Original Paper

Experimental Nephrology

Exp Nephrol 2002;10:34–42

Received: December 22, 2000
Accepted: April 24, 2001

$P2X_7$ Receptors Are Expressed during Mouse Nephrogenesis and in Collecting Duct Cysts of the *cpk/cpk* Mouse

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Key Words

$P2X_7$ receptors · Mouse nephrogenesis · *cpk/cpk* mice · Western blotting · Congenital polycystic kidney disease · Immunolocalization of $P2X_7$

Abstract

Background: Purinergic receptors are cell-surface molecules that bind extracellular nucleotides, notably ATP. The P2X family includes seven nonselective ion channels with one member, $P2X_7$, implicated in cytolytic pore formation and cell death. ***Materials and Methods:*** We sought $P2X_7$ expression in mouse nephrogenesis and *cpk/cpk* renal cyst growth, conditions in which both proliferation and apoptosis are prominent. ***Results:*** $P2X_7$ immunolocalized to condensed metanephric mesenchyme: both proliferation and apoptosis were detected in this compartment, assessed by proliferating cell nuclear antigen expression and propidium iodide-stained pyknotic nuclei respectively. Later in nephrogenesis, $P2X_7$ was detected in collecting ducts, a pattern persisting to maturity. A mesenchymal to epithelial shift of $P2X_7$ expression was also documented in ureter development. In *cpk/cpk* kidneys, $P2X_7$-expressing collecting duct cysts dominated histology from two weeks until four weeks after birth, when animals die from uremia. In polycystic kidneys pyknotic nuclei were rarely identified in $P2X_7$-expressing epithelia, but were detected between cysts, consistent with a non-apoptotic role for $P2X_7$ in cyst enlargement. ***Conclusion:*** $P2X_7$ is expressed during normal nephrogenesis and in a model of congenital polycystic kidney disease. Further experiments are necessary to define possible functions of $P2X_7$ in these settings.

Introduction

Signalling roles for extracellular nucleotides have been suggested by the cloning of specific cell-surface receptors, initially identified in the nervous and immune systems [1, 2], and by the demonstration that nucleotides, like ATP, can be released from cells under physiological and pathological conditions [3, 4]. Nucleotide receptors are classified into P2X receptors, which are ligand-gated ion channels, and P2Y receptors, which are G protein-coupled. Of the first seven members of the P2X family, $P2X_7$ is unique. As well as being a nonselective cation channel, it

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possesses an extended carboxy-terminal which, at high concentrations of ligands or sustained exposure to these molecules, is implicated in cytolytic pore formation [5].

In vitro, $P2X_7$ transduces several ATP-induced responses including cell fusion and apoptosis with caspase and NF-κB activation in cells endogenously expressing $P2X_7$ including monocytes/macrophages, leukocytes and microglia [6–9]. One study has reported enhanced proliferation of lymphoid cells after $P2X_7$ transfection [10]. In vivo, $P2X_7$ is expressed in brain and the immune system [11], and in epithelia of salivary glands [12], skin [13], gastrointestinal tract [14] and uterus [15]. In some of these organs, the receptor is expressed at sites of tissue remodelling, differentiation and cell deletion by shedding or apoptosis.

In nephrogenesis, the interplay between mesenchyme and ureteric bud is poorly understood. Renal mesenchymal cells are highly proliferative but some, which fail to incorporate into nephrons, undergo apoptosis [16–18]. Additionally, in polycystic kidney diseases, epithelia lining cysts are less well differentiated and also have high cell turnover, and structures between cysts undergo apoptosis [19–23]. Since $P2X_7$ has been implicated in cell death in a variety of cell types, we sought to determine the expression of $P2X_7$ in mouse nephrogenesis and the *cpk/cpk* model of renal cystic kidney disease.

Materials and Methods

Animals

Protocols were approved by the Home Office. Normal C57BL/6J mice were maintained in our colony. *cpk/cpk* polycystic mice on a C57BL/6J background were generated by mating *cpk*/+ parents (Jackson Laboratories, Bar Harbor, Me., USA). C57BL/6J metanephroi and kidneys were examined on embryonic day 11 (E11; E0 was the day of the vaginal plug), E13, E16, E19, neonatally (within 24 h of birth) and at 1, 2, 3 and 4 weeks postnatally; this period spans the inception of the organ, nephron generation (E14 to 1 week postnatal), ending with early adulthood. Kidneys were collected from neonatal and 1, 2, 3 and 4 weeks postnatal *cpk/cpk* mice, and from phenotypically normal (i.e. +/+ and *cpk*/+) littermates. The cystic status of organs was confirmed histologically, although *cpk/cpk* kidneys are grossly enlarged from 2 weeks postnatally [21]. At least 3 animals were examined at each age, for each group.

Chemicals and $P2X_7$ Antibodies

Chemicals were obtained for Sigma (Poole, UK) unless otherwise specified. We used two $P2X_7$ antibodies. '$P2X_7$ antibody 1', a rabbit antibody (APR-OO4; Alomone Laboratories, Jerusalem, Israel) raised against the 20 carboxy-terminal amino acids (576–595; KIRKEFPKTQGQYSGFKYPY) of rat $P2X_7$ [5, 11]: this epitope is highly conserved between rat, mouse and humans and is not present in other P2X receptors, or in any other known protein. '$P2X_7$ antibody 2', recognising amino acids 555 to 569 (TWRFVSQDMADFAIL) [13, 14, 24]. Since both antibodies gave similar immunohistochemical results, data are shown for the APR-004 antibody, unless otherwise stated. As negative controls for immunohistochemistry and Western blots, we pre-reacted antibody with excess immunizing peptide (1:2, weight for weight).

Western Blotting

For SDS-polyacrylamide electrophoresis, 12% gels were run in a Mini-PROTEAN II cell (Gibco BRL, Paisley, UK). Using E19 C57BL/6J metanephroi, one week postnatal *cpk/cpk* kidneys and organs from phenotypically normal littermates, proteins were extracted at 4°C in lysis buffer (150 m*M* NaCl, 50 m*M* Tris pH 8, 1% Nonidet P40, 0.5% sodium deoxycholate, 1% sodium lauryl sulphate) to which protease inhibitors had been added (30 mg/l of aprotinin, 0.1 *M* sodium orthovanadate, 0.1 *M* phenoxymethyl sulphoxide). Proteins (40 μg) were mixed with equal volume of sample buffer (1 ml glycerol, 0.5 ml α-mercaptoethanol, 3 ml 10% SDS, 1.25 ml Tris pH 6.7 and 0.001 g bromophenol blue); this was heated at 95°C for 5 min, quenched on ice, and equal quantities of protein loaded. Electrophoresed proteins were transferred using a Mini-PROTEAN trans-blot cell (Gibco BRL) onto hybond-C pure nitrocellulose membranes (Amersham Life Science, Buckinghamshire, UK). Blots were incubated overnight at 4°C in blocking solution (5% non-fat milk in PBS 0.1% Tween). $P2X_7$ was detected by applying anti-$P2X_7$ antibody at 1:1,000 at 4°C for 1 h. Goat anti-rabbit horseradish-peroxidase conjugated secondary antibody (DAKO, High Wycombe, UK) was applied at 1:2,000 for 30 min at room temperature. Signals were generated using enhanced chemiluminescence reagent.

Immunohistochemistry

Organs were fixed in 4% paraformaldehyde, wax-embedded and sectioned at 5 μm. Sections were rehydrated, immersed in citric acid buffer (2.1 g/l, 6.0) and microwaved for 5 min. For brightfield studies, endogenous peroxidase was quenched with 3% hydrogen peroxide for 30 min and sections blocked in 10% fetal calf serum for 45 min at room temperature. Sections were incubated at 37°C with either $P2X_7$ antibody (1:50–1:100) followed by anti-rabbit secondary antibody and a streptavidin-biotin peroxidase system (ABC kit; DAKO, High Wycombe, UK); peroxidase activity was visualized as a brown color generated with diaminobenzidine (DAB; 0.5 g/l with 0.03% hydrogen peroxide). Negative controls comprised pre-reaction of primary antibody with immunizing peptide or omission of primary antibody. Some of these, and other, sections were reacted with: (1) hematoxylin, a blue nuclear stain; (2) periodic acid-Schiff (PAS) to stain proximal tubule brush borders red; (3) biotinylated Dolichos biflorus agglutinin (DBA), a marker of collecting ducts [25], applied at 1:100 for 30 min at room temperature, followed by reaction with streptavidin-peroxidase and DAB; (4) biotinylated antibody to human proliferating cell nuclear antigen (PCNA; 32552A, BD-Pharmingen, San Diego, Calif., USA), a surrogate marker of mitosis [26] applied at 1:50 overnight at 4°C, followed by reaction with streptavidin-peroxidase and metal-enhanced DAB, resulting in positive blue nuclei. Slides were examined with a Zeiss Axiophot microscope. Preliminary studies identified $P2X_7$ immunoreactivity in mouse skin and bladder urothelium (not shown), known sites of expression [13, 24]. For immunofluorescent detection of $P2X_7$, a similar protocol was followed, but hydrogen peroxide was omitted and primary antibody was detected by fluorescein isothiocyanate (FITC)-conjugated second antibody. Nuclei were stained with pro-

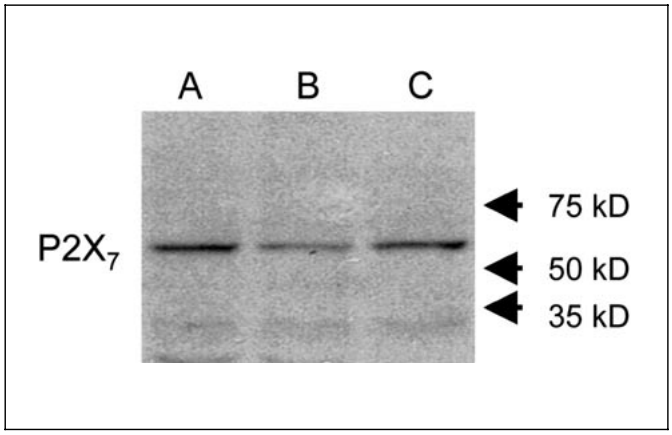


Fig. 1. Western blot. A representative result from three experiments is shown, using $P2X_7$ antibody 1. A major 70-kD band was detected in C57BL/6J E19 metanephroi (lane A), 1 week postnatal phenotypically normal kidneys (lane B) and littermate *cpk/cpk* kidneys (lane C). It was abolished after pre-reacting $P2X_7$ antibody with immunizing peptide (not shown).

pidium iodide (100 μg/l) applied for 5 min followed by washes with phosphate-buffered saline (pH 7.4) [17, 22]. Sections were mounted in Citifluor™ (Chemical Labs, University of Kent, Canterbury, UK) and viewed with a confocal laser scanning microscope (Leica, Heidelberg, Germany). Using appropriate wavelengths, $P2X_7$ protein was detected as a green color and nuclei as a red color. Apoptotic cells were identified by their small and fragmented (pyknotic), bright nuclei [17, 22], nucleated red blood cells also have bright nuclei but these are larger and regular in outline.

Results

Western Blotting

In Western blots of C57BL/6J metanephroi and postnatal phenotypically-normal and *cpk/cpk* littermate kidneys (fig. 1), a major 70-kD band was detected, as described in other tissues [11]; moreover, this band was abolished after pre-reacting primary antibody with immunizing peptide.

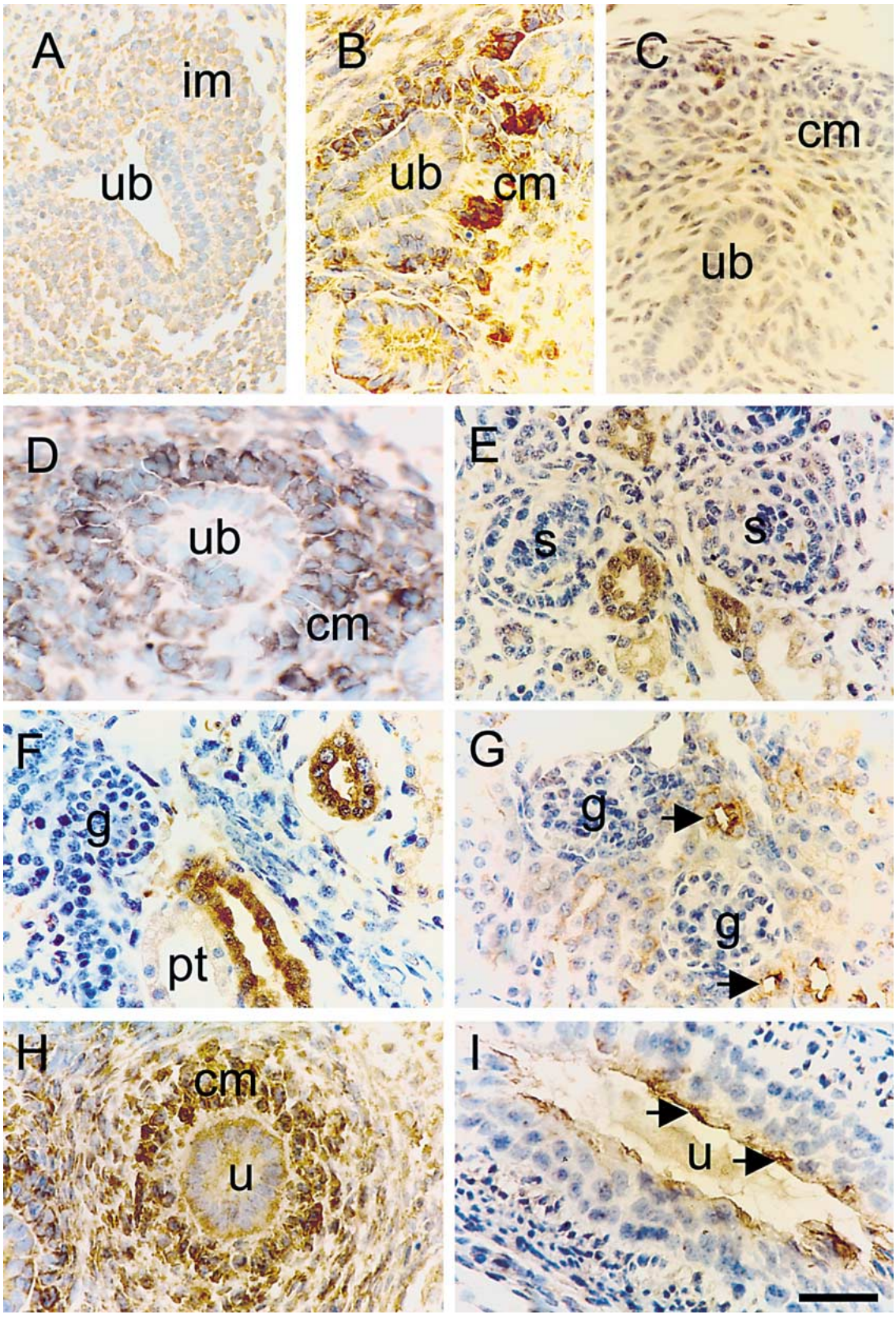
$P2X_7$ in Normal Nephrogenesis

Mouse metanephrogenesis begins at E11 with induction between metanephric mesenchyme, a section of intermediate mesoderm, which aggregates and polarises to form nephron tubules, and ureteric bud epithelium, which arborises to form collecting ducts and also forms the renal pelvic and ureter urothelium. At E11, immunohistochemical $P2X_7$ signal was not detected in the metanephros (fig. 2A). At E13 (fig. 2B), the receptor was immunolocalized to condensing mesenchyme but was downregulated in polarised vesicles, the next step in nephron formation. A positive signal was absent after pre-reacting primary antibody with immunizing peptide (fig. 2C). Double staining with $P2X_7$ and PCNA antibodies (fig. 2D) revealed that most cells in condensates expressed both proteins. At E16, $P2X_7$ immunostaining was detected in collecting ducts in the outer (fig. 2E) and inner (fig. 2F) cortex, and in medullary collecting ducts (not shown), a pattern maintained postnatally (fig. 2G). In embryonic tubules, immunostaining was predominantly cytoplasmic but postnatally the receptor was apical. Hence, in kidney development, $P2X_7$ was initially detected in condensing mesenchyme but was later expressed in epithelia. A similar pattern was observed during ureter maturation, with initial expression (E13; fig. 2H) in condensing mesenchyme enveloping the ureteric bud stalk, whereas, as development proceeded, $P2X_7$ was downregulated in smooth muscle but became prominent apically in urothelium (fig. 2I).

$P2X_7$ in Congenital Polycystic Kidney Disease

Renal pathology in *cpk/cpk* mice resembles human autosomal recessive polycystic disease, with initial proximal tubular dilation followed by formation of large collecting duct cysts and destruction of normal architecture; in mice, uremic death occurs around 30 days after birth

Fig. 2. $P2X_7$ immunolocalization in C57BL/6J mouse nephrogenesis. All sections were hematoxylin stained except **D**. All were probed with the $P2X_7$ antibody (positive signal appears brown), except **C** which was probed with the primary antibody pre-reacted with immunizing peptide. **D** was also reacted with PCNA antibody (positive blue nuclear signal). **A** At E11, immunohistochemical $P2X_7$ signal above background was not detected in ureteric bud (ub) or the intermediate mesenchyme (im) forming the renal mesenchyme. **B** At E13, $P2X_7$ was detected in condensing mesenchyme (cm), with downregulation in primitive nephron vesicles: adjacent ureteric bud branches (ub) did not show immunostaining above background levels. **C** E13: no signal after pre-reacting primary antibody with immunizing peptide. **D** E13: Double staining with $P2X_7$ and PCNA antibodies: most cells in mesenchymal condensates expressed both proteins. **E, F** At E16, $P2X_7$ immunolocalized to collecting ducts in superficial (**E**) and deep (**F**) cortex. S-shaped bodies (s), primitive glomeruli (g) and proximal tubules (pt) did not express $P2X_7$. **G** Four weeks postnatally, $P2X_7$ protein was detected apically in a subset of cortical tubules, probably collecting ducts. **H** At E13, $P2X_7$ was detected in condensing mesenchyme (cm) around the ureter (u). **I** Neonatally, $P2X_7$ was detected apically in urothelia. Bar is 40 μm in all frames apart from 20 μm in **D**.



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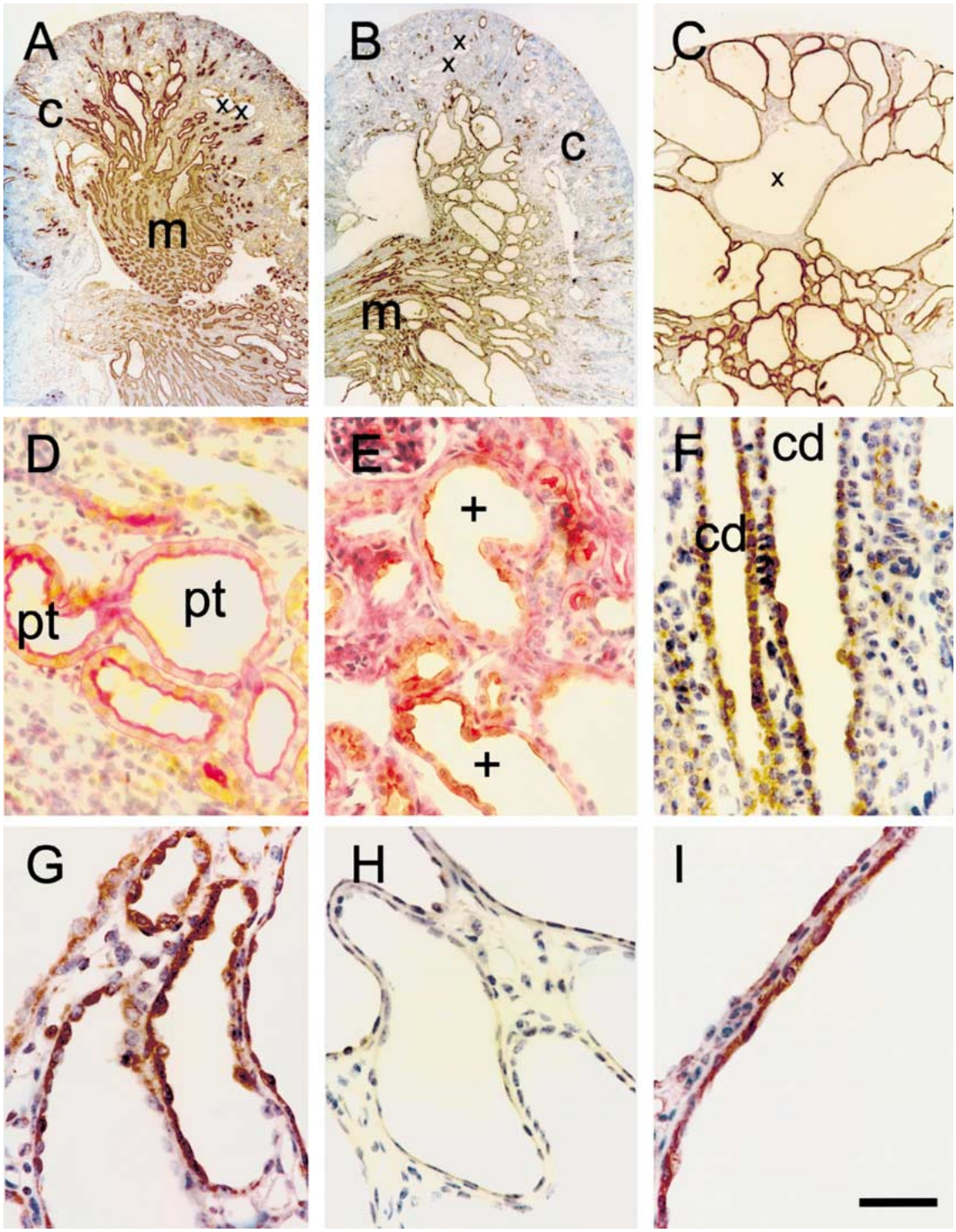

Fig. 3. Immunolocalization of $P2X_7$ in *cpk/cpk* kidneys. All sections stained with hematoxylin. **A–C** were DNA stained (positive signal appears brown). **D–I** were probed with $P2X_7$ antibody apart from H which was probed with antibody pre-reacted with immunizing peptide (positive signal appears brown). **D** and **E** were also stained with PAS to color proximal tubule brush borders red. **A–C** Low magnification of neonatal (**A**), 1 week postnatal (**B**) and 3 weeks postnatal (**C**) *cpk/cpk* kidneys demonstrated progressive enlargement of DBA-positive collecting ducts to form large cysts. Note that differentiation between cortex (c) and medulla (m) was progressively lost and that a subset of cysts (x), probably of proximal tubule origin, failed to stain with DBA. **D, E** Cortex of neonatal *cpk/cpk* kidneys: PAS-positive proximal tubule cysts (pt in **D**) did not express $P2X_7$, whereas some cells in dilating tubules which were PAS-negative (+ in **E**) expressed

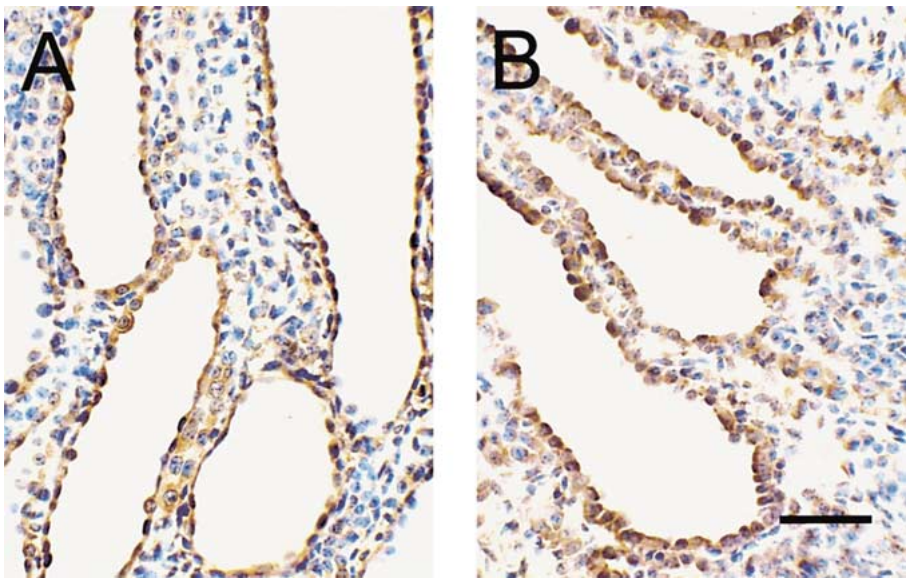

Fig. 4. $P2X_7$ immunostaining comparing two antibodies. Sections of a neonatal *cpk/cpk* kidney counterstained with hematoxylin. Note dilated medullary collecting ducts with similar epithelial immunostaining with $P2X_7$ antibody 1 (**A**) and 2 (**B**). Bar = 40 μm.

[19–21, 27]. Figure 3A–C shows DBA-stained sections of *cpk/cpk* kidneys. In neonates (fig. 3A), a subset of collecting ducts were dilated and small, DBA-negative cortical cysts of presumed proximal tubule origin were noted. At 1 week (fig. 3B), medullary collecting ducts expanded to form medium-sized cysts, with a few DBA-negative cysts remaining in the cortex. At 3 weeks (fig. 3C), the organ was greatly enlarged and histology dominated by large cysts, >95% DBA-positive; from this stage, only a few glomeruli and normal calibre tubules were identified between cysts. In neonatal and 1-week-old *cpk/cpk* mice, proximal tubule cysts, identified by PAS-positive brush borders, did not express $P2X_7$ (fig. 3D), whereas the protein was detected in a subset of cells in dilating cortical and medullary collecting ducts (fig. 3E, F). By three weeks, epithelial $P2X_7$ immunostaining was detected in most cells in most cysts (fig. 3G–I), whereas the same cells only rarely stained for PCNA (not shown). No staining was observed when primary antibody was pre-reacted with immunizing peptide (fig. 3H). Phenotypically normal littermate kidneys showed similar $P2X_7$ patterns as wild-type C57BL/6J mice (not shown).

the receptor. **F** At one week after birth, dilating medullary collecting ducts (cd) expressed $P2X_7$. **G–I** Medium-sized and large cysts in a 3-week-old *cpk/cpk* mouse kidney. Note epithelial $P2X_7$ immunostaining in cysts whereas protein was not detected between cysts. No immunostaining was observed when the primary antibody was pre-reacted with immunizing peptide (**H**). Bar is 240 μm in **A–C** and 40 μm in other frames.

We found that immunostaining was similar with both anti-$P2X_7$ antibodies described in 'Materials and Methods'. A representative comparison is shown in figure 4, with immunostaining in dilated medullary collecting ducts from a *cpk/cpk* kidney.

Immunolocalization of $P2X_7$ and Apoptosis

To determine the spatial relationship of $P2X_7$ expression and apoptosis, we used confocal microscopy to record receptor immunolocalization, detected by an FITC-conjugated second antibody (green), together with pyknotic nuclei detected by propidium iodide staining (bright red). In early nephrogenesis (E13; fig. 5A–C), scattered pyknotic nuclei with morphological features of apoptotic death were visualised within mesenchyme, a region with $P2X_7$ immunoreactivity. As previously reported [22], pyknotic nuclei were detected in *cpk/cpk* kidneys between $P2X_7$-expressing cysts (fig. 5D–F); by contrast, pyknotic nuclei were very rarely noted in cystic epithelia.

Discussion

A complex pattern of renal P2 nucleotide receptor expression is emerging [28]. Bailey et al. [29] identified $P2Y_1$, $P2Y_2$ and $P2Y_4$ transcripts in proximal tubules, outer medullary collecting ducts and thin ascending limbs of loops of Henle dissected from adult rats. P2X receptors are expressed in vitro, including $P2X_1$ and $P2X_4$ in proximal tubule-like cells [30, 31], $P2X_4$ in outer medullary col-

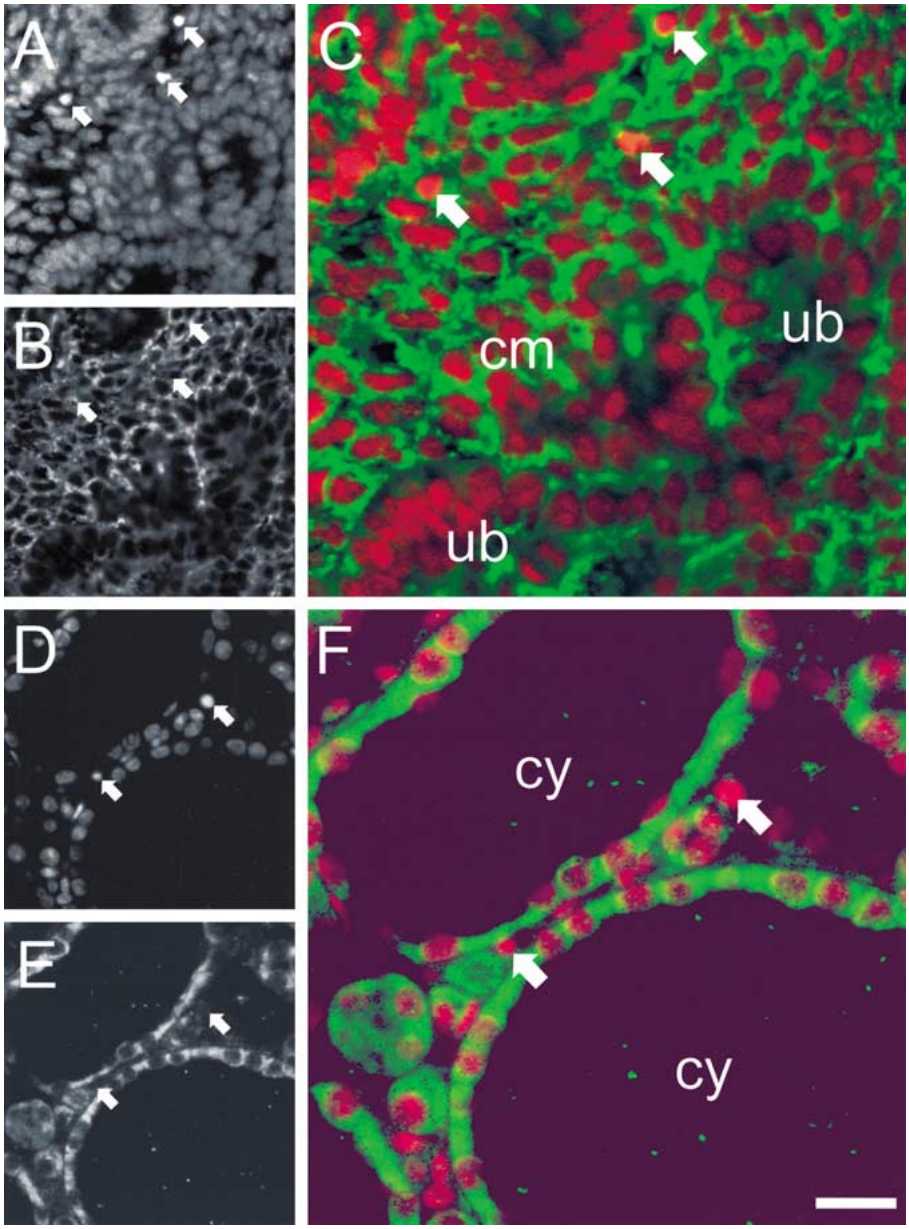

Fig. 5. Localization of $P2X_7$ immunostaining and pyknotic nuclei by confocal microscopy. **A–C** Sections from a normal C57BL/6J E13 metanephros. **D–F** Sections from a 3-week-postnatal *cpk/cpk* mouse kidney. Both sections were stained with propidium iodide and with $P2X_7$ primary antibody detected by an FITC-conjugated second antibody. **A** and **D** show the propidium iodide wavelength only, **B** and **E** show the FITC wavelength only, while **C** and **F** are merged colour images with $P2X_7$ immunostaining in green and pyknotic nuclei appearing as bright red. **A–C** In the normal metanephros, three pyknotic nuclei (arrows in **A–C**) were detected within condensing mesenchyme (cm) which itself expressed $P2X_7$ protein. Ureteric bud branch epithelia (ub) did not express significant levels of the receptor. **D–F** In the section of the *cpk/cpk* kidney, two pyknotic nuclei (arrows in **D–F**) were detected between cyst (cy) epithelia, which themselves expressed $P2X_7$ protein. Bar = 20 μm in **C** and **F**.

lecting duct cells [31] and $P2X_3$ and $P2X_4$ in inner medullary collecting duct cells [32]. Chan et al. [33] immunolocalized $P2X_1$ to large calibre and afferent glomerular arteries in mature rat kidneys. Of relevance to the current study, Rassendren et al. [34] reported a low level of $P2X_7$ mRNA in normal adult human kidneys on Northern blot, although the cells expressing this receptor were not defined, and Harada et al. [35] detected low levels of $P2X_7$ immunostaining in glomeruli of normal adult rats; this may represent mesangial expression since mesangial cultures express $P2X_7$ transcripts after cytokine exposure. Additionally, $P2X_7$ mediates apoptosis of cultured mesangial cells derived from adult rats [35, 36].

The current study brings a developmental perspective to renal $P2X_7$ expression. Metanephric mesenchymal condensates form around ureteric bud branches and subsequently differentiate into nephrons. Most condensate cells are proliferative and express survival molecules including PAX2 and BCL2 [37–39], while a subset of condensing cells which fail to become integrated into nephrons undergo apoptosis [16, 17, 22]. We detected $P2X_7$ protein in condensing mesenchymal cells, some of which

also expressed PCNA; however, in addition, a minority of nearby mesenchymal cells, which also appeared to express $P2X_7$, were apoptotic. Hence, $P2X_7$ may have roles in modulating cell turnover, but functional experiments, for example manipulating $P2X_7$ signalling in metanephric organ culture [18], are required to test this hypothesis.

$P2X_7$ protein was no longer detected as nephrons matured through the S-shape stage to glomerular and proximal tubule epithelia. However, receptor expression became detectable in collecting duct derivatives of the ureteric bud, a pattern maintained until four weeks postnatally, the limit of this study. Since apoptosis and proliferation are rare postnatally, we speculate that the receptor might have a role in epithelial ion transport in mature epithelia. However, although Deetjen et al. [40] provided functional evidence for luminal P2Y receptors ($P2Y_2$ or $P2Y_4$) in isolated perfused adult mouse cortical collecting ducts, benzoyl-benzoyl ATP, a specific $P2X_7$ agonist, had no apparent physiological effect when applied apically or basolaterally; the exact age of experimental animals, however, was not stated nor was the receptor sought using immunohistochemistry. Finally, our observation that mouse ureteric epithelium expresses $P2X_7$ is consistent with immunoreactivity in adult rat bladder and ureter urothelium reported by Lee et al. [24].

In *cpk/cpk* polycystic kidneys, previous reports have observed marked epithelial proliferation at the onset of cystogenesis, but less proliferation in later stages of collecting duct cyst enlargement [19, 20, 41]. Additionally, cells between cysts undergo apoptosis, a process that may contribute to the loss of functioning nephrons [22, 23]; this pattern of death was confirmed in the current study. In fact, the generation of renal cysts in bcl2-deficient mice argues that, in certain circumstances, cyst formation may be secondary to apoptotic deletion of cells [38]. In the current study we demonstrated $P2X_7$ protein in epithelia of expanding collecting duct tubules and cysts at all stages of the disease, whereas there was no immunoreactivity between cysts; this pattern suggests a role for $P2X_7$ other than the induction of death. Recently, a different role for ATP in cyst enlargement has been considered [4]: cultured epithelia from human autosomal dominant kidney cysts secrete high levels of ATP apically compared to normal cells, and ATP levels sufficient to stimulate purinergic receptors were measured in cyst fluid. Although the expression of purinergic receptors was not sought in this study [4], it was suggested that nucleotides might stimulate luminal receptors causing enhanced solute and water transport, resulting in rapid expansion of cysts. In future, it would be interesting to measure extracellular ATP levels in *cpk/cpk* cysts and to assess the effects of the nucleotide on, for example, *cpk/cpk* collecting duct cell lines in culture in order to provide evidence of a functional role for the $P2X_7$ receptor. Such experiments are necessary to prove that $P2X_7$ has a role in cyst formation, rather than simply being a non-functional marker for the origin of collecting duct cysts.

Acknowledgements

This work was supported by a National Kidney Research Fund Training Fellowship to K.A.H., a Studentship (S10/98) to T.M.J. and a Project Grant (R3/2/99) to A.S.W. and P.J.D.W.

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