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Extracellular nucleotides activate the p38-stress-activated protein kinase cascade in glomerular mesangial cells

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1 Extracellular ATP and UTP have been reported to activate a nucleotide receptor ($P2Y_2$-receptor) that mediates arachidonic acid release with subsequent prostaglandin formation, a reaction critically depending on the activity of a cytosolic phospholipase A_2. In addition, extracellular nucleotides trigger activation of the classical mitogen-activated protein kinase (MAPK) cascade and cell proliferation as well as of the stress-activated protein kinase (SAPK) cascade.

2 In this study, we report that ATP and UTP are also able to activate the p38-MAPK pathway as measured by phosphorylation of the p38-MAPK and its upstream activators MKK3/6, as well as phosphorylation of the transcription factor ATF_2 in a immunocomplex-kinase assay.

3 Time courses reveal that ATP and UTP induce a rapid and transient activation of the p38-MAPK activity with a maximal activation after 5 min of stimulation which declined to control levels over the next 20 min.

4 A series of ATP and UPT analogues were tested for their ability to stimulate p38-MAPK activity. UTP and ATP were very effective analogues to activate p38-MAPK, whereas ADP and γ-thio-ATP had only moderate activating effects. 2-Methyl-thio-ATP, $\beta\gamma$-imido-ATP, AMP, adenosine and UDP had no significant effects of p38-MAPK activity. In addition, the extracellular nucleotide-mediated effect on p38-MAPK was almost completely blocked by 1 mM of suramin, a putative P2-purinoceptor antagonist.

5 In summary, these results demonstrate for the first time that extracellular nucleotides are able to activate the MKK3/6- p38-MAPK cascade most likely *via* the $P2Y_2$-receptor. Moreover, this finding implies that all three MAPK subtypes are signalling candidates for extracellular nucleotide-stimulated cell responses.

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Abbreviations: ATF_2, activator of transcription factor-2; BSA, bovine serum albumine; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; MBP, myelin basic protein; PBS, phosphate-buffered saline; SAPK, stress-activated protein kinase; TPA, 12-O-tetradecanoyl-phorbol 13-acetate

Introduction

Extracellular nucleotides, like ATP and UTP, exert diverse functions on intact cells that are caused by activation of plasma membrane receptors, termed P2 purinoceptors. These receptors are either part of ligand-gated ion channels (P2X subtypes) or couple through G-proteins to their intracellular effector enzymes (P2Y subtypes). To date seven mammalian P2X receptors ($P2X_{1-7}$) and five P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ have been cloned and characterized. (For review, see: Barnard *et al*., 1994; Boarder & Hourani, 1998; Ralevic & Burnstock, 1998).

In renal mesangial cells, ATP and UPT act on a common nucleotide receptor ($P2Y_2$ receptor) and mediate phosphoinositide hydrolysis and generation of 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (Pfeilschifter, 1990a,b) with a subsequent mobilization of intracellular calcium (Pavenstädt *et al*., 1993; Gutierrez *et al*., 1999), activation of protein kinase C (Pfeilschifter & Huwiler, 1996), activation of the classical mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), cascade (Huwiler & Pfeilschifter, 1994) and the stress-activated protein kinase (SAPK) cascade (Huwiler *et al*., 1997a) as well as stimulation of prostaglandin E_2 synthesis (Pfeilschifter, 1990a; Schulze-Lohoff *et al*., 1992).

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p38 is a member of the MAPK family of protein kinases showing sequence similarity and structural conservation with the other MAPK members like the ERKs, and the SAPKs or c-Jun N-terminal kinases (JNKs). ERKs are mainly activated by mitogens and growth factors, whereas SAPK/JNKs and p38-MAPK are most often activated by cellular stresses (chemical, heat and osmotic shock, UV irradiation, inhibitors of protein synthesis), by bacterial lipopolysaccharide and by inflammatory cytokines, like interleukin-1β and tumour necrosis factor α (Cano & Mahadevan, 1995).

In this study, we show that extracellular nucleotides are able to activate the stress-activated protein kinase p38-MAPK cascade, thus signifying all three MAPK members as potential candidates for extracellular nucleotide-mediated cell responses.

Methods

Cell-culture

Rat renal mesangial cells were cultivated and characterized as previously described (Pfeilschifter, 1990a,b). In a second step, single cells were cloned by limited dilution on 96-microwell

plates. Clone MZ B_1 with apparent mesangial cell morphology was used for further processing (Pfeilschifter & Vosbeck, 1991). For the experiments in this study passages 5–25 were used. Human mesangial cells were cultivated exactly as described for rat mesangial cells (Pfeilschifter, 1990a,b). Bovine glomerular endothelial cells were isolated and cultivated as previously described (Briner & Kern, 1994; Huwiler *et al*., 1997b). The macrophage cell line RAW 264.7 was cultivated as described (Messmer *et al*., 1994).

Detection of p38-MAPK protein by Western blot analysis

Quiescent cells were washed with phosphate-buffered saline (PBS) and scraped into lysis buffer containing 50 mM HEPES, pH 7.4, (mM) NaCl 150, $MgCl_2$ 1.5, EDTA 1, EGTA 1, Na_3VO_4 1, NaF 50, β-glycerophosphate 20, 10% (v v^{-1}) glycerol, 1% (v v^{-1}) Triton X-100, 25 μg ml^{-1} leupeptin, 200 units ml^{-1} aprotinin, 1 μM pepstatin A, 1 mM PMSF, and homogenized by ten passes through a 26G-needle fitted to a 1 ml syringe. Thereafter extracts were centrifuged for 10 min at 14 000 $\times g$ at 4°C and the supernatant was taken for protein determination. Equal amounts of protein were subjected to SDS–PAGE (10% acrylamide gel), and proteins were transferred on to nitrocellulose paper for 1 h at 12 V using a semi-dry blotting apparatus. The blotting buffer used was 25 mM Tris, 190 mM glycine in 20% (v v^{-1}) methanol. After the transfer, nitrocellulose filters were washed extensively in distilled water and blocked for 1 h in blocking buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.2% Triton X-100, 3% (w v^{-1}) bovine serum albumin (BSA)). Filters were then incubated for 4 h with the indicated antibodies (at a dilution of 1 : 300 for p38α, 1 : 100 for p38β and 1 : 50 for p38δ). After washing in buffer A (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.2% (v v^{-1}) Triton X-100; 4 × 5 min), the filters were incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit IgG antibodies in blocking buffer. Thereafter, filters were washed again (4 × 5 min) in buffer A and finally colour reaction was carried out in a solution containing 0.4 mg ml^{-1} nitroblue tetrazolium chloride, 0.19 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-phosphate toluidine salt in 100 mM Tris, pH 9.5, 50 mM $MgSO_4$ and washed extensively in distilled water to stop the reaction. Alternatively, blots were stained with an enhanced chemiluminescence (ECL) system according to the manufacturer's recommendations.

Activation of p38-MAPK by detection of phosphorylated p38-MAPK

Since it has been shown that phosphorylation of p38-MAPK is always accompanied by an increased activity of the enzyme (Raingeaud *et al*., 1995), the activation of p38-MAPK can also be detected by measuring the phosphorylation state of the enzyme, i.e. in Western blot analysis using antibodies which specifically recognize the dual phosphorylated (on residues Thr^{180} and Tyr^{182}) active form of p38-MAPK. Confluent mesangial cells were incubated for 2 days in Dulbecco's modified Eagle medium (DMEM) containing 0.1 mg ml^{-1} of fatty acid-free BSA and then stimulated at 37°C as indicated. To stop the reaction, the medium was removed and the cells washed with ice-cold PBS. Cells were then scraped and homogenized as described above. The homogenate was centrifuged for 10 min at 14 000 × g and the supernatant taken for protein determination. Cell extracts containing 100 μg of protein were subjected to SDS–PAGE (10% acrylamide gel), proteins were transferred on to nitrocellulose paper and Western blot analysis was performed using a polyclonal phospho-p38-MAPK-selective antibody at a dilution of 1 : 1000. Bands were visualized by horseradish peroxidase using the ECL method according to the manufacturer's recommendation, and exposed to a Hyperfilm MP for 20 min.

Activation of p38-MAPK in an immunocomplex-kinase assay

Confluent mesangial cells in 100 mm-diameter dishes were incubated for 2 days in DMEM containing 0.1 mg ml^{-1} of fatty acid-free BSA and then stimulated at 37°C with various agents as indicated. To stop the reaction, the medium was removed and the cells washed with ice-cold PBS. Cells were then scraped directly into lysis buffer and homogenized by ten passes through a 26-gauge needle fitted to a 1 ml syringe. The homogenate was centrifuged for 10 min at 14 000 × g and the supernatant taken for immunoprecipitation. Samples containing 500 μg of protein and 5% fetal calf serum in lysis buffer, were incubated with a phospho-specific antibody against the p38-MAPK (at a dilution of 1 : 100) overnight at 4°C. Then 20 μl of a 50% slurry of protein A-sepharose 4B-CL in PBS was added and the mixture was incubated for 1 h on a rotation wheel. After centrifugation for 5 min at 3000 × g immunocomplexes were washed 3 × with a low salt buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.1% SDS) and 3 × with a high salt buffer ((mM) Tris-HCl 50, pH 7.4, NaCl 500, 0.2% Triton X-100, EDTA 2, EGTA 2, 0.1% SDS) and once with 20 mM HEPES, pH 7.4, 20 mM $MgCl_2$ before the kinase reaction was started by addition of 30 μl of kinase buffer (25 mM HEPES, pH 7.4, 25 mM $MgCl_2$, 25 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 mM DTT, 20 μM ATP, 2 μCi [^{32}P-γ]ATP, 100 ng of GST-ATF_2 for 30 min at 37°C). The reaction was stopped by adding SDS-Laemmli-buffer and the proteins were separated by SDS–PAGE (13% acrylamide gels). Coomassie-stained gels were dried and analysed on a phosphoimager (Molecular Dynamics)

Activation of MKK3/6 by detection of phosphorylated MKK3/6

The procedure was performed exactly as described for the p38-MAPK, except for a few modifications. In brief, cell extracts containing 200 μg of protein were separated on SDS–PAGE (10% acrylamide gel) and transferred to nitrocellulose membranes. For Western blot analysis a phospho-MKK3/6-selective antibody was used at a dilution of 1 : 300. Bands were visualized by horseradish peroxidase using the EC method according to the manufacturer's recommendation, and exposed to a Hyperfilm MP for 20 min.

Statistical analysis

Statistical analysis was performed by 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.

Chemicals

ATP and UTP were obtained from Boehringer Mannheim, Rotkreuz, Switzerland; anti-rabbit alkaline phosphatase-linked IgG was from Bio-Rad Labs., Veenendaal, the Netherlands; [^{32}P-γ]-ATP (specific activity: > 5000 Ci $mmol^{-1}$),

ECL-system and Hyperfilm were purchased from Amersham International, Amersham, Bucks., U.K.; p38α-MAPK-, phospho-p38 and phospho-MKK3/6-specific antibodies were from New England Biolabs, Schwalbach, Germany; p38β-MAPK antibodies were from Santa Cruz Biotechnology Inc., Heidelberg, Germany; the p38δ-MAPK antibody was from Transduction Laboratories, Lexington, KY, U.S.A.; protein A-sepharose 4B-CL was from Pharmacia Fine Chemicals, Uppsala, Sweden; SB 203580 was from Alexis, Laeufelfingen, Switzerland; 12-O-tetradecanoyl-phorbol 13-acetate (TPA) was from Calbiochem, Lucerne, Switzerland; IL-1β and TGFβ$_2$ were kindly provided by Novartis Pharma Inc., Basel, Switzerland; the plasmid construct encoding GST-ATF$_2$ (Gupta *et al*., 1995) was kindly provided by Prof. Roger Davis, University of Massachusetts Medical School, Worcester, Massachusetts, U.S.A. The generation and characterization of the MAPK- and SAPK-specific antisera have been reported previously (Huwiler *et al*., 1995; 1997a).

Results

Expression pattern of p38-MAPK isoenzymes in mesangial cells

Western blot analysis of mesangial cell lysates with a polyclonal antiserum specific for human p38α-MAPK reveals a single band at 38–40 kDa in human and rat mesangial cells as well as in the human epidermoid carcinoma cell line A 431 indicating that the isoenyzme p38α is expressed in mesangial cells (Figure 1, upper panel). Since the p38-MAPK exists as a family of different isoenzymes (Wang *et al*., 1997; Jiang *et al*., 1997) we also tested for protein expression of the other p38-isoforms, i.e. p38β and p38δ. As seen in Figure 1 middle panel, p38β showed positive staining in rat mesangial cells, glomerular endothelial cells and RAW 264.7 macrophages. In contrast, no detection of p38δ protein in human or rat mesangial cells was observed (Figure 1, lower panel), whereas the human epidermoid carcinoma cell line A 431 which serves as a positive control (Wang *et al*., 1997; Jiang *et al*., 1997) showed a clear band at approximately 40 kDa. Since no selective p38γ antibodies were available, no statement on the expression of this isoform in mesangial cells can be made.

Activation of p38-MAPK by stress factors and G protein-coupled agonists in mesangial cells

In a next step we investigated the activation pattern of the p38-MAPK in mesangial cells by measuring its ability to phosphorylate the activator of transcription factor-2 (ATF$_2$). Since the discovery of p38-MAPK as a stress-activated protein kinase it has generally been assumed that only stress stimuli, like high osmolarity, UV irradiation, nitric oxide and cytokines are potent activators of the p38-MAPK pathway (Cano & Mahadevan, 1995; Huwiler & Pfeilschifter, 1999). As shown in Figure 2A stimulation of mesangial cells for 10 min with anisomycin (lane 2), interleukin 1β (lane 4) and 0.5 M NaCl (lane 6) cause a marked phosphorylation of ATF$_2$ consistent with the fact that stress stimuli are able to trigger p38-MAPK activation. UV irradiation of cells for 10 min at 20 Jm^{-2} (lane 5) and TGFβ$_2$ treatment (50 ng ml^{-1} for 10 min; lane 7) causes only a marginal activation of the p38-MAPK. Surprisingly, we found that agonists like angiotensin II (lane 3), ATP (lane 8) and the phorbol ester TPA (lane 9), all substances which either directly or indirectly activate PKC, were highly potent activators of the p38-MAPK cascade.

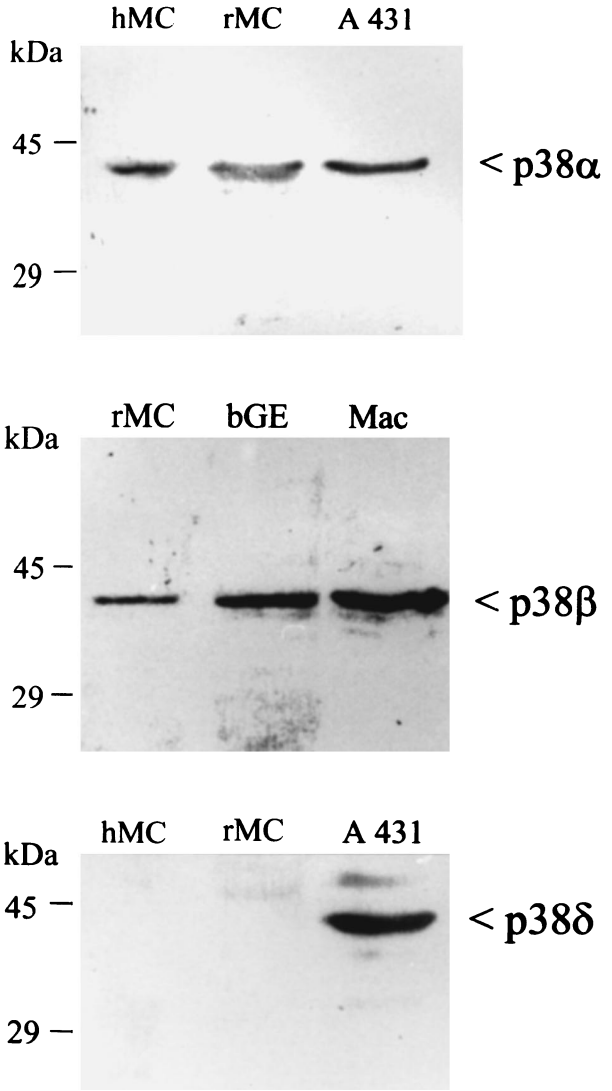

Figure 1 Western blot analysis of p38α, p38β and p38δ MAPK in glomerular mesangial cells. Cell lysates containing 200 μg of protein from quiescent human mesangial cells (hMC), rat mesangial cells (rMC), a human epidermoid carcinoma cell line (A 431), glomerular endothelial cells (bGE), a macrophage cell line RAW 264.7 (Mac) were separated on SDS–PAGE (10% acrylamide gel), transferred to a nitrocellulose membrane and Western blot analysis was performed using polyclonal antibodies against p38α-MAPK at a dilution of 1 : 300 and against p38β at dilution of 1 : 100, and a monoclonal antibody against p38δ at a dilution of 1 : 50. Bands were visualized by alkaline phosphatase.

Involvement of the P2Y$_2$ purinoceptor in ATP- and UTP-induced p38-MAPK activation

Figure 2B shows that stimulation of cells for 10 min with 100 μM UTP is equally potent in activating p38-MAPK as ATP, suggesting the involvement of the P2Y$_2$-receptor in the p38-MAPK activation as already previously shown for other signalling pathways in mesangial cells (Pfeilschifter, 1990a,b; Pavenstädt *et al*., 1993; Pfeilschifter & Merriweather, 1993; Huwiler & Pfeilschifter, 1994). As seen in Figure 2C upper panel, both ATP and UTP increase dual phosphorylation of the p38-MAPK in a concentration dependent manner as detected in a Western blot analysis using a phospho-specific antibody against p38-MAPK. In contrast, the total amount of p38-MAPK is unaffected by ATP or UTP treatment as shown

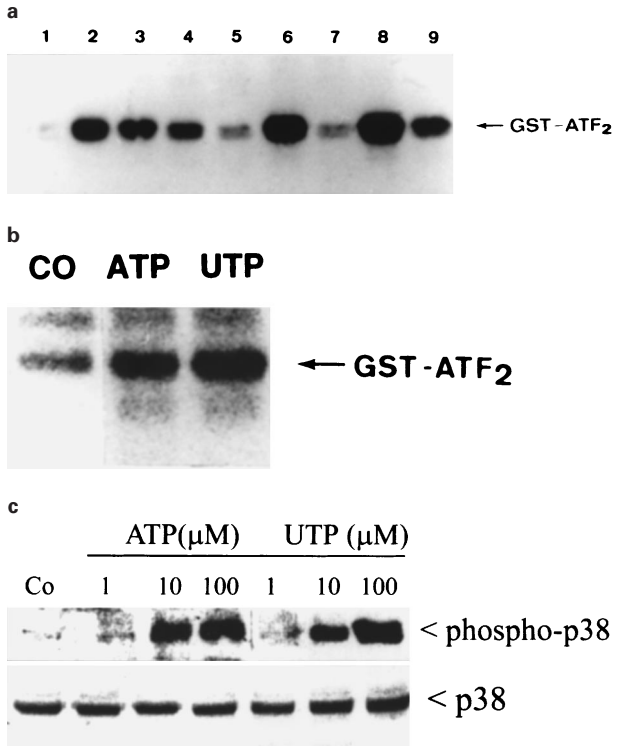

Figure 2 Effect of different stimuli on p38-MAPK activity in mesangial cells. (A) Quiescent mesangial cells were treated for 10 min with either vehicle (lane 1), anisomycin (50 ng ml^{-1}; lane 2), angiotensin II (500 nM; lane 3), interleukin-1β (1 nM; lane 4), UV (20 Jm^{-2}; lane 5), NaCl (0.5 M; lane 6), TGFβ_2 (50 ng ml^{-1}; lane 7), ATP (100 μM; lane 8) and TPA (500 nM; lane 9). (B) Quiescent mesangial cells were treated for 10 min with either vehicle (co), ATP (100 μM) or UTP (100 μM). Thereafter cells were harvested and p38-MAPK activity was determined by measuring the phosphorylation of ATF_2 in an immunocomplex kinase assay as described in the Methods section. (C) Cells were stimulated with the indicated concentrations (in μM) or ATP and UTP for 10 min. Thereafter, cell lysates (containing 100 μg of protein) were subjected to Western blot analysis using either a phospho-specific antibody against the p38-MAPK at a dilution of 1 : 1000 (upper panel) or an antibody recognizing total p38-MAPK at a dilution of 1 : 300 (lower panel). Bands were visualized by horseradish peroxidase using the ECL method, and exposed to a Hyperfilm MP for 20 min. Data are representative of three independent experiments giving similar results.

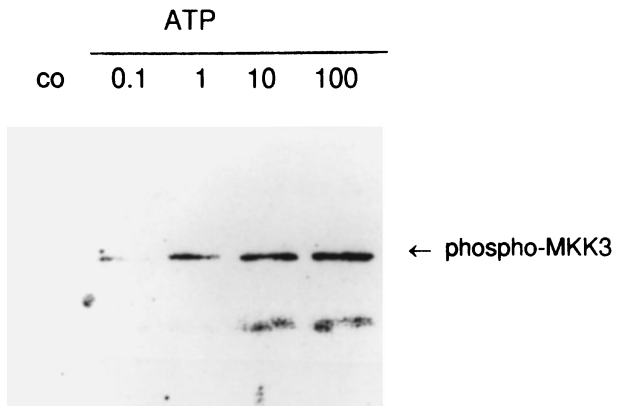

Figure 3 Effect of ATP on the upstream p38-MAPK activator MKK3/6 phosphorylation in mesangial cells. Quiescent mesangial cells were treated with the indicated concentrations of ATP (in μM) for 10 min. Thereafter, cell lysates (containing 200 μg of protein) were subjected to Western blot analysis using a phospho-specific antibody against the MKK3/6 at a dilution of 1 : 300. Bands were visualized by horseradish peroxidase using the ECL method, and exposed to a Hyperfilm MP for 20 min. Similar results were obtained in two independent experiments.

in Figure 2C lower panel. In addition, also the phosphorylation, and thus activity (Raingeaud *et al*., 1996), of the upstream activators of p38-MAPK, the MKK3/6 are concentration-dependently enhanced by ATP, as detected in a Western blot analysis (Figure 3).

Time course analyses reveal a rapid and transient phosphorylation of the p38-MAPK after 5 min of stimulation with ATP. This elevated activity level was maintained for 10 min and thereafter rapidly declined to basal levels (Figure 4, upper panel). A similar time course of p38-MAPK activation was observed by UTP stimulation (Figure 4, lower panel).

Figure 5A shows that the trypanoside suramin a putative P2 receptor antagonist (Dunn & Blakeley, 1988; Boarder & Hourani, 1998; Ralevic & Burnstock, 1998) almost completely blocked ATP and UTP-induced p38-MAPK phosphorylation. Furthermore, the commonly used p38-MAPK inhibitor SB 203580 (Cuenda *et al*., 1995) was tested for its ability to reduce p38-MAPK phosphorylation. When intact mesangial cells were stimulated with ATP in the presence of SB 203580 no significant reduction of p38-MAPK phosphorylation was detected (Figure 5B). However, active p38-MAPK which was obtained by immunoprecipitation of ATP-stimulated mesangial cells, was markedly inhibited *in vitro* by 1 μM and 10 μM SB203580 when measuring ATF_2 phosphorylation by an immunocomplex kinase assay (Figure 5C). This finding is consistent with a previous report showing that SB 203580 does not interfere with the phosphorylation of p38-MAPK, but

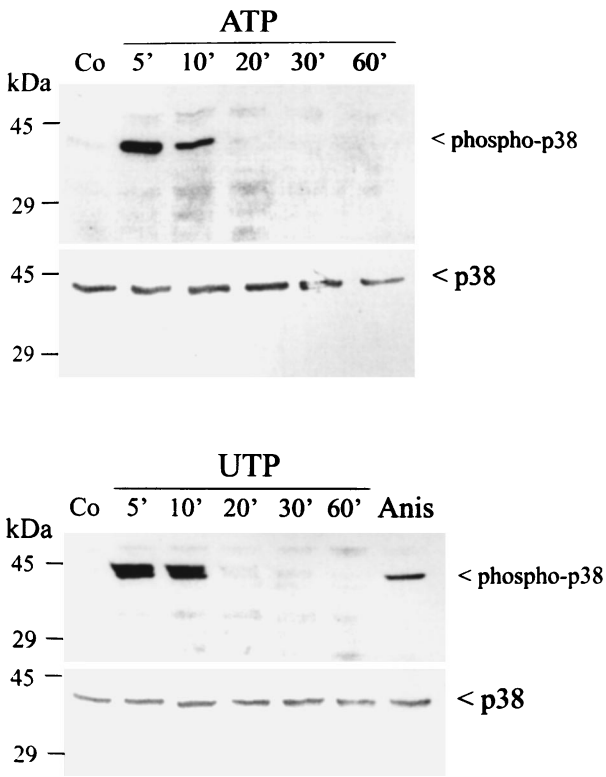

Figure 4 Time-course of ATP-stimulated p38-MAPK activity in mesangial cells. Quiescent mesangial cells were treated with either vehicle for 5 min (co) or 100 μM of ATP (upper panel) and 100 μM of UTP (lower panel) for the indicated time periods (in minutes). As a positive control, cells were stimulated for 10 min with anisomycin (Anis; 50 ng ml^{-1}). Thereafter, cell lysates (containing 100 μg of protein) were subjected to Western blot analysis using either a phospho-specific antibody against the p38-MAPK at a dilution of 1 : 1000 or an antibody against total p38-MAPK at a dilution of 1 : 300. Bands were visualized by horseradish peroxidase using the ECL method, and exposed to a Hyperfilm MP for 20 min. Similar results were obtained in three independent experiments.

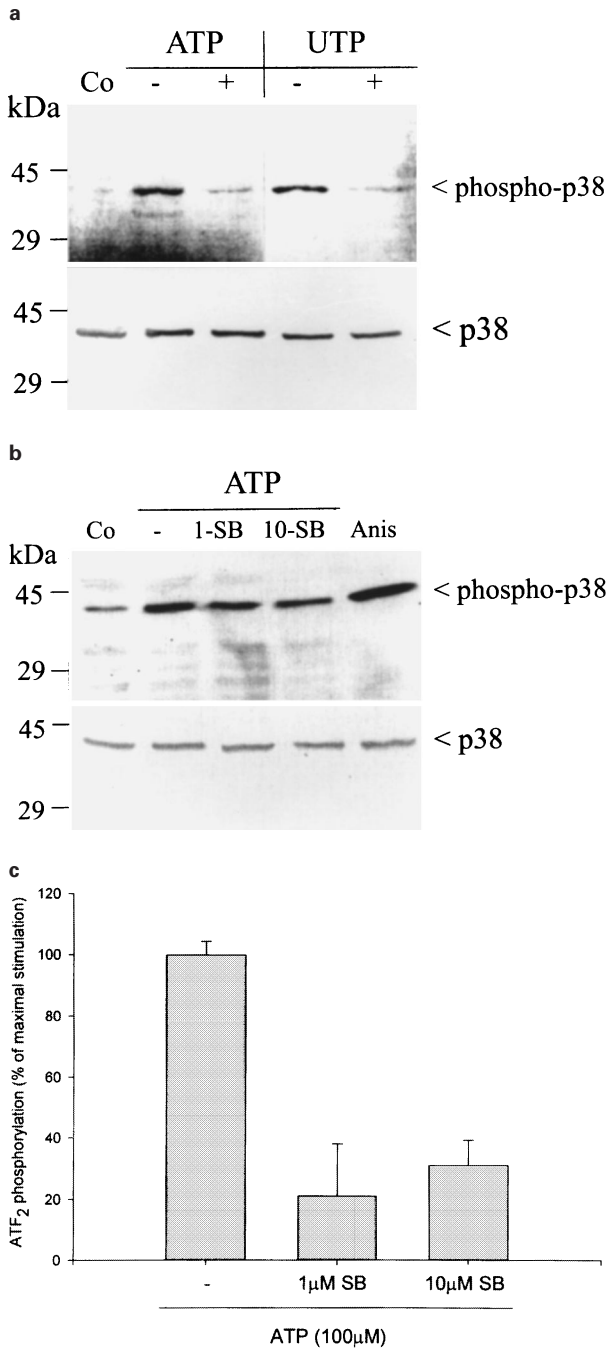

Figure 5 Effect of suramin and SB 203580 on ATP-induced p38-MAPK activity in mesangial cells. (A) Quiescent mesangial cells were stimulated with vehicle (co) or pretreated for 20 min in the absence (−) or presence (+) of suramin (1 mM) before stimulation with ATP or UTP (100 μM each). (B) Quiescent mesangial cells were stimulated with vehicle (co) or pretreated for 20 min in the absence (−) or presence of 1 μM SB 203580 (1-SB) and 10 μM SB 203580 (10-SB) before stimulation with ATP (100 μM) for 10 min. As a positive control cells were stimulated with anisomycin (Anis; 50 ng ml^{-1}) for 10 min. Thereafter, cell lysates (containing 100 μg of protein) were subjected to Western blot analysis using either a phospho-specific antibody against the p38-MAPK at a dilution of 1 : 1000 or an antibody against total p38-MAPK at a dilution of 1 : 300. Bands were visualized by horseradish peroxidase using the ECL method, and exposed to a Hyperfilm MP for 20 min. Similar results were obtained in three independent experiments. (C) Active p38-MAPK obtained by immunoprecipitation of phospho-p38-MAPK from ATP-stimulated mesangial cells was incubated in the absence (−) or presence of 1 μM and 10 μM SB 203580 during the kinase assay as described in the Methods section. Thereafter the phosphorylation of ATF_2 was analysed and quantitated on a Phosphorimager. Results are expressed as percentage of maximal ATP stimulation and are means ± s.d.mean, $n = 3$.

rather interferes with the ATP binding site of the enzyme (Young *et al*., 1997) and thus the activity of p38-MAPK.

In order to define the receptor subtype involved in extracellular nucleotide-mediated p38-MAPK activation, a series of ATP analogues were tested on their ability to activate the p38-MAPK. As seen in Figure 6, UTP and ATP were the most potent activators of p38-MAPK, whereas, ADP and γ-thio-ATP had only moderate activating effects on p38-MAPK activity. 2-Methyl-thio-ATP, $\beta\gamma$-imido-ATP, AMP, adenosine and UDP showed no significant effects.

Discussion

Considerable efforts have been made to describe the physiological actions of the purine nucleoside, adenosine, in the control of epithelial transport, renin secretion and regulation of renal blood flow (Spielman & Arend, 1991). More recently it became evident that extracellular ATP also markedly contributes to the control of renal vascular function, tubuloglomerular feedback mechanism, mesangial cell activation and epithelial transport (for review see Inscho *et al*., 1994; Chan *et al*., 1998). Moreover, evidence for potential pathophysiological roles of extracellular ATP is accumulating. In the Thy-1 model of mesangioproliferative glomerulonephritis extracellular ATP released after platelet aggregation has proinflammatory activity and consequently nucleotidases may exert anti-inflammatory effects (Poelstra *et al*., 1992; 1993).

It is well accepted that extracellular adenine nucleotides mediate their effects by several subtypes of P2 receptors (Communi & Boeynaems, 1997; King *et al*., 1998; Ralevic & Burnstock, 1998; Boarder & Hourani, 1998). Pfeilschifter (1990b) reported that in mesangial cells UTP apparently produces its biological effects by binding to a receptor which has equivalent sensitivity to ATP. Studying the characteristics of UTP and ATP-stimulated inositoltrisphosphate production in glomerular mesangial cells revealed that responses to both ligands were inhibited equally by pertussis toxin, modulated

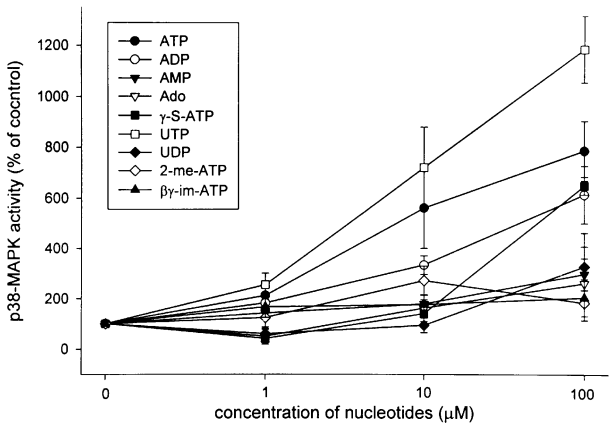

Figure 6 Effect of different ATP and UTP analogues on p38-MAPK phosphorylation in mesangial cells. Quiescent mesangial cells were stimulated for 10 min with the indicated concentrations (in μM) of ATP, ADP, AMP, adenosine (Ado), γ-thio-ATP (γ-S-ATP), UTP, UDP, 2-methyl-thio-ATP (2-me-ATP) and $\beta\gamma$-imino-ATP ($\beta\gamma$-im-ATP). Thereafter cell lysates, containing 100 μg of protein were subjected to SDS–PAGE (10% acrylamide gel) and Western blot analysis was performed using a specific phospho-p38-MAPK at a dilution of 1 : 1000. Bands were visualized by horseradish peroxidase using the ECL method, exposed to a Hyperfilm MP, and evaluated on a densitometer. The data are means ± s.d.mean, $n = 3$–6.

similarly by short-term and long-term phorbol ester pretreatment, non-additive at maximal concentrations, similarly inhibited by reactive blue 2 and showed complete cross-desensitization, observations which all provided no evidence for separate purino- and pyrimidinoceptor on mesangial cells. In contrast, it was suggested that ATP and UTP may 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. 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 inositoltrisphosphate and 1,2-diacylglycerol formation (Pfeilschifter, 1990a,b), and the subsequent Ca^{2+} mobilization (Pavenstädt *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), the activation of the classical MAPK pathway and subsequent cell proliferation (Huwiler & Pfeilschifter, 1994). Indeed, Mohaupt *et al*. (1998) have reported that rat mesangial cells express $P2Y_2$ receptor mRNA. Interestingly, the stimulation of the SAPK pathway in mesangial cells was not only stimulated by ATP and UTP but also markedly by UDP and ADP (Huwiler *et al*., 1997). These observations together with the recent finding that $P2Y_2$ receptors are activated selectively by nucleoside triphosphates but not by nucleoside diphosphates (Nicholas *et al*., 1996a,b) points to a possible heterogeneity of P2Y receptors on rat mesangial cells. Whether these cells express a $P2Y_6$ receptor in addition to the $P2Y_2$ receptor remains to be investigated. Further evidence for a heterogeneity of P2Y receptors on mesangial cells comes from a report by Huber-Lang *et al*. (1997) showing that ATP and UTP differentially affect membrane voltage V_m and ion currents in the cells. Repetitive application of ATP desensitized the $P2Y_2$ receptor-mediated effects on V_m. However, despite the complete loss of ATP-triggered changes in V_m, the depolarizing action of UTP was still preserved, although at a reduced level. These observations suggest that mesangial cells may express a separate nucleotide receptor which is selectively activated by UTP but not by ATP. This is further supported by the fact that suramin at the very high concentration of 1 mM almost completely blocked ATP- and UTP-stimulated p38-MAPK activation (Figure 5A) in mesangial cells whereas it did not influence the V_m response to UTP although the depolarization induced by ATP was completely inhibited (Huber-Lang *et al*., 1997). Whether this suggested additional receptor subtype corresponds to the $P2Y_4$ subtype has not been evaluated.

Multiple P2Y receptors have been reported to coexist on endothelial cells (Boarder & Hourani, 1998) and vascular smooth muscle cells (Harper *et al*., 1998). In the latter case transcripts coding for $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors were detected by reverse transcriptase-polymerase chain reaction (Harper *et al*., 1998). Preliminary data from our laboratory suggest that mesangial cells indeed do express the mRNA coding for $P2Y_2$ and $P2Y_6$ but not for $P2Y_4$ receptor subtypes. In this context it is important to note that the $P2Y_4$ receptor that is stimulated by UTP is not sensitive to suramin (Ralevic & Burnstock, 1998; King, 1998; Bogdanov *et al*., 1998).

What is urgently needed in order to unequivocally appoint certain signalling pathways and functional cell responses to specific P2Y receptor subtypes is the development of selective antagonists. Concerning the reported activation of p38-MAPK in mesangial cells and P2Y receptor subtype(s) involved, we cannot presently be sure whether this is triggered solely by a $P2Y_2$ receptor or whether other P2Y receptor subtypes will also contribute to observed activation.

The physiological role of the p38-MAPK in the cell is still unclear, but the discovery that p38-MAPK exists as a family of at least four isoforms, α,β γ and δ, which all display slightly different substrate specificities and tissue distribution (Wang *et al*., 1997) makes it tempting to speculate that each isoform may exert an individual function in stress signalling. In this context it is worth noting that p38δ is activated *in vivo* in rats with crescentic glomerulonephritis (Jiang *et al*., 1997). Whether extracellular nucleotides contribute to p38δ activation under pathological conditions is under investigation in our laboratory.

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