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Ecto-nucleotide pyrophosphatase modulates the purinoceptor-mediated signal transduction and is inhibited by purinoceptor antagonists

[1]Bert Grobben, [1]Patrik Claes, [1]Dirk Roymans, [3]Edgard L. Esmans, [2]Harry Van Onckelen & *,[1]Herman Slegers

[1]Department of Biochemistry, Cellular Biochemistry, University of Antwerp, Universitaire Instelling Antwerpen, Universiteitplein 1, B-2610 Antwerpen-Wilrijk, Belgium; [2]Department of Biology, Plant Biochemistry, University of Antwerp, Universitaire Instelling Antwerpen, Universiteitplein 1, B-2610 Antwerpen-Wilrijk, Belgium and [3]Department of Chemistry, Nucleoside Research, University of Antwerp, Universitair Centrum Antwerpen, B-2020 Antwerpen, Belgium

1 The effect of ecto-nucleotide pyrophosphatase (ecto-NPPase; EC 3.6.1.9) on the ATP- and ADP-mediated receptor activation was studied in rat C6 glioma cells. The P2-purinoceptor antagonists pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and reactive blue (RB2) are potent inhibitors ($IC_{50} = 12 \pm 3$ μM) of the latter enzyme. 4,4′-diisothiocyanatostilbene-2,2′ disulfonic acid (DIDS), 5′-phosphoadenosine 3′-phosphate (PAP) and suramin were less potent inhibitors with an IC_{50} of 22 ± 4, 36 ± 7 and 72 ± 11 μM respectively.

2 P1-purinoceptor antagonists CGS 15943, cyclo-pentyl theophylline (CTP) and theophylline did not affect the activity of the ecto-NPPase.

3 ATP- and ADP-mediated $P2Y_1$-like receptor activation inhibited the (−)-isoproterenol-induced increase of intracellular cyclic AMP concentration. PPADS, an ineffective P2Y-antagonist in C6, potentiated the ATP and ADP effect approximately 3 fold due to inhibition of nucleotide hydrolysis by the ecto-NPPase.

4 We conclude that ecto-NPPase has a modulatory effect on purinoceptor-mediated signalling in C6 glioma cell cultures.

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Abbreviations: AMPCP, α,β-methylene-ADP; AMPCPP, α,β-methylene-ATP; CPT, cyclo-pentyl theophylline; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid; ecto-ATPDase, ecto-ATP diphosphohydrolase; ecto-NPPase, ecto-nucleotide pyrophosphatase; IBMX, iso-butyl methylxanthine; MEM, minimal essential medium; 2MeS-ATP, 2-methylthio-ATP; PAP, 5′-phosphoadenosine-3′-phosphate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; RB2, reactive blue 2

Introduction

Purinoceptors were originally classified by Burnstock (1978) as P1- and P2-purinergic receptors depending on the activities of adenosine and adenine nucleotides. Based on the rank order of agonist potency and antagonist affinity P1-receptors were further subclassified into A1, A2 and A3 receptors and the P2-receptors into ionotropic P2X- and metabotropic P2Y-receptors (for review see Collis and Hourani, 1993; Fredholm *et al*., 1994; Chen *et al*., 1995; Nicholas *et al*., 1996). The subclassification of P2-receptors also reflects their signal transduction mechanisms. The P2X receptors are ligand-gated ion channels while P2Y-receptors have G_q- or G_i-protein-linked signal transduction pathways coupled to activation of phospholipase C or to inhibition of adenylate cyclase respectively (for review see Boarder *et al*., 1995; Nicholas *et al*., 1996).

Potencies of agonists such as ATP, UTP, ATPγS, 2-methylthio-ATP (2MeS-ATP) and α,β-methylene-ATP (AMPCPP) may be affected by ecto-nucleotidase hydrolysis leading to an incorrect estimation of the agonist potency order (Kennedy & Leff, 1995; Chen & Lin, 1997; Vigne *et al*., 1998). Pharmacological classification is further hampered by the fact that receptor selective P_2-antagonists are not yet available, although antagonists like reactive blue (RB2), suramin, 4,4′-diisothiocyanatostilbene-2,2′ disulphonic acid (DIDS) and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) are commonly used to block P2-receptor subtypes. RB2 and suramin, two non-selective P2-receptor antagonists, interact with several proteins unrelated to purinoceptor signaling and also inhibit ecto-ATPase (E.C. 3.6.1.3) and ecto-ATP diphosphohydrolase (ecto-ATPDase, E.C. 3.6.1.5) degradation of P2-receptor agonists (Crack *et al*., 1994; Chen *et al*., 1996; Brown *et al*., 1997; Heine *et al*., 1999). PPADS, a more selective P2X- and $P2Y_1$-antagonist, and DIDS, a selective P2Y-antagonist, are also reported as inhibitors of ecto-ATPase (Knowles, 1988; Chen *et al*., 1996). Besides their antagonizing effect on purinoceptor activation, their inhibition of agonist degradation by ecto-nucleotidases further complicates receptor characterization based on the ranking profiles of agonists and antagonists.

Rat C6 glioma is a bipotential cell line often used as a model system for astrocytes. Early passages of C6 exhibit oligodendrocytic and astrocytic progenitor properties and have an astrocyte type 2 phenotype after treatments that increase the

*Author for correspondence; E-mail: slegers@uia.ua.ac.be

intracellular concentrations of cyclic AMP (Lee *et al*., 1992; Messens & Slegers, 1992; Anciaux *et al*., 1997). The cell is responsive to extracellular nucleotides and expresses a $P2Y_1$-like purinoceptor, a putative A2- and the pyrimidinergic $P2Y_6$-receptor (Elfman *et al*., 1984; Boyer *et al*., 1993; Lazarowski & Harden, 1994; Lin & Chuang, 1994). The $P2Y_1$-like receptor is G_i-protein-coupled to adenylate cyclase and its activation inhibits the β-adrenergic receptor-mediated stimulation of cyclic AMP synthesis. The purinoceptor is not antagonized by PPADS like its $P2Y_1$ counterpart (Boyer *et al*., 1994; Schachter *et al*., 1997).

In rat C6 glioma cells we recently identified an ecto-nucleotide pyrophosphatase (ecto-NPPase) as one of the main enzymes involved in the extracellular metabolization of nucleotides (Grobben *et al*., 1999). This ecto-NPPase is expressed on the plasma membrane of many cell types, e.g. osteoblasts, chondrocytes, lymphocytes, fibroblasts, smooth muscle cells and hepatocytes (Huang *et al*., 1994; Scott *et al*., 1997; Kettenhofen *et al*., 1998). The latter enzyme accounted for the hydrolysis of more than 75% of the ATP or α,β-bond hydrolyzable ATP analogues (2MeS-ATP, ATPβS). It hydrolyzes ATP into AMP and PP_i and therefore may have an important function in a shift of P2- to P1-receptor activation. In this communication we demonstrated that purinergic and pyridiminergic receptor antagonists s.a. RB2, DIDS, PPADS and suramin are inhibitors of the ecto-NPPase. The presented data extent the inhibitory effect of some purinoceptor antagonists to the ecto-enzymes mainly involved in the extracellular ATP metabolization. In C6 glioma cells, inhibition of the ecto-NPPase resulted in a potentiation of the $P2Y_1$-like purinoceptor-mediated inhibition of cyclic AMP synthesis.

Methods

Materials

Nucleotides, nucleotide derivatives, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS), theophylline, cyclo-pentyl theophylline (CPT), 5′-phosphoadenosine-3′-phosphate (PAP) and (−)-isoproterenol were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pyridoxal phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), CGS 15943 and Reactive Blue 2 (RB2) were from RBI (Köln, Germany). Suramin was from BIOMOL Research Laboratories (PA, U.S.A). [γ-^{32}P]-ATP (spec. act. 3000 Ci mmol^{-1}) was from NEN (Boston, MA, U.S.A.).

Cell culture

Rat C6 glioma cells (ATCC no. CCL 107) were obtained from ATCC (Manassas, VA, U.S.A.) and maintained in monolayer culture as described previously (Slegers & Joniau, 1996). Phosphorylation assays and ATP hydrolysis experiments were performed in 96-well plates 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 nonessential amino acids (100×), 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (GIBCO, Paisley, U.K.), and 30 nM sodium selenite (Sigma Chemical Co.). Cell numbers were measured in a haemocytometer after cell detachment with trypsin/EDTA in phosphate-buffered saline.

Measurement of extracellular nucleotide hydrolysis

Assays were performed in the logarithmic growth phase and at a cell density of approximately 10^5 cells cm^{-2} (3.3×10^4 cells well^{-1}). Reactions were initiated by addition of [γ-^{32}P]-ATP to the cells. The added ATP concentration is indicated in the figure legends. Samples were taken from the medium at the indicated time points and the hydrolysis stopped by addition of 100 mM EDTA. Nucleotide containing samples of 1 μl were analysed by TLC on polyethyleneimine cellulose plates (Merck, Darmstadt, Germany) by ascending chromatography in 750 mM KH_2PO_4 (pH 3.0). Radioactive spots were visualized by autoradiography, excised, and counted in a liquid scintillation counter Tricarb 1600 (Packard, Meriden, CT, U.S.A.).

Degradation products of ATP and ADP were also quantified by ion-pairing hydrophobic interaction chromatography. At the time points indicated in the figures legends, samples were diluted 1 : 2 with buffer A [KH_2PO_4 (pH 6.0) 200 mM, tetrabutylammonium hydrogen sulphate 8 mM (Fluka, Bornem, Belgium)] and 100 μl was injected onto a C18 column (5 μm, 250×4.6 mm; Vydac, Hesperia, CA, U.S.A.). Bound nucleotides were eluted with a gradient from buffer A to buffer B [KH_2PO_4 (pH 6.0) 200 mM, tetrabutylammonium hydrogen sulphate 8 mM, 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 0% A, 100% B and at a flow rate of 1 ml min^{-1}. Nucleotides were detected by absorbance measurement at 254 nm and eluted with a retention time of 11.55 min (AMP), 21.79 min (ADP) and 26.14 min (ATP).

Ecto-NPPase activity

Ecto-NPPase activity was assayed in buffer A [3-(cyclohexylamine)-1-propane sulphonic acid (pH 10) 50 mM, NaCl 100 mM, $CaCl_2$ 2 mM] containing 20 μM [γ-^{32}P]ATP (s.a. 10^8 c.p.m. nmol^{-1}). The reaction mixtures were incubated at 37°C for 30 min and stopped by addition of 100 mM EDTA. Experiments were performed in the linear range of ATP hydrolysis. Samples of 1 μl were analysed by TLC as described above.

Measurement of intracellular cyclic AMP concentration

C6 glioma cells were cultivated in 96-well plates in chemically defined medium up to a cell density of 2.5×10^5 cells cm^{-2}. Intracellular cyclic AMP synthesis was induced by addition of 5 μM (−)-isoproterenol. The effect of extracellular nucleotides on adenylate cyclase activity was determined by simultaneous addition of the nucleotide and (−)-isoproterenol. A 15 min incubation with the indicated effector of the ecto-NPPase preceded the (−)-isoproterenol stimulation. After a 30 min incubation at 37°C, the medium was removed and lysis buffer added. The intracellular cyclic AMP concentration was subsequently determined with a cyclic AMP-enzyme immunoassay kit [Amersham Pharmacia Biotech, Buckinghamshire, U.K.] according to the manufacturer's instructions.

Statistical analysis

Results are represented as the means ± s.d. calculated from at least three independent experiments. Statistically significant differences ($P<0.05$) were calculated using a paired Student's *t*-test.

Results

Inhibition of ecto-nucleotide pyrophosphatase by purinoceptor antagonists

In cultures of C6 glioma cells extracellular ATP is mainly hydrolyzed by an ecto-NPPase, previously identified as a homologue of the plasma cell differentiation antigen PC-1 (Grobben *et al*. 1999). The latter enzyme is potently inhibited by purinoceptor antagonists. Cellular ATP hydrolysis in the presence of 50 μM PPADS, PAP, suramin, RB2 and DIDS is shown in Figure 1. In the absence of purinoceptor antagonists, the added ATP was completely hydrolyzed after approximately 8 h (Figure 1A). The ecto-NPPase and ecto-ATPase activities accounted for 85 and 15% of the hydrolysis respectively. The rate of ATP hydrolysis by the ecto-NPPase and ecto-ATPases was calculated from the linear part of the ATP degradation into PP_i and P_i as a function of time (Figure 1B). In the presence of 50 μM purinoceptor antagonist, the initial rate of the ecto-NPPase-dependent ATP hydrolysis decreased from 0.205 ± 0.007 fmol min^{-1} cell^{-1} to 0.152 ± 0.023 fmol min^{-1} cell^{-1} (PAP), 0.134 ± 0.023 fmol min^{-1} cell^{-1} (suramin), 0.083 ± 0.001 fmol min^{-1} cell^{-1} (DIDS), 0.064 ± 0.001 fmol min^{-1} cell^{-1} (PPADS) and 0.032 ± 0.001 fmol min^{-1} cell^{-1} (RB2). Concomitantly, the P_i-release increased 1.8 fold ($P<0.05$) in the presence of PPADS and DIDS, and 2.1 fold ($P<0.05$) in the presence of suramin. Ecto-ATPase activities did not increase statistically significant ($P>0.05$) in the presence of PAP and RB2 (Figure 1B). In the presence of the most potent inhibitors DIDS, PPADS and RB2, the time for complete hydrolysis of 10 μM ATP increased approximately 2 fold.

PPADS and RB2 were equipotent inhibitors of the ecto-NPPase ($IC_{50} = 12 \pm 3$ μM) whereas DIDS, PAP and suramin exhibited IC_{50} values of 22 ± 4, 36 ± 7 and 72 ± 11 μM respectively (Figure 2A). In comparison, P1-purinoceptor antagonists CGS 15943, CPT, theophylline, 8-phenyltheophylline and IBMX were also analysed for inhibition of the ecto-NPPase (Figure 2B). In contrast to the P2Y-purinoceptor antagonists, the latter did not inhibit the enzyme.

Effect of ATP and ADP on (−)-isoproterenol-induced cyclic AMP-production

Stimulation of C6 with 5 μM of the β-adrenergic receptor agonist (−)-isoproterenol, induced a transient accumulation of intracellular cyclic AMP. The latter concentration attained a maximal value after 30 min and returned to the basal level after 2 h (Figure 3). Basal and (−)-isoproterenol-induced cyclic AMP levels, determined at 30 min by a cyclic AMP-enzyme immunoassay, were 3.7 ± 0.6 and 214 ± 25 pmol 10^{-6} cells^{-1} respectively. The latter values have been confirmed by (−) electrospray mass spectrometry. Pre-incubation of C6 with 100 μM IBMX, an intracellular phosphodiesterase inhibitor, did not affect the maximal attained cyclic AMP concentrations (Figure 4, inset). Addition of ATP and ADP resulted in a dose-dependent inhibition of the (−)-isoproterenol-induced adenylate cyclase activation (Figures 3 and 4). ATP and ADP exhibited IC_{50} values of 9 and 3 μM respectively demonstrating that ADP, in comparison to ATP, is a 3 fold more potent agonist of the P2Y$_1$-like receptor of C6 (Boyer *et al*., 1993). PPADS has been reported as an ineffective P2Y$_1$-like purinoceptor antagonist (Boyer *et al*., 1994). In addition, we have shown that the latter compound is a potent inhibitor of the ecto-NPPase. These properties allowed us to evaluate the effect of the ecto-NPPase on the ATP/ADP-mediated inhibitory effect on the (−)-isoproterenol-induced activation of adenylate cyclase. PPADS did not antagonize the inhibitory effect of ATP and ADP on the activation of adenylate cyclase. On the contrary, when cells were pre-incubated with 50 μM

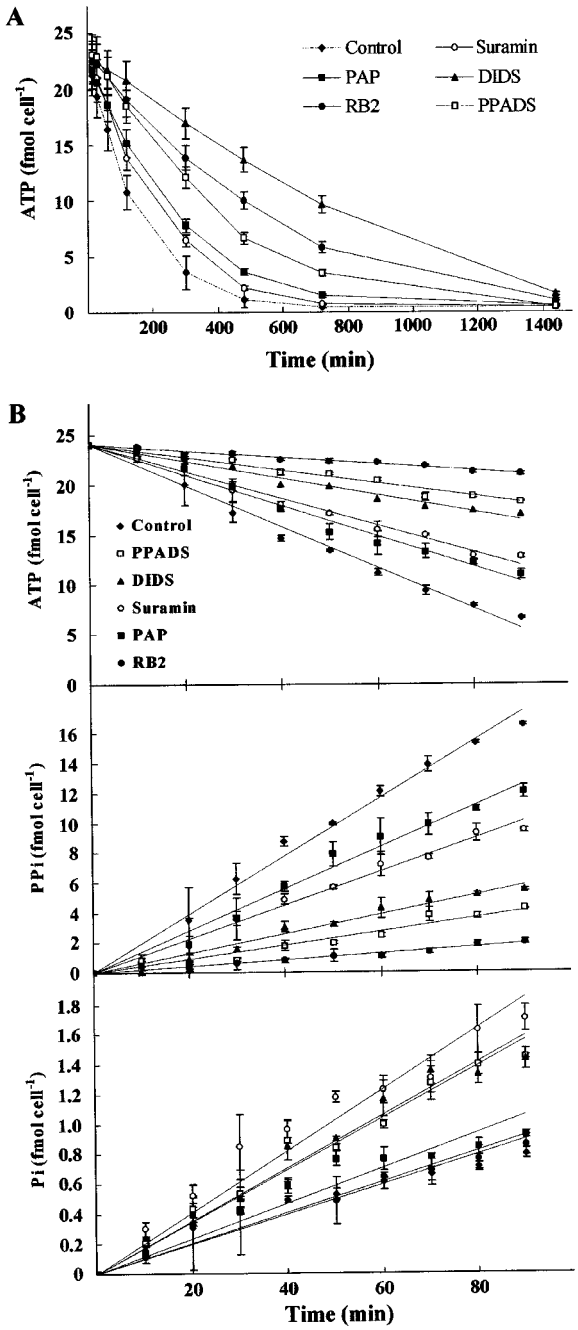

Figure 1 Time-dependent hydrolysis of ATP. Cells were grown in chemically defined medium up to a density of approximately 2.15×10^5 cells cm^{-2}. Cells were pre-incubated for 30 min with 50 μM of the respective antagonist. Hydrolysis was initiated by addition of 10 μM [γ-^{32}P]-ATP (s.a. 10^8 c.p.m. nmol^{-1}). At the indicated time points, samples were taken from the medium and analysed by TLC for the hydrolysis of ATP into P_i and PP_i. Time dependence for complete hydrolysis of ATP is shown in (A), the linear phase of the ATP hydrolysis is shown in (B). The remaining amount of ATP and the amount of formed PP_i and P_i, representative for the ecto-NNPase and ecto-ATPase/ecto-ATPDase activities respectively, are expressed as fmol cell^{-1}. Data are the means $\pm$ s.d. determined from three independent measurements. In the presence of P2-purinoceptor antagonists, the hydrolysis of ATP and the release of PP_i from ATP was significantly different from the control ($P<0.05$). With the exception of PAP and RB2, the P_i-release was also significantly different from the control.

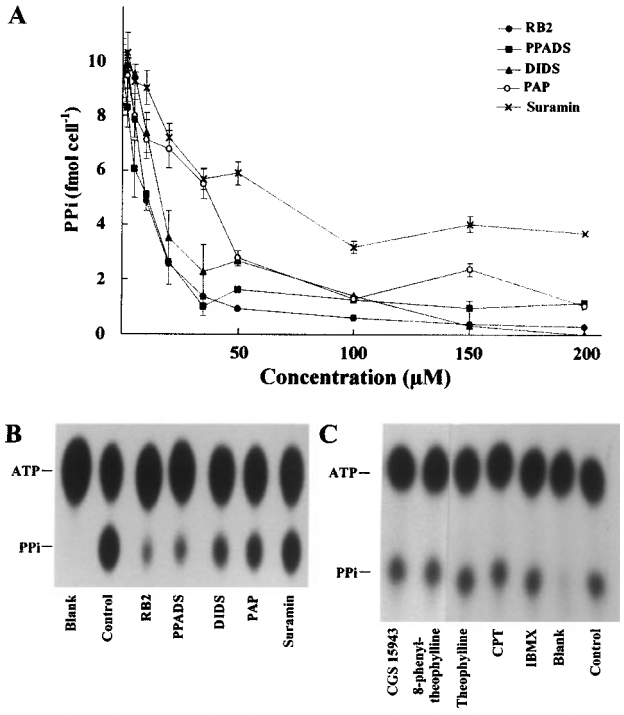

Figure 2 Inhibition of ecto-NPPase by purinoceptor antagonists. Cells were grown in chemically defined medium up to a density of approximately 2.15×10^5 cells cm^{-2}. Cells were pre-incubated for 30 min with the indicated antagonist concentrations before addition of 10 μM [γ-^{32}P]-ATP (s.a. 10^8 c.p.m. nmol^{-1}). After an incubation of 90 min, samples were taken from the medium and analysed by TLC for the hydrolysis of ATP into P_i and PP_i (A). The amount of formed PP_i is expressed as fmol cell^{-1}. Data are the means$\pm$s.d. determined from three independent measurements. (B) shows the autoradiograph of the TLC-plate of samples incubated for 45 min in the presence of 50 μM P2-purinoceptor antagonist. Blank: hydrolysis in the absence of C6 glioma cells. Control: hydrolysis in the absence of antagonist. (C) shows the autoradiograph of the TLC-plate after analysis of samples hydrolyzed in the presence of P1-purinoceptor antagonists (100 μM antagonist, 30 min incubation).

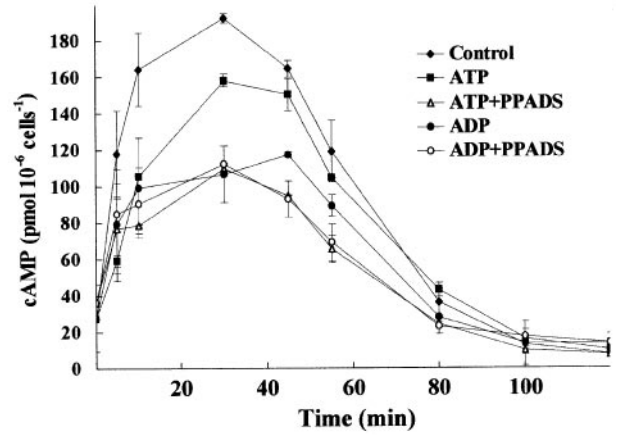

Figure 3 Effect of nucleotides on the (−)-isoproterenol-induced cyclic AMP synthesis. Cells were grown in chemically defined medium up to a density of 2.5×10^5 cells cm^{-2}. Cyclic AMP synthesis was initiated by the simultaneous addition of 5 μM (−)-isoproterenol and 2.5 μM ATP or ADP with or without a pre-incubation with 50 μM PPADS for 30 min. At the indicated times after addition of nucleotides, the medium was removed and the intracellular cyclic AMP concentration assayed as described in Methods. Intracellular cyclic AMP concentrations are expressed as pmol in 10^6 cells. Data are the means$\pm$s.d. determined from three independent measurements.

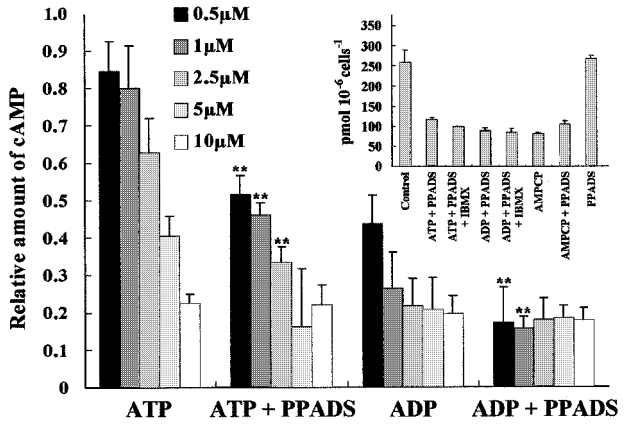

Figure 4 Dose-dependent ATP- and ADP-mediated inhibition of (−)-isoproterenol-induced cyclic AMP synthesis. Cells were grown in chemically defined medium up to a density of 2.5×10^5 cells cm^{-2}. Cyclic AMP synthesis was initiated by the simultaneous addition of 5 μM (−)-isoproterenol and the indicated ATP or ADP concentration with 50 μM PPADS. After an incubation for 30 min, the medium was removed and the intracellular cyclic AMP concentration assayed as described in Methods. The results are expressed relative to the amount of cyclic AMP formed in the control reaction (259 ± 30 pmol 10^{-6} cells^{-1}). Data are the means$\pm$s.d. determined from four independent measurements. Statistically significant differences in the amount of cyclic AMP upon addition of PPADS are indicated (**$P<0.05$). Inset: the potentiated inhibitory effect on the cyclic AMP synthesis (pmol 10^{-6} cells^{-1}) of ATP (2.5 μM) and ADP (0.25 μM) with 50 μM PPADS in the presence or absence of 100 μM IBMX (pre-incubated for 30 min). Addition of IBMX did not result in a statistically significant increase of intracellular cyclic AMP concentrations ($P>0.05$). Inhibition of cyclic AMP synthesis by 100 μM AMPCP in the presence or absence of 50 μM PPADS. Data are the means$\pm$s.d. determined from three independent measurements.

PPADS, the effect of ATP and ADP was potentiated more than 3 fold (Figure 4). In contrast to PPADS, pre-incubation of C6 with DIDS and RB2 resulted in a competitive antagonizing effect on the induced cyclic AMP synthesis (data not shown).

To confirm that the observed effect of PPADS was due to inhibition of the ecto-NPPase, the hydrolysis of ATP and ADP was analysed by TLC and ion-pairing hydrophobic interaction chromatography respectively. During the 30 min incubation of [γ-^{32}P]-ATP in the absence of PPADS, and depending on the added ATP concentration, ATP was hydrolyzed approximately 37 and 78% for 10 and 0.5 μM ATP respectively (Figure 5). The ecto-NPPase accounted for at least 75% of the latter hydrolysis as indicated by the results shown in Figure 1B. Addition of 50 μM PPADS decreased the ATP hydrolysis to less than 20%, resulting in a higher ATP concentration in the medium due to a potent inhibition of the ecto-NPPase activity.

Conditioned media of ADP-stimulated cells were analysed for the amount of ADP and AMP by ion-pairing hydrophobic interaction chromatography. During a 30 min incubation and in the absence of PPADS, 0.5 and 10 μM ADP were hydrolyzed for 75 and 31% respectively (Figure 5). The latter hydrolysis is inhibited after incubation with 50 μM PPADS. Therefore, the potentiating effect of PPADS on the ADP-mediated inhibition of the (−)-isoproterenol-induced activation of adenylate cyclase is due to a higher ADP concentration in the medium.

In addition, 100 μM AMPCP, a non-hydrolyzable ADP-analog and a less potent $P2Y_1$-like receptor agonist, inhibited the cyclic AMP synthesis to the same extent as 1 μM ADP. Pre-incubation of C6 glioma cells with 100 μM IBMX did not

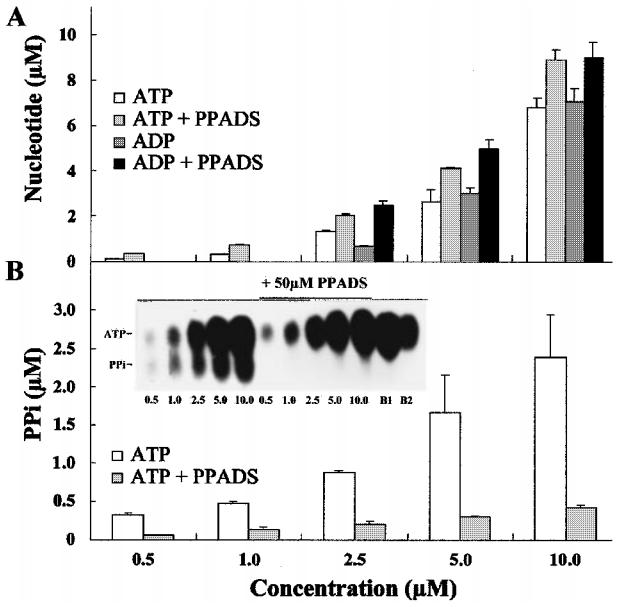

Figure 5 Inhibition of the extracellular ATP and ADP hydrolysis by PPADS. Cells were grown in chemically defined medium up to a density of 2.5×10^5 cells cm^{-2}. Cells were pre-incubated for 15 min with 50 μM PPADS before the addition of the indicated [γ-^{32}P]-ATP (s.a. 10^8 c.p.m. nmol^{-1}) or ADP concentration. After a 30 min incubation, samples were analysed by TLC for the hydrolysis of ATP (A) into PP_i (B), and by ion-pairing hydrophobic interaction chromatography for the hydrolysis of ADP (A). Hydrolysis products could not be measured below concentrations of 2.5 μM ADP, due to the detection limit of the chromatographic procedure. The inset shown in (B) is the autoradiograph after TLC-analysis of the hydrolyzed [γ-^{32}P]-ATP in the absence or presence of 50 μM PPADS. ATP concentrations are indicated in the inset. B1 and B2: hydrolysis of respectively 10 and 2.5 μM [γ-^{32}P]-ATP in the absence of C6 glioma cells. Data are expressed as μM and are the means$\pm$s.d. determined from three independent measurements. The hydrolysis of ATP and ADP in the presence or absence of PPADS are significantly different ($P<0.05$).

reverse the potentating effect of PPADS, indicating that it does not result from an increased rate of intracellular cyclic AMP hydrolysis (Figure 4 insert). In addition PPADS had no effect on the AMPCP-induced inhibition of cyclic AMP accumulation, providing further evidence that during the stimulation period, the hydrolysis of nucleotides by the ecto-NPPase modulates the (−)-isoproterenol-induced increase in intracellular cyclic AMP.

Discussion

Extracellular ATP hydrolysis proceeds through a cascade of ecto-nucleotidases involving ecto-ATPase, ecto-ATPDase, ecto-NPPase and ecto-5′-nucleotidase (Zimmerman, 1996). ATP is subsequently degraded into ADP, AMP and finally adenosine. Previously, we reported that an ecto-NPPase was the main ATP hydrolyzing enzyme of C6 glioma cells (Grobben *et al*., 1999). This enzyme bypasses the formation of ADP and hydrolyzes ATP into AMP. The ecto-NPPase was identified as a PC-1-related enzyme, a type II glycoprotein with nucleotide phosphodiesterase I/pyrophosphatase activity, originally identified as a plasma cell differentiation antigen. The enzyme was found to be expressed in a wide variety of tissues and cell types (van Driel & Goding, 1987; Harahap & Goding, 1988; Huang *et al*., 1994; Scott *et al*., 1997; Goding *et al*., 1998; Kettenhofen *et al*., 1998). The function of PC-1 on plasma cells remains unclear but it has been shown that in serum ATP is mainly hydrolyzed by a NPPase, proposed to be the soluble form of PC-1 (Belli *et al*., 1993; Park *et al*., 1996). Furthermore, the catalytic site of autotaxin, a PC-1-homolog, has been shown to be linked to its stimulation of the motility of tumor cells (Lee *et al*., 1996). PC-1 is highly expressed on chondrocytes and osteoblasts, and has an important role in bone mineralization (Huang *et al*., 1994, Johnson *et al*., 1999).

The ecto-NPPase has a broad substrate specificity, capable of hydrolyzing most P2-purinoceptor agonists (Grobben *et al*., 1999). The enzyme has been proposed to play a role in the regulation of purinoceptor signalling by modulation of the extracellular nucleotide concentrations and by shifting P2- into P1-purinoceptor activated signal transduction. In this communication we report the inhibition of the ecto-NPPase by generally used P2Y-purinoceptor antagonists and its effect on the ATP- and ADP-mediated inhibition of the (−)-isoproterenol-induced cyclic AMP-signalling through the P2Y$_1$-like purinoceptor expressed on C6 glioma (Schachter *et al*., 1996).

In C6 glioma cells, the existence of a purinergic P2Y$_1$-like and a pyrimidinergic P2Y$_6$-receptor has been reported (Boyer *et al*., 1993; Lazarowski & Harden, 1994; Lin & Chuang, 1994). The P2Y$_1$-like purinoceptor, activated by ADP > ATP but not by UTP and UDP, is negatively coupled to adenylate cyclase whereas the P2Y$_6$-pyrimidinoceptor (UDP > > UTP and weakly by ATP and ADP) activates phospholipase C. Our results indicated that commonly used P2Y antagonists (e.g. DIDS, RB2, PPADS, PAP and suramin) inhibit the ecto-NPPase of C6 glioma cells to a different extent. The purinoceptor antagonists interfere with the extracellular metabolization of added purine/pyrimidine agonists demonstrating that secondary effects on the purinoceptor-mediated signalling must therefore be taken into account when antagonist and/or agonist rankings are used to characterize purinergic/pyrimidinergic receptors.

Previously, Chen *et al*. (1996) reported the inhibition of ecto-ATPase of C6 by PPADS, suramin and RB2. In contrast to their findings, we did not observe inhibition of the activity of ecto-ATPases at receptor antagonist concentrations that completely abolished the ecto-NPPase activity. On the contrary, our data indicated a significant increase in the activity of ecto-ATPases in the presence of DIDS, PPADS and suramin. These apparently contradictory results are explained by the use of different assays to measure the enzymatic activities. The use of activated charcoal does not allow a discrimination between ecto-ATPase and ecto-NPPase activities. Our protocol based on TLC on PEI-cellulose plates enabled the quantification of ecto-ATPase and ecto-NPPase activities. The decreased ecto-ATPase activity observed by Chen *et al*. (1996) most likely resulted from an inhibition of the ecto-NPPase as demonstrated by our data.

By the use of PPADS, reported as an ineffective antagonist for the P2Y$_1$-like purinoceptor (Boyer *et al*., 1994) but a potent inhibitor of the ecto-NPPase, we were able to show that its effect on the hydrolysis of ATP and ADP modulates the (−)-isoproterenol-induced cyclic AMP synthesis. Previously Pianet *et al*. (1989) demonstrated that (−)-isoproterenol, a β-adrenergic receptor agonist, induced a marked increase of the intracellular cyclic AMP concentration in C6. ATP and ADP inhibited the latter response through activation of a P2Y$_1$-like purinoceptor. In this communication we have been able to demonstrate that inhibition of ecto-NPPase potentiated the effect of ATP and ADP on the (−)-isoproterenol-induced increase in cyclic AMP synthesis. The 3 fold potentiation is due to a higher extracellular nucleotide concentration.

In summary our results support the hypothesis that ecto-NPPase has a modulatory effect on purinoceptor-mediated

signalling in C6 glioma cell cultures. The use of PPADS enabled us to evaluate the effect of nucleotide hydrolysis on the (−)-isoproterenol-induced intracellular cyclic AMP synthesis. Our data also demonstrated that P2Y-purinoceptor antagonists interfered with the extracellular nucleotide metabolism. Therefore, these secondary effects must be taken into account when analysing purinoceptor-mediated signal transduction in C6 glioma cell cultures and may also be extended to a wide variety of cell types expressing ecto-NPPase and other purinoceptor subtypes.

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