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$P2Y_{AC}^{-}$-receptor agonists enhance the proliferation of rat C6 glioma cells through activation of the p42/44 mitogen-activated protein kinase

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1 Extracellularly added P^1,P^3-di(adenosine-5′) triphosphate (Ap_3A), P^1,P^4-di(adenosine-5′) tetraphosphate (Ap_4A), ATP, ADP, AMP and adenosine are growth inhibitory for rat C6 glioma cells. Analysis of nucleotide hydrolysis and the use of nucleotidase inhibitors demonstrated that the latter inhibition is due to hydrolysis of the nucleotides to adenosine.

2 Agonists of the $P2Y_{AC}^{-}$-receptor enhance the growth of C6 cells if their hydrolysis to adenosine is inhibited by pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS). In these conditions, the potency to stimulate cell growth parallels the ranking of the receptor agonists, i.e. 2-methylthioadenosine-5′-diphosphate (2MeSADP) > Ap_3A > Ap_4A. ATP and ADP are still hydrolysed in the presence of PPADS and have no proliferative effect on C6 cells.

3 The enhanced growth is due to a $P2Y_{AC}^{-}$-receptor-mediated activation of p42/44 mitogen-activated protein kinase (MAPK) as shown by immunoblotting and protein kinase assays for active MAPK and the use of the MAPK/extracellular signal-regulated kinase kinase (MEK) inhibitor PD98059.

4 The UTP-induced enhancement of the growth of C6 cells is due to activation of MAPK by a PPADS sensitive nucleotide receptor.

5 In conclusion, the effect of nucleotides on the growth of C6 cells is determined by ecto-nucleotidases and by activation of nucleotide receptors. Hydrolysis of nucleotides to adenosine induces growth inhibition while inhibition of the hydrolysis of agonists of the $P2Y_{AC}^{-}$-receptor enhances cell growth by activation of MAPK.

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Abbreviations: Ap_3A,P^1,P^3-di(adenosine-5′) triphosphate; Ap_4A,P^1,P^4-di(adenosine-5′) tetraphosphate; ATPase, adenosine triphosphatase; ATPDase, adenosine triphosphate diphosphohydrolase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK/extracellular-signal regulated kinase kinase; MEM, minimal essential medium, 2MeSADP, 2-methylthioadenosine-5′-diphosphate; NPPase, ecto-nucleotide pyrophosphatase/phosphodiesterase I; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; RB2, reactive blue 2; TCA trichloroacetic acid

Introduction

Nucleotides, and in particular ATP and adenosine, have been reported to affect the proliferation of different cell types. The effects are not always unequivocal, e.g. ATP stimulates the proliferation of primary cultures of astrocytes and rat aortic smooth muscle cells (Abbracchio *et al*., 1994; Harper *et al*., 1998), is ineffective on the growth of transformed mouse fibroblasts (Weisman *et al*., 1988) and has an inhibitory effect on the growth of several human tumour cell lines (Seetulsingh-Goorah & Stewart., 1998; Rapaport *et al*., 1983). Adenosine has a growth inhibitory effect on most of the examined cell types (Seetulsingh-Goorah & Stewart, 1998; Fishman *et al*., 1998), although a growth stimulation is also reported (Lelievre *et al*., 1998). This apparent discrepancy may be explained by the existence of several mechanisms that take place at the cell surface: firstly, extracellular nucleotides are degraded by a cascade of cell surface-bound enzymes, i.e. ecto-ATPase, ecto-apyrase, ecto-nucleotide pyrophosphatase/phosphodiesterase I (NPPase) and ecto-5′-nucleotidase, hydrolysing ATP into ADP, AMP and adenosine (Zimmermann, 1996). Cellular uptake of the latter by adenosine transporters can induce an adenosine-dependent pyrimidine starvation resulting in inhibition of proliferation (Lasso de la Vega *et al*., 1994). Secondly, the added nucleotides and their breakdown products affect cell proliferation by activation of nucleotide receptors, coupled to phospolipase C (PLC) or adenylate cyclase (AC). In addition, ATP may activate ecto-protein kinases that modulate the activity of autocrine growth factors and growth inhibitors (Vilgrain & Baird, 1991; Friedberg *et al*., 1995). The inhibitory effect of ATP and other adenosine phosphates on cell proliferation may be explained by one or a combination of these mechanisms.

Two main classes of nucleotide receptors, P1 and P2, have been described (Burnstock, 1978). The P1-receptors are mainly responsive to adenosine. The P1-receptor subtypes A1 and A3 are negatively, and A2 is positively coupled to

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AC. The P2-receptors, activated by ATP/ADP or UTP/UDP, are subdivided in metabotropic P2Y- and ionotropic P2X-receptors. P2Y-receptors are coupled to a PLC-dependent Ca^{2+} mobilization and activation of protein kinase C (PKC) or to AC ($P2Y_{11}$, $P2Y_{12}$), while activation of the P2X-receptors, ligand gated ion-channels, generate a Ca^{2+}-influx (Fredholm *et al*., 1994; Communi *et al*., 1997; Boeynaems *et al*., 2000; Hollopeter *et al*., 2001).

We investigated the effect of adenosine and its mono- and dinucleotides on the proliferation of rat C6 glioma cells. The latter is a bipotential cell line often used as a model system for astrocytes. Several purinergic receptors are reported to be present on C6 cells: an $A2_b$-receptor, a 'nucleotide receptor' coupled to PLC and activated by all nucleoside triphosphates, a PLC-coupled $P2Y_6$-receptor activated by UDP, and a P2Y-receptor negatively coupled to AC presumed to be the $P2Y_{12}$-receptor (Ohkubo *et al*., 2001; Lin & Chuang, 1994; Nicholas *et al*., 1996; Boyer *et al*., 1993; Hollopeter *et al*., 2001). The agonist potency of the latter AC-coupled receptor is more or less the same as that of the $P2Y_1$-receptor and has hitherto been called the $P2Y_1$-like receptor (Albert *et al*., 1997; Grobben *et al*., 2000). However, in contrast to the $P2Y_1$-receptor, this receptor is not coupled to PLC and it is not sensitive to the antagonists PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid) and 5′-phosphoadenosine 3′-phosphate (Boyer *et al*., 1994). Although Webb *et al*. (1996) concluded that the $P2Y_1$-like- and the $P2Y_1$-receptor are identical but coupled to a different second messenger system, Schachter *et al*. (1997) disproved this finding. To describe more accurately the pharmacological properties of the $P2Y_1$-like receptor we propose the name $P2Y_{AC}^{-}$ for future reference to this receptor. The recently cloned $P2Y_{12}$-receptor has been proposed to be present on C6 glioma cells (Hollopeter *et al*., 2001). However, ATP, an antagonist for the $P2Y_{12}$-receptor, is an agonist for the $P2Y_{AC}^{-}$-receptor (Grobben *et al*., 2000), suggesting that $P2Y_{12}$ and $P2Y_{AC}^{-}$ are different receptors. When the $P2Y_{AC}^{-}$-receptor is cloned and appears to be a unique receptor, and not the $P2Y_1$-receptor coupled to a different second messenger system, the $P2Y_n$ nomenclature can be used.

In this study we examined the effect of adenosine nucleotides on the growth of C6 cells. Both growth inhibitory and proliferative effects were observed, depending on whether the added adenosine nucleotides were hydrolysed by ecto-nucleotidases or not. Hydrolysis to adenosine proved to be essential for growth inhibition. We previously studied the hydrolysis of extracellular nucleotides, and identified NPPase as the main ATP hydrolysing ecto-enzyme of C6 cells (Grobben *et al*., 1999). When nucleotide hydrolysis was inhibited by PPADS, an enhanced proliferation was observed for all potent agonists of the $P2Y_{AC}^{-}$-receptor, except for ADP and to a lesser extent ATP, which are hydrolysed even in the presence of PPADS. The enhanced proliferation is due to a $P2Y_{AC}^{-}$-receptor-mediated activation of p42/44 MAPK.

Methods

Materials

Nucleotides, nucleotide derivatives and (−)-isoproterenol were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), and Reactive Blue 2 (RB2) were from RBI (Köln, Germany). [^{3}H]-myo-inositol (21 Ci mmol^{-1}) was from NEN (Boston, MA, U.S.A.). PD98059 was from Calbiochem (La Jolla, CA, U.S.A.).

Cell culture

Rat C6 glioma cells (ATCC CCL 107) were obtained from ATCC (Manassas, VA, U.S.A.) and maintained in monolayer culture as described previously (Slegers & Joniau, 1996). Experiments were performed in 96-well plates or 34 mm petri dishes on cells cultured in serum-free chemically defined medium containing Ham's F10/minimal essential medium (MEM, 1 : 1 v v^{-1}), 2 mM L-glutamine, 1% (v v^{-1}), MEM vitamines (100 ×), 1% (v v^{-1}) MEM non-essential amino acids (100 ×), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (GIBCO, Paisley, Scotland), and 30 nM sodium selenite (Sigma Chemical Co.). Cell numbers were measured in a haemocytometer after cell detachment with trypsin/EDTA in Dulbecco's phosphate-buffered saline (1 : 50).

Measurement of intracellular cyclic AMP concentration

C6 cells were cultivated in 96-well plates in serum-free chemically defined medium up to a cell density of 2.5×10^5 cells cm^{-2}. Cyclic AMP synthesis was induced by addition of 5 μM (−)-isoproterenol. The effect of extracellular nucleotides on the activity of adenylate cyclase was determined by simultaneous addition of the nucleotide and (−)-isoproterenol. After a 30 min incubation at 37°C, the intracellular cyclic AMP concentration was determined with a cyclic AMP-enzyme immunoassay kit [Amersham Pharmacia Biotech, Buckinghamshire, England] according to the manufacturer's instructions.

Measurement of phosphatidylinositol hydrolysis

C6 cells were grown in 35 mm petri dishes. At a density of 1×10^5 cells cm^{-2}, cells were labelled overnight with 2 μCi ml^{-1} of [^{3}H]-myo-inositol in serum-free chemically defined medium. Subsequently, cells were washed with 2 × 2 ml chemically defined medium supplemented with 10 mM LiCl and incubated for 30 min. After addition of the indicated effectors, cells were incubated for another 30 min. The reactions were terminated by aspiration of the medium and addition of 1 ml of 5% (w v^{-1}) trichloroacetic acid (TCA). Dishes were shaken for 15 min and the supernatant was collected. TCA was removed by washing with 3 × 1 ml diethylether. The aqueous fraction was applied on a column containing 0.5 g dowex AG 1-X8 resin (100–200 mesh) in formate form (BioRad, Hertfordshire, U.K.). Free inositol was eluted with 2 × 10 ml 50 mM ammoniumformate. Inositol mono-, di- and triphosphates were eluted with 10 ml 1 M ammoniumformate, 0.1 M formic acid. The remaining radioactivity was eluted with 4 M ammoniumformate, 0.1 M formic acid. Radioactivity was measured in a liquid scintillation counter (Tri-Carb 1600 TR, Packard, Meriden, U.S.A.).

Measurement of nucleotide hydrolysis

Assays were performed in the logarithmic growth phase and at a cell density of approximately 1.35×10^5 cells cm^{-2}. Cells

were incubated for 15 min with PPADS or RB2 prior to addition of the indicated nucleotides. The medium was aspirated at the indicated time points. Samples were diluted 1 : 1 with buffer A [200 mM potassium phosphate (pH 6.0), 8 mM tetrabutylammonium hydrogen sulphate (Fluka, Bornem, Belgium)] and 100 μl was injected onto a reverse phase C18 column (Vydac, Hesperia, CA, U.S.A.). Bound nucleotides were eluted with a gradient from buffer A to buffer B [100 mM potassium phosphate (pH 6.0), 8 mM tetrabutylammonium hydrogen sulphate, 30% (v v^{-1}) methanol]: 0 min 100% A, 0% B; 9 min 100% A, 0% B; 15 min 75% A, 25% B; 22.5 min 10% A, 90% B; 25 min 0% A, 100% B; 30 min 100% A, 0% B. Nucleotides were detected by absorption measurement at 254 nm and eluted with a retention time of 8.5 min (AMP), 9.6 min (adenosine), 16.1 min (ADP) and 24.1 min (ATP).

Detection of p42/44 MAPK activation

C6 cells were cultivated in 96-well plates in serum-free chemically defined medium up to a cell density of 1.2×10^5 cells cm^{-2}. Cells were incubated for 15 min with nucleotide receptor antagonists before stimulation with nucleotides. Incubation was at 37°C for 10 min. The reaction was stopped by aspiration of the medium and addition of 1 μl 10^{-3} $cells^{-1}$ SDS–PAGE sample buffer (6 mM Tris/HCl (pH 6.8), 0.5% (w v^{-1}) SDS, 10% (v v^{-1}) glycerol, 0.5% (v v^{-1}) 2-mercaptoethanol]. Samples were boiled for 5 min, and 20 μl was analysed by SDS–PAGE on a 12.5% (w v^{-1}) polyacrylamide gel. Proteins were electroblotted overnight onto a nitrocellulose membrane (Hybond-C pure, Amersham Pharmacia Biotech, Buckinghamshire, U.K.). Immunodetection was performed using pAbs ($1:5 \times 10^3$) raised against p44 (ERK1) and p42 (ERK2) (Anti-ACTIVE® MAPK, Promega, Leiden, The Netherlands). The nitrocellulose membrane was incubated with horseradish peroxidase conjugated donkey anti-rabbit antibody ($1:4 \times 10^4$) and active MAPK was visualized on film (X-OMAT blue, Kodak, Rochester, N.Y., U.S.A.) by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

In gel *assay for p42/44 MAP kinase*

The assay was performed as described by Kameshita & Fujisawa (1989) with slight modifications. Cell lysates were prepared as described in the previous paragraph, and proteins were seperated on a SDS-polyacrylamide gel consisting of a stacking gel and a 12.5% (w v^{-1}) separation gel containing 0.5 mg ml^{-1} myelin basic protein (MBP) added prior to polymerization. After electrophoresis, SDS was removed by incubation of the gel at room temperature in 2×100 ml of 20% (v v^{-1}) 2-propanol, 50 mM Tris-HCl (pH 8.0) for 1 h, and incubation in 250 ml of 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol for 1 h. Subsequently, proteins were denatured by incubation of the gel in 2×100 ml of 6M guanidine-HCl, 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol for 1 h, and renatured by addition of 6×250 ml 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 0.04% (v v^{-1}) Tween 40 at 4°C for 16 h. After renaturation of the proteins, the gel was preincubated at room temperature for 30 min with 100 ml 40 mM HEPES–NaOH (pH 8.0), 2 mM dithiotreitol, 0.1 mM EGTA, 5 mM $MgCl_2$. The *in gel* kinase assay was initiated by addition of 25 μM [γ-^{32}P]ATP (25 μCi) in the same buffer. After incubation for 1 h, the non-incorporated radioactivity was removed by repeated washes with 5% (w v^{-1}) TCA for 4 h. The gel was dried and the incorporated radioactivity was detected using a phosphoimager (PhosphoImager SI, Molecular Dynamics, Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Statistical analysis

Results are represented as the means ± s.e.mean calculated from at least three independent experiments. Statistically significant differences were calculated using the Student's *t*-test.

Results

Hydrolysis of nucleotides to adenosine determines the growth inhibition of C6 cells

Adenosine, AMP, ADP, ATP, Ap_3A and Ap_4A inhibit the growth of C6 cells (Figure 1). Previously we demonstrated that adenosine nucleotides, as well as diadenosine polyphosphates (Ap_nA), are rapidly hydrolysed to adenosine by this cell line (Grobben *et al*., 1999, 2000). To investigate whether hydrolysis of the added nucleotides is essential for growth inhibition, several experiments were conducted. To inhibit ecto-NPPase, the main mono- and dinucleotide hydrolysing ecto-enzyme of C6 cells (Grobben *et al*., 1999), we used inhibitors of this enzyme, i.e. Reactive Blue 2 (RB2) and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS). PPADS as well as RB2 blocked the growth inhibitory effect of ATP and Ap_nA, while the effect of ADP was only blocked by RB2. No effect was observed on the AMP- and adenosine-induced growth inhibition. Except for

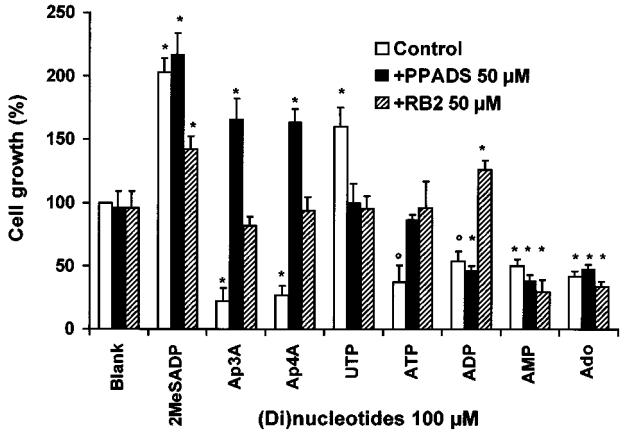

Figure 1 Effect of mono- and dinucleotides on cell proliferation in the presence and absence of purinergic receptor antagonists. Cells were grown in serum-free chemically defined medium in 96-well plates. At a density of $1.0-1.4 \times 10^5$ cells cm^{-2} nucleotides (100 μM) were added to the cells, preceded by a 15 min incubation with PPADS or RB2 (50 μM) as indicated. After 48 h of cultivation, cell numbers were counted in a haemocytometer. The number of cells grown without stimulation was taken as 100%. Data are the means ± s.e.mean of three independent experiments. The statistically significant difference from control is indicated (*$P<0.01$; °$P<0.02$).

the result of ADP, these data indicated that the hydrolysis by ecto-nucleotidases is required for inhibition of the cell growth induced by adenine nucleotides. In contrast, UTP, a rapidly hydrolysed nucleotide (Grobben *et al*., 1999) and 2-methylthioADP (2MeSADP), a non-hydrolysable ADP analogue, did not inhibit but even enhanced the growth of C6 cells (Figure 1).

To investigate further the growth inhibitory effect of ADP even in the presence of PPADS, we compared the hydrolysis rate of 100 μM ADP (1.28 fmol min^{-1} cell^{-1}) and ATP (1.10 fmol min^{-1} cell^{-1}) in the presence of 50 μM PPADS or RB2 (Figure 2). The hydrolysis of ADP was reduced 5.4 fold in the presence of RB2 (0.24 fmol min^{-1} cell^{-1}) but only 1.7 fold in the presence of PPADS (0.75 fmol min^{-1} cell^{-1}). The hydrolysis of ADP in the presence of PPADS was 1.8 fold faster than that of ATP in the presence of PPADS (0.42 fmol min^{-1} cell^{-1}) and explains the adenosine-mediated growth inhibition by ADP in the presence of PPADS. RB2 almost completely inhibited the hydrolysis of ADP and reversed growth inhibition into a moderate growth enhancement (Figure 1). These data demonstrated that hydrolysis of nucleotides into AMP and/or adenosine is a prerequisite for the growth inhibition of C6 cells.

PPADS and RB2 are widely used P2Y-receptor antagonists, and it is not excluded that the effect of these compounds on the nucleotide-mediated growth inhibition is due to their antagonizing effect on nucleotide receptors. To demonstrate that P2Y-receptor activation is not involved in the induction of growth inhibition, we tested whether the growth inhibition is correlated with the nucleotide potency to activate P2Y-receptors. Therefore we determined the potency of the adenosine nucleotides and UTP to activate PLC- or AC-coupled receptors in C6 cells (Table 1). Ap_4A (EC_{50} 12 ± 2 μM) and ATP (EC_{50} 19 ± 1 μM) appeared to be the most potent P2 receptor-agonist for PLC activation and stimulated PI-turnover more potently than ADP (EC_{50} 50 ± 3 μM). Since the PLC-coupled $P2Y_2$-receptor was recently reported to enhance the proliferation of C6 cells (Tu *et al*., 2000), we also tested the effect of the $P2Y_2$ agonist

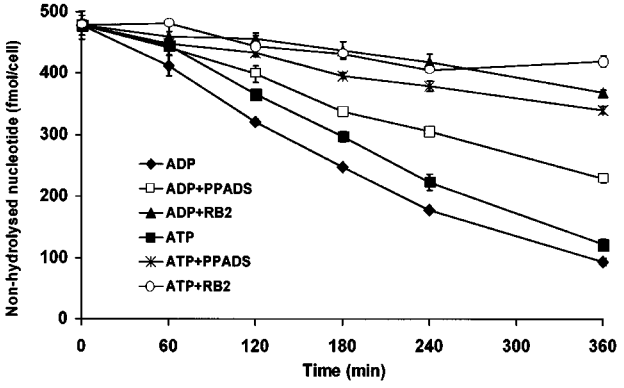

Figure 2 Hydrolysis of ADP and ATP in the presence of PPADS and RB2. Cells were grown in serum-free chemically defined medium in 96-well plates. At a density of 1.35×10^5 cells cm^{-2} nucleotides (100 μM) were added to the cells, preceded by a 15 min incubation with PPADS or RB2 (50 μM) as indicated. The medium was aspirated at the indicated time points and the remaining amount of ATP or ADP (fmol cell^{-1}) determined by hydrophobic ion-pairing chromatography. Data are the means$\pm$s.e.mean of three independent experiments.

Table 1 Effect of nucleotide receptor agonists on second messenger systems

Agonists	*EC_{50} for inhibition of AC*	*EC_{50} for PI-turnover*
2MeSADP	250 ± 37 pM	>100 μM
ADP	300 ± 45 nM	50 ± 3 μM
Ap3A	1 ± 0.5 μM	>100 μM
Ap4A	10 ± 1 μM	12 ± 2 μM
ATP	5 ± 1.5 μM	19 ± 1 μM
AMP	n.d.	n.d.
Ado	n.d.	n.d.
UTP	>50 μM	18 ± 5 μM

n.d.: not detectable

UTP. The EC_{50} for PLC activation by UTP was 18 ± 5 μM. Agonists of the P2Y$_{AC}^{-}$-receptor inhibit the (−)-isoproterenol-induced activation of AC. The obtained EC_{50} values for P2Y$_{AC}^{-}$-activation by adenosine mono- and dinucleotides are compiled in Table 1. Adenosine and AMP had no measurable effect on PI-turnover and on AC activity, excluding the involvement of PLC- and AC-coupled P2Y-receptors in the mechanism of growth inhibition of C6 cells.

Activation of the P2Y$_{AC}^{-}$-receptor enhances cell proliferation

Based on inhibition of the (−)-isoproterenol-induced AC activation, the agonist potency for the P2Y$_{AC}^{-}$-receptor was determined to be 2MeSADP > ADP > Ap_3A > ATP > Ap_4A (Table 1). With the exception of ATP and ADP, all these agonists enhanced the growth of C6 cells when their hydrolysis was blocked with PPADS. 2MeSADP had a strong proliferative effect in the absence and presence of PPADS (210 ± 20%) and in the presence of RB2 (142 ± 11%) (Figure 1). In the case of Ap_3A and Ap_4A, growth inhibition was inverted into growth stimulation when the cells were preincubated with PPADS, but not with RB2. In the presence of RB2, ATP and ADP had no effect and a minor growth stimulatory effect respectively. Activation of MAPK is correlated with cell proliferation. Therefore, we measured the effect of P2Y$_{AC}^{-}$-receptor agonists on p42/44 MAPK activation in the absence and presence of PPADS and RB2 (50 μM). We determined this activation by immunoblotting against phosphorylated MAPK, and also demonstrated that the phosphorylation was correlated with the enzymatic activity by an *in gel* kinase assay using MBP as a substrate (Figure 3).

Agonists of the P2Y$_{AC}^{-}$-receptor activated p42/44 MAPK in the presence of PPADS. The observed activation also correlated with the observed growth stimulation by 2MeSADP and the minor growth stimulation by ADP in the presence of the antagonist RB2. Indeed 2MeSADP, the most potent agonist of the P2Y$_{AC}^{-}$-receptor, is still partially activating MAPK in the presence of RB2 (Figure 3), while the effect of the less potent P2Y$_{AC}^{-}$-agonists ADP, ATP, Ap_3A and Ap_4A was blocked by RB2.

Since 2MeSADP does not induce PI-turnover in C6 cells and thus is a specific and potent agonist of the P2Y$_{AC}^{-}$-receptor of these cells, we determined the concentration-response of 2MeSADP on cell growth (Figure 4). An EC_{50} of 250–500 pM was measured for the activation of MAPK. This value corresponds with the EC_{50} for the inhibition of AC by

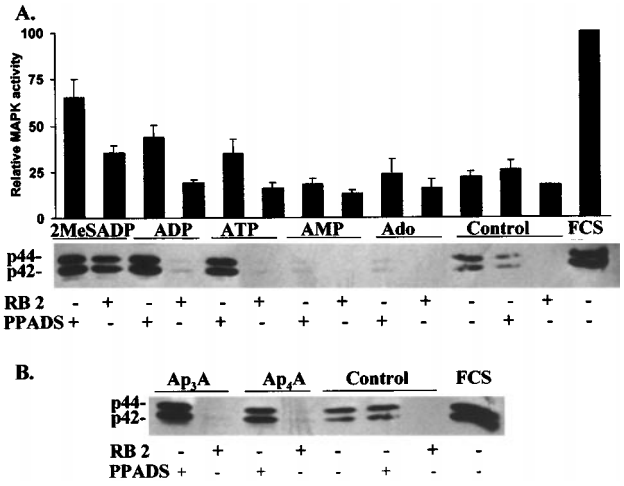

Figure 3 Nucleotide-mediated activation of p42/44 MAPK. (A) Cells were grown in serum-free chemically defined medium in 96-well plates. At a density of $1.0-1.4\times10^5$ cells cm^{-2} mononucleotides (100 μM) were added to the cells, preceded by a 15 min incubation with PPADS or RB2 (50 μM) as indicated. After 10 min, the medium was removed, the cells were dissolved in SDS–PAGE buffer and analysed for MAPK activation by immunoblotting. The blot shown is representative for three independent experiments. Samples of the blot were analysed for MAPK activation by an *in gel* kinase assay using MBP as a substrate as described in Methods. Stimulation of the cells with 10% (v v^{-1}) foetal calf serum was used as a positive control and was taken as 100% for the *in gel* kinase assay. Data are the mean±s.e.mean of three independent experiments. (B) Cells were grown as described in A. Dinucleotides (100 μM) were added to the cells preceded by a 15 min incubation with PPADS or RB2 (50 μM) as indicated. Immunoblotting was performed as described in A.

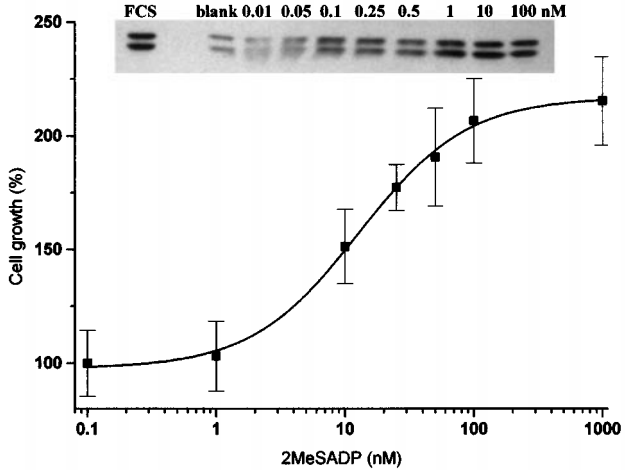

Figure 4 Concentration-response of 2MeSADP on cell proliferation and p42/44 MAPK activation. Cells were grown in serum-free chemically defined medium in 96-well plates. At a density of 1.0×10^5 cells cm^{-2} a varying concentration of 2MeSADP was added to the cells. After 10 min the medium was removed, the cells were dissolved in SDS–PAGE buffer and analysed for MAPK activation by immunoblotting (insert). Stimulation of the cells with 10% (v v^{-1}) foetal calf serum was used as a positive control. The blot shown is representative for three independent experiments. In parallel, cells were grown for 48 h and cell numbers were counted in a haemocytometer. The number of cells grown without stimulation was taken as 100%. Data are the means±s.e.mean of three independent experiments.

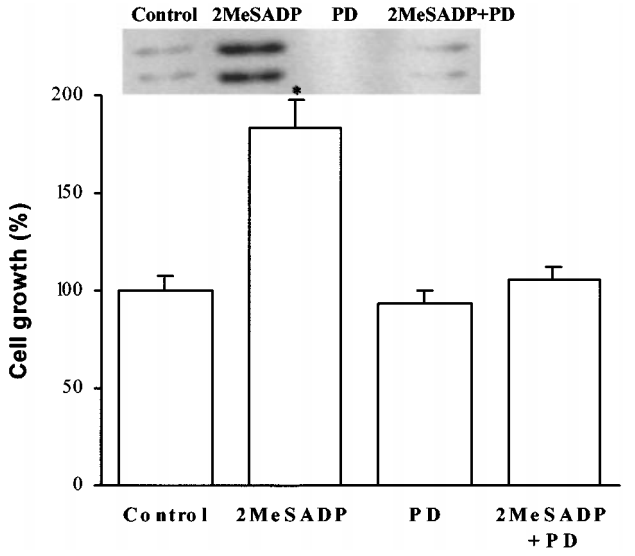

Figure 5 The nucleotide-mediated growth enhancement is p42/44 MAPK dependent. Cells were grown in serum-free chemically defined medium in 96-well plates. At a density of $1.0-1.4\times10^5$ cells cm^{-2}, 2MeSADP (100 nM) was added to the cells, preceded by a 3 h incubation with the MEK inhibitor PD98059 (5 μM). After a 48 h incubation, cell numbers were counted in a haemocytometer. The number of cells grown without stimulation was taken as 100%. Data are the means±s.e.mean of three independent experiments. The statistically significant difference from control is indicated ($^*P<0.05$). Insert: In parallel, cells were dissolved in SDS–PAGE buffer 10 min after stimulation with 2MeSADP and analysed for p42/44 MAPK activation by immunoblotting.

$P2Y_{AC}^{-}$-activation (Table 1), resulting in an EC_{50} of 10 nM for the stimulation of proliferation measured after 48 h. The MEK inhibitor PD98059 was used to demonstrate that the activation of p42/44 MAPK is necessary for the growth enhancement (Figure 5). The enhanced proliferation induced by 2MeSADP was reduced to the basal level when C6 cells were stimulated in the presence of PD98059, proving that the $P2Y_{AC}^{-}$-mediated growth enhancement is p42/44 MAPK dependent.

Recently, Tu *et al.* (2000) have reported that MAPK is activated by ATP and UTP in C6 cells through activation of the $P2Y_2$-receptor. In agreement with these data, UTP had a stimulatory effect on cell proliferation. This effect was blocked by RB2 and PPADS (Figure 1), in contrast to the growth enhancement induced by activation of the PPADS insensitive $P2Y_{AC}^{-}$-receptor.

Discussion

Hydrolysable adenosine mono- and dinucleotides have a growth inhibitory effect on C6 cells. The inhibition is due to hydrolysis of the added nucleotides to AMP and adenosine. All growth inhibitory nucleotides have been shown to be substrates of the ecto-NPPase, the main nucleotide hydrolysing ecto-enzyme of C6 cells. This enzyme is potently inhibited by RB2 and PPADS (Grobben *et al.*, 1999; 2000), and RB2 abolished the growth inhibition of all hydrolysable nucleotides tested. With the exception of ADP, addition of PPADS also blocked the growth inhibition of all nucleotides. Determination of the hydrolysis rate of ATP and ADP in the absence or presence of PPADS and RB2 demonstrated that ADP is still hydrolysed in

the presence of PPADS. This indicated the presence of a RB2 sensitive ADP hydrolysing enzyme on the plasma membrane of C6 cells that is only weakly inhibited by PPADS. This enzyme could be an ADPase or an ATPDase preferentially hydrolysing ADP. In this context, it has already been reported that ATPDase is more potently inhibited by Evans Blue than by PPADS (Heine *et al*., 1999).

The observation that both the ADP-induced growth inhibition as well as the ADP hydrolysis are more potently blocked by RB2 than by PPADS, supports the proposition that hydrolysis of adenosine nucleotides to AMP and adenosine is the key mechanism for the nucleotide-induced growth inhibition. As AMP is rapidly converted into adenosine by 5′-nucleotidase, and PPADS as well as RB2 do not inhibit the latter enzyme, adenosine is the active compound in the nucleotide-mediated growth inhibition of C6 cells. Adenosine has been reported to inhibit cell proliferation by two different mechanisms. Firstly, adenosine uptake by adenosine transporters may cause an adenosine-dependent pyrimidine starvation (Weisman *et al*., 1988; Lasso de la Vega *et al*., 1994). Secondly, adenosine activates A_2-receptors present on the plasma membrane (Jackson & Carlson, 1992), inducing growth inhibition by inactivation of the MAPK cascade (Hirano *et al*., 1996). It remains to be determined which mechanism is responsible for the observed growth inhibition of C6 cells.

PPADS inhibits the hydrolysis of diadenosine polyphosphates to such an extent that growth inhibition was converted into an enhanced proliferation comparable to the one observed with the nonhydrolysable nucleotide 2MeSADP. We have recently demonstrated that diadenosine polyphosphates are $P2Y_{AC}^{-}$-agonists, and that this PPADS insensitive receptor activates p42/44 MAPK. Since 2MeSADP is the most potent agonist of the $P2Y_{AC}^{-}$-receptor (Boyer *et al*., 1994), the presented data demonstrate that the enhanced growth is correlated with an activation of this receptor. In addition 50 μM RB2, an antagonist of the $P2Y_{AC}^{-}$-receptor, is not sufficient to inhibit the nucleotide receptor-mediated p42/44 MAPK-activation of 100 μM 2MeSADP completely. We thus proved that activation of the P2Y-receptor, negatively coupled to AC, enhances cell growth by activation of p42/44 MAPK. Also, a basal level of p42/44 MAPK activity was observed in the cells. Since inhibition of this basal activity by PD98059 did not affect the growth rate of C6 cells (Figure 5), p42/44 MAPK activity appears to be non-essential for cell growth under normal conditions. In addition, RB2 blocks the basal activity of p42/44 MAPK, suggesting that C6 cells are permanently stimulated by an efflux of nucleotides. Indeed, a release of nucleotides by C6 cells has been reported (Lazarowski & Harden, 1999; Lazarowski *et al*., 2000). As is the case for PD98059, inhibition by RB2 of the basal autocrine p42/44 MAPK stimulation does not affect the normal growth of C6. Therefore, it is unlikely that the inhibition of basal p42/44 MAPK activity by AMP and adenosine as observed in Figure 4 is the cause of the growth inhibition.

Activation of the MAPK-signal transduction cascade by nucleotide receptors has been reported previously (Harper *et al*., 1998; Neary *et al*., 1998; 1999; Lenz *et al*., 2000). $P2Y_2$ has been identified as the receptor through which the mitogenic signal of ATP and UTP is transduced into the cell (Miyagi *et al*., 1996; Soltoff *et al*., 1998; Tu *et al*., 2000). PPADS, an antagonist for several P2Y-receptors, blocked the growth stimulatory effect of UTP in C6 cells. Furthermore, Tu *et al*. (2000) reported that the UTP-mediated p42/44 MAPK activation is pertussis toxin insensitive. Since the $P2Y_2$-receptor is PLC-coupled through the pertussis toxin sensitive Gi protein (Lustig *et al*., 1996; Soltoff *et al*., 1998), and the $P2Y_{AC}^{-}$-receptor is PPADS insensitive, UTP activates MAPK by a different still unknown mechanism. As UTP in the absence of inhibitors of ecto-nucleotidases is still capable of enhancing cell proliferation, we may conclude that UMP and uridine, UTP-hydrolysis products generated by NPPase and 5′-nucleotidase, are not growth inhibitory for C6 cells. ATP is an agonist of both the PPADS sensitive $P2Y_2$-receptor and the PPADS insensitive $P2Y_{AC}^{-}$-receptor, and can thus activate MAPK by binding to both receptors. Notwithstanding this activation, no growth enhancement was observed after stimulation of C6 cells with ATP in the absence or presence of PPADS. In contrast to Ap_3A and Ap_4A, ATP is hydrolysed by a PPADS insensitive ecto-ATPase present on the plasma membrane of C6 cells (Grobben *et al*., 1999). Hydrolysis of ATP results in the formation of adenosine, a growth inhibitor of C6 cells, which explains the lack of growth enhancement by ATP.

In conclusion, the presented data demonstrated that $P2Y_{AC}^{-}$-receptor agonists induce an enhanced growth of C6 cells. If these nucleotides are hydrolysed into the growth inhibitory adenosine, the growth enhancement is neutralized. In addition, the nucleotide-mediated cell growth is due to a $P2Y_{AC}^{-}$-induced p42/44 MAPK activation.

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