

# Extracellular ATP and UTP activate the protein kinase B/Akt cascade *via* the P2Y<sub>2</sub> purinoceptor in renal mesangial cells

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**1** Extracellular nucleotides can activate a common purinoceptor mediating various cell responses. In this study we report that stimulation of rat mesangial cells with ATP and UTP leads to a rapid activation of the protein kinase B/Akt (PKB) pathway. Time-course studies reveal a rapid and transient phosphorylation of both Ser<sup>473</sup> and Thr<sup>308</sup> of PKB with a maximal effect after 5 min of stimulation. The response is concentration-dependent with a maximal effect at 30  $\mu$ M of ATP and UTP. Western blot analysis of mesangial cells reveals the expression of the isoenzymes PKB- $\alpha$  and PKB- $\gamma$ , but not the PKB- $\beta$ .

**2** ATP and UTP also activate the upstream located PI 3-kinase-dependent kinase. Furthermore, the ATP- and UTP-induced PKB phosphorylation is abolished by two inhibitors of the PI 3-kinase. In addition, suramin, a putative P2Y<sub>2</sub> receptor antagonist, and pertussis toxin, an inhibitor of G<sub>i</sub>/G<sub>o</sub> activation, markedly block ATP- and UTP-induced PKB phosphorylation.

**3** A series of ATP and UTP analogues were tested for their ability to stimulate PKB phosphorylation. UTP, ATP and  $\gamma$ -thio-ATP are the only compounds capable of activating PKB.

**4** Stress-induced apoptosis of mesangial cells is reduced by the stable ATP analogue,  $\gamma$ -thio-ATP, and this inhibitory effect is reversed in the presence of LY 294002.

**5** In summary, these results demonstrate that extracellular nucleotides are able to activate the PI 3-kinase/PDK/PKB cascade *via* the P2Y<sub>2</sub>-receptor and a pertussis toxin-sensitive G<sub>i</sub> protein. Moreover, in mesangial cells this cascade may have an important role in the antiapoptotic response but not in the mitogenic or inflammatory response produced by extracellular nucleotides.

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**Keywords:** Protein kinase B/Akt; PDK; PI 3-kinase; P2Y<sub>2</sub> purinoceptor; ATP; UTP; apoptosis; mesangial cell

**Abbreviations:** BSA, bovine serum albumin; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF, insulin-like growth factor; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PDGF-BB, platelet-derived growth factor-BB; PDK, PI 3-kinase dependent kinase; PIP<sub>3</sub>, phosphatidylinositol trisphosphate; PKB, protein kinase B; SAPK, stress-activated protein kinase; SGK, serum- and glucocorticoid-regulated protein kinase; TNF $\alpha$ , tumour-necrosis factor- $\alpha$

## Introduction

Extracellular nucleotides, such as ATP and UTP, exert diverse effects on intact cells by activation of plasma membrane receptors, termed P2 purinoceptors. These receptors either couple through G-proteins to their intracellular effector enzymes (P2Y subtypes) or are themselves part of ligand-gated ion channels (P2X subtypes) (Ralevic & Burnstock, 1998; Boarder & Hourani, 1998).

In renal mesangial cells, ATP acts on a nucleotide receptor (P2Y<sub>2</sub> receptor) and mediates phosphoinositide hydrolysis with generation of 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (Pfeilschifter, 1990a, b) leading to mobilization of intracellular calcium (Pavenstädt *et al.*, 1993), activation of protein kinase C (Pfeilschifter & Huwiler, 1996), activation of the classical mitogen-activated protein kinase cascade (Huwiler & Pfeilschifter, 1994) and the cytosolic phospholipase A<sub>2</sub>, and stimulation of prostaglandin E<sub>2</sub> synthesis (Pfeilschifter, 1990a; Schulze-Lohoff *et al.*, 1992). Further-

more, in rat mesangial cells ATP can activate the stress-activated protein kinase cascade (Huwiler *et al.*, 1997a) as well as the p38-MAPK cascade in rat mesangial cells (Huwiler *et al.*, 2000). The serine-threonine-specific kinase protein kinase B/Akt<sup>1</sup> (PKB) was first identified as the human homologue of a transforming oncogene (Staal, 1987; Coffey & Woodgett, 1991), and has now gained interest because of its possible function in cell survival signalling (Chan *et al.*, 1999). Classical activators of PKB include growth factors, including platelet-derived growth factor and insulin-like growth factor, which act through tyrosine kinase receptors and involve the small G protein p21<sup>ras</sup> and phosphatidylinositol 3-kinase (PI 3-kinase). The lipid products of PI 3-kinase such as phosphatidylinositol trisphosphate (PIP<sub>3</sub>), are important cofactors for PKB activation. Additionally, PKB requires phosphorylation at two sites for full activation, namely at Thr<sup>308</sup> in the activation loop, and at Ser<sup>473</sup> in a hydrophobic part at the C-terminus. The phosphoinositide-dependent kinase-1 (PDK-1) was identified as the kinase responsible for Thr<sup>308</sup> phosphorylation whereas the kinase

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phosphorylating Ser<sup>473</sup>, tentatively named PDK-2, has not yet been identified. A recent report suggests that Ser<sup>473</sup> is an autophosphorylation site of PKB (Toker & Newton, 2000).

In this study in rat renal mesangial cells, we show that extracellular nucleotides, such as ATP and UTP, can activate the PKB cascade in a suramin- and pertussis toxin-sensitive manner. We also show that this pathway may play an important role in extracellular nucleotide-triggered cytoprotection against stress-induced cell death, but that it does not play an important role in the proliferative and inflammatory responses triggered by ATP and UTP.

## Methods

### Chemicals

All nucleotides were obtained from Fluka or Sigma Aldrich Fine Chemicals; [<sup>3</sup>H-methyl]-thymidine (specific activity: 25 Ci mmol<sup>-1</sup>), [<sup>3</sup>H]-arachidonic acid (specific activity: 240 Ci mmol<sup>-1</sup>), [<sup>32</sup>P- $\gamma$ ]-ATP (specific activity: >5000 Ci mmol<sup>-1</sup>), anti-rabbit and anti-mouse horseradish peroxidase-linked IgGs, protein A-sepharose 4B-CL, protein G-sepharose fast flow and Hyperfilm were purchased from Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany; donkey anti-sheep horseradish peroxidase-linked IgG was from Sigma Aldrich Chemie GmbH, Deisenhofen, Germany; the PKB $\alpha$ /Akt1-specific antibody and the phospho-PKB antibodies were from New England Biolabs, Frankfurt, Germany; PDK1 activity assay kit was from Upstate Biotechnology, Lake Placid, NY, U.S.A.; PKB $\beta$ /Akt-2 and PKB $\gamma$ /Akt-3 antibodies, wortmannin, LY 294002 and pertussis toxin were obtained from Calbiochem-Novabiochem, Schwalbach, Germany; all cell culture nutrients were from Life Technologies, Karlsruhe, Germany.

### Cell-culture

Rat renal mesangial cells were cultivated and characterized as described previously (Pfeilschifter, 1990a, b). In a second step, single cells were cloned by limited dilution on 96-microwell plates. Clones with apparent mesangial cell morphology were used for further processing. In this study Passages 5–18 were used for the experiments. Glomerular endothelial cells were isolated and cultivated as previously described (Briner & Kern, 1994; Huwiler *et al.*, 1997b). Glomerular parietal epithelial cells were cultivated in Dulbecco's modified Eagle medium containing 10% foetal bovine serum (Huwiler *et al.*, 1993). The macrophage cell line P388D1 was cultivated in RPMI 1640 medium containing 10% foetal bovine serum.

### Cell stimulation and Western blot analysis

Confluent mesangial cells in 60 mm-diameter dishes were stimulated with the indicated substances in Dulbecco's modified Eagle medium (DMEM) containing 0.1 mg ml<sup>-1</sup> of fatty acid-free bovine serum albumin (BSA). Thereafter the medium was withdrawn and the cells washed once with ice-cold phosphate-buffered saline (PBS) solution. Cells were scraped into ice-cold lysis buffer (50 mM Tris, HCl, pH 7.4,

150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 40 mM  $\beta$ -glycerophosphate, 50 mM sodiumfluoride, 10  $\mu$ g ml<sup>-1</sup> leupeptin, 10  $\mu$ g ml<sup>-1</sup> aprotinin, 1  $\mu$ M pepstatin A, 1 mM phenylmethyl sulphonyl fluoride) and homogenized by 10 passes through a 26G-needle fitted to a 1 ml syringe. Samples were centrifuged for 10 min at 14,000  $\times g$  and the supernatant taken for protein determination. Cell extracts containing 70  $\mu$ g of protein were prepared in SDS-sample buffer and subjected to SDS-PAGE. Proteins were transferred onto nitrocellulose paper for 1 h at 11 V using a semi-dry blotting apparatus. The blotting buffer used was 25 mM Tris, 190 mM glycine in 20% methanol. After the transfer, immunostaining was performed as previously described in detail (Huwiler *et al.*, 1995; 2000). Antibodies were diluted in blocking buffer as indicated in the legends of the figures. Bands were detected by the enhanced chemiluminescence (ECL) method as recommended by the manufacturer.

### PDK1 Immunocomplex-kinase activity assay

The PDK1 Immunocomplex kinase activity assay was performed according to the manufacturer's (Upstate Biotechnology, Lake Placid, NY, U.S.A.) recommendations with slight modifications. In brief, mesangial cells in 100 mm-diameter dishes were incubated for 2 days in DMEM containing 0.1 mg ml<sup>-1</sup> of fatty acid-free BSA and then stimulated at 37°C with various agents as indicated. The medium was removed and the cells were washed with ice-cold PBS to stop the reaction. Cells were then scraped directly into lysis buffer and homogenized by 10 passes through a 26-gauge needle fitted to a 1 ml syringe. The homogenate was centrifuged for 10 min at 14,000  $\times g$  and the supernatant taken for immunoprecipitation. Samples containing 500  $\mu$ g of protein and 5% foetal calf serum in lysis buffer, were incubated with the various antibodies overnight at 4°C. 20  $\mu$ l of a 50% slurry of protein G-sepharose in PBS was then added and the mixture incubated for 1 h on a rotating wheel. After centrifugation for 3 min at 2000  $\times g$  immunocomplexes were washed three times with a low salt buffer and 3  $\times$  with a high salt buffer and once with 50 mM Tris, HCl pH 7.4. The beads were incubated in 30  $\mu$ l of 1  $\times$  PDK1 assay dilution buffer containing 500 ng of inactive serum- and glucocorticoid-regulated protein kinase (SGK) for 30 min at 30°C. Thereafter a SGK substrate peptide (RPRAATF; 66  $\mu$ M final concentration) and 10  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]-ATP were added and a second kinase reaction was allowed to continue for 10 min at 30°C. 25  $\mu$ l was spotted onto a P81 paper to stop the reaction, washed three times with 0.75% phosphoric acid and once with acetone and then counted in a  $\beta$ -counter.

### Reverse transcriptase-PCR

Total RNA was isolated using guanidinium isothiocyanate solution. 1.5  $\mu$ g of RNA was used for reversed transcriptase-PCR (First Strand cDNA Synthesis Kit, MBI). The following sequences were performed for PCR (Taq DNA Polymerase, recombinant, MBI): 94°C for 5 min (1 cycle), and 94°C for 30 s, 55°C (50°C for p110 $\alpha$ ) for 1.5 min, 72°C for 1 min (with variable numbers of cycles) and final extension at 72°C for 7 min. The number of cycles were: 30 for p110 $\alpha$  and 35 for

p110 $\delta$  and p110 $\gamma$ . Sequences of the primers for analysis of mRNA: mouse p110 $\alpha$ : forward: GAA AAT GGC TTT GAA TCT CTG G; reverse: GAT ACA TCC CAC AGG CAC G; mouse p110 $\delta$ : forward: GAA AAG TGA ATG CTG ACG AGC; reverse: ACT TCG TGG CGC ATC TTC; mouse p110 $\gamma$ : forward: ATA TCC CTG TCC TGC CTC G; reverse: AGA GCA ATT CTT TGT CCT CTG C; GAPDH: forward: AAT GCA TCC TGC ACC ACC AA; reverse: GTC ATT GAG AGC AAT GCC AGC. PCR products (length: 779 bp for p110 $\alpha$ , 619 bp for p110 $\delta$ , 621 bp for p110 $\gamma$  and 470 bp for GAPDH) were run on a 1.5% agarose gel containing 0.5  $\mu\text{g ml}^{-1}$  ethidium bromide.

### Proliferation assay

Confluent mesangial cells in 24-well plates were incubated for 2 days in serum-free DMEM. Thereafter, cells were stimulated for 24 h with the agonists in the presence of 1  $\mu\text{Ci ml}^{-1}$  of [ $^3\text{H}$ -methyl]-thymidine. To stop the reaction, medium was withdrawn and the cells washed twice with ice-cold PBS and incubated in 5% trichloroacetic acid for 30 min at 4°C. Thereafter, cells were washed twice with 5% trichloroacetic acid and then incubated in 0.5 M NaOH for 30 min at 37°C to solubilize the DNA. [ $^3\text{H}$ ]-thymidine incorporated into the DNA was then counted in a  $\beta$ -counter (Packard).

### Determination of arachidonic acid release

Confluent mesangial cells in 16 mm-diameter wells were labelled for 24 h with [ $^3\text{H}$ ]-arachidonic acid (1  $\mu\text{Ci ml}^{-1}$ ) in DMEM, containing 0.1 mg  $\text{ml}^{-1}$  fatty acid-free BSA. Thereafter cells were washed three times to remove all non-incorporated [ $^3\text{H}$ ]-arachidonic acid. Approximately 80–90% of the added [ $^3\text{H}$ ]-arachidonic acid was incorporated by this method. The labelled cells were incubated in DMEM containing 1 mg  $\text{ml}^{-1}$  BSA as a trap for the released [ $^3\text{H}$ ]-arachidonic acid. The cells were then stimulated with vehicle or the indicated agonists for 30 min. Thereafter, the medium was removed and centrifuged. Cells were dissolved in 0.5 M NaOH, and radioactivity was counted in the supernatants and cell extracts in a scintillation counter. The percentage [ $^3\text{H}$ ]-arachidonic acid released from total incorporated radioactivity was calculated.

### Apoptosis assay

Confluent mesangial cells in 30 mm-diameter dishes were incubated with the indicated stimulants in Dulbecco's modified Eagle medium (DMEM) containing 0.1 mg  $\text{ml}^{-1}$  fatty acid-free BSA for 24 h. Thereafter, oligonucleosomal DNA fragmentation, a characteristic biochemical feature of apoptotic cell death, was measured using a nucleosome DNA ELISA (Roche Diagnostics), which quantitatively records histone-associated DNA fragments.

### Statistical analysis

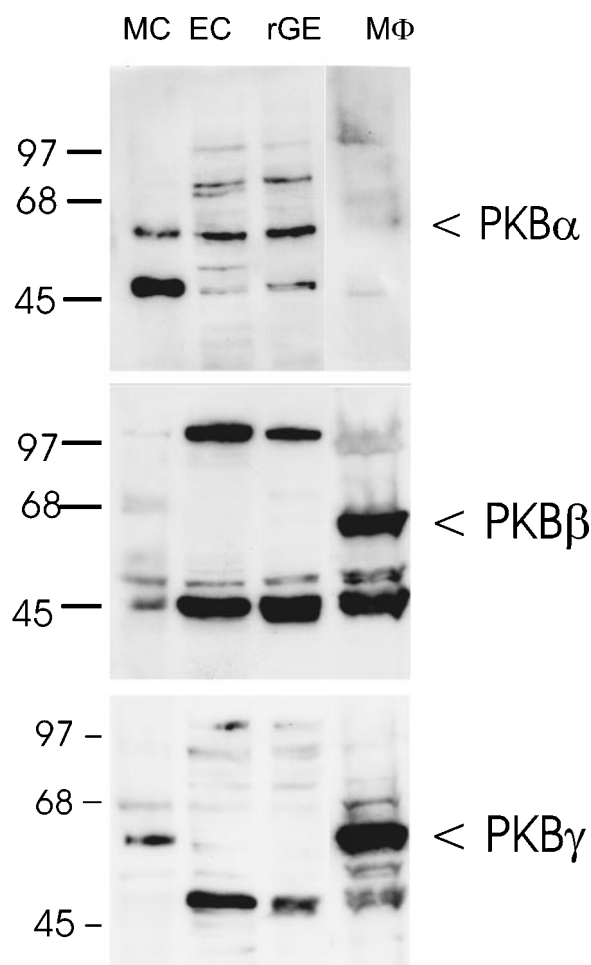
Statistical analysis was performed by using one way analysis of variance (ANOVA). For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni. One-

way ANOVA with Bonferroni's post test was performed using GraphPad InStat version 3.00 for Windows NT, GraphPad Software, San Diego California U.S.A.

## Results

### Expression of PKB isoenzymes in rat mesangial cells

To investigate the pattern of expression of PKB isoenzymes in renal mesangial cells, Western blot analysis of glomerular cell lysates was performed using polyclonal antisera selective for the three known isoforms of PKB. According to sequence information, PKB- $\alpha$  and - $\beta$  run at a molecular size of approximately 60 kDa, whereas PKB- $\gamma$  has a 25 amino acid C-terminal deletion and runs at approximately 58 kDa (Nakatani *et al.*, 1999). As seen in Figure 1, PKB- $\alpha$  is expressed in all cell types present in the renal glomerulus, i.e.



**Figure 1** Western blot analysis of PKB isoforms in glomerular cells. Lysates of glomerular mesangial cells (MC), parietal epithelial cells (EC), endothelial cells (rGE) and the mouse macrophage cell line P388D1 (M $\Phi$ ) were separated on SDS-PAGE (10% acrylamide gel), transferred to a nitrocellulose filter and Western blot analysis was performed using specific PKB $\alpha$ , PKB $\beta$  and PKB $\gamma$  antibodies at a dilution of 1:1600, 1:1000 and 1:1000, respectively. Bands were detected by the ECL method according to the manufacturer's recommendation. Molecular size standards are indicated on the left side (in kDa).

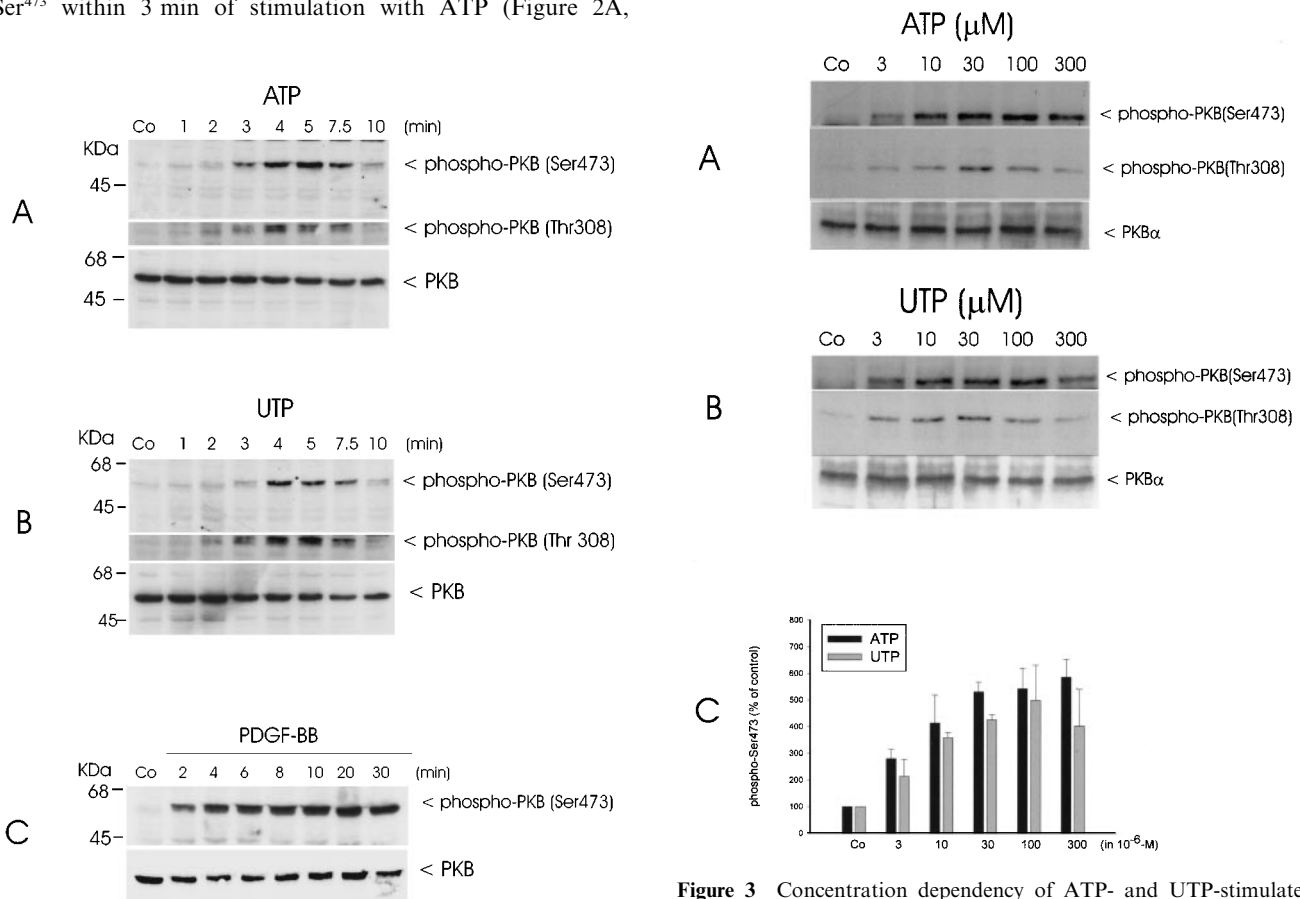
mesangial cells (MC), epithelial cells (EC) and endothelial cells (rGE), whereas the mouse macrophage cell line P388D1 (MΦ) does not express the  $\alpha$ -isoform (Figure 1, upper panel). The identity of the intensive band at 45 kDa in mesangial cells is unclear but could represent a degradation product of the PKB- $\alpha$ . In contrast, PKB- $\beta$  is not expressed in any of the glomerular cells, but shows a high expression in P388D1 macrophages (Figure 1, middle panel). PKB- $\gamma$  is differentially expressed in glomerular cells, i.e. expression is found in mesangial cells but not in epithelial or endothelial cells. Again, PKB- $\gamma$  shows a high expression in P388D1 macrophages (Figure 1, lower panel).

#### *ATP and UTP stimulate the PI 3-kinase/PDK/PKB cascade in mesangial cells*

We investigated next the effect of extracellular nucleotides on the phosphorylation pattern of PKB in mesangial cells, because phosphorylation of PKB is considered to reflect activation of the enzyme (Andjelkovic *et al.*, 1996).

Time-course experiments reveal a rapid phosphorylation of Ser<sup>473</sup> within 3 min of stimulation with ATP (Figure 2A,

upper panel). Maximal phosphorylation is obtained after 5 min which is followed by a rapid decrease after 10 min. A similar increase in phosphorylation is observed in the case of Thr<sup>308</sup> (Figure 2A, middle panel), whereas the total level of PKB- $\alpha$  is not affected by stimulation (Figure 2A, lower panel). Stimulation with UTP causes a rapid and transient phosphorylation of Ser<sup>473</sup> (Figure 2B upper panel) and Thr<sup>308</sup> (middle panel) similar to that produced by ATP. Again, the total PKB level does not change on stimulation with UTP (Figure 2B, lower panel). Long-term stimulation of mesangial cells with ATP and UTP (30 min til 24 h) gives no second peak of phosphorylation (data not shown). In contrast to the transient activation of PKB by ATP and UTP, PDGF-BB causes a more sustained activation of PKB, which is still maximal after 30 min of stimulation (Figure 2C). The phosphorylation of PKB by ATP and UTP occurs in a concentration-dependent manner, with a maximal effect with 30  $\mu$ M ATP (Figure 3A) or UTP (Figure 3B) after 5 min of stimulation.



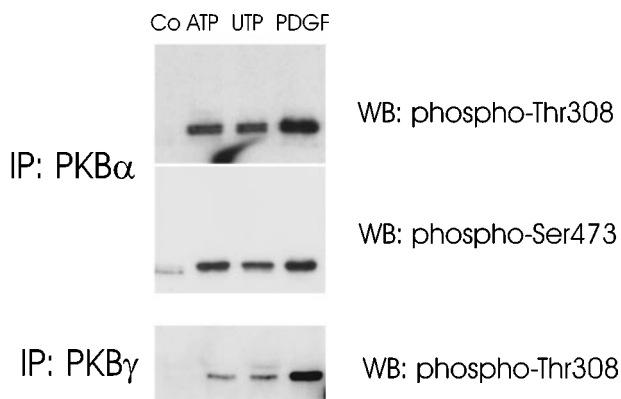
**Figure 2** Time-course of ATP- and UTP-stimulated PKB phosphorylation in mesangial cells. Quiescent mesangial cells were treated with either vehicle (co), 100  $\mu$ M of ATP (A), 100  $\mu$ M of UTP (B) or 25 ng ml<sup>-1</sup> of PDGF-BB (C) for the indicated time periods. Thereafter cells were harvested and Western blot analyses were performed using specific phospho-Ser<sup>473</sup>-PKB (upper panel), phospho-Thr<sup>308</sup>-PKB (middle panel) and total PKB $\alpha$  (lower panel) antibodies at a dilution of 1:1000, 1:1000 and 1:1600, respectively. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative of 2 (C) or 3 (A and B) independent experiments giving similar results.

**Figure 3** Concentration dependency of ATP- and UTP-stimulated PKB phosphorylation in mesangial cells. Quiescent mesangial cells were treated with either vehicle (co) or the indicated concentrations of ATP (A) and UTP (B) for 5 min. Thereafter cells were harvested and Western blot analysis was performed using specific phospho-Ser<sup>473</sup>-PKB (upper panel), phospho-Thr<sup>308</sup>-PKB (middle panel) and total PKB $\alpha$  antibodies at a dilution of 1:1000, 1:1000 and 1:1600, respectively. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative of three independent experiments giving similar results. (C): The phospho-Ser<sup>473</sup>-PKB bands of the ATP and UTP stimulated samples were densitometrically evaluated. Results are expressed as per cent of vehicle-stimulated control values and are means  $\pm$  s.d.. ( $n=3$ ).

Since mesangial cells do express PKB- $\alpha$  and PKB- $\gamma$ , the possibility that extracellular nucleotides can activate both isoforms was investigated. For this purpose, the two isoforms were immunoprecipitated from stimulated cells and subjected to Western blot analysis using phospho-specific PKB antibodies. As seen in Figure 4, immunoprecipitated PKB- $\alpha$  is clearly phosphorylated on residue Thr<sup>308</sup> (upper panel) and also on Ser<sup>473</sup> (middle panel) after ATP, UTP and PDGF-BB stimulation. Immunoprecipitated PKB- $\gamma$ , which lacks the C-terminal Ser<sup>473</sup>, still shows a marked phosphorylation on Thr<sup>308</sup> (lower panel). These findings suggest that both isoforms are activated by extracellular nucleotides.

The possibility that ATP and UTP also activate the upstream kinase PDK-1 was examined using an immunocomplex kinase assay in an *in vitro* two-step kinase reaction, i.e. immunoprecipitated PDK-1 is incubated with recombinant inactive serum- and glucocorticoid-regulated protein kinase (SGK) in the first step and then with a peptide fragment of a SGK substrate in a second step. Thereafter, <sup>32</sup>P-phosphorylated SGK substrate peptide is measured in a  $\beta$ -counter. Figure 5 shows that ATP within 2 min does indeed induce a rapid activation of PDK-1 in mesangial cells.

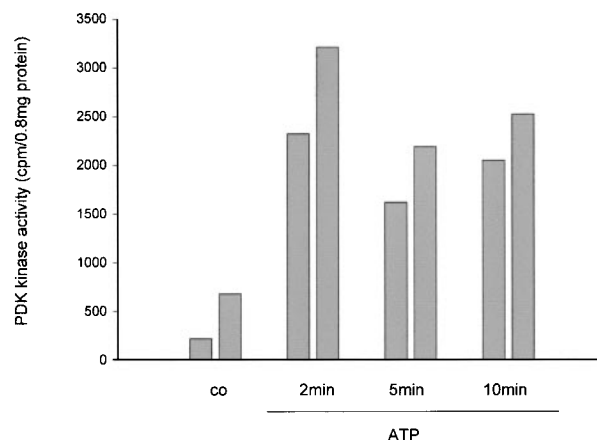
In order to trace the cascade one step further upstream, the PI 3-kinase was investigated since it is well accepted that PDK-1 and also PKB require the lipid products of PI 3-kinase, particularly PIP<sub>3</sub>, for full activation. By using reverse transcriptase-PCR of mouse mesangial cells, the PI 3-kinase subtypes p110 $\alpha$ , p110 $\gamma$  and p110 $\delta$  could be identified (Figure 6A). Evidence for the involvement of PI 3-kinase in ATP and UTP-induced PKB activation is obtained using the two inhibitors of PI 3-kinase, wortmannin (Powis *et al.*, 1994; Okada *et al.*, 1994) and LY 294002 (Vlahos *et al.*, 1994), which block the ATP- and UTP-stimulated PKB activation in a dose-dependent manner (Figure 6B).



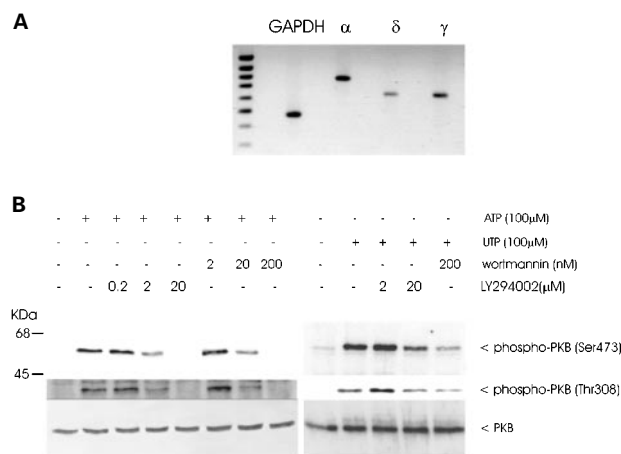
**Figure 4** Phosphorylation of PKB isoforms  $\alpha$  and  $\gamma$  by ATP, UTP and PDGF-BB in mesangial cells. Quiescent mesangial cells were stimulation for 5 min with either vehicle (Co), ATP (100  $\mu$ M), UTP (100  $\mu$ M) or PDGF-BB (25 ng ml<sup>-1</sup>). Thereafter, PKB $\alpha$  and PKB $\gamma$  were immunoprecipitated from cell lysates with isoform-selective antibodies (in a dilution of 1:250 each) and subjected to Western blot analysis using either a phospho-Ser<sup>473</sup>-PKB- or a phospho-Thr<sup>308</sup>-PKB-specific antibody as indicated. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative of two independent experiments giving similar results.

### Involvement of the P2Y<sub>2</sub> receptor and a pertussis toxin sensitive G<sub>i</sub> protein in PKB activation

In the next step, suramin, a putative P2Y<sub>2</sub> purinoceptor antagonist without actions on the P2Y<sub>4</sub> receptor (Boarder &



**Figure 5** Effect of ATP on PDK-1 activity in rat mesangial cells. Quiescent mesangial cells were treated with either vehicle (co) or 100  $\mu$ M of ATP for the indicated time periods. Thereafter cells were harvested and PDK-1 was immunoprecipitated and subjected to an *in vitro* two-step kinase reaction as described in the Methods section. Data are expressed as c.p.m. per 0.8 mg of protein and show the individual values of two independent experiments.



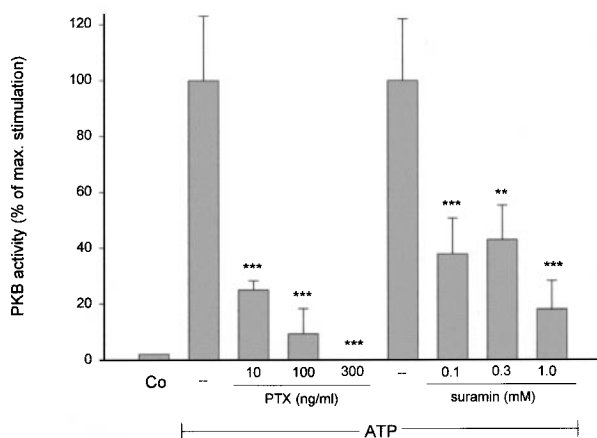
**Figure 6** PI 3-kinase isoform expression in mesangial cells and the effect of wortmannin and LY 294002 on ATP-induced PKB phosphorylation. (A): RNA was prepared from quiescent mouse mesangial cells and reversed transcriptase-PCR of the p110 $\alpha$  ( $\alpha$ ), p110 $\delta$  ( $\delta$ ), p110 $\gamma$  ( $\gamma$ ) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as described in the Experimental Procedure Section. GAPDH runs at 470 bp, p110 $\alpha$  at 779 bp, p110 $\delta$  at 619 bp and p110 $\gamma$  at 621 bp. (B): Quiescent mesangial cells were pretreated with the indicated concentrations of wortmannin and LY 294002 for 20 min before stimulation for 5 min with 100  $\mu$ M of ATP. Thereafter cell lysates, containing 70  $\mu$ g of protein were subjected to SDS-PAGE (8% acrylamide gel) and Western blot analysis was performed using specific phospho-Ser<sup>473</sup>-PKB (upper panel), phospho-Thr<sup>308</sup>-PKB (middle panel) and total PKB $\alpha$  antibodies at a dilution of 1:1000, 1:1000 and 1:1600, respectively. Bands were visualized by the ECL method and evaluated on a densitometer. Data are representative of at least 3 independent experiments giving similar results.

Hourani, 1998) was tested and found to block ATP and UTP-induced PKB phosphorylation in a dose-dependent manner as shown in Figure 7. In addition, the stimulatory action of ATP and UTP is almost completely blocked by pertussis toxin (Hewlett *et al.*, 1983), a potent inhibitor of the  $G_i/G_o$  proteins (Figure 7).

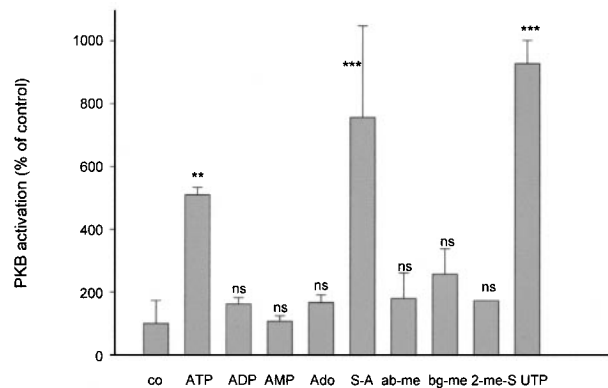
A series of ATP analogues were tested for their ability to induce PKB phosphorylation. As seen in Figure 8, ATP, UTP and  $\gamma$ -thio-ATP are the only substances that stimulate PKB phosphorylation. Neither ADP, AMP nor adenosine cause significant PKB phosphorylation. Furthermore, 2-methyl-thio-ATP,  $\alpha\beta$ -methylene-ATP and  $\beta\gamma$ -imido-ATP were also without effect.

### *Involvement of the PKB pathway in cell responses of mesangial cells to extracellular nucleotides*

The fact that extracellular nucleotides trigger the release of arachidonic acid by a cPLA<sub>2</sub> (Pfeilschifter, 1990a) and also stimulate cell proliferation (Huwiler & Pfeilschifter, 1994; Schulze-Lohoff *et al.*, 1992), prompted us to investigate the contribution of the PKB pathway in nucleotide-induced arachidonic acid release and the proliferation of mesangial cells. Stimulation for 30 min with ATP and UTP causes a several fold increase in arachidonic acid release (Figure 9A) consistent with previous data (Pfeilschifter, 1990a, b). However, neither wortmannin nor LY 294002 can block the extracellular nucleotide-induced arachidonic acid release, thus clearly arguing against a role of the PKB pathway in nucleotide-induced cPLA<sub>2</sub> activation and arachidonic acid release. Similarly, extracellular nucleotide-stimulated cell proliferation is not inhibited by either wortmannin or LY 294002 when measuring [<sup>3</sup>H]-thymidine incorporation into



**Figure 7** Effect of pertussis toxin and suramin on ATP- and UTP-induced PKB phosphorylation in mesangial cells. Quiescent mesangial cells were pretreated with the indicated concentrations of suramin (A) for 20 min or pertussis toxin (B) for 20 h before stimulation for 5 min with 100  $\mu$ M of ATP. Thereafter cell lysates, containing 70  $\mu$ g of protein were subjected to SDS-PAGE (8% acrylamide gel) and Western blot analysis was performed using specific phospho-Ser<sup>473</sup>-PKB (upper panel), phospho-Thr<sup>308</sup>-PKB (middle panel) and total PKB $\alpha$  antibodies at a dilution of 1:1000, 1:1000 and 1:1600, respectively. Bands were visualized by the ECL method and evaluated on a densitometer. Results are expressed as per cent of control value and are means  $\pm$  s.d. ( $n = 3-4$ ).



**Figure 8** Effect of different ATP analogues on PKB phosphorylation in mesangial cells. Quiescent mesangial cells were stimulated for 5 min with 100  $\mu$ M of ATP, ADP, AMP, adenosine (Ado),  $\gamma$ -S-ATP (S-A),  $\alpha\beta$ -methylene-ATP (ab-me),  $\beta\gamma$ -methylene-ATP (bg-me), 2-methyl-S-ATP (2-me-S) or UTP. Thereafter cell lysates, containing 70  $\mu$ g of protein were subjected to SDS-PAGE (8% acrylamide gel) and Western blot analysis was performed using a specific phospho-Ser<sup>473</sup>-PKB antibody at a dilution of 1:1000. Bands were visualized by the ECL method and evaluated on a densitometer. Results are expressed as per cent of control value and are means  $\pm$  s.d. ( $n = 2-4$ ). \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , statistically significant difference compared to unstimulated control value.

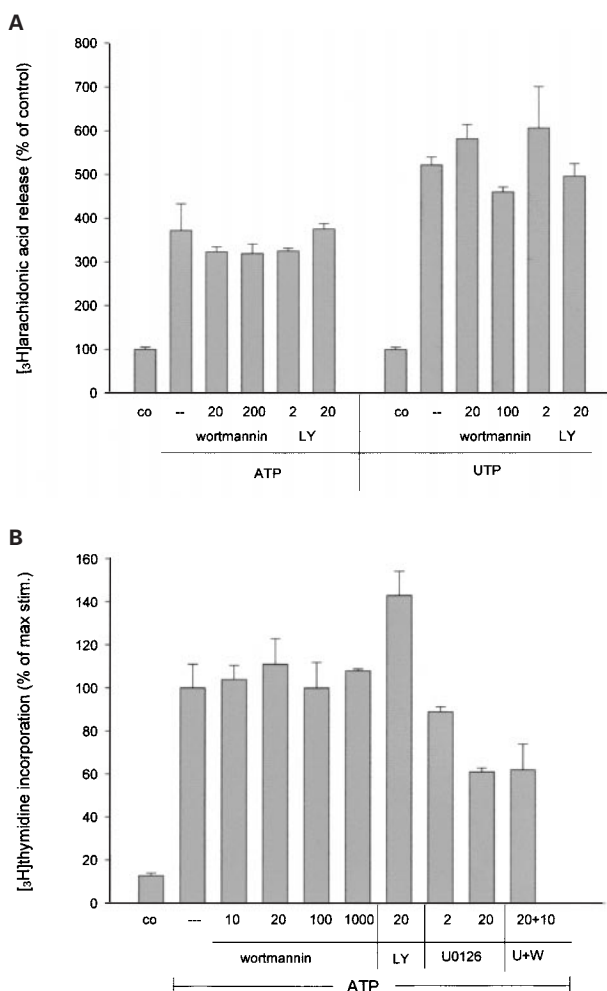
DNA (Figure 9B). However, a maximal 50% inhibition is seen with the classical mitogen-activated protein kinase (MAPK) cascade inhibitor U0126, which is not further increased in the presence of wortmannin (Figure 9B).

In contrast to these findings, extracellular nucleotides may exert a cytoprotective effect on stress-induced apoptosis in mesangial cells. As seen in Figure 10, cotreatment of cells with tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) and cycloheximide (CHX) causes a marked increase in oligonucleosomal DNA fragmentation, which is a typical feature of programmed cell death (apoptosis). This increased DNA fragmentation is reduced in a dose-dependent manner in the presence of the stable ATP analogue,  $\gamma$ -thio-ATP, and this protective effect in turn can be reversed with the PI 3-kinase inhibitor, LY294002 (Figure 10). Stimulation of cells with LY 294002 alone, at a concentration of 20  $\mu$ M, has no effect on apoptosis (Figure 10).

## Discussion

It has recently become apparent that ATP not only functions as an intracellular energy source, but also acts as an extracellular signalling molecule mediating cell-cell communication *via* activation of plasma membrane receptors, termed P2 purinoceptors. These receptors have been classified into two main classes, the G protein-coupled receptors (P2Y) and the ligand-gated ion channels (P2X) (Ralevic & Burnstock, 1998; Boarder & Hourani, 1998; Abbracchio & Burnstock, 1994).

In renal mesangial cells, ATP acts on a nucleotide receptor (P2Y<sub>2</sub> receptor), that can equally well be activated by UTP, and which mediates phosphoinositide hydrolysis and synthesis of prostaglandin E<sub>2</sub> (Pfeilschifter, 1990a, b). In addition, it was observed that extracellular nucleotides stimulate



**Figure 9** Effect of the PI 3-kinase inhibitors wortmannin and LY 294002 on ATP- and UTP-induced arachidonic acid release and cell proliferation. (A): [ $^3$ H]-arachidonic acid-labelled mesangial cells were stimulated for 30 min with 100  $\mu$ M of ATP or UTP in the presence of the indicated concentrations of LY 294002 (in  $\mu$ M) and wortmannin (in nM), which were preincubated for 30 min. Thereafter, the radioactivity released into the medium was counted as described in the Methods section. Results are expressed as per cent of control value and are means  $\pm$  s.d. ( $n=4$ ). (B): Quiescent mesangial cells were stimulated for 24 h with 100  $\mu$ M of ATP or UTP in the presence of the indicated concentrations of LY 294002 (in  $\mu$ M) and wortmannin (in nM), and 1  $\mu$ Ci ml $^{-1}$  of [ $^3$ H]-thymidine. Thereafter, the radioactivity incorporated into the DNA was counted as described in the Methods section. Results are expressed as per cent of control value and are means  $\pm$  s.d. ( $n=4$ ).

growth of mesangial cells (Huwiler and Pfeilschifter, 1994; Schulze-Lohoff *et al.*, 1992), a hallmark of chronic progressive glomerular diseases. Moreover, ATP and UTP were shown to trigger the classical MAPK cascade (Huwiler and Pfeilschifter, 1994), as well as the stress-activated protein kinase (SAPK) cascade (Huwiler *et al.*, 1997a) and the p38-MAPK cascade (Huwiler *et al.*, 2000). However, the detailed molecular mechanisms by which ATP and UTP trigger the above mentioned cell responses is still unknown.

In this study we show that ATP and UTP are able to activate the PKB/Akt pathway, which is considered to play a central role in cell growth and survival (Chan *et al.*, 1999).

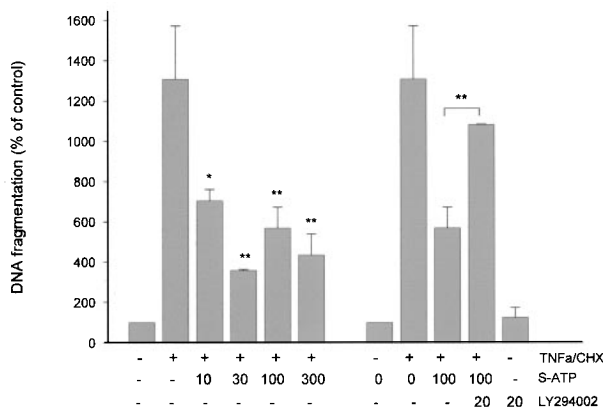
Classical activators of this pathway include various growth factors, such as platelet-derived growth factor and (PDGF) and insulin-like growth factor (IGF), that act through receptor tyrosine kinases and involve p21<sup>ras</sup> and the PI 3-kinase. In early studies, neither lysophosphatidic acid, which acts through G $_i$  protein-coupled receptors, nor phorbol ester, which directly activates protein kinase C, were found to activate the PKB pathway in fibroblasts (Burgering & Coffey, 1995; Franke *et al.*, 1995).

However, with the identification of the  $\gamma$ -isoform of PI 3-kinase, which is primarily activated by G $\beta\gamma$  subunits (Stoyanov *et al.*, 1995; Hirsch *et al.*, 2000), a link between G protein-coupled agonists and PKB activation was evident. Subsequently, several studies reported that G protein-coupled receptors can indeed activate the PKB pathway (Murga *et al.*, 2000; Bommakanti *et al.*, 2000). Murga *et al.* (2000) showed that PI3K- $\beta$  or - $\gamma$  subtypes are essential for signaling between G protein-coupled receptors and PKB. In cells lacking these subtypes, e.g. NIH 3T3 mouse fibroblasts, no signal propagation from G protein-coupled receptors to PKB could be detected without PI3K- $\beta$  or - $\gamma$  being transfected into the cells.

In this study we show that rat mesangial cells do express the  $\alpha$ ,  $\gamma$  and  $\delta$  isoforms of PI 3-kinase as detected by reverse transcriptase-PCR. The  $\beta$ -isoform could not be analysed because rat and mouse sequences are not available. The involvement of PI 3-kinase in nucleotide-induced PKB activation is further corroborated by finding that the PI 3-kinase inhibitors, LY 294002 and wortmannin, completely block nucleotide-induced PKB activation.

Our data further indicate that a pertussis toxin-sensitive G $_i$  protein is involved in ATP- and UTP-signalling to the PKB pathway. This is in line with earlier reports showing the involvement of a pertussis toxin-sensitive G $_i$  protein in nucleotide-induced inositol trisphosphate production in mesangial cells (Pfeilschifter, 1990a). The G protein-coupled agonist N-formyl-Met-Leu-Phe consistently triggered pertussis toxin-sensitive PIP $_3$  accumulation in neutrophils (Traynor-Kaplan *et al.*, 1989).

Molecular cloning and functional characterization have established that extracellular nucleotides mediate their effects via several subtypes of P2 receptors (Ralevic & Burnstock, 1998; Boarder & Hourani, 1998; Communi & Boeynaems, 1997; King *et al.*, 1998). Thus the identification of the P2Y receptor subtype responsible for signaling to the PKB cascade is of importance. It was suggested that ATP and UTP use a common nucleotide or P2u receptor for transducing their signals in mesangial cells (Pfeilschifter, 1990b). The P2u receptor has been cloned (Lustig *et al.*, 1993; Parr *et al.*, 1994) and renamed P2Y $_2$  receptor by the NC-IUPHAR subcommittee on P2Y receptors (Fredholm *et al.*, 1994). The signal transduction pathways and functional cell responses triggered by ATP and UTP in mesangial cells fit to a P2Y $_2$  receptor subtype. This includes nucleotide-stimulated inositol trisphosphate and 1,2-diacylglycerol formation (Pfeilschifter, 1990a, b), and the subsequent Ca $^{2+}$  mobilization (Pavenstadt *et al.*, 1993; Gutierrez *et al.*, 1999), protein kinase C activation (Pfeilschifter & Huwiler, 1996) and prostaglandin E $_2$  synthesis (Pfeilschifter, 1990a), the stimulation of phospholipase D (Pfeilschifter & Merriweather, 1993), and the activation of the classical MAPK pathway and subsequent cell proliferation (Huwiler & Pfeilschifter, 1994).



**Figure 10** Effect of the stable ATP analog,  $\gamma$ -thio-ATP, on TNF $\alpha$ /cycloheximide-induced DNA fragmentation in mesangial cells. Confluent mesangial cells were treated for 20 h with tumour necrosis factor- $\alpha$ /cycloheximide (TNF $\alpha$ /CHX; 2 nM and 10  $\mu$ M) in the presence of  $\gamma$ -thio-ATP (S-ATP; in  $\mu$ M) and where indicated in the presence of LY 294002 (20  $\mu$ M). Thereafter, DNA fragmentation was measured as described in the Methods section. Results are expressed as per cent of control values and are means  $\pm$  s.d. ( $n = 2-4$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , statistically significant difference compared to TNF $\alpha$ /CHX-stimulated value.

Indeed, Mohaupt *et al.* (1998) have reported that rat mesangial cells express P2Y<sub>2</sub> receptor mRNA, although other receptors, such as the P2Y<sub>4</sub> (Harada *et al.*, 2000) and P2Y<sub>6</sub> (A. Huwiler, unpublished results) have also been identified in these cells.

Our finding that suramin, which antagonizes P2Y<sub>2</sub> receptor activation, but not P2Y<sub>4</sub> receptor activation (Ralevic & Burnstock, 1998; Boarder & Hourani, 1998; King *et al.*, 1998; Bogdanov *et al.*, 1998), is able to block nucleotide-induced PKB activation, strongly suggests the involvement of the P2Y<sub>2</sub> receptor and not the P2Y<sub>4</sub> receptor, in the triggering of PKB activation. The involvement of the P2Y<sub>6</sub> receptor, which is also expressed in rat mesangial cells according to reverse transcriptase-PCR (data not shown), can be excluded here because of the lack of effect of UDP and ADP, the strongest agonists on this receptor subtype (Ralevic & Burnstock, 1998; Boarder & Hourani, 1998). Furthermore, the P2Y<sub>1</sub> receptor as well as P2X<sub>1-7</sub> receptors can also be excluded because  $\alpha\beta$ -methylene-ATP, a potent agonist at these receptors (Boarder & Hourani, 1998) is unable to stimulate the PKB pathway.

The additional finding that adenosine is unable to stimulate the PKB pathway, gives further evidence that ATP acts *via* a purinoceptor and not *via* degradation to adenosine, binding to adenosine receptors (A-receptors) and signalling through the adenylate cyclase pathway (Scholz-Pedretti *et al.*, 2001). In contrast, Gao *et al.* (2001) reported that adenosine selectively triggers PKB activation in mast cells by A<sub>3</sub> adenosine receptor activation, an effect which also involves a pertussis toxin sensitive G<sub>i</sub> protein.

Our data further indicate that the PKB pathway, which is rapidly activated by ATP and UTP, is not involved in either nucleotide-triggered cell proliferation or in arachidonic acid release and subsequent prostaglandin synthesis, two cell responses that are potently activated by nucleotides in

mesangial cells (Pfeilschifter, 1990a; Huwiler & Pfeilschifter, 1994). However, the PKB pathway seems to play an important role in the anti-apoptotic cell response of mesangial cells, since TNF $\alpha$ /CHX-induced cell death is reduced in a dose-dependent manner in the presence of the stable ATP analogue  $\gamma$ -thio-ATP (Figure 10). This anti-apoptotic effect of  $\gamma$ -thio-ATP is most probably mediated by PKB, but not by the classical MAPK, because the PI 3-kinase inhibitor LY 294002 (Vlahos *et al.*, 1994), and not the highly selective MAPK kinase (MEK) inhibitor U0126 (Davies *et al.*, 2000) (data not shown), can reverse the inhibitory effect of  $\gamma$ -thio-ATP on stress-induced apoptosis. Furthermore, in addition to the biochemical indication of apoptosis we have analysed for morphological changes indicative for programmed cell death, by staining with the H $\delta$ -33342 dye, which showed a similar protective effect of  $\gamma$ -thio-ATP (data not shown). Of course these are only first steps in elucidating the mechanism of protection by extracellular nucleotides. One mechanism by which PKB promotes cell survival involves the phosphorylation of Ser<sup>136</sup> of the proapoptotic Bcl-2 family member Bad, which subsequently leads to a dissociation from Bcl-2/Bcl-X<sub>L</sub> and protection of mitochondria from undergoing functional impairment, i.e. prevention of cytochrome *c* release and facilitation of mitochondrial ATP/ADP exchange (Datta *et al.*, 1997). We did indeed find that ATP stimulation of mesangial cells leads to a phosphorylation of Bad at Ser<sup>136</sup> in mesangial cells (data not shown).

In contrast to this cytoprotective effect of nucleotides involving the P2Y<sub>2</sub> receptor, Schulze-Lohoff *et al.* (1998) reported that mesangial cells exposed to high concentrations (in the millimolar range) of ATP, but not UTP, undergoes apoptosis and necrosis. This effect is due to activation of the P2X<sub>7</sub> receptor since the effect can be mimicked by the P2X<sub>7</sub> receptor agonist, 3-O-(4-benzoylbenzoyl)-ATP.

Obviously, the concentration of ATP is a critical determinant of which purinoceptor signalling pathway is initiated and therefore of the cell response: low physiological concentrations of ATP trigger the P2Y<sub>2</sub> receptor signalling cascade leading to proliferation *via* MAPK activation and an anti-apoptotic effect *via* PKB activation, whereas high and toxic concentrations trigger the P2X<sub>7</sub> receptor signalling cascade leading directly to apoptosis and/or necrosis.

In summary, we have shown that extracellular nucleotides in mesangial cells can activate the PKB/Akt pathway *via* the P2Y<sub>2</sub> receptor coupling to a G<sub>i</sub> protein, and potentially mediate cytoprotection against stress-induced cell death. Future work will have to delineate whether P2Y<sub>2</sub> receptor agonists and antagonists will be efficient in treatment of glomerular disease which is characterized by mesangial cell proliferation and apoptosis.

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