mice glomeruli with either non-immune serum or pre-absorbed antibody (Figure 6.2 E). To identify the immunopositive cell type, co-localisation studies were carried out. $P2X_7$ receptor immunoreactivity co-localised with the podocyte nuclear protein WT-1 (Figure 6.2 F), however mesangial cells were also immunopositive for $P2X_7$ receptors. The mesangial cell marker Thy-1 was not suitable for this study, since an antibody raised in mouse was not suitable to probe mouse tissue. A suitable alternative was not available at this time.

6.3.2 Immuno-electronmicroscopy

$P2X_7$ receptor immunoreactivity was located predominantly on podocytes of diabetic kidneys, rather than mesangial cells in accordance with the immunohistochemical data. Minimal immuno-precipitation was detected in healthy controls (Figure 6.3 A) compared with 3-week (Figure 6.3 B) and 9-week (Figure 6.3 C) diabetic animals. $P2X_7$ receptor immunoreactivity appeared as small clumps of immuno-precipitate in both the cytoplasm and on the cell membrane of podocytes (Figure 6.3 B). Particularly dense immuno-precipitate was noted on some podocyte foot-processes at the glomerular filtration barrier (Figure 6.3 C). Immunoreactivity was abolished if the primary antibody was replaced with non-immune serum (Figure 6.3 D), and when the primary antibody was pre-absorbed with homologous peptide.

Figure 6. 2 **$P2X_7$ receptor expression in murine glomerulonephritis**

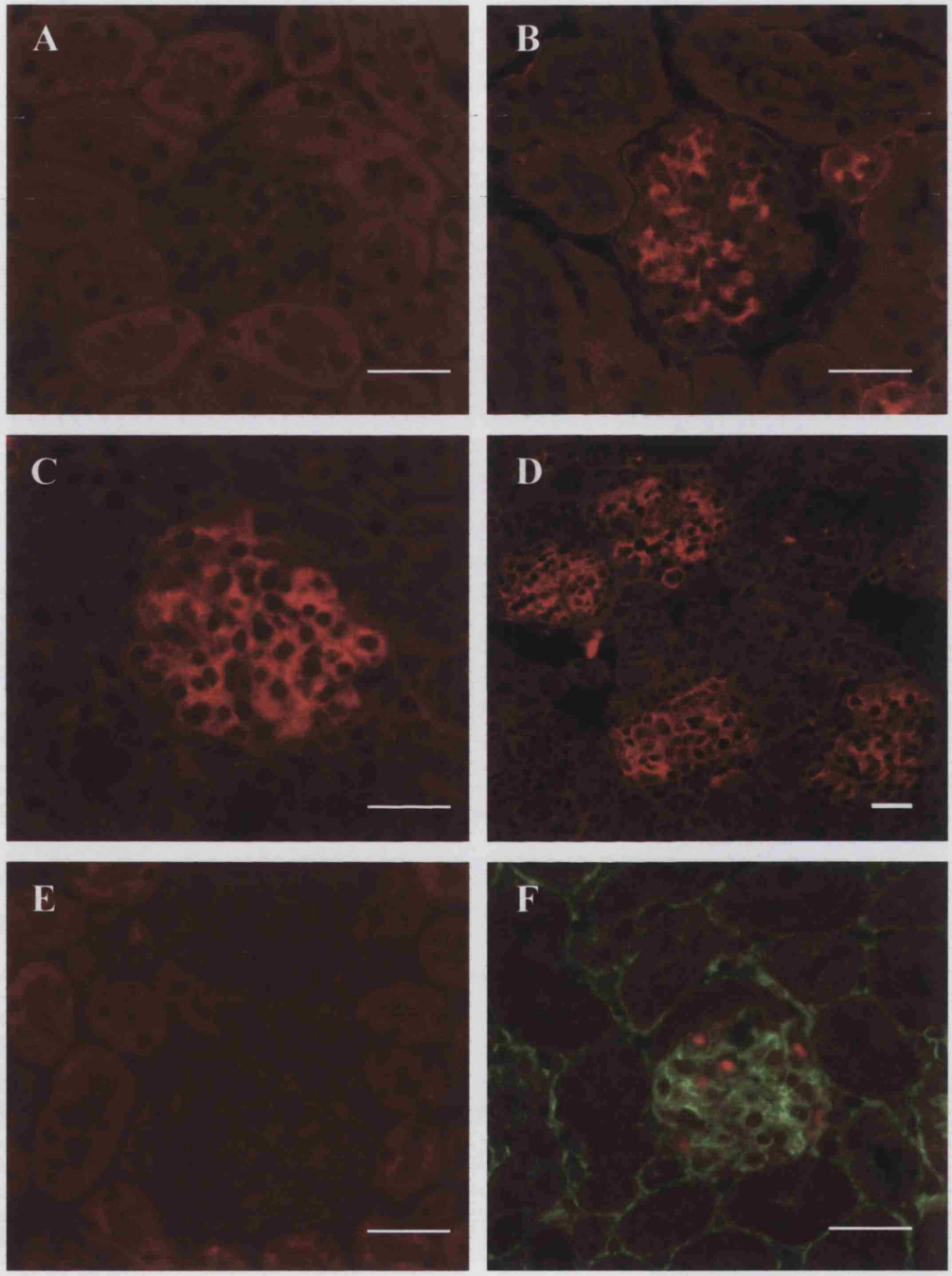


Figure 6.2 legend

A. Control mouse glomerulus showing no $P2X_7$ receptor immunoreactivity (Scale bar = 50μm).

B. Mouse glomerulus, 24 hours post-injection of nephrotoxic serum showing increased $P2X_7$ receptor immunoreactivity (Scale bar = 50μm).

C. and D. Mouse glomeruli, 8 days post-injection of nephrotoxic serum showing increased $P2X_7$ receptor immunoreactivity (Scale bars = 50μm).

E. An immunohistochemical control showing no immunostaining of mouse glomerulus, 24 hours post-injection, pre-absorbed with excess peptide showing (Scale bar = 50μm).

F. $P2X_7$ receptor immunoreactivity (green) 24 hours post-injection, co-stained with the podocyte marker WT-1 (red) (Scale bar = 50μm).

Figure 6. 3 **Ultra-structural localisation of $P2X_7$ receptor to podocytes**

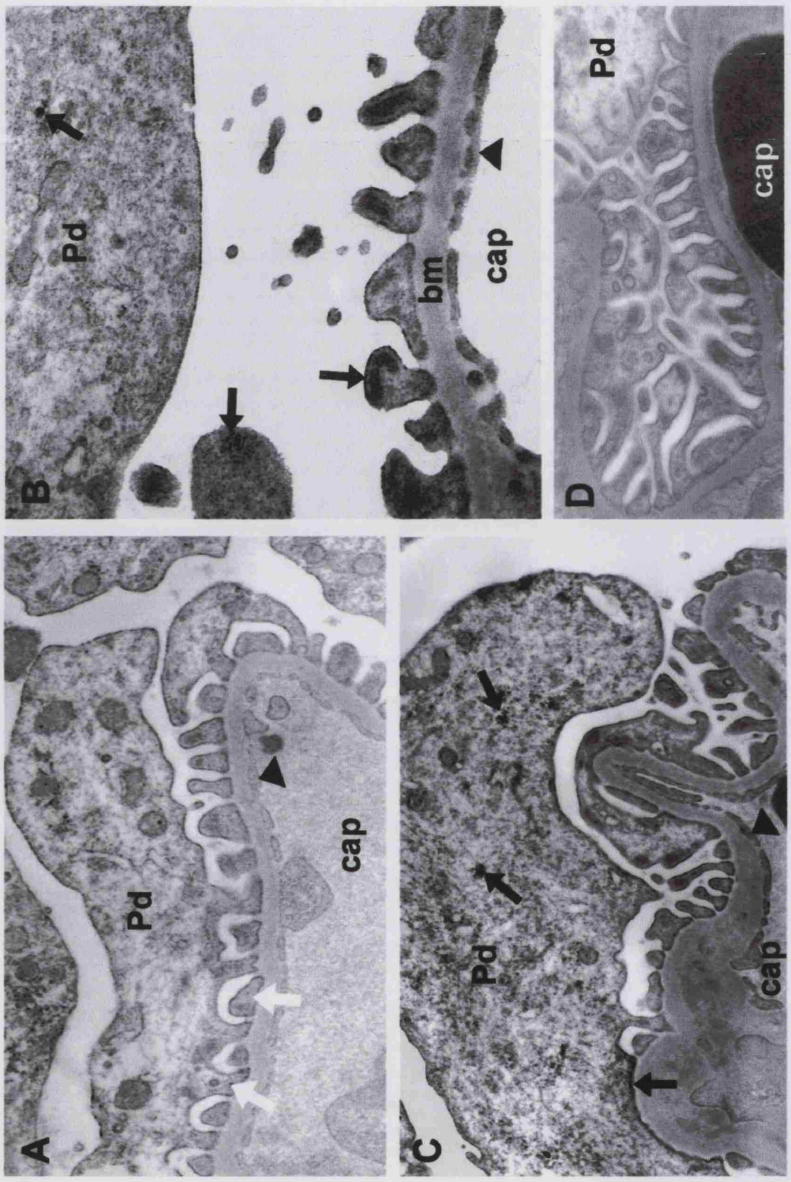


Figure 6.3 legend

A. Age-matched control kidney showing no $P2X_7$ receptor immunoreactivity on podocytes (Pd), podocyte foot processes (white arrows), and fenestrated endothelium (black arrow head) of glomerular capillaries (cap), (magnification x 16,000).

B. $P2X_7$ receptor immuno-precipitate located intracellularly and on the cell membrane of podocyte (Pd) foot processes (black arrows) of 3-week diabetic glomerulus. Glomerular capillary (cap), basement membrane (bm), fenestrated endothelium (black arrow head) (magnification x 15,000).

C. Higher magnification of the glomerular filtration barrier of a 9-week glomerulus, showing $P2X_7$ receptor immuno-precipitate located intracellularly and in association with the cell membrane of secondary foot processes (black arrows). Glomerular capillary (cap), fenestrated endothelium (black arrow head) (magnification x 28,000).

D. No immunoprecipitate when the $P2X_7$ receptor antibody was replaced with non-immune serum (magnification x 15,000).

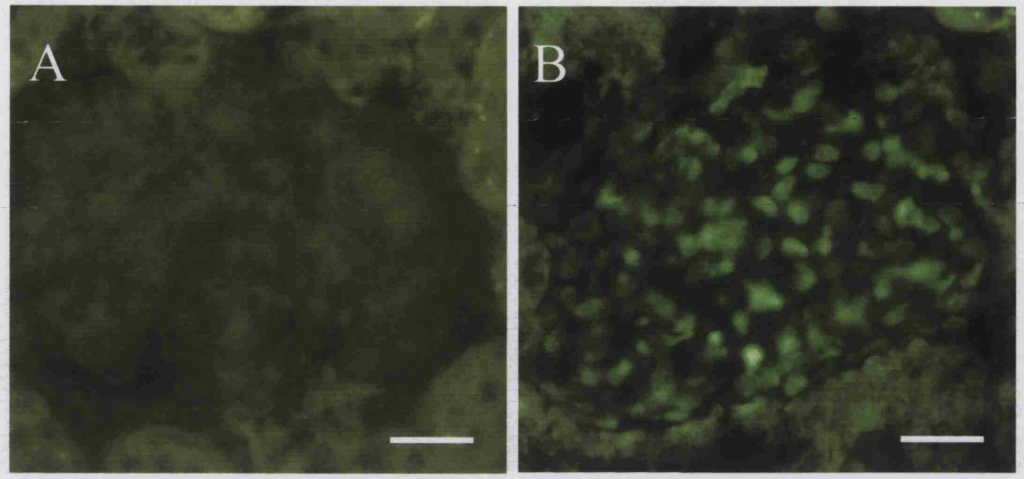
6.3.3 Detection of apoptotic cells

The presence of apoptotic cells in diabetic glomeruli was sought using the TUNEL assay as described in Chapter 4. In normal control glomeruli, there were no detectable apoptotic cells (Figure 6.4A). However, in diabetic glomeruli apoptotic nuclei were readily detectable (Figure 6.4B).

6.3.4 Levels of $P2X_7$ receptor mRNA in crescentic glomerulonephritis

To determine the relative abundance of $P2X_7$ receptor mRNA in kidney tissue, a ratio was calculated for $P2X_7$ receptor RNA to the constitutively expressed housekeeping gene HPRT. Experiments were repeated in triplicate on 6 animals at each time point. On day 2, $P2X_7$ receptor mRNA was unchanged when compared to control. On day 4, $P2X_7$ receptor mRNA was increased by 266% ($p<0.001$) compared to control animals (Figure 6.5) On day 7, $P2X_7$ receptor mRNA was increased but it was not significant.

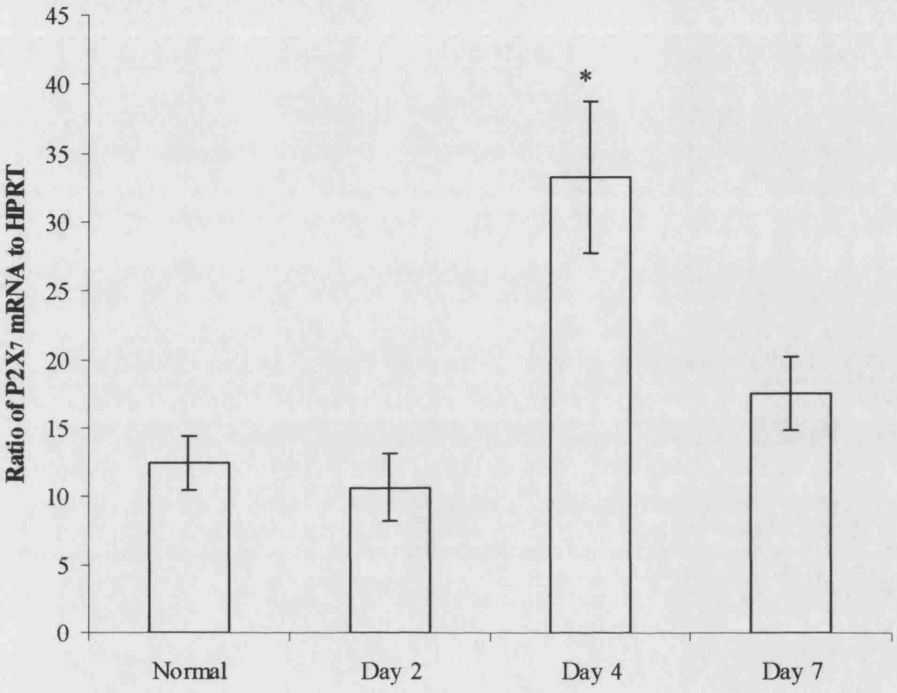
Figure 6. 4 **Identification of apoptotic cells in diabetic glomeruli**



A. Control glomerulus with no detectable apoptotic cells.

B. Increase in TUNEL- positive nuclei in a 9–week diabetic rat glomerulus (scale bar = 30μm)

Figure 6. 5 **Relative abundance of P2 receptor mRNA in kidneys from the rat model of crescentic glomerulonephritis expressed as a ratio to that of the housekeeping gene HPRT**



*Results are presented for unaffected control animals (normal) and 2, 4, and 7 days after anti-GBM nephrotoxic injection. (n=6, * p<0001)*

6.4 Discussion

The current study provides evidence for increased glomerular expression of the $P2X_7$ receptor protein in three models of chronic glomerular injury: the STZ- induced model of diabetes, the transgenic (mRen2)27 renin-related model of hypertension and the murine model of accelerated nephrotoxic nephritis. The characteristic pathological features of glomerulonephritis are inflammatory cell infiltration, extracellular matrix accumulation, and glomerular cell proliferation. These result in glomerulosclerosis and scarring and eventually permanent damage to the ultrafiltration apparatus (Wolf & Shankland, 2003). Similarly, diabetes mellitus may contribute to glomerular basement membrane damage caused by hypertension or may induce it independently through alteration of basement membrane proteins (Ljutic & Kes, 2003). Glomerular hypertension results in glomerular capillary wall stretch, endothelial damage and a rise in protein glomerular filtration. These processes cause changes in mesangial and proximal tubule cells resulting in the replacement of functional by non-functional connective tissue and the development of fibrosis (Ljutic & Kes, 2003). Glomerular hypertension is also associated with oxidative stress that can cause activation of circulating leukocytes and can stimulate the release of cytokines and growth factors (Ha & Kim, 1999). This leads to extracellular matrix formation, progressive sclerosis of both the glomerulus and tubules, and ultimately loss of nephron units. When the compensatory capacity of the remaining nephrons is exceeded, renal function progressively deteriorates and renal failure develops.

The results presented in this Chapter show elevated $P2X_7$ receptor expression located primarily in the glomeruli of the three models of chronic renal injury. Electron-microscopy confirmed expression on mainly podocytes although there was some minimal endothelial and mesangial cell staining. The physiological function of the $P2X_7$ receptor is still under investigation but a number of roles have been proposed. Expression in shedding epithelia such as skin (Groschel-Stewart *et al.*, 1999a), duodenum (Groschel-Stewart *et al.*, 1999b), vagina and uterus (Bardini *et al.*, 2000) suggests it may have a role in normal cell turnover, and in particular with cell death. There are several reports of $P2X_7$ receptor activation causing either necrosis or apoptosis of cells of haemopoietic origin such as macrophages and lymphocytes (Di Virgilio, 1995;Surprenant *et al.*, 1996;Grahames *et al.*, 1999). Furthermore, the ability of extracellular ATP to trigger apoptosis via the $P2X_7$ receptor has been reported in a number of other 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, dramatic membrane blebbing and micro-vesiculation have been observed within seconds to minutes of receptor activation, a phenomenon in which large membrane-bound vesicles protrude rapidly from the cell surface associated with cells undergoing apoptosis (Wilson *et al.*, 2002). Normally, apoptosis is a tightly regulated mechanism for maintaining normal and healthy cell numbers and, in the adult kidney, occurs at a low level (Wolf & Shankland, 2003). However, apoptosis increases following several forms of glomerular injury including ischemia, glomerulosclerosis (Sugiyama *et al.*, 1996) and diabetic nephropathy (Zhang *et al.*, 1997). In the present study, apoptotic cells

were detected in diabetic rat glomeruli but not normal controls, although it was not possible to determine whether the same cells express the $P2X_7$ receptor.

More prolonged activation of the $P2X_7$ receptor results in the formation of a non-selective pore, permeable to large molecular weight fluorescent dyes such as YO-PRO-1 (Virginio *et al.*, 1999). Opening of this pore in mouse macrophages leads to complete depolarisation of the membrane potential and ultimately, cell death (Buisman *et al.*, 1988). However, Virginio and co-workers report that pore formation and membrane blebbing are events that occur independently of each other, since the rate of membrane disruption could be slowed by low extracellular Na^+, but pore formation was accelerated under the same conditions (Virginio *et al.*, 1999). A recent study linked pore formation to the p38MAPK pathway since uptake of the dye YO-PRO-1 by monocytes was blocked by direct inhibition of this pathway (Donnelly-Roberts *et al.*, 2004). However, this pathway has previously been associated with activation of $P2Y_2$ receptors in cultured mesangial cells (Huwiler *et al.*, 2000), although this receptor was not detected on rat mesangial cells by immunohistochemistry (see Chapter 3). In studies reported to date, the response generated by activation of the $P2X_7$ receptor seems to be determined by the length of exposure to the agonist. Activation of the ligand-gated ion channel after brief exposure to ATP, may lead to membrane blebbing and apoptosis (Surprenant *et al.*, 1996), whilst formation of the membrane pore after more prolonged exposure to ATP is likely to result in cell death by lysis and necrosis (Buisman *et al.*, 1988). ATP is released from virtually every cell when cell damage occurs, as seen in the mesangiolytic stage of anti-Thy-1 glomerulonephritis (Poelstra *et al.*, 1992). Whether a cell undergoes apoptosis in response to ATP may depend on the cell

type, the level of $P2X_7$ receptor expression at the cell membrane, the concentration of released ATP and the duration of exposure. A recent study demonstrated enhanced sensitivity of the $P2X_7$ receptor in the retinal microvessicles of STZ-induced diabetic rats in which pore formation could be triggered by a significantly lower concentration of BzATP (Sugiyama *et al.*, 2004). Therefore it may be possible that diabetes increases the sensitivity of the $P2X_7$ receptor to ATP not only in the retina but also in the glomerulus.

Of relevance to the present study and to glomerular injury and inflammation is that brief stimulation of the $P2X_7$ receptor rapidly promotes activation of caspase-1 and release of IL-1β from activated macrophages and HEK-293 cells, independently of membrane blebbing events (Ferrari *et al.*, 1997a;Perregaux *et al.*, 2000;Verhoef *et al.*, 2003). When stimulated by an inflammatory insult such as bacterial lipopolysaccharide (LPS), monocytes and macrophages generate large quantities of proIL-1β which is cleaved by caspase-1 into the mature form IL-1β (Dinarello, 1998). Macrophages isolated from $P2X_7$ receptor knock-out mice fail to generate mature IL-1β in response to ATP, thus confirming the role of $P2X_7$ in this response (Solle *et al.*, 2001). IL-1β is a key component of the innate immune response to infection or tissue damage and its circulating levels are tightly regulated to prevent aberrant activation of inflammatory pathways. However, when macrophages are exposed to LPS and ATP they generate approximately 20-35 times more mature IL-1β than if stimulated with LPS alone (Le Feuvre *et al.*, 2002). Previous reports have suggested a $P2X_7$-induced rise in intracellular Ca^{2+} initiates release of IL-1β–containing microvessicles (Gudipaty *et al.*, 2003), or that $P2X_7$ receptor activation via ATP accelerates caspase-1 processing and activation by

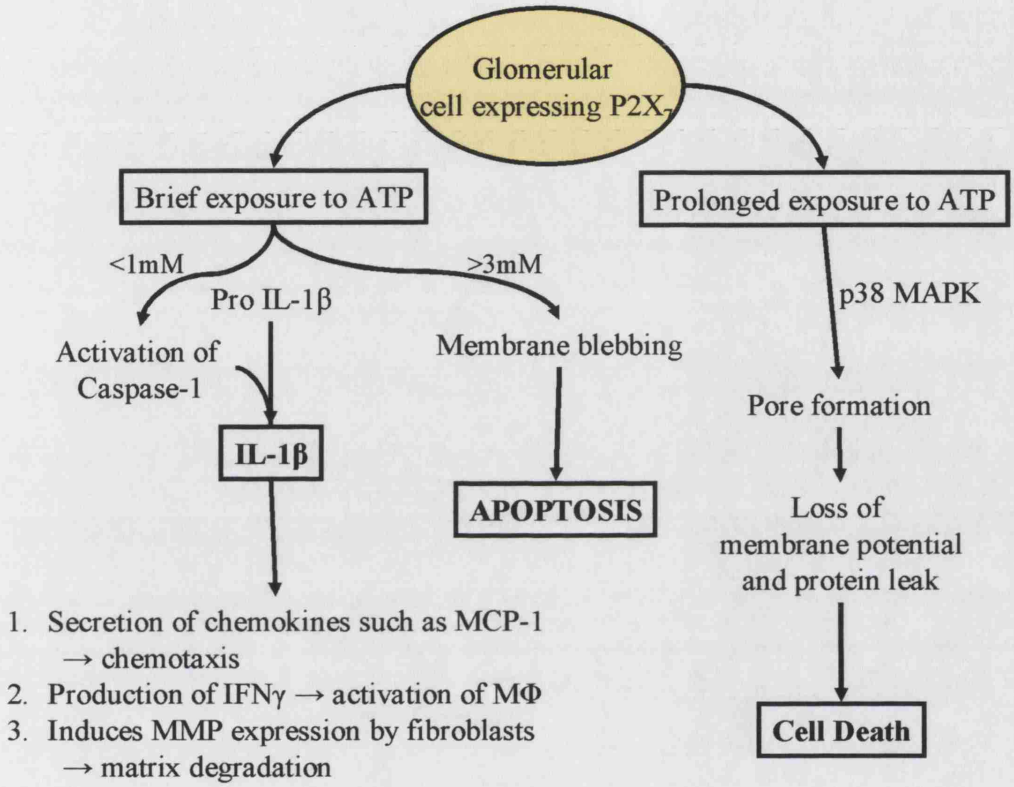
stimulating increased K^+ efflux via a phospholipase A2 dependent mechanism (Kahlenberg & Dubyak, 2004). However, the exact mechanisms of caspase-1 activation are not well defined. Glomerular mesangial cells and podocytes are both capable of producing IL-1β (Niemir *et al.*, 1997;Tesch *et al.*, 1997), and it is thought that this cytokine is largely responsible for the leukocyte infiltration in anti-glomerular basement membrane antibody glomerulonephritis (Tang *et al.*, 1994). When released from producing cells, IL-1β binds to receptors on target cells and elicits signalling cascades that enhance the inflammatory response leading to cell death (Dinarello, 1998). Target cells include endothelial cells which, when exposed to IL-1β, are induced to secrete chemokines such as monocyte chemoattractant protein-1 (MCP-1) (Sica *et al.*, 1990), and to upregulate the expression of vascular adhesion molecules such as E-selectin (Savage *et al.*, 1997). MCP-1 provides a stimulus for chemotaxis in glomerulonephritis thus facilitating immune cell infiltration into an area of early inflammation (Fujinaka *et al.*, 1997). In cultured astrocytes, $P2X_7$ receptor activation increases expression of MCP-1 via a MAP kinase dependent mechanism which includes the p38 MAP kinase (Panenka *et al.*, 2001). This kinase pathway plays a role in the cascade to programmed cell death (Koul, 2003) and may provide a link with the apoptotic function of the $P2X_7$ receptor.

Interestingly, in the rat model of crescentic glomerulonephritis, maximal $P2X_7$ receptor mRNA expression was detected on the fourth day after injection of nephrotoxic serum. This coincides with a previous report of glomerular infiltration of CD8+ monocytes and macrophages which were maximal on day 4 and the beginning of glomerular cell damage in this model (Tam *et al.*, 1999). These observations provide increasing evidence for an association between the

$P2X_7$ receptor, macrophage chemotaxis and glomerular inflammation at least in this model. There are several other inflammatory cytokines which have been associated with the $P2X_7$ receptor. Increased $P2X_7$ receptor expression has been reported in cultured fibroblasts exposed to high concentrations of extracellular glucose and this has been linked to release of the autocrine growth factor IL-6 (Solini *et al.*, 2000), which is also increased in diabetic glomeruli (Harada *et al.*, 1993). In microglial cells, $P2X_7$ receptor stimulation potently activates the transcription factor NF-κB which is an important transcriptional activator involved in proinflammatory cytokine synthesis and apoptosis (Ferrari *et al.*, 1997b). Furthermore, LPS and cytokines such as interferon γ (IFN-γ) and tumour necrosis factor-α (TNF-α) can increase expression of the $P2X_7$ receptor (Di Virgilio, 1995;Harada *et al.*, 2000), and extracellular ATP can also stimulate TNF-α release (Tonetti *et al.*, 1995) further linking the inflammatory response to $P2X_7$ receptor activation. In glomerulonephritis, immune deposits attract inflammatory cells and result in local release of TNF-α and IL-1β (Kluth & Rees, 1999). Moreover, IL-1β is released from podocytes with glomerulosclerosis (Niemir *et al.*, 1997), and IL-β and TNF-α may also be increased in diabetic glomeruli (Hasegawa *et al.*, 1991). IL-1β may also, in conjunction with macrophage-derived IL-12, induce IFNγ secretion (Hunter *et al.*, 1995), and induce the expression of matrix metalloproteinase-9 (MMP-9) from mesangial cells and this effect can be potentiated by ATP (Huwiler *et al.*, 2003). Camp and co-workers demonstrated that levels of MMP-9 were increased and contributed to the interstitial fibrosis and glomerular basement membrane thickening in spontaneously hypertensive rats (Camp *et al.*, 2003).

The findings presented in the present study of increased $P2X_7$ receptor expression in three models of glomerular cell damage compared with age-matched control animals may indicate a role for the $P2X_7$ receptor in glomerular repair by deleting damaged cells. Consistent with this role, and in contrast to other P2 receptor subtypes, $P2X_7$ receptor activation requires relatively high concentrations of ATP which are most likely to be found during platelet aggregation, thrombosis and cell injury (Born & Kratzer, 1984). Extracellular ATP, via $P2X_7$ receptors, may interact with and regulate inflammatory cytokine release playing an important role in the inflammatory response. Consequently, the level of receptor expression, together with the concentration of locally released ATP probably determines the final outcome. Brief exposure to ATP leads to IL-1β release, membrane blebbing and apoptosis, whilst more prolonged activation results in pore formation via the MAPK pathway and cell death by necrosis (Figure 6.6). Understanding how $P2X_7$ receptor activity is regulated may provide useful therapeutic strategies for controlling inflammatory response mechanisms.

Figure 6. 6 **Proposed mechanisms for the relationship between $P2X_7$ receptor and the inflammatory response**



The response generated by the $P2X_7$ receptor is determined by the length of exposure to the agonist. Brief exposure to a low concentration of ATP (<1mM) promotes activation of caspase-1 and conversion of the inflammatory cytokine from pro-IL-1β to mature IL-1β (Verhoef et al., 2003). IL-1β induces secretion of chemokines, monocyte and macrophage (MΦ) infiltration and subsequent activation, and production of matrix metalloproteinases (MMP). Brief exposure of the $P2X_7$ receptor to a higher concentration of ATP (>3mM) may also induce membrane blebbing and apoptosis (Verhoef et al., 2003). It is not clear whether production of IL-1β and membrane blebbing events can occur simultaneously. More prolonged exposure to ATP results in formation of a large

membrane pore which allows leakage of vital cellular components and ultimately cell death.

Chapter 7

General Discussion

7.1 Extracellular nucleotide signalling to epithelial cells

Extracellular ATP is now widely accepted as a signalling molecule that may function as a paracrine or autocrine factor and can cause a wide range of biological effects. In this respect, both P2X and P2Y receptors have been identified as having widespread and abundant distribution in mammalian epithelia. The earliest studies of P2 receptor expression in kidney initially reported that P2X receptors were confined to blood vessels and controlled renal blood flow, while P2Y receptors were present on the tubule epithelium. However, this broad division of their localisation and function has proved to be an over simplification. This thesis provides a detailed overview of the distribution and complexity of the P2 receptor system in mammalian kidney.

It is clear from the studies presented that both P2X and P2Y receptors are strongly represented in the intrarenal vasculature, cells of the glomerulus and normal kidney tubule epithelium. Data presented in Chapter 3 demonstrate that $P2X_1$, $P2X_2$ and $P2Y_1$ were the only receptors detected in the intrarenal vasculature where, based on several lines of evidence presented in the literature, they may modulate tubuloglomerular feedback. Interestingly, the dominant receptor subtypes in the glomerulus and early nephron are of the P2Y type and possibly regulate salt and water balance, whereas $P2X_5$ receptors dominate in the collecting duct. $P2X_4$ and $P2X_6$ receptors were expressed at low level throughout the nephron. The P2 receptor system is complicated further by the existence of multiple receptor subtypes and individual variations within each segment of the nephron.

The roles postulated for P2 receptors in the kidney are multiple and diverse (see section 1.6), including cell cycle regulation, as mediators of

hormonal control in the proximal tubule, as mediators or moderators of TGF, and as regulators of ion transport processes along the nephron. Clues can also be obtained by studying other epithelial tissues, for example, several P2Y receptors have been identified in the gastrointestinal tract where they promote secretion of K^+, HCO_3^- and Cl^- (Kottgen *et al.*, 2003;Robaye *et al.*, 2003), and inhibition of Na^+ absorption (Yamamoto & Suzuki, 2002); and $P2X_7$ receptors have been identified in duodenal villus tip cells, which undergo apoptosis before being exfoliated into the intestinal lumen (Groschel-Stewart *et al.*, 1999b). In pancreatic ducts, which express several P2X and P2Y receptor subtypes (Luo *et al.*, 1999), luminal ATP and UTP stimulate HCO_3^- secretion (Ishiguro *et al.*, 1999) and inhibition of K^+ channels (Hede *et al.*, 1999). In respiratory epithelium, P2 receptors contribute to mucociliary clearance by secretion of mucin from goblet cells (Kemp *et al.*, 2004), by increasing ciliary beat frequency (Ma *et al.*, 1999) and by promoting secretion of Cl^- and inhibition of Na^+ absorption, helping to maintain increased hydration of the respiratory surface (Inglis *et al.*, 1999). It is tempting, given their ubiquitous expression, to assign a common underlying function to P2 receptors in epithelia. However, it is likely that the different organ systems in which these receptors have been identified have developed P2 receptors to carry out very different roles, the complexity of which are just beginning to be discovered.

There can be several P2 receptor subtypes expressed by a single cell type and they can be apical, basolateral or expressed in both membrane domains. Consequently, this multiplicity has made identification of subtypes based on agonist or antagonist induced calcium transients difficult. The development of subtype-selective agonists and antagonists that work *in vivo* is essential for a

proper analysis of P2 receptor function in any organ system of the body. Although the function of P2 receptors in the kidney is still largely speculative, clues can be obtained by studying their expression in various renal diseases.

7.2 Expression of P2 receptors in ADPKD: Are animal models and cell cultures appropriate to study a clinical disease?

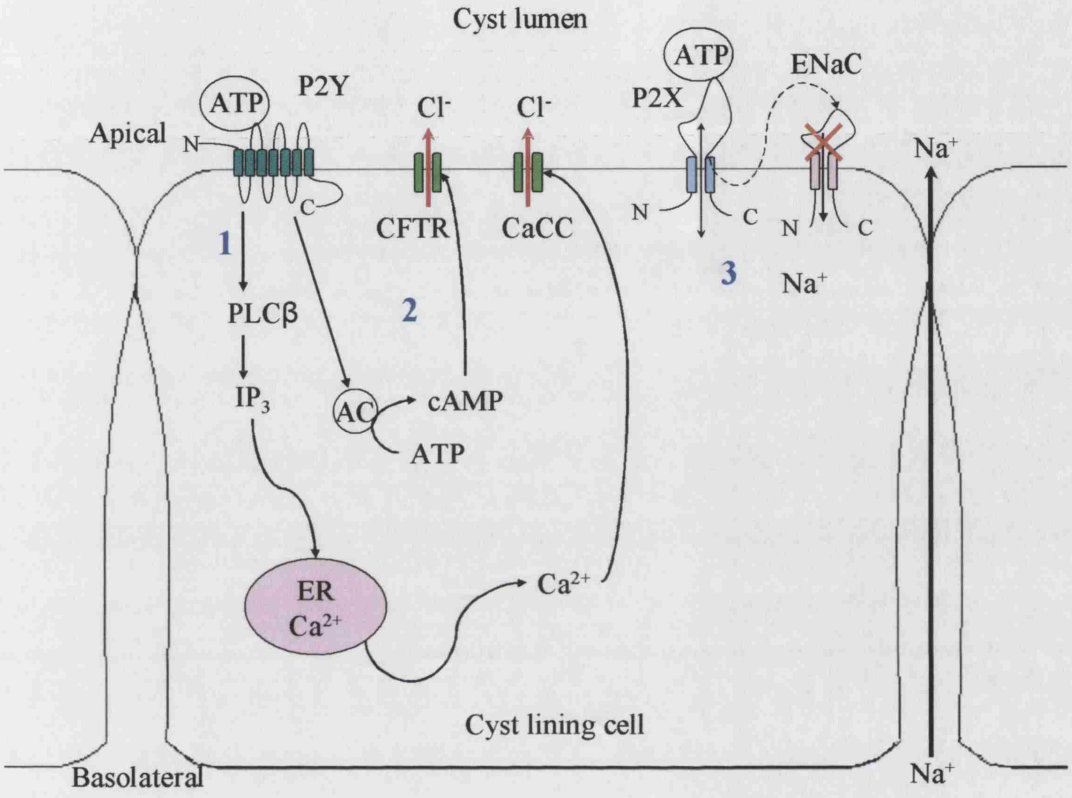
Since ATP is a normal constituent of the extracellular environment, it is highly probable that alterations in extracellular levels of ATP and thus over or under-stimulation of P2 receptors, or alterations in receptor number may be associated with disease. One possible disease is autosomal dominant polycystic kidney disease; an inherited disorder in which multiple cysts develop in the kidney and their growth and enlargement reduce renal function. Recently, 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). Furthermore, Schwiebert and co-workers reported expression of P2X and P2Y receptor protein and mRNA transcripts in human ADPKD cell cultures, although they did not make any quantitative assessments of P2 receptor expression in ADPKD cells *versus* normal kidney cells (Schwiebert *et al.*, 2002). In this thesis, the expression and localisation of P2 receptor protein and a quantitative analysis of P2 receptor mRNA in polycytsic *versus* normal kidney tissue was sought in the Han:SPRD rat model of ADPKD. P2 receptor expression has been detected on cyst epithelial cells of patients with ADPKD and these results are documented in Appendix 3. However, there was limited availability of normal kidney for comparison of receptor expression and the ADPKD tissue removed for clinical

reasons was often in the advanced stages of the disease. The Han:SPRD rat model bears a certain degree of similarity to human ADPKD: autosomal dominant inheritance, slow disease progression and similar histological changes, therefore it was ideal for the purposes of this study. However, there are important differences between this model and human ADPKD to be considered such as the predominant involvement of proximal tubules while in human ADPKD, cysts arise from all nephron segments; the lack of extrarenal manifestations and the fact that the mutated locus that carries the disease is not yet identified (Schafer *et al.*, 1994;Bihoreau *et al.*, 1997). Therefore, it is important to bear in mind that animal models are only as good as their resemblance to human disease. It is evident from the studies presented in Chapter 4 that both P2X and P2Y receptors are expressed on cyst epithelial cells in the Han:SPRD rat model. Increased mRNA and protein expression of $P2Y_2$ and $P2Y_6$ receptor subtypes suggests that these receptors could be involved with cyst development. However, at present the contribution that P2 receptors might make in enhancing cyst growth in the Han:SPRD rat is speculative and based on related *in vitro* observations. For fluid-filled cysts to increase in size there must be an increase in the number of epithelial cells lining the cyst to allow for expansion and an increase in volume of fluid within cysts. These factors can be linked to P2 receptor activity in other cell types: P2 receptor mediated proliferation has been demonstrated in cultured rat mesangial cells (Harada *et al.*, 2000;Vonend *et al.*, 2003) and human keratinocytes (Greig *et al.*, 2003b), and P2 receptor-mediated fluid secretion has been demonstrated in several renal cell types (Cuffe *et al.*, 2000;Deetjen *et al.*, 2000;Lehrmann *et al.*, 2002;Rubera *et al.*, 2000).

Although, the mechanism of P2Y-induced fluid secretion is not known, a hypothesis based on evidence presented in the literature (see section 1.9), is illustrated in Figure 7.1: P2Y receptor activation and coupling through either Gq or Gs leads to production of phospholipase C-β and cAMP, respectively. PLC-β stimulates production of IP_3 and subsequent release of Ca^{2+} from intracellular stores which may activate calcium-activated chloride channels, whereas cAMP can affect several receptors including CFTR (Morales *et al.*, 2000). Activation of one or both chloride channels can lead to facilitated movement of chloride into the cyst lumen where water follows by osmosis. In addition to control of chloride channels, it is also known that P2Y receptor activation can release G protein βγ subunits which can regulate other receptors such as outwardly rectifying K^+ channels (O'Grady *et al.*, 1996). Finally, P2X receptor mediated inhibition of the epithelial sodium channel (Wildman *et al.*, 2003a) or other, as yet unidentified ion channels may also contribute to elevated solute concentrations in cyst fluid and further stimulation of fluid accumulation.

Cell cycle regulation, and therefore the rate of cell proliferation, differentiation and death, is an important consideration in ADPKD. Cell turnover in the normal adult kidney occurs at a very low rate (Wolf & Shankland, 2003); however in ADPKD cyst cells, cell proliferation is increased as cysts expand (Ramasubbu *et al.*, 1998). Several P2Y receptor subtypes identified on cyst epithelial cells of the Han:SPRD rat model have been associated with proliferation of renal cells. $P2Y_2$ and $P2Y_4$ receptors were both detected on Han:SPRD cy/+ cyst lining cells. In mesangial cells, these receptors are linked to the mitogen-activated protein kinase cascade mediated through protein kinase

Figure 7. 1 **A possible mechanism for involvement of P2 receptors in ADPKD fluid secretion**



The facilitated transcellular transport of Cl^- in conjunction with paracellular passive transport of Na^+ creates a salt gradient that promotes the osmotic flow of water into the cyst lumen. P2Y receptor coupling to Gq, activates phospholipase C-β generating the second messenger inositol 1,4,5-triphosphate (IP_3), which docks with IP_3 receptor on the endoplasmic reticulum (ER) triggering release of Ca^{2+}. The increase in intracellular Ca^{2+} activates calcium-sensitive chloride channels (CaCC) and chloride is released into the cyst lumen. 2. P2Y receptor coupling to Gs stimulates production of adenylate cyclase (AC) which converts ATP to cAMP. Cyclic AMP activates protein kinase A which in turn phosphorylates CFTR increasing its permeability to Cl^- and thus

releasing Cl^- into the cyst lumen. 3. P2X-mediated inhibition of the epithelial sodium channel (ENaC) which normally absorbs Na^+ from the apical medium, contributing to elevated solute in cyst lumen fluid.

C, which stimulates cell proliferation (Huwiler & Pfeilschifter, 1994;Ishikawa *et al.*, 1994;Vonend *et al.*, 2003). Furthermore, expression of the $P2X_7$ receptor on cyst epithelial cells in this rat model is also of interest since the $P2X_7$ receptor has been linked to apoptosis in several *in vitro* cell types (Schulze-Lohoff *et al.*, 1998;Coutinho-Silva *et al.*, 1999;Harada *et al.*, 2000). Apoptosis is postulated to be a contributing factor to the loss of renal tissue and the progressive deterioration in renal function with ADPKD (Woo, 1995;Ali *et al.*, 2000;Ecder *et al.*, 2002). $P2X_7$ receptor expression has also been detected in *cpk* mouse kidney cyst epithelia (Hillman *et al.*, 2002). However, in the study presented in Chapter 4 and that of Hillman and co-workers, $P2X_7$ receptor expression could not unequivocally be linked to cells undergoing apoptosis. Another postulated role for the $P2X_7$ receptor, and of some relevance to ADPKD, is with recruitment of inflammatory cytokines a subject discussed in more detail in Chapter 6. In cystic epithelia of the Han:SPRD rat model, and in urine from patients with ADPKD, there is increased expression of monocyte chemoattractant protein-1 (MCP-1)(Zheng *et al.*, 2003;Cowley, Jr. *et al.*, 2001). MCP-1 is a chemotactic factor for monocytes and macrophages, which are known to express $P2X_7$ receptors and to secrete IL-1β which is thought to be converted to its active form in a $P2X_7$-dependent manner (Le Feuvre *et al.*, 2002;Verhoef *et al.*, 2003). Thus the $P2X_7$ receptor may be part of a larger chain of events that recruits cytokines and leads to inflammation in renal cysts.

To explore further the role that P2 receptors play in cyst growth and development an *in vitro* renal cell culture model was used that, using P2 receptor agonists and antagonists, enabled the study of altered P2 receptor activity on cyst growth. This model of renal cyst formation incorporates the growth of MDCK

cells in hydrated collagen gel and yields spherical monolayered cysts filled with clear fluid (McAteer *et al.*, 1987). These cells exhibit epithelial cell proliferation and accumulation of fluid within the lumen (Grantham *et al.*, 1989), providing an *in vitro* cell system for studying the unique properties of renal cyst formation. Identification of the exact receptor subtypes involved could not be achieved with the agonists and antagonists available and used in this study. This was because the availability of more selective antagonists is limited and, since the effects would be monitored over several days, only non-hydrolysable drugs were used also limiting the drugs available. A second problem arose with the interpretation of the data due to the presence of a mixed population of P2Y and probably P2X receptors, including P2X homomers and heteromers. This problem could be addressed using RT-PCR and immunoblotting to detect P2 receptor mRNA transcripts and proteins, respectively. What still remains to be addressed are the localisation of P2 receptor subtypes (apical *versus* basolateral) and whether the drugs used in this study can pass through the cyst monolayer into the lumen. This can be achieved using a coloured marker dye to track movement of substances from the basolateral medium into the lumen. Nevertheless, several of the P2Y receptor subtypes were identified as potential mediators of MDCK cyst enlargement.

Cyst growth was reduced mainly by P2Y receptor antagonists and, along with reports that MDCK cells express P2Y receptors (Post *et al.*, 1998;Brindikova *et al.*, 2003) and the demonstration of P2Y-mediated cAMP production in these cells (Post *et al.*, 1998), suggests that MDCK cyst expansion may be related to P2Y receptor stimulation. Previous studies using this cell culture model have shown the dependence on cAMP for formation of cysts and

for Cl^--stimulated fluid secretion and that inhibition of either of these processes results in significantly smaller cysts (Grantham *et al.*, 1989;Mangoo-Karim *et al.*, 1989;Li & Sheppard, 2003). In Chapter 5, the dependence of ATP on cyst growth was demonstrated and indicative of the presence of ATP-sensitive P2 receptors. It has long been established that ATP can increase Cl^- secretion in this cell line (Simmons, 1981b). Thus, the components for Cl^--stimulated fluid secretion, including expression of several Cl^- channels (Kolb *et al.*, 1985;Li & Sheppard, 2003) mediated by P2Y receptors, as outlined in the hypothesis (Figure 7.1), have been demonstrated in MDCK type I cysts.

7.3 Expression of the $P2X_7$ receptor and renal cell injury

Expression of the $P2X_7$ receptor may also be associated with the recruitment of inflammatory cytokines to sites of tissue injury. In this context, $P2X_7$ receptor activation promotes release of IL-1β from activated macrophages, and both interferon-γ and TNFα can increase expression of the $P2X_7$ receptor (Di Virgilio, 1995;Labasi *et al.*, 2002;Verhoef *et al.*, 2003). These studies led to the idea that the $P2X_7$ receptor could mediate inflammatory cytokine processing and release. This is relevant to the results presented in Chapter 5 since macrophage infiltration and release of inflammatory cytokines are characteristic components of the glomerular damage seen in diabetes mellitus, hypertension and glomerulonephritis (Kluth & Rees, 1999). $P2X_7$ receptor expression was elevated in the glomeruli, predominantly on podocytes, of all three models of glomerular injury. The descriptive nature of this study means that the potential role of the $P2X_7$ receptor in the pathophysiology of renal cell injury is speculative. However, when taken together with previous studies of involvement

of the $P2X_7$ receptor in recruitment of inflammatory cytokines, such as IL-1β, the conclusions are not implausible. Damaged podocytes, for example with glomerulosclerosis, have been shown to release IL-1β (Niemir *et al.*, 1997), and IL-1β and TNF-α may be increased in diabetic glomeruli (Hasegawa *et al.*, 1991). The immune deposits found in glomerulonephritis attract inflammatory cells and promote local release of TNFγ and IL-1β (Kluth & Rees, 1999). These cytokines have been shown to increase the expression of $P2X_7$ receptors in glomerular cells (Harada *et al.*, 2000). Thus ATP, via the $P2X_7$ receptor, could interact with and regulate the inflammatory response, eventually leading to cell death as a mechanism for deleting damaged cells. It is interesting to note that the increase in $P2X_7$ receptor mRNA on day 4 in the rat model of glomerulonephritis coincided with macrophage infiltration and the first appearance of cellular damage in this model. This lends support to the concept that the $P2X_7$ receptor has a role in the inflammatory response and also in apoptosis.

Three main conclusions can be drawn from this thesis. Firstly, a multitude of P2 receptors are expressed in the kidney, implying that there are important roles for these receptors in kidney function. Second, P2 receptors that are linked to secretion of ions such as chloride, and are potentially relevant to cell cycle control were detected on cyst lining cells of the Han:SPRD rat and probably contributed to MDCK cyst growth. This suggests a detrimental role for P2 receptors with increasing cyst size in ADPKD epithelia. Nevertheless, the intracellular signalling pathways of P2Y receptors are complex and not fully elucidated, and the contribution that P2X receptors may make is not clear, therefore the exact mechanisms by which P2 receptor activation leads to cyst enlargement are unknown. Third, expression of the $P2X_7$ receptor on cells lining

renal cysts and in three models of renal cell damage suggest a role with tissue repair by recruitment of cytokines and with apoptosis by deleting damaged cells.

7.4 Future experiments

Experiments throughout this thesis have been largely descriptive, cataloguing P2 receptor expression in native rat kidney and rat models of renal disease. The question arises whether expression of P2 receptors on cyst lining cells functionally affects cyst growth. This was partially addressed in Chapter 5 with the use of an *in vitro* model of renal cyst growth and P2 receptor inhibitors. However, further experimentation is required to assess the ability of agonists and antagonists to move from the basolateral medium into the cyst lumen. Indeed, if this can be demonstrated, the working hypothesis of stimulation of P2 receptors by nucleotides trapped within cysts would hold.

The recent development of RNA interference (RNAi) technology could be considered a useful tool to silence the expression of specific P2 receptor subtype genes *in vitro* and monitor the effects on MDCK cyst growth. RNAi is utilised by most eukaryotes *in vivo* as a protecting mechanism against invasion by foreign genes, for example viral genes (Dorsett & Tuschl, 2004), and was first described in the nematode, *Caenorhabditis* elegans, in response to double stranded RNA (Fire *et al.*, 1998). Post-transcriptional gene silencing was initiated by a double-stranded RNA (dsRNA) that was homologous to the gene being suppressed. Upon introduction into the cell, the dsRNA is degraded to small interfering RNAs (siRNA) that join an RNase complex. The RNase complex binds to a complementary mRNA that is cleaved and undergoes sequence specific degradation (Dorsett & Tuschl, 2004). RNAi can be applied in

mammalian cells by direct transfection of siRNAs designed and synthesised in the laboratory. This emerging technology is a powerful tool for selective knock-down of genes and is especially useful in *in vitro* cell systems. By using an siRNA complementary to, for example $P2Y_2$, and then monitoring cyst growth in the manner described in this thesis, a direct comparison could be made between cyst growth and the role of the receptor.

It would be intriguing to explore further the association between the $P2X_7$ receptor and glomerular cell damage. This could be achieved by induction of experimental crescentic glomerulonephritis using anti-GBM antibody in mice with selective knock-down of the $P2X_7$ receptor. Knockout mice are a useful tool for studying gene function since the resulting mutant phenotype can provide clues to gene function (Table 7.1). It would be interesting to note whether the severity of anti-GBM crescentic glomerulonephritis is affected by loss of the $P2X_7$ receptor. In this respect, immunohistochemistry can be used to establish the degree of macrophage infiltration, assessed at different time points, using techniques established by Tam and co-workers (Tam *et al.*, 1999). Evidence for enhanced cell proliferation and/or apoptosis using immunohistochemistry, and the production of inflammatory cytokines such as IL-1β, TNF-α and MCP-1, using immunoblotting and real- time PCR, could also be assessed in this model. Furthermore, expression of other P2 receptors, in particular the $P2Y_1$ and $P2Y_2$ subtypes, could be assessed in a model of crescentic glomerulonephritis since these receptors are expressed on glomerular cells and have potential roles in cell proliferation.

Another approach to examine the role for P2 receptors in renal disease is a comparative gene expression study using gene array technology. This

Table 7. 1 **Genotype to phenotype relationship of known P2 receptor knockout mice**

Genotype	Phenotype	Reference
$P2X_1$ -/-	Male infertility Impaired TGF	(Mulryan *et al.*, 2000) (Inscho *et al.*, 2004)
$P2X_2$ -/-	Inhibition of GI peristalsis	(Ren *et al.*, 2003)
$P2X_3$ -/-	Reduced response to pain. Increased bladder capacity Delayed micturition reflex	(Souslova *et al.*, 2000) (Cockayne *et al.*, 2000;Vlaskovska *et al.*, 2001)
$P2X_7$ -/-	Reduced IL-1β production from stimulated macrophages; attenuated inflammatory response. Deficient bone formation	(Labasi *et al.*, 2002) (Ke *et al.*, 2003)
$P2Y_1$ -/-	Prolonged bleeding time; defective platelet aggregation	(Leon *et al.*, 1999)
$P2Y_4$ -/-	Reduced Cl^- secretion in jejunum	(Robaye *et al.*, 2003)
$P2Y_{12}$ -/-	Prolonged bleeding time; reduced platelet aggregation	(Foster *et al.*, 2001)

TGF, tubuloglomerular feedback; GI, gastointestinal

technique allows the simultaneous determination of expression levels of a large number of genes from different samples or populations of cells (Brown & Botstein, 1999;Duggan *et al.*, 1999). Complementary DNA of the genes of interest, for example P2 receptor genes, are synthesised and robotically applied in microscopic quantities and in defined positions on a glass slide. This slide, or microarray, is then probed with cDNA that has been generated from the two populations of mRNA that are to be compared, for example normal kidney and ADPKD kidney. A different fluorescent dye is used to make the cDNA for each of the two mRNAs to allow direct comparison on a single array. Complementary cDNA from the samples binds to DNA on the slide of the genes of interest and is detected by fluorescence emission following laser excitation. The relative fluorescence intensity for each gene reflects the relative abundance of the particular mRNA in that sample and a ratio can be calculated for, for example, normal *versus* disease. This technique can be used to identify genes that are switched on or off and genes that are up or down regulated in the two samples (Brown & Botstein, 1999). Identification of these genes can then allow a hypothesis to be developed and provide a starting point for further investigations.

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Appendicies

Appendix 1

Alignment of rat amino acid sequences for P2X receptor subunits. Conserved amino acids are shown in red, conserved cysteines are shown in blue. Alignments were carried out using the ClustalW sequence alignment tool (Web address: http://www.ebi.ac.uk/clustalw/index.html), sequences were deduced from NCBI accession numbers; NP037129 ($rP2X_1$), NP446108 ($rP2X_2$), NP112337 ($rP2X_3$), NP113782 ($rP2X_4$), NP542958 ($rP2X_5$), JC4843 ($rP2X_6$), NP062129 ($rP2X_7$). Putative transmembrane domains are shown by blue arrows.

```
                                                TM1
P2X1        -MARRLQDELSAFFFEYDTPRMVLVRNKKVGVIFRLIQLVVLVYVIGWVF 49
P2X2        MVRRLARGCWSA-FWDYETPKVIVVRNRRLGFVHRMVQLLILLYFVWYVF 49
P2X3        ------MNCISD-FFTYETTKSVVVKSWTIGIINRAVQLLIISYFVGWVF 43
P2X4        --MAGCCSVLGSFLFEYDTPRIVLIRSRKVGLMNRAVQLLILAYVIGWVF 48
P2X5        MGQAAWKGFVLS-LFDYKTAKFVVAKSKKVGLLYRVLQLIILLYLLIWVF 49
P2X6        MASAVAAALVSWGFLDYKTEKYVMTRNCWVGISQRLLQLGVVVYVIGWAL 50
P2X7        ----MPACCSWNDVFQYETNKVTRIQSVNYGTIKWILHMTVFSYVS-FAL 45

                                    →
P2X1        VYEKGYQTS-SDLISSVSVKLKGLAVTQL-------QGLGPQVWDVADYV 91
P2X2        IVQKSYQDSETGPESSIITKVKGITMS------------EDKVWDVEEYV 87
P2X3        LHEKAYQVRDTAIESSVVTKVKGFGRY------------ANRVMDVSDYV 81
P2X4        VWEKGYQETDSVVS-SVTTKAKGVAVTNT-------SQLGFRIWDVADYV 90
P2X5        LIKKSYQDIDTSLQSAVVTKVKGVAYTNT-------TMLGERLWDVADFV 92
P2X6        LAKKGYQEWDMDPQISVITKLKGVSVTQV-------KELEKRLWDVADFV 93
P2X7        MSDKLYQRK-EPLISSVHTKVKGVAEVTENVTEGGVTKLVHGIFDTADYT 94


P2X1        FPAHGDSSFVVMTNFIVTPQQTQGHCAENP--EGGICQDDSGCTPGKAER 139
P2X2        KPPEGGSVVSIITRIEVTPSQTLGTCPESMRVHSSTCHSDDDCIAGQLDM 137
P2X3        TPPQGTSVFVIITKIIVTENQMQGFCPENEEKYR--CVSDSQC--GPERF 127
P2X4        IPAQEENSLFIMTNMIVTVNQTQSTCPEIPDKTS-ICNSDADCTPGSVDT 139
P2X5        IPSQGENVFFVVTNLIVTPNQRQGICAEREGIPDGECSEDDDCHAGESVV 142
P2X6        RPSQGENVFFLVTNFLVTPAQVQGRCPEHPSVPLANCWADEDCPEGEMGT 143
P2X7        LPLQG-NSFFVMTNYLKSEGQEQKLCPEYPSRGK-QCHSDQGCIKGWMDP 142


P2X1        KAQGIRTGNCVP-FNGTVKTCEIFGWCPVEVDDKIPSPALLREAENFTLF 188
P2X2        QGNGIRTGHCVPYYHGDSKTCEVSAWCPVEDG-TSDNHFLGKMAPNFTIL 186
P2X3        PGGGILTGRCVN-YSSVLRTCEIQGWCPTEVD-TVEMPIM-MEAENFTIF 174
P2X4        HSSGVATGRCVP-FNESVKTCEVAAWCPVENDVGVPTPAFLKAAENFTLL 188
P2X5        AGHGLKTGRCLRVGNSTRGTCEIFAWCPVETK-SMPTDPLLKDAESFTIS 191
P2X6        YSHGIKTGQCVAFNGTHR-TCEIWSWCPVESS-AVPRKPLLAQAKNFTLF 191
P2X7        QSKGIQTGRCIP-YDQKRKTCEIFAWCPAEEGKEAPRPALLRSAENFTVL 191
```

```
P2X1       IKNSISFPRFKVNRRNLVEEVNGTYMKKCLYHKIQHPLCPVFNLGYVVRE 238
P2X2       IKNSIHYPKFKFSKGNIASQKSD-YLKHCTFDQDSDPYCPIFRLGFIVEK 235
P2X3       IKNSIRFPLFNFEKGNLLPNLTDKDIKRCRFHPEKAPFCPILRVGDVVKF 224
P2X4       VKNNIWYPKFNFSKRNILPNITTSYLKSCIYNAQTDPFCPIFRLGTIVGD 238
P2X5       IKNFIRFPKFNFSKANVLETDNKHFLKTCHFSSTN-LYCPIFRLGSIVRW 240
P2X6       IKNTVTFNKFNFSRTNALDTWDNTYFKYCLYDSLSSPYCPVFRIGDLVAM 241
P2X7       IKNNIDFPGHNYTTRNILP----GMNISCTFHKTWNPQCPIFRLGDIFQE 237

P2X1       SGQDFRSLAEKGGVVGITIDWKCDLDWHVRHCKPIYQFHGLYG---EKNL 285
P2X2       AGENFTELAHKGGVIGVIINWNCDLDLSESECNPKYSFRRLD--PKYDPA 283
P2X3       AGQDFAKLARTGGVLGIKIGWVCDLDKAWDQCIPKYSFTRLDGVSEKSSV 274
P2X4       AGHSFQEMAVEGGIMGIQIKWDCNLDRAASLCLPRYSFRRLDTRDLEHNV 288
P2X5       AGADFQDIALKGGVIGIYIEWDCDLDKAASKCNPHYYFNRLDN-KHTHSI 289
P2X6       TGGDFEDLALLGGAVGINIHWDCNLDTKGSDCSPQYSFQLQE-------- 283
P2X7       IGENFTEVAVQGGIMGIEIYWDCNLDSWSHRCQPKYSFRRLDDKYTNESL 287

                                                      TM2
P2X1       SPGFNFRFARHF-VQNGTNRRHLFKVFGIHFDILVDGKAGKFDIIPTMTT 334
P2X2       SSGYNFRFAKYYKINGTTTTRTLIKAYGIRIDVIVHGQAGKFSLIPTIIN 333
P2X3       SPGYNFRFAKYYKMENGSEYRTLLKAFGIRFDVLVYGNAGKFNIIPTIIS 324
P2X4       SPGYNFRFAKYYRDLAGKEQRTLTKAYGIRFDIIVFGKAGKFDIIPTMIN 338
P2X5       SSGYNFRFARYYRDPNGVEFRDLMKAYGIRFDVIVNGKAGKFSIIPTVIN 339
P2X6       -RGYNFRTANYWWAASGVESRSLLKLYGIRFDILVTGQAGKFALIPTAIT 332
P2X7       FPGYNFRYAKYY-KENGMEKRTLIKAFGVRFDILVFGTGGKFDIIQLVVY 336

P2X1       IGSGIGIFGVATVLCDLLLLHILP-----------------KRHYYKQK 366
P2X2       LATALTSIGVGSFLCDWILLTFMN-----------------KNKLYSHK 365
P2X3       SVAAFTSVGVGTVLCDIILLNFLK-----------------GADHYKAR 356
P2X4       VGSGLALLGVATVLCDVIVLYCMK-----------------KKYYYRDK 370
P2X5       IGSGLALMGAGAFFCDLVLIYLIR-----------------KSEFYRDK 371
P2X6       VGTGAAWLGMVTFLCDLLLLYVDR-----------------EAGFYWRT 364
P2X7       IGSTLSYFGLATVCIDLIINTYASTCCRSRVYPSCKCCEPCAVNEYYYRK 386

P2X1       KFKYA----------------------------EDMGPGEGEHDP---- 383
P2X2       KFDKVRTPKHPSSRW----------------PVTLALVLGQIPPPPSHYS 399
P2X3       KFEEV----------------------------TETTLKGTASTNP-VFA 377
P2X4       KYKYV------------------------------------------- 375
P2X5       KFEKVRGQKEDAN--------------------VEVEANEMEQERPEDEP 401
P2X6       KYEEARAPKATTN--------------------SA--------------- 379
P2X7       KCEPIVEPKPTLKYVSFVDEPHIWMVDQQLLGKSLQDVKGQEVPRPQTDF 436

P2X1       ---------------------------------VATSSTLGLQENMRT 398
P2X2       QDQPPSPPSGEGPTLGEGAELPLAVQSPRPCSISALTEQVVDTLGQHMGQ 449
P2X3       SDQ-----------------------------ATVEKQSTDSGAYSIGH 397
P2X4       ------------------------------------EDYEQGLSGEMNQ 388
P2X5       LERVRQDEQSQELAQSGRKQNSNCQVLLEPARFGLRENAIVNVKQSQILH 451
P2X6       ------------------------------------------------
P2X7       LELSRLSLSLHHSPPIPGQPEEMQLLQIEAVPRSRDSPDWCQCGNCLPSQ 486

P2X1       S------------------------------------------------ 399
P2X2       RPPVPEPSQQDSTSTDPKGLAQL--------------------------- 472
P2X3       ------------------------------------------------
P2X4       ------------------------------------------------
P2X5       PVKT----------------------------------------------- 455
P2X6       ------------------------------------------------
P2X7       LPENRRALEELCCRRKPGQCITTSELFSKIVLSREALQLLLLYQEPLLAL 536
P2X7       EGEAINSKLRHCAYRSYATWRFVSQDMADFAILPSCCRWKIRKEFPKTQG 586
P2X7       QYSGFKYPY 595
```

Appendix 2

Alignment of rat amino acid sequences for P2Y receptor subunits. Conserved amino acids are shown in red, conserved cysteines are shown in blue. Alignments were carried out using the ClustalW sequence alignment tool (Web address: http://www.ebi.ac.uk/clustalw/index.html), sequences were deduced from NCBI accession numbers; NP036932 ($rP2Y_1$), NP058951 ($rP2Y_2$), NP113868 ($rP2Y_4$), NP476465 ($rP2Y_6$), NP073637 ($rP2Y_{12}$), XP227178 ($rP2Y_{13}$). Sequences for rat $P2Y_{11}$ and $P2Y_{14}$ were unavailable. Putative transmembrane domains are shown by blue arrows.

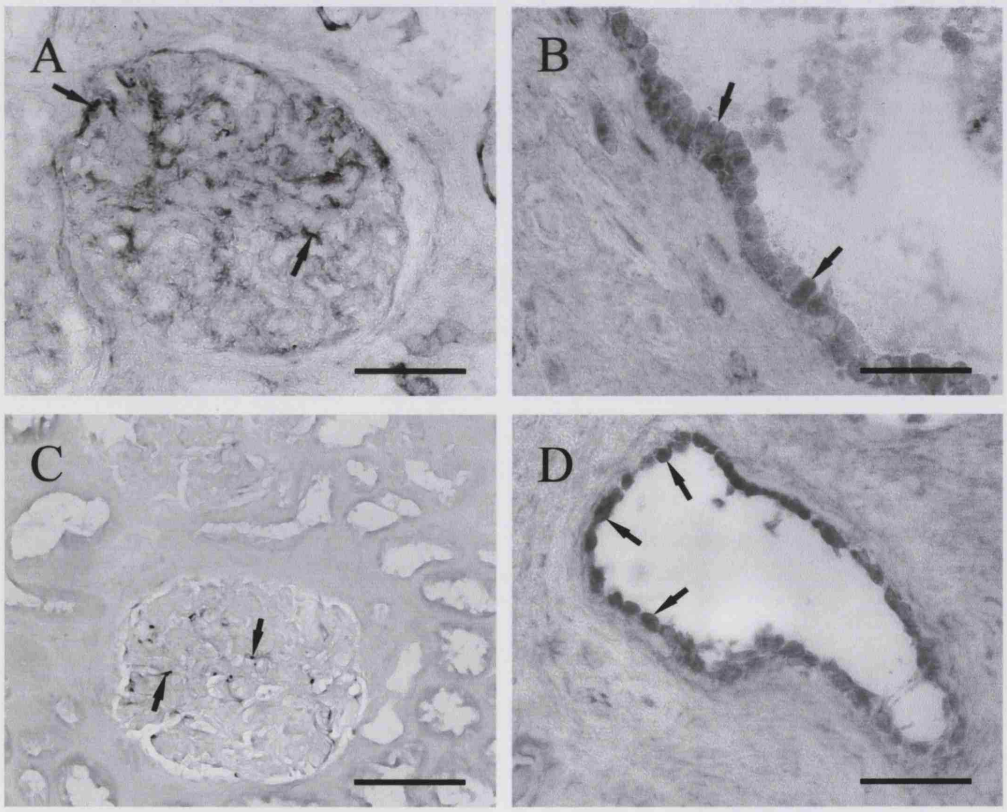
```
P2Y1       MTEVPWSAVPNGTDAAFLAGLGSLWGNSTIASTAAVSSSFRCALIKTGFQ 50
P2Y2       ----------------MAAGLDSWNST-INGTWEGDELGYKCRFNEDFK 32
P2Y4       ----------------MTSAESLLFTS-LGPSPSSGDG--DCRFNEEFK 30
P2Y6       ----------------MERDNGTIQAPGLPPT--------TCVYREDFK 25
P2Y12      ----------------MEVPGANATSANTTSIPG--TSTLCSRDYKIT 30
P2Y13      ----------------------MLGTVNTTGMQGFNKSERCPRDTRMT 26

                    TM1                          TM2
P2Y1       FYYLPAVYILVFIIGFLGNSVAIWMFVFHMKPWSGISVYMFNLALADFLY 100
P2Y2       YVLLPVSYGVVCVLGLCLNVVALYIFLCRLKTWNASTTYMFHLAVSDSLY 82
P2Y4       FILLPMSYAVVFVLGLALNAPTLWLFLFRLRPWDATATYMFHLALSDTLY 80
P2Y6       RLLLPPVYSVVLVVGLPLNVCVIAQICASRRTLTRSAVYTLNLALADLLY 75
P2Y12      QVLFPLLYTVLFFAGLITNSLAMRIFFQ-IRSKSNFIIFLKNTVISDLLM 79
P2Y13      QLLFPVLYTVVFFTGVLLNTLALWVFIH-IPSNSTFIIYLKNTLVADLIM 75

                                TM3
P2Y1       VLTLPALIFYYFNKTDWIFGDVMCKLQRFIFHVNLYGSILFLTCISAHRY 150
P2Y2       AASLPLLVYYYAQGDHWPFSTVLCKLVRFLFYTNLYCSILFLTCISVHRC 132
P2Y4       VLSLPTLVYYYAARNHWPFGTGLCKFVRFLFYWNLYCSVLFLTCISVHRY 130
P2Y6       ACSLPLLIYNYARGDHWPFGDLACRLVRFLFYANLHGSILFLTCISFQRY 125
P2Y12      ILTFPFKILSDAKLGAGHLRTLVCQVTSVTFYFTMYISISFLGLITIDRY 129
P2Y13      TLMLPFKILSDSRLAPWQLRGFVCTFSSVVFYETMYVGIMMLGLIAFDRF 125

                                TM4
P2Y1       SGVVYPLKSLG-RLKKKNAIYVSVLVWLIVVVAISPILFYSGTGIRKNKT 199
P2Y2       LGVLRPLHSLS-WGHARYARRVAAVVWVLVLACQAPVLYFVTTSVRGT-R 180
P2Y4       LGICHPLRAIR-WGRPRFASLLCLGVWLVVAGCLVPNLFFVTTNANGT-T 178
P2Y6       LGICHPLAPWHKRGGRRAAWVVCGVVWLVVTAQCLPTAVFAATGIQRN-R 174
P2Y12      LKTTRPFKTSS-PSNLLGAKILSVAIWAFMFLLSLPNMILTNRRPKDK-D 177
P2Y13      LKIVVPFRKTF-VKKTAFAKIVSISIWLLMFLISLPNMILN-KEATAS-T 172

                                TM5
P2Y1       VTCYDSTSDEYLRSYFIYSMCTTVAMFCIPLVLILGCYGLIVRALIYKDL 249
P2Y2       ITCHDTSARELFSHFVAYSSVMLGLLFAVPFSIILVCYVLMARRLLKPAY 230
P2Y4       ILCHDTTLPEEFDHYVYFSSAVMVLLFGLPFLITLVCYGLMARRLYRPLP 228
P2Y6       TVCYDLSPPILSTRYLPYGMALTVIGFLLPFTALLACYCRMARRLCR-QD 223
P2Y12      ITKCSFLKSEFGLVWHEIVNYICQVIFWINFLIVIVCYSLITKELYRSYV 227
```

```
P2Y13     VKKCASLKSPLGLLWHQVVSHTCQFIFWTVFILMLLFYTVIAKKVYDSYR 222

                        TM6
                  ------------>
P2Y1      D---NSPLRRKS--IYLVIIVLTVFAVSYIPFHVMKTMNLRARLDFQTPE 294
P2Y2      GTTGLPRAKRKS--VRTIALVLAVFALCFLPFHVTRTLYYSFRSLD---L 275
P2Y4      G-AGQSSSRLRS--LRTIAVVLTVFAVCFVPFHITRTIYYQARLLQ---A 272
P2Y6      GPAGPVAQERRSKAARMAVVVAAVFVISFLPFHITKTAYLAVRSTPG--V 271
P2Y12     RTRGSAKAPKKR-VNIKVFIIIAVFFICFVPFHFARIPYTLSQTRAV--F 274
P2Y13     KFK-SRDSKHKR-LEAKVFIVMAVFFVCFAPFHFVRVPYTHSQTTNK--T 268

                        TM7
                  ------------>
P2Y1      MCDFNDRVYATYQVTRGLASLNSCVDPILYFLAGDTFRRRLSRATRKASR 344
P2Y2      SCHTLNAINMAYKITRPLASANSCLDPVLYFLAGQRLVRFARDAKPATEP 325
P2Y4      DCHVLNIVNVVYKVTRPLASANSCLDPVLYLFTGDKYRNQLQQLCRGSKP 322
P2Y6      SCPVLETFAAAYKGTRPFASANSVLDPILFYFTQQKFRRQPHDLLQKLTA 321
P2Y12     DCNAENTLFYVKESTLWLTSLNACLDPFIYFFLCKSFRNSLMSMLRCSTS 324
P2Y13     DCRLENQLFLAKESTLFLATTNICMDPLIYIILCKKFTRKVPCMRWRTKT 318


P2Y1      RSEANLQSKSEEMTLN----------ILSEFKQNGDTSL---------- 373
P2Y2      TPSPQARRKLGLHRPNRTDTVRKDLSISSDDSRRTESTPAGSETKDIRL 374
P2Y4      KPR-TAASSLALVTLH-------EESISRWADTHQDSTFSAYEGDRL-- 361
P2Y6      KWQRQRV------------------------------------------ 328
P2Y12     GANKKKGQEGG--------------------DPSEETPM---------- 343
P2Y13     AASSDEHHSSQ--------------------TDNITLS---------- 336
```

Appendix 3

Kidney tissue was obtained with consent from patients who had undergone partial or total nephrectomy. Ten-micrometer thick sections of renal tissue were incubated overnight with primary polyclonal antibody to P2X subtypes 1-7 and $P2Y_1$ and $P2Y_2$. Positive staining was visualised by the nickel-intensified DAB technique that produced a black precipitate (described in detail in Chapter 2).

Figure 1 **$P2Y_1$ and $P2Y_2$ receptor expression on human kidney tissue**



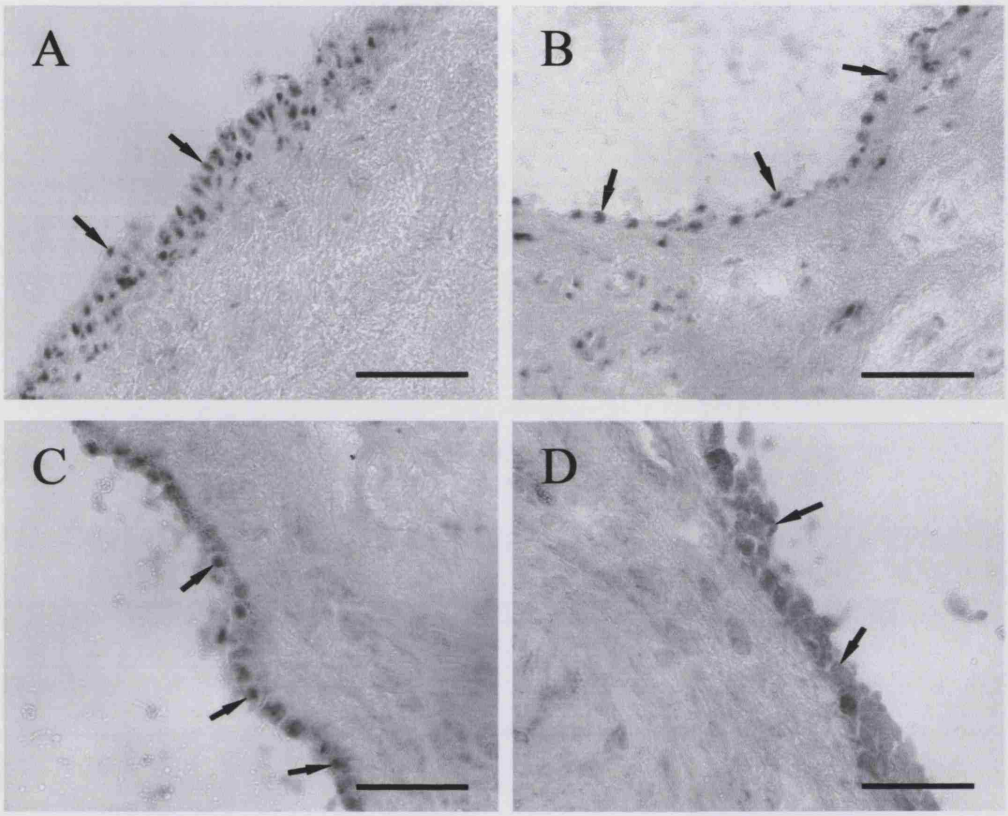
A. $P2Y_1$ receptor immunoreactivity on glomerular mesangial cells (indicated by arrows) of non-ADPKD kidney tissue (scale bar = 50μm).

B. $P2Y_1$ receptor immunoreactivity on epithelial cells lining an ADPKD cyst (scale bar = 50μm).

C. $P2Y_2$ receptor immunoreactivity on glomerular cells (arrows) of non-ADPKD kidney tissue (scale bar = 50μm).

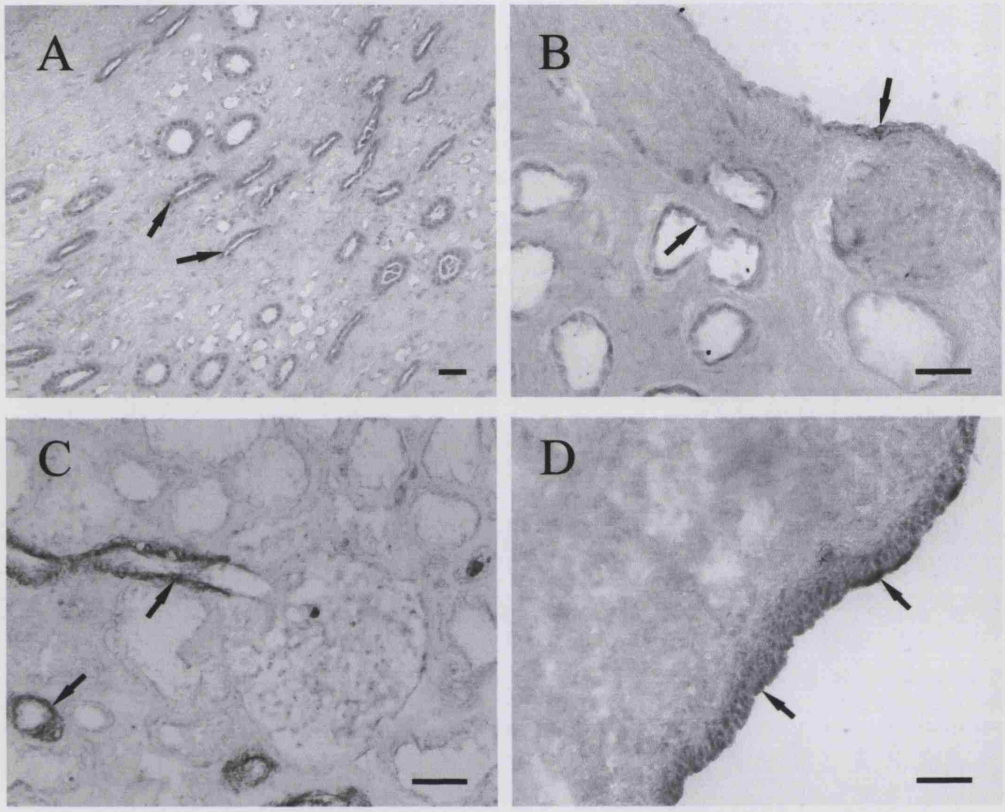
D. $P2Y_2$ receptor immunoreactivity on epithelial cells lining an ADPKD cyst (scale bar = 50μm).

Figure 2 **P2 receptor expression on ADPKD kidney tissue**



ADPKD cyst epithelial cells with: A. Intracellular $P2Y_6$ receptor immunoreactivity (arrows) (scale bar = 50μm), B. Sporadic $P2Y_{11}$ receptor immunoreactivity (scale bar = 50μm), C. $P2X_3$ receptor immunoreactivity, and D. $P2X_4$ receptor immunoreactivity (scale bars = 50μm)

Figure 3 **$P2X_5$ and $P2X_7$ receptor expression in human kidney tissue**



A. $P2X_5$ receptor immunoreactivity on collecting duct cells (arrows) in the inner medulla of non-ADPKD kidney tissue (scale bar = 50μm).

B. $P2X_5$ receptor immunoreactivity on epithelial cells (arrows) lining ADPKD cysts (scale bar = 50μm).

C. $P2X_7$ receptor immunoreactivity (arrows) on the afferent arteriole and interlobular artery of non-ADPKD kidney tissue (scale bar = 50μm).

D. $P2X_7$ receptor immunoreactivity on epithelial cells (arrows) lining an ADPKD cyst (scale bar = 50μm).