Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 474 (2003) 43–51
www.elsevier.com/locate/ejphar

Functional expression of adenosine A_{2A} and A_3 receptors in the mouse dendritic cell line XS-106

John M. Dickenson[a,*], Steve Reeder[a], Bob Rees[a], Steve Alexander[b], Dave Kendall[b]

[a] Department of Life Sciences, Faculty of Science and Mathematics, School of Science, Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS, UK
[b] School of Biomedical Sciences, University of Nottingham, Queen's Medical Centre, Nottingham, NG7 2UH, UK

Received 24 March 2003; received in revised form 20 June 2003; accepted 27 June 2003

Abstract

There is increasing evidence to suggest that adenosine receptors can modulate the function of cells involved in the immune system. For example, human dendritic cells derived from blood monocytes have recently been described to express functional adenosine A_1, A_{2A} and A_3 receptors. Therefore, in the present study, we have investigated whether the recently established murine dendritic cell line XS-106 expresses functional adenosine receptors. The selective adenosine A_3 receptor agonist 1-[2-chloro-6[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N*-methyl-β-D-ribofuranuronamide (2-Cl-IB-MECA) inhibited forskolin-mediated [^{3}H]cyclic AMP accumulation and stimulated concentration-dependent increases in p42/p44 mitogen-activated protein kinase (MAPK) phosphorylation. The selective adenosine A_{2A} receptor agonist 4-[2-[[-6-amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzene-propanoic acid (CGS 21680) stimulated a robust increase in [^{3}H]cyclic AMP accumulation and p42/p44 MAPK phosphorylation. In contrast, the selective adenosine A_1 receptor agonist CPA (N^6-cyclopentyladenosine) did not inhibit forskolin-mediated [^{3}H]cyclic AMP accumulation or stimulate increases in p42/p44 MAPK phosphorylation. These observations suggest that XS-106 cells express functional adenosine A_{2A} and A_3 receptors. The non-selective adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) inhibited lipopolysaccharide-induced tumour necrosis factor-α (TNF-α) release from XS-106 cells in a concentration-dependent fashion. Furthermore, treatment with Cl-IB-MECA (1 μM) or CGS 21680 (1 μM) alone produced a partial inhibition of lipopolysaccharide-induced TNF-α release (when compared to NECA), whereas a combination of both agonists resulted in the inhibition of TNF-α release comparable to that observed with NECA alone. Treatment of cells with the adenosine A_{2A} receptor selective antagonists 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5ylamino]ethyl)phenol (ZM 241385; 100 nM) and 5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5*c*]pyrimidine (SCH 58261; 100 nM) and the adenosine A_3 receptor selective antagonist *N*-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-*c*]quinazolin-5-benzeneacetamide (MRS 1220; 100 nM) partially blocked the inhibitory effects of NECA on lipopolysaccharide-induced TNF-α release. Combined addition of MRS 1220 and SCH 58261 completely blocked the inhibitory effects of NECA on lipopolysaccharide-induced TNF-α release. In conclusion, we have shown that the mouse dendritic cell line XS-106 expresses functional adenosine A_{2A} and A_3 receptors, which are capable of modulating TNF-α release.

Keywords: Adenosine A_3 receptor; Adenosine A_{2A} receptor; Dendritic cell; TNF-α (tumour necrosis factor-α)

1. Introduction

There is increasing evidence to suggest that the purine adenosine can modulate the function of cells involved in the immune system (Di Virgilio et al., 1996). The physiological responses to adenosine are mediated via the activation of specific cell surface receptors and, to date, four adenosine receptors (A_1, A_{2A}, A_{2B} and A_3), belonging to the G protein-coupled receptor superfamily, have been cloned and pharmacologically characterised (Fredholm et al., 2001). Adenosine A_{2A} and A_{2B} receptors are positively coupled to adenylyl cyclase via G_s proteins, whereas adenosine A_1 and A_3 receptors are negatively coupled to adenylyl cyclase via pertussis toxin-sensitive G_i/G_o proteins. Activation of the adenosine A_1 receptor promotes the phagocytic and chemotactic responses of neutrophils, indicating that the adenosine A_1 receptor is pro-inflammatory (Cronstein et al., 1990;

* Corresponding author. Tel.: +44-115-948-6683; fax: +44-115-948-6636.
E-mail address: john.dickenson@ntu.ac.uk (J.M. Dickenson).

0014-2999/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0014-2999(03)02041-7

Zahler et al., 1994). In contrast, the anti-inflammatory actions of adenosine are predominantly mediated through the activation of adenosine A_2 receptors. For example, in human neutrophils, activation of the adenosine A_{2A} receptor mediates the inhibition of neutrophil superoxide generation, adhesion and phagocytosis (Cronstein, 1994). Furthermore, adenosine (probably via adenosine A_2 receptors) has been shown to inhibit the release of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) from activated human monocytes (a precursor of dendritic cells), human endothelial cells and rat Kuppfer cells (an antigen-presenting cell found in the liver) (Bouma et al., 1994, 1996; Reinstein et al., 1994). It has also been reported that activation of the adenosine A_3 receptor inhibits TNF-α release from macrophages (McWhinney et al., 1996). The above findings suggest that adenosine receptors represent potentially important targets for therapeutic intervention in a wide variety of inflammatory disorders such as asthma, rheumatoid arthritis and inflammatory bowel disease.

Dendritic cells are professional antigen presenting cells and, as such, they are vitally important in the activation of the immune response (Banchereau et al., 2000). Understanding basic dendritic cell biology and the signalling molecules involved in their regulation is essential for the future potential use of dendritic cell based therapies for the treatment of cancer and infectious diseases (Banchereau et al., 2000). Interestingly, it has recently been shown that human dendritic cells derived from blood monocytes express functional adenosine A_1, A_{2A} and A_3 receptors (Panther et al., 2001, 2003; Fossetta et al., 2003). We have recently obtained a murine-derived dendritic cell line (XS-106), which may represent a useful model system for studying dendritic cells in culture. Therefore, in the present study we have investigated whether the murine dendritic cell line XS-106 expresses functional adenosine receptors.

2. Materials and methods

2.1. Cell culture

Murine XS-106 dendritic cells and murine NS46 fibroblasts were a generous gift from Dr.A.Takashima (University of Texas). XS-106 cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) foetal calf serum, 0.5 ng/ml murine recombinant granulocyte-macrophage colony-stimulating factor and 5% (v/v) NS46 fibroblast supernatant. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere and subcultured by harvesting floating cells from confluent 75-cm^2 flasks. NS46 fibroblasts were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum and subcultured (1:5 split ratio) using trypsin (0.05% w/v)/EDTA (0.02% w/v). NS46 fibroblast supernatant was obtained by maintaining confluent cells in 75-cm^2 flasks in a minimal volume of RPMI 1640 medium for four days. The supernatant was centrifuged and filtered prior to use.

2.2. Measurement of [^{3}H]cyclic AMP accumulation

XS-106 cells were grown in 24-well plate cluster dishes and, when 80–90% confluent, were incubated for 2 h at 37 °C with 500 μl of Hanks/HEPES buffer (pH 7.4) containing [^{3}H]adenine (37 kBq/well). [^{3}H]Adenine-labelled cells were washed once and then incubated in 1 ml/well Hanks/HEPES buffer containing the cyclic AMP phosphodiesterase inhibitor, rolipram (10 μM) for 15 min at 37 °C. Agonists were added (in 10 μl of medium) 5 min prior to incubation with 3 μM forskolin (10 min). Incubations were terminated by the addition of 50 μl concentrated HCl. [^{3}H]Cyclic AMP was isolated by sequential Dowex-alumina chromatography as previously described (Donaldson et al., 1988). After elution, the levels of [^{3}H]cyclic AMP were determined by liquid scintillation counting.

2.3. Western blot analysis of p42/p44 mitogen-activated protein kinase (MAPK) activation

XS-106 cells were grown in 6-well plate cluster dishes and when 80–90% confluent placed in RPMI 1640 medium containing 0.1% bovine serum albumin for 16 h. Serum-starved cells were then washed once with Hanks/HEPES buffer, pH 7.4, and incubated at 37 °C for 30 min in 500 μl/well of the same medium. Agonists were subsequently added in 500 μl of medium and the incubation continued for 5 min (unless otherwise stated) at 37 °C. Stimulations were terminated by aspiration of the medium and the addition of 300 μl of ice-cold lysis buffer [150 mM NaCl, 50 mM Tris·HCl, 5 mM EDTA, 1% (v/v) IGEPAL CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM Na_3VO_4, 1 mM NaF, 1 mM benzamidine, 0.1 mM phenylmethylsulphonylfluoride, 10 μg/ml aprotinin and 5 μg/ml leupeptin]. Cells were then incubated on ice for 5 min, after which the cell lysates were removed and placed into Eppendorf microcentrifuge tubes and vortexed. Insoluble material was removed by centrifugation and 250 μl of the cell lysate removed and stored at −20 °C until required. Protein determinations were made using the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Protein samples (20 μg) were separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE; 10% acrylamide gel) using a Bio-Rad Mini-Protean III system (1 h at 200 V). Proteins were transferred to nitrocellulose membranes using a Bio-Rad Trans-Blot system (1 h at 100 V in 25 mM Tris, 192 mM glycine and 20% MeOH). Following transfer, the membranes were washed with Tri-buffered saline (TBS) and blocked for 1 h at room temperature with 5% (w/v) skimmed milk powder in TBS. Blots were then incubated overnight at 4 °C with primary antibodies in 5% (w/v) skimmed milk powder dissolved in TBS-Tween 20 (0.1% by volume). Primary antibodies were

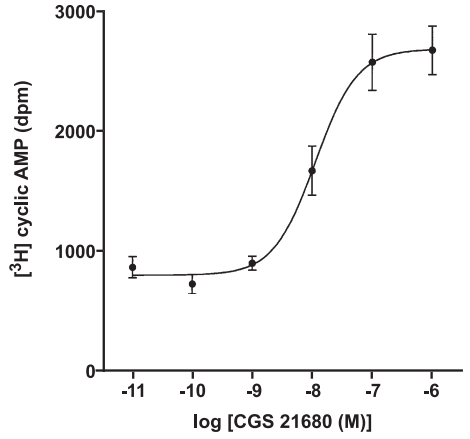

Fig. 1. Adenosine A_{2A} receptor-induced simulation of cyclic AMP accumulation in XS-106 cells. Cells were stimulated with the indicated concentrations of CGS 21680 for 15 min. Data represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in three other independent experiments.

removed and the blot extensively washed with TBS/Tween 20. Blots were then incubated for 2 h at room temperature with the secondary antibodies (swine anti-mouse or anti-rabbit antibody coupled to horseradish peroxidase) at 1:1000 dilution in 5% (w/v) skimmed milk powder dissolved in TBS/Tween 20. Following removal of the secondary antibody, blots were extensively washed as above and developed using the Enhanced Chemiluminescence detection system (Amersham) and quantified using the programme QuantiScan (BioSoft). The uniform transfer of proteins to the nitrocellulose membrane was routinely monitored by transiently staining the membranes with Ponceau S stain (Sigma) prior to application of the primary antibody.

2.4. Measurement of TNF-α release

XS-106 cells (1×10^5/well) in complete RPMI 1640 medium were plated in 24-well plate cluster dishes and left for two days. Cells were then washed with serum-free RPMI 1640 medium and incubated with lipopolysaccharide (1 μg/ml) in the absence or presence of 5′-*N*-ethylcarboxamidoadenosine (NECA) or prostaglandin E_1 for 18 h in RPMI 1640 medium containing 2 mM L-glutamine, 1% (v/v) foetal calf serum and 5% (v/v) NS46 fibroblast supernatant. Where appropriate, adenosine receptor antagonists were added 30 min prior to incubation with lipopolysaccharide and NECA. Following stimulation, supernatants were collected and analysed for TNF-α release using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to the manufacturer's instructions.

2.5. Data analysis

Agonist pEC_{50} values ($-\log EC_{50}$; concentration of drug producing 50% of the maximal response) were obtained by computer assisted curve fitting by use of the computer programme Prism (GraphPAD, CA, USA). Statistical significance was determined by Student's unpaired *t*-test ($P < 0.05$ was considered statistically significant). All data are presented as mean + S.E.M. The *n* in the text refers to the number of separate experiments.

2.6. Materials

[2,8-^{3}H]Adenine was from Amersham International (Aylesbury, Bucks). Aprotinin, bovine serum albumin, N^6-cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dipropyl xanthine (DPCPX), foetal calf serum, forskolin, leupeptin, NECA, prostaglandin E_1 and RPMI 1640 medium were obtained from Sigma (Poole, Dorset, UK). 4-[2-[[-6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzene-propanoic acid (CGS 21680), 1-[2-chloro-6[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N*-methyl-β-D-ribofuranuronamide (2-Cl-IB-MECA), 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5ylamino]ethyl)phenol (ZM 241385) and *N*-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-*c*]quinazolin-5-benzeneacetamide (MRS 1220) were from Tocris Cookson (Bristol, UK). Rolipram was purchased from Calbiochem (Nottingham, UK). Phospho-specific p42/p44 MAPK (Thr^{202}/Tyr^{204}) and total (unphosphorylated) p42/p44 MAPK antibodies were purchased from Sigma. 5-Amino-2-(2-furyl)-7-phenylethyl-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5*c*]pyrimidine (SCH58261) was a kind gift from Dr. Silvio Dionisotti, Schering-Plough, Milan, Italy. All other chemicals were of analytical grade.

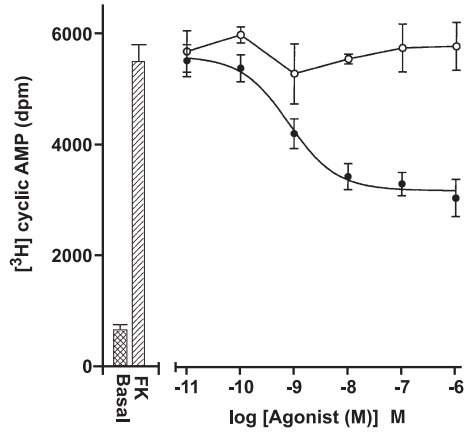

Fig. 2. Effect of Cl-IB-MECA and CPA on forskolin-stimulated cyclic AMP accumulation in XS-106 cells. Cells were initially pre-stimulated for 5 min with the indicated concentrations of Cl-IB-MECA (closed circles) and CPA (open circles) prior to stimulation with 3 μM forskolin for 10 min in the continued presence of agonists. The histograms represent basal and 3 μM forskolin-stimulated [^{3}H]cyclic AMP accumulation. Data represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in three other independent experiments.

3. Results

3.1. Effect of adenosine receptor agonists on cyclic AMP accumulation

In order to establish whether the murine dendritic cell line XS-106 expresses functional adenosine A_1, A_{2A} and A_3 receptors we determined the effects of selective adenosine receptor agonists on intracellular cyclic AMP levels. The selective adenosine A_{2A} receptor agonist CGS 21680 stimulated a robust increase in [^{3}H]cyclic AMP accumulation in XS-106 cells (p[EC_{50}] = 7.9 ± 0.1; n = 4; Fig. 1) suggesting functional expression of adenosine A_{2A} receptors. The selective adenosine A_3 receptor agonist Cl-IB-MECA elicited a concentration-dependent inhibition of forskolin-stimulated [^{3}H]cyclic AMP accumulation in XS-106 cells (p[EC_{50}] = 9.1 ± 0.2; n = 4; Fig. 2). Cl-IB-MECA (1 μM) inhibited 55 ± 7% (n = 4) of the cyclic AMP accumulation induced by 3 μM forskolin. In contrast, the selective adenosine A_1 receptor agonist CPA had no significant effect on forskolin-induced [^{3}H]cyclic AMP accumulation in XS-106 cells (Fig. 2). Overall, these data indicate that the murine dendritic cell line XS-106 expresses functional adenosine A_{2A} and A_3 receptors.

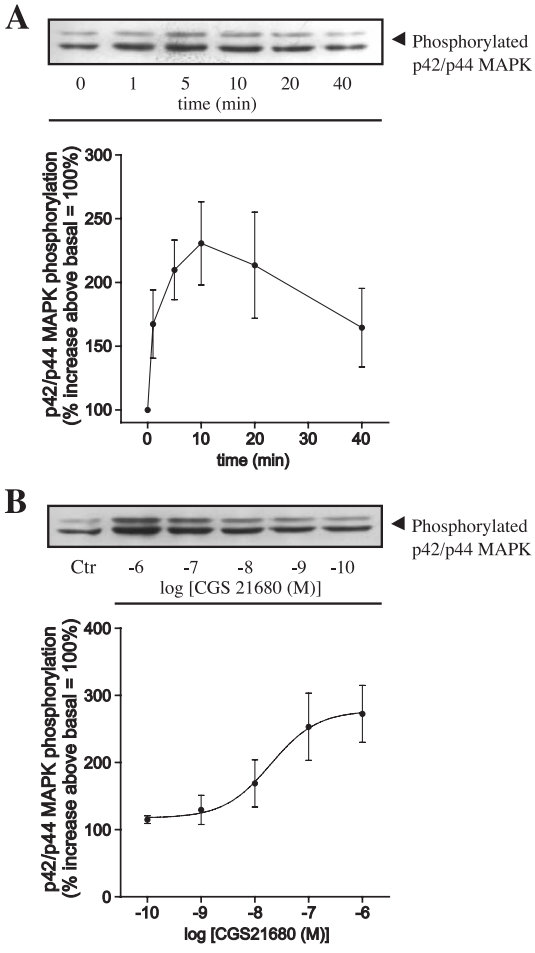

Fig. 3. Adenosine A_{2A} receptor-induced increases in p42/p44 MAPK phosphorylation in XS-106 cells. Cell lysates (20 μg) were analysed for p42/p44 MAPK activation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Representative Western blots for each experiment are shown in the upper panels. (A) Time-course profile for CGS 21680-induced p42/p44 MAPK phosphorylation in XS-106 cells treated with vehicle (time zero) or CGS 21680 (1 μM) for the indicated periods of time. (B) Concentration–response curve for CGS 21680 in XS-106 cells treated with vehicle (control) or the indicated concentrations of CGS 21680 for 10 min. Combined results represent the mean ± S.E.M. from five independent experiments.

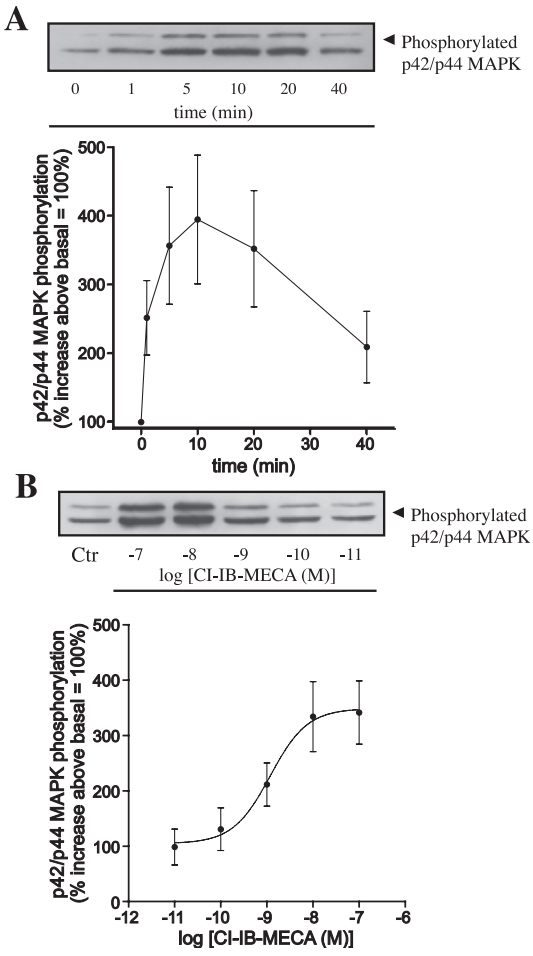

Fig. 4. Adenosine A_3 receptor-induced increases in p42/p44 MAPK phosphorylation in XS-106 cells. Cell lysates (20 μg) were analysed for p42/p44 MAPK activation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Representative Western blots for each experiment are shown in the upper panels. (A) Time-course profile for Cl-IB-MECA-induced p42/p44 MAPK phosphorylation in XS-106 cells treated with vehicle (time zero) or Cl-IB-MECA (100 nM) for the indicated periods of time. (B) Concentration–response curve for Cl-IB-MECA in XS-106 cells treated with vehicle (control) or the indicated concentrations of Cl-IB-MECA for 10 min. Combined results represent the mean ± S.E.M. from five independent experiments.

3.2. Effect of adenosine receptor agonists on p42/p44 MAPK phosphorylation

Previous studies have reported coupling of adenosine A_1, A_{2A} and A_3 receptors to p42/p44 MAPK signalling in a

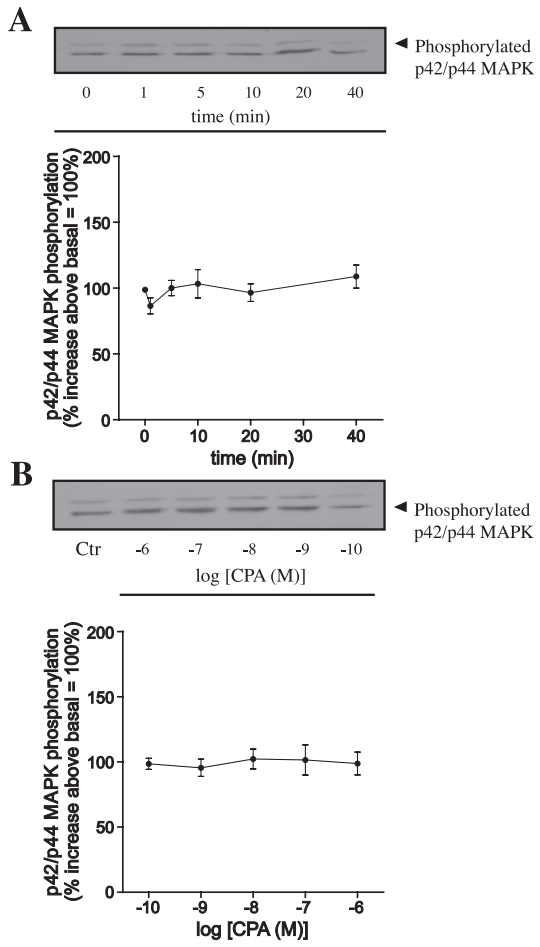

Fig. 5. Effect of the selective adenosine A_1 receptor agonist CPA on p42/p44 MAPK phosphorylation in XS-106 cells. Cell lysates (20 μg) were analysed for p42/p44 MAPK activation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Representative Western blots for each experiment are shown in the upper panels. (A) Time-course profile for the effects of CPA on p42/p44 MAPK phosphorylation in XS-106 cells treated with vehicle (time zero) or CPA (1 μM) for the indicated periods of time. (B) Concentration–response curve for CPA in XS-106 cells treated with vehicle (control) or the indicated concentrations of CPA for 10 min. Combined results represent the mean ± S.E.M. from four independent experiments.

variety of cell types (Seidel et al., 1999; Dickenson et al., 1998; Graham et al., 2001; Schulte and Fredholm, 2002). Therefore, the functional expression of adenosine A_1, A_{2A} and A_3 receptors in XS-106 cells was also explored by measuring levels of p42/p44 MAPK phosphorylation following stimulation of cells with selective adenosine receptor agonists. Increases in p42/p44 MAPK phosphorylation were monitored by Western blot analysis using a phospho-specific p42/p44 MAPK (Thr^{202}/Tyr^{204}) antibody. Stimulation of XS-106 cells with the selective adenosine A_{2A} receptor agonist CGS 21680 produced a rapid and transient increase in p42/p44 MAPK phosphorylation (Fig. 3). The response to CGS 21680 was time-dependent with peak activation occurring at 10 min (Fig. 3A) and concentration-dependent ($p[EC_{50}] = 7.7 \pm 0.1$; $n = 5$; Fig. 3B). Similarly, the selective adenosine A_3 receptor agonist Cl-IB-MECA also stimulated a robust increase in p42/p44 MAPK phosphorylation. The response to Cl-IB-MECA was time-dependent with peak activation occurring at 10 min (Fig. 4A) and concentration-dependent ($p[EC_{50}] = 8.9 \pm 0.2$; $n = 5$; Fig. 4B). Finally, no significant increases in p42/p44 MAPK phosphorylation were observed using the selective adenosine A_1 receptor agonist CPA (see Fig. 5). These observations confirm that the murine dendritic cell line XS-106 expresses functional adenosine A_{2A} and A_3 receptors. This is in contrast to human dendritic cells derived from blood monocytes, which express functional adenosine A_1, A_{2A} and A_3 receptors (Panther et al., 2001).

3.3. Effect of adenosine receptor agonists on TNF-α release

Having established that XS-106 cells express functional adenosine A_{2A} and A_3 receptors, we then investigated whether activation of these receptors modulates lipopolysaccharide-induced TNF-α release. Previous studies have shown that increasing intracellular cyclic AMP levels with prostaglandin E_2 inhibits lipopolysaccharide-stimulated release of TNF-α and interleukin-12 from human monocyte-derived dendritic cells (Gantner et al., 1999; Rieser et al., 1997). In agreement with previous reports, prostaglandin E_1 profoundly inhibited lipopolysaccharide-induced TNF-α release from XS-106 cells in a concentration-dependent fashion ($p[EC_{50}] = 8.0 \pm 0.1$; $n = 4$; see Fig. 6). Similarly, the non-selective adenosine receptor agonist NECA ($p[EC_{50}] = 6.4 \pm 0.2$; $n = 4$) also inhibited TNF-α release in a concentration-dependent manner (Fig. 6). Selective adenosine receptor agonists were then used in order to

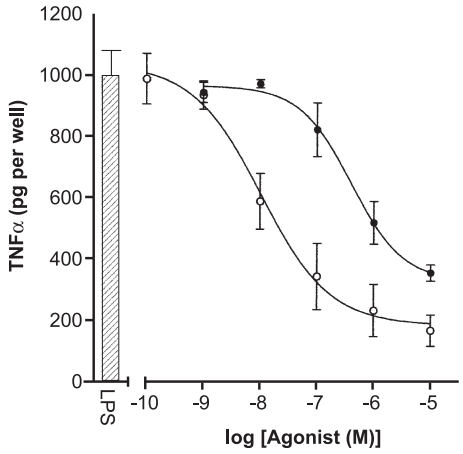

Fig. 6. Effect of prostaglandin E_1 and the non-selective adenosine receptor agonist NECA on lipopolysaccharide-induced TNF-α release from XS-106 cells. Cells were incubated with lipopolysaccharide (1 μg/ml) in the absence or presence of the indicated concentrations of NECA (closed circles) or prostaglandin E_1 (open circles) for 18 h. Following stimulation supernatants were collected and analysed for TNF-α release using an ELISA kit. The results represent mean ± S.E.M. of four independent experiments each performed in duplicate.

determine which adenosine receptor subtype(s) are involved in the inhibition of lipopolysaccharide-induced TNF-α release by NECA. The selective adenosine A_1 receptor agonist CPA (1 μM) had no effect on lipopolysaccharide-induced TNF-α release (Fig. 7). In contrast, Cl-IB-MECA and CGS 21680, selective adenosine A_3 and A_{2A} receptor agonists, respectively, partially inhibited lipopolysaccharide-induced TNF-α release (see Fig. 7). When combined Cl-IB-MECA and CGS 21680 completely blocked lipopolysaccharide-induced TNF-α production. We also investigated the effects of selective adenosine receptor antagonists on NECA-mediated inhibition of lipopolysaccharide-induced TNF-α release. As shown in Fig. 8, the selective adenosine A_1 receptor antagonist DPCPX had no significant effect on the inhibition of lipopolysaccharide-induced TNF-α release by NECA. In contrast, the adenosine A_{2A} receptor selective antagonists ZM 241385 (100 nM) and SCH 58261 (100 nM) and the adenosine A_3 receptor selective antagonist MRS 1220 (100 nM) partially blocked the inhibitory effects of NECA on lipopolysaccharide-induced TNF-α release. The non-selective adenosine receptor antagonist XAC also partially blocked the effects of NECA on lipopolysaccharide-induced TNF-α release. Finally, a combination of MRS 1220 (100 nM) and SCH 58261 (100 nM) completely blocked the inhibitory effects of NECA on lipopolysaccharide-induced TNF-α release. These data indicate that adenosine A_{2A} and A_3 receptors mediate the inhibitory effects of NECA on lipopolysaccharide-induced TNF-α release from XS-106 cells.

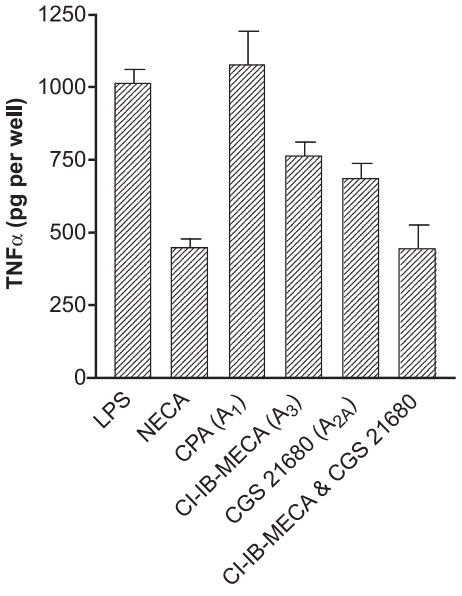

Fig. 7. Effect of selective adenosine receptor agonists on lipopolysaccharide-induced TNF-α release from XS-106 cells. Cells were exposed to lipopolysaccharide (1 μg/ml; 18 h) alone or in the presence of the following adenosine receptor agonists NECA (1 μM; non-selective), CPA (1 μM; A_1 receptor selective), Cl-IB-MECA (1 μM; A_3 receptor selective) and CGS 21680 (1 μM; A_{2A} receptor selective). The results represent mean ± S.E.M. of three independent experiments each performed in duplicate.

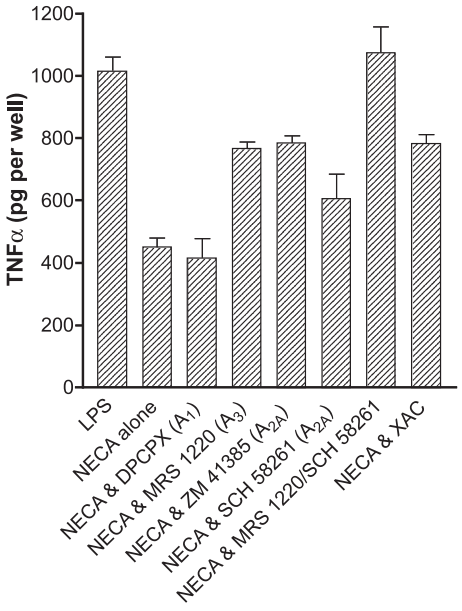

Fig. 8. Effect of selective adenosine receptor antagonists on lipopolysaccharide-induced TNF-α release from XS-106 cells. Cells were pre-treated for 30 min (100 nM each; alone or in combination) with the following adenosine receptor antagonists: DPCPX (A_1 receptor selective), ZM 241385 and SCH 58261 (A_{2A} receptor selective), MRS 1220 (A_3 receptor selective) and XAC (non-selective). Cells were then exposed to NECA (1 μM) in the presence of lipopolysaccharide (1 μg/ml) for 18 h. The results represent mean ± S.E.M. of three independent experiments each performed in duplicate.

4. Discussion

Adenosine is an endogenous immunomodulator that has been shown to exhibit anti-inflammatory and immunosuppressive properties via the activation of adenosine receptors. Four subtypes of adenosine receptor (A_1, A_{2A}, A_{2B} and A_3) have been cloned and are variably expressed on immune cells including neutrophils, lymphocytes, eosinophils, monocytes/macrophages and mast cells (Sullivan and Linden, 1998). However, to date, very few studies have addressed the role of adenosine receptors in modulating dendritic cell function. Dendritic cells are antigen-presenting cells specialised to activate naïve T-lymphocytes and initiate primary immune responses (Banchereau et al., 2000). At present, the vast majority of investigations employing dendritic cells involve the use of cells harvested from spleen or bone marrow of rats and mice or alternatively human blood monocytes differentiated in vitro with interleukin-4, granulocyte-macrophage colony-stimulating factor and TNF-α to produce a dendritic cell phenotype (Romani et al., 1994; Inaba et al., 1992). Unfortunately, these time-consuming methods impose severe constraints upon the amount of information that can be generated concerning

dendritic cell function. Interestingly, recent studies have shown that human monocyte-derived dendritic cells express functional adenosine A_1, A_{2A} and A_3 receptors (Panther et al., 2001, 2003; Fossetta et al., 2003).

There is now available a murine derived dendritic cell line (XS-106), which is capable of processing and presenting antigen to lymphocytes. Previous studies have shown that XS-106 cells express major histocompatibility complex class II antigen as well as CD40, CD80 and CD86 (Matsue et al., 1999). In addition, XS-106 cells express the dendritic cell marker CD11C and induce a robust proliferation of allogeneic T cells. Furthermore, when pulsed with antigen, XS-106 cells induce antigen specific humoral and T cell-mediated immune responses (Timares et al., 1998; Hayashi et al., 2000). Finally, their adhesive properties and cytokine profiles are consistent with dendritic cells, resembling closely "mature" dendritic cells such as cultured Langerhans cells or $CD11C^+$ splenic dendritic cells (Mummert et al., 2000). Therefore, the aim of the present study was to determine whether the murine dendritic cell line XS-106 expresses functional adenosine A_1, A_{2A} and A_3 receptors using receptor selective agonists. The selective adenosine A_{2A} receptor agonist CGS 21680 induced concentration-dependent increases in [^{3}H]cyclic AMP accumulation ($EC_{50} = 12$ nM) and p42/p44 MAPK phosphorylation ($EC_{50} = 19$ nM) in XS-106 cells. These EC_{50} values are comparable to values previously reported for human adenosine A_{2A} receptor-mediated stimulation of adenylyl cyclase (32 nM) and p42/p44 MAPK (circa 10 nM) in transfected Chinese hamster ovary cells (Seidel et al., 1999). Furthermore, the EC_{50} values for CGS 21680 mediated increases in [^{3}H]cyclic AMP and p42/p44 MAPK phosphorylation are similar to K_i values obtained from CGS 21680 binding to rat and human adenosine A_{2A} receptors, 15 and 27 nM, respectively (Klotz, 2000). Unfortunately, although all four adenosine receptor subtypes have been cloned from the mouse (Marquardt et al., 1994), very little is known about their pharmacology. However, these observations indicate that XS-106 cells express functional adenosine A_{2A} receptors.

The selective adenosine A_3 receptor agonist Cl-IB-MECA was used to determine the functional expression of adenosine A_3 receptors in XS-106 cells. Measurements of intracellular [^{3}H]cyclic AMP clearly revealed that Cl-IB-MECA inhibited forskolin-induced [^{3}H]cyclic AMP accumulation suggesting presence of functional adenosine A_3 receptors. In addition, Cl-IB-MECA stimulated increases in p42/p44 MAPK phosphorylation, which is in agreement with previous studies showing coupling of the adenosine A_3 receptor to p42/p44 MAPK signalling (Graham et al., 2001; Schulte and Fredholm, 2002). The EC_{50} values for Cl-IB-MECA-mediated inhibition of forskolin-induced [^{3}H]cyclic AMP accumulation (0.8 nM) and stimulation of p42/p44 MAPK phosphorylation (1.2 nM) are similar to the EC_{50} value recently reported for Cl-IB-MECA-induced Ca^{2+} responses (circa 1 nM) in human immature monocyte-derived dendritic cells (Fossetta et al., 2003). The EC_{50} values for Cl-IB-MECA-mediated functional responses differ from the K_i value of 11 nM reported for Cl-IB-MECA binding to human adenosine A_3 receptors (Klotz, 2000). However, on the basis of published sequences, it is apparent that the mouse adenosine A_3 receptor shows much greater identity with the rat receptor than the human receptor. Blast (http://www.ncbi.nlm.nih.gov/blast/) comparison of the protein sequences (excluding N- and C-termini since ligand binding has been modelled to the intramembrane regions of the adenosine A_3 receptor (Van Galen et al., 1994) indicates 94% identity for mouse:rat receptor comparisons, while mouse/human comparison indicates a much lower degree of homology (76% identity). Thus, although not directly assessed, it is likely that the pharmacology of the mouse adenosine A_3 receptor more closely resembles the rat receptor than the human receptor. Cl-IB-MECA binds to the rat adenosine A_3 receptor with a K_i value of 0.33 nM (Fredholm et al., 2001). Overall, these data clearly indicate that XS-106 cells express functional adenosine A_3 receptors. Finally, the selective adenosine A_1 receptor agonist CPA did not inhibit forskolin-stimulated [^{3}H]cyclic AMP accumulation or increase levels of p42/p44 MAPK phosphorylation indicating that murine XS-106 cells do not express functional adenosine A_1 receptors. These data contrast to those obtained using immature human (monocyte-derived) dendritic cells, which do express adenosine A_1 receptors (Panther et al., 2001).

In order to determine the effect of adenosine receptor activation on XS-106 cell function, we measured TNF-α release from the cells in response to a challenge with lipopolysaccharide. Previous studies have shown that increasing intracellular cyclic AMP levels with prostaglandin E_2 inhibits lipopolysaccharide-stimulated release of TNF-α and interleukin-12 from human monocyte-derived dendritic cells (Gantner et al., 1999; Rieser et al., 1997). In this study, stimulation of XS-106 cells with the non-selective adenosine receptor agonist NECA inhibited lipopolysaccharide-stimulated release of TNF-α. Furthermore, Cl-IB-MECA and CGS 21680, selective adenosine A_3 and A_{2A} receptor agonists respectively, partially blocked lipopolysaccharide-induced TNF-α release, whereas when combined Cl-IB-MECA and CGS 21680 inhibited TNF-α release comparable to that observed with NECA alone. These observations suggest that the inhibitory effects of the non-selective adenosine receptor agonist NECA are mediated via the activation of adenosine A_3 and A_{2A} receptors. The inhibitory effects of NECA were partially reversed by the selective adenosine A_{2A} receptor antagonists ZM 241385 and SCH 58261 and the adenosine A_3 receptor antagonist MRS 1220. It is notable that the combined addition of MRS 1220 and SCH 58261 completely blocked the effects of NECA. These observations are in agreement with previous studies that have reported adenosine A_{2A} receptor-mediated inhibition of pro-inflammatory cytokines such as TNF-α, interleukin-6 and interleukin-8 (Bouma et al., 1994, 1996; Reinstein et al., 1994). The inhibitory effects of the adenosine A_{2A} receptor

stimulation on TNF-α release from XS-106 cells presumably reflect coupling of this receptor to increases in the immunosuppressive second messenger cyclic AMP. The studies of Panther et al. (2001) showed that adenosine A_{2A} receptor activation inhibited interleukin-12 release from human monocyte-derived dendritic cells.

The results presented indicate that adenosine A_3 receptor activation also inhibits lipopolysaccharide-induced TNF-α release from XS-106 cells. The adenosine A_3 receptor has also been shown to inhibit TNF-α release from macrophages (McWhinney et al., 1996). At present, the signal transduction pathway involved in adenosine A_3 receptor-mediated inhibition of TNF-α release from XS-106 cells (this study) and macrophages (McWhinney et al., 1996) is unclear. As mentioned previously, inhibition of TNF-α release is usually associated with G_s-protein-coupled receptor-mediated cyclic AMP production. Interestingly, adenosine A_3 receptors have been shown to induce an increase in intracellular calcium and potentiate Ca^{2+} currents via protein kinase A activation in A6 renal cells (Reshkin et al., 2000) and hippocampal CA3 pyramidal neuronal cells (Fleming and Mogul, 1997). In addition, the adenosine A_3 receptor stimulates cyclic AMP production in human eosinophils (Ezeamuzie and Philips, 2003). However, in this study, we did not observe any stimulatory effects of Cl-IB-MECA on [^{3}H]cyclic AMP accumulation (data not shown) indicating that the adenosine A_3 receptor is not directly coupled to G_s-protein/cyclic AMP accumulation in XS-106 cells. The molecular mechanisms responsible for adenosine A_3 receptor-mediated inhibition of TNF-α release remain to be established.

In conclusion, this study has shown that the murine dendritic cell line XS-106 expresses functional adenosine A_{2A} and A_3 adenosine receptors. XS-106 cells may therefore represent a useful model system cell line for studying further the role of adenosine receptors in modulating dendritic cell function.

Acknowledgements

The work was funded by The Nottingham Trent University.

References

Bouma, M.G., Stad, R.K., Van den Wildenberg, F.A.J.M., Buurman, W.A., 1994. Differential regulatory effects of adenosine on cytokine release by activated human monocytes. J. Immunol. 153, 389–396.

Bouma, M.G., Van den Wildenberg, F.A.J.M., Buurman, W.A., 1996. Adenosine inhibits cytokine release and expression of adhesion molecules by activated human endothelial cells. Am. J. Physiol. 270, C522–C529.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.-J., Pulendran, B., Palucka, K., 2000. Immunology of dendritic cells. Annu. Rev. Immunol. 18, 767–811.

Cronstein, B.N., 1994. Adenosine, an endogenous anti-inflammatory agent. J. Appl. Physiol. 76, 5–13.

Cronstein, B.N., Daguma, L., Nichols, D., Hutchison, A.J., Williams, M., 1990. The adenosine/neutrophil paradox resolved: human neutrophils possess both A_1 and A_2 receptors that promote chemotaxis and inhibit O_2 generation, respectively. J. Clin. Invest. 85, 1150–1157.

Dickenson, J.M., Blank, J.L., Hill, S.J., 1998. Human adenosine A_1 receptor and $P2Y_2$-purinoceptor-mediated activation of the mitogen-activated protein kinase cascade in transfected CHO cells. Br. J. Pharmacol. 124, 1491–1499.

Di Virgilio, F., Ferrari, D., Chiozzi, P., Falzoni, S., Sanz, J.M., dal Susino, M., Mutini, C., Hanau, S., Baricordi, O.R., 1996. Purinoceptor function in the immune system. Drug Dev. Res. 39, 319–329.

Donaldson, J., Brown, A.M., Hill, S.J., 1988. Influence of rolipram on the cyclic 3′, 5′-adenine monophosphate response to histamine and adenosine in slices of guinea-pig cerebral cortex. Biochem. Pharmacol. 37, 715–723.

Ezeamuzie, C.I., Philips, E., 2003. Positive coupling of atypical adenosine A3 receptors on human eosinophils to adenylyl cyclase. Biochem. Biophys. Res. Commun. 300, 712–718.

Fleming, K.M., Mogul, D.J., 1997. Adenosine A_3 receptors potentiate hippocampal calcium current by a PKA-dependent/PKC-independent pathway. Neuropharmacol. 36, 353–362.

Fossetta, J., Jackson, J., Deno, G., Fan, X., Du, K.K., Bober, L., Soude-Bermejo, A., de Bouteiller, O., Caux, C., Lunn, C., Lundell, D., Palmer, R.K., 2003. Pharmacological analysis of calcium responses mediated by the human A_3 adenosine receptor in monocyte-derived dendritic cells and recombinant cells. Mol. Pharmacol. 63, 342–350.

Fredholm, B.B., Ijzerman, A.P., Jacobson, K.A., Klotz, K.-N., Linden, J., 2001. International Union of Pharmacology: XXV. Nomenclature and classification of adenosine receptors. Pharmacol. Rev. 53, 527–552.

Gantner, F., Schudt, C., Wendel, A., Hatzelmann, A., 1999. Characterization of the phosphodiesterase (PDE) pattern of in vitro-generated human dendritic cells (DC) and the influence of PDE inhibitors on DC function. Pulm. Pharmacol. Ther. 12, 377–386.

Graham, S., Combes, P., Crumiere, M., Klotz, K.N., Dickenson, J.M., 2001. Regulation of p42/p44 mitogen-actvated protein kinase by the human adenosine A_3 receptor in transfected CHO cells. Eur. J. Pharmacol. 420, 19–26.

Hayashi, S., Johnston, S.A., Takashima, A., 2000. Induction of Th2-directed immune response by Il-4 transduced dendritic cells in mice. Vaccine 18, 3097–3105.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., Steinman, R.M., 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony stimulating factor. J. Exp. Med. 176, 1693–1702.

Klotz, K.-N., 2000. Adenosine receptors and their ligands. Naunyn-Schmiedeberg's Arch. Pharmacol. 362, 382–391.

Lowry, O.H., Rosebrough, N.J., Farr, A.C., Randall, R.J., 1951. Protein measurements with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Marquardt, D.L., Walker, L.L., Wasserman, S.I., 1994. Adenosine receptors on mouse bone marrow-derived mast cells: functional significance and regulation by aminophylline. J. Immunol. 133, 932–937.

Matsue, H., Matsue, K., Okumura, K., Yagita, H., Takashima, A., 1999. Induction of antigen-specific immunosuppression by CD95L cDNA-transfected 'killer' dendritic cells. Nat. Med. 5, 930–937.

McWhinney, C.D., Dudley, M.W., Bowlin, T.L., Peet, N.P., Schook, L., Bradshaw, M., De, M., borcherding, D.R., Edwards III, C.K., 1996. Activation of adenosine A_3 receptors on macrophages inhibits tumor necrosis factor-α. Eur. J. Pharmacol. 310, 209–216.

Mummert, M.E., Mohamadzadeh, M., Mummert, D.I., Mizumoto, N., Takashima, A., 2000. Development of a peptide inhibitor of hyaluronan-mediated leukocyte trafficking. J. Exp. Med. 192, 769–779.

Panther, E., Idzko, M., Herouy, Y., Rheinen, H., Gebicke-Haerter, P.J., Mrowietz, U., Dichmann, S., Norgauger, J., 2001. Expression and function of adenosine receptors in human dendritic cells. FASEB J. 15, 1963–1970.

Panther, E., Corinti, S., Idzko, M., Herouy, Y., Napp, M., La Sala, A., Girolomoni, G., Norgauer, J., 2003. Adenosine affects expression of membrane molecules, cytokine, and chemokine release, and the T cell stimulatory capacity of human dendritic cells. Blood 101, 3985–3990.

Reinstein, L.J., Lichtman, S.N., Currin, R.T., Wang, J., Thurman, R.G., Lemasters, J.J., 1994. Suppression of lipopolysaccharide-stimulated release of tumor necrosis factor by adenosine: evidence for A2 receptors on rat Kupffer cells. Hepatology 19, 1445–1452.

Reshkin, S.J., Guerra, L., Bagorda, A., Debellis, L., Cardone, R., Li, A.H., Jacobson, K.A., Casavola, V., 2000. Activation of A_3 adenosine receptor induces calcium entry and chloride secretion in A6 cells. J. Membr. Biol. 178, 103–113.

Rieser, C., Bock, G., Klocker, H., Bartsch, G., Thurnher, M., 1997. Prostaglandin E2 and tumor necrosis factor alpha cooperate to activate human dendritic cells: synergistic activation of interleukin 12 production. J. Exp. Med. 186, 1603–1608.

Romani, N., Grumer, S., Brang, D., Kampgen, E., Lenz, A., Trockenbacher, B., Konwalinka, G., Fritsch, P., Steinman, R.M., Schuler, G., 1994. Proliferating dendritic cells progenitors in human blood. J. Exp. Med. 180, 83–93.

Schulte, G., Fredholm, B.B., 2002. Signaling pathway from the human adenosine A_3 receptor expressed in Chinese hamster ovary cells to the extracellular signal-regulated kinase 1/2. Mol. Pharmacol. 62, 1137–1146.

Seidel, M.G., Klinger, M., Freissmuth, M., Holler, C., 1999. Activation of mitogen-activated protein kinase by the A_{2A} adenosine receptor via a rap1-dependent and via a $p21^{ras}$-dependent pathway. J. Biol. Chem. 274, 25833–25841.

Sullivan, G.W., Linden, J., 1998. Role of A_{2A} adenosine receptors in inflammation. Drug Dev. Res. 45, 103–112.

Timares, L., Takashima, A., Johnston, S.A., 1998. Quantitative analysis of the immunopotency of genetically transfected dendritic cells. Proc. Natl. Acad. Sci. U. S. A. 95, 13147–13152.

Van Galen, P.J., van Bergen, A.H., Gallo-Rodriguez, C., Melman, N., Olah, M.E., Ijzerman, A.P., Stiles, G.L., Jacobson, K.A., 1994. A binding site model and structure-activity relationships for the rat A_3 adenosine receptor. Mol. Pharmacol. 45, 1101–1111.

Zahler, S., Becker, B.F., Raschke, P., Gerlach, E., 1994. Stimulation of endothelial adenosine A_1 receptors enhances adhesion of neutrophils in the intact guinea-pig coronary system. Cardiovasc. Res. 28, 1366–1372.