JPP Journal of Pharmacy and Pharmacology

JPP 2001, 53: 1153–1156

Received February 12, 2001
Accepted April 30, 2001
ISSN 0022-3573

Inhibition of NF-κB-mediated gene transcription by the human A_{2B} adenosine receptor in Chinese hamster ovary cells

Patricia Combes and John M. Dickenson

Abstract

NF-κB is a transcription factor that plays a vital role in regulating inducible gene expression in immune and inflammatory responses. In view of the well documented effects of adenosine on immune and inflammatory responses, we have explored whether adenosine A_1, A_{2B} and A_3 receptors regulate NF-κB activity in transfected Chinese hamster ovary (CHO) cells using a luciferase reporter gene construct. No increases in NF-κB activity were observed in CHO-A_1, -A_{2B} and -A_3 cells stimulated with the non-selective adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine. Elevating intracellular cyclic AMP (cAMP) levels using forskolin (direct activator of adenylyl cyclase) and rolipram (type IV, cAMP-specific phosphodiesterase inhibitor), inhibited NF-κB activity in CHO cells. Adenosine A_{2B} receptor stimulation also inhibited NF-κB activity, whereas adenosine A_1 and A_3 receptor activation had no effect. These data reflect the known coupling of adenosine A_{2B} receptors to increases in cAMP. In conclusion, adenosine A_1, A_{2B} and A_3 receptors do not directly activate NF-κB in CHO cells. However, adenosine A_{2B} receptor activation significantly inhibited NF-κB activity. Inhibition of NF-κB activity by the adenosine A_{2B} receptor may contribute to the anti-inflammatory effects of adenosine.

Introduction

The purine adenosine regulates a number of physiological functions through the activation of specific cell surface receptors (Ralevic & Burnstock 1998). Four adenosine receptors (A_1, A_{2A}, A_{2B} and A_3), belonging to the G protein-coupled receptor (GPCR) superfamily, have been cloned and pharmacologically characterized. 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.

Recent studies have shown that adenosine is a potent regulator of immune and inflammatory responses (Cronstein 1994; Di Virgilio et al 1996). The anti-inflammatory action of adenosine is partially related to its ability to modulate the release of pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin (IL-)6 and IL-8 (Bouma et al 1994, 1996; Reinstein et al 1994; McWhinney et al 1996), and attenuate the expression of cell surface adhesion molecules (involved in the development of an inflammatory response) on human endothelial cells (Bouma et al 1996).

The transcription factor NF-κB plays a key role in the regulation of immune and inflammatory responses by modulating gene expression (Barnes & Karin 1997). Inducible genes regulated by NF-κB include pro-inflammatory cytokines, cell

Department of Life Sciences, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK

Patricia Combes, John M. Dickenson

Correspondence: J. M. Dickenson, Department of Life Sciences, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK. E-mail: john.dickenson@ntu.ac.uk

Funding: This work was funded by The Royal Society.

adhesion molecules, cell surface inflammatory receptors and inflammatory enzymes, such as inducible cyclooxygenase-2, nitric oxide synthetase and cytosolic phospholipase A_2 (Barnes & Karin 1997).

Recent studies have indicated that GPCRs are capable of regulating NF-κB activity (Cowen et al 1997; Kranzhöfer et al 1999; Xie et al 2000). Therefore, the aim of the present study was to investigate whether adenosine receptor subtypes regulate NF-κB activity in Chinese hamster ovary (CHO) cells expressing adenosine A_1, A_{2B} and A_3 receptors, together with a NF-κB-dependent luciferase reporter gene plasmid.

Materials and Methods

Materials

5′-*N*-ethylcarboxamidoadenosine (NECA) was obtained from Sigma Chemical Co. (Poole, Dorset, UK). Forskolin and rolipram were from Calbiochem (Nottingham, UK). Dulbecco's modified Eagle's medium (DMEM)/nutrient mix F-12 (1 : 1) and fetal calf serum (FCS) were from Sigma Chemical Co. All other chemicals were of analytical grade.

Cell culture

CHO cells transfected with the human A_1 adenosine receptor (CHO-A_1) were a generous gift from Dr Andrea Townsend-Nicholson and Professor John Shine (Garvan Institute, Sydney, Australia). CHO cells transfected with the human adenosine A_{2B} and A_3 receptors (CHO-A_{2B} and CHO-A_3, respectively) were kindly provided by Professor Klotz (Klotz et al 1998). Cells were cultured in DMEM/nutrient mix F-12 (1 : 1) supplemented with 2 mM L-glutamine and 10 % (v/v) FCS. Cells were maintained at 37°C in a humidified 5 % CO_2 atmosphere and subcultured (1 : 10 split ratio) using trypsin (0.05 %, w/v)/EDTA (0.02 %, w/v).

NF-κB-dependent luciferase measurements

CHO-A_1, -A_{2B} and -A_3 cells were stably transfected with a NF-κB-dependent luciferase reporter plasmid (pkBtkluc) containing three copies of an NF-κB binding site from the immunoglobin κ light chain (a gift from Professor Takemori; see Kashiwada et al 1998). CHO-A_1, -A_{2B} and -A_3 cells were co-transfected with pkBtkluc and pZEOSV (Invitrogen) and then selected and maintained using 500 μg mL^{-1} G418 and 250 μg mL^{-1} zeocin (Invitrogen). Cells were grown in 24-well plate cluster dishes, and when 80–90 % confluent were washed once with serum-free DMEM/F-12 medium before incubating for 24 h in serum-free DMEM/F-12 medium (1 mL/well). Serum-starved cells were stimulated with appropriate agents for 6 h and then washed twice with Dulbecco's phosphate-buffered saline solution (Sigma Chemical Co.) before the addition of 120 μL cell culture lysis reagent (Promega). After shaking for 15 min, the cell lysates were removed and placed into Eppendorf microcentrifuge tubes and vortexed. Cell lysates (20 μL) were then transferred to opaque 96-well plates and luminescence measured using a Dynex Microtiter Plate Luminometer. The luminometer injects 100 μL luciferase assay substrate (Promega) into each sample and determines luminescence over 10 s.

Data analysis

Statistical significance was determined by analysis of variance followed by Dunnett's multiple comparison tests using the computer program Prism (GraphPAD) and $P < 0.05$ was considered statistically significant. All data are presented as mean $\pm$ s.e.m., where n refers to the number of separate experiments.

Results

Potential activation of NF-κB activity by adenosine receptor subtypes

The potential regulation of NF-κB-mediated transcriptional activity by human adenosine A_1, A_{2B} and A_3 receptors was explored in CHO cells using an NF-κB luciferase reporter construct. Stimulation of serum-starved CHO cells (expressing adenosine A_1, A_{2B} and A_3 receptors) with 10 % FCS produced a robust increase in luciferase activity, confirming expression of the NF-κB reporter gene in these cells. However, treatment of CHO-A_1, -A_{2B} and -A_3 cells with the non-selective adenosine receptor agonist NECA (10 μM) did not increase luciferase expression (Table 1). These data indicate that adenosine A_1, A_{2B} and A_3 receptors do not directly activate NF-κB in transfected CHO cells.

Inhibition of NF-κB activity by the adenosine A_{2B} receptor

Elevated levels of cyclic AMP (cAMP) are known to inhibit the activation of NF-κB (see Haraguchi et al 1995). In this study, we investigated the effects of elevating intracellular cAMP levels using forskolin (direct activator of adenylyl cyclase) and rolipram (type IV,

Table 1 Potential regulation of NF-κB after adenosine A_1, A_{2B}, A_3 receptor activation in Chinese hamster ovary (CHO) cells.

Treatment	% Response to FCS		
	CHO-A_1	CHO-A_{2B}	CHO-A_3
10% FCS	100	100	100
Basal	14±2	14±3	19±2
NECA	16±2	15±2	16±2

CHO-A_1, -A_{2B} and -A_3 cells transfected with the NF-κB-dependent luciferase reporter plasmid were serum-starved for 24 h before stimulation for 6 h with 10% fetal calf serum (FCS) or the non-selective adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA; 10 μM). The data are expressed as a percentage of the response to 10% FCS (= 100%) and represent the mean±s.e.m. of four independent experiments each measured in triplicate. No significant changes in luciferase activity were observed after treatment of cells with NECA.

Table 2 Effect of forskolin, rolipram and 5′-*N*-ethylcarboxamidoadenosine (NECA) on NF-κB-mediated luciferase expression in CHO-A_{2B} cells.

Treatment	% Inhibition of response to FCS
Forskolin	60±4*
Rolipram	23±3*
Forskolin and rolipram	56±6*
NECA	46±7*
NECA and rolipram	60±3*

CHO-A_{2B} cells transfected with the NF-κB-dependent luciferase reporter plasmid were serum-starved for 24 h before stimulation for 6 h with 10% fetal calf serum (FCS) alone or in the presence of forskolin (10 μM), rolipram (10 μM) or the non-selective adenosine receptor agonist NECA (10 μM). The effects of forskolin and NECA were also investigated in the presence of rolipram (10 μM). The data are expressed as the percentage inhibition of the response obtained with 10% FCS alone (after subtracting the appropriate basal value). Data represent the mean±s.e.m. of four independent experiments each measured in triplicate. No significant changes in basal luciferase activity were observed after treatment with rolipram or forskolin. *$P < 0.05$, significantly different compared with the response obtained with 10% FCS alone.

cAMP-specific phosphodiesterase inhibitor) on NF-κB activity in CHO cells. As summarized in Table 2, forskolin (10 μM) and rolipram (10 μM) significantly reduced FCS-induced increases in NF-κB activation in CHO-A_{2B} cells. Treating CHO-A_{2B} cells with both forskolin and rolipram produced a similar degree of inhibition of FCS-induced increases in NF-κB as observed for forskolin alone (Table 2). Similar results were also obtained using CHO-A_1 and CHO-A_3 cells (data not shown). Having established that increases in intracellular cAMP levels can regulate NF-κB activity, we then examined whether adenosine A_1, A_{2B} and A_3 receptor stimulation modulates FCS-induced NF-κB activation. Stimulating CHO-A_1 and -A_3 cells with NECA (10 μM) had no significant effect on FCS-induced increases in luciferase activity (data not shown). This presumably reflects the negative coupling of adenosine A_1 and A_3 receptors to adenylyl cyclase via G_i proteins. However, stimulating CHO-A_{2B} cells with NECA (10 μM) produced a significant inhibition of FCS-induced NF-κB activation (summarized in Table 2). Treatment of CHO-A_{2B} cells with NECA (10 μM) in the presence of rolipram (10 μM), further inhibited FCS-induced increases in NF-κB activation (Table 2). These observations indicate that the G_s protein-coupled adenosine A_{2B} receptor is capable of modulating NF-κB transcriptional activity in CHO cells.

Discussion

Previous studies have shown that adenosine can inhibit pro-inflammatory cytokine release (TNF-α, IL-6 and IL-8) and the expression of cell surface adhesion molecules (Bouma et al 1994, 1996; Reinstein et al 1994; McWhinney et al 1996). In this study, we used an NF-κB luciferase reporter gene construct to determine whether the adenosine A_1, A_{2B} and A_3 receptor subtypes regulate NF-κB activity in transfected CHO cells, given that the well documented effects of adenosine on immune and inflammatory responses may involve regulation of NF-κB activity. We found that stimulation of adenosine A_1, A_{2B} and A_3 receptor subtypes had no significant effect on NF-κB activity in transfected CHO cells. Interestingly, the G_i protein-coupled 5-HT_{1A} receptor activates NF-κB in CHO cells (Cowen et al 1997). The inability of adenosine A_1 and A_3 receptors (both G_i protein-linked) to directly stimulate NF-κB activity may be a consequence of receptor number. In the study of Cowen et al (1997) the 5-HT_{1A} receptor was expressed at 2.8 pmol mg^{-1} of protein, whereas in this study the adenosine A_1 receptor and adenosine A_3 receptor were expressed at approximately 1000 and 800 fmol mg^{-1} of protein, respectively (Klotz et al 1998). Alternatively, and in view of the high level of receptor expression, NF-κB activation by the 5-HT_{1A} receptor in CHO cells may reflect coupling of the 5-HT_{1A} receptor to other types of G proteins, for example G_q. Indeed, reports published to date describing GPCR coupling to NF-κB mainly involve G_q protein-coupled receptors, for ex-

ample muscarinic M3 receptors, bradykinin B_2 receptors and α_{1A}-adrenergic receptors (Todisco et al 1999; Minneman et al 2000; Xie et al 2000).

Previous studies have shown that NF-κB activity can be inhibited by increases in intracellular cAMP levels (see Haraguchi et al 1995). In this study we have shown that treatment of CHO cells with agents that elevate intracellular cAMP levels (independent of receptor activation) are capable of inhibiting NF-κB activity. Furthermore, adenosine A_{2B} receptor activation also significantly inhibits NF-κB activity (induced by FCS) in CHO cells. This presumably reflects the coupling of the adenosine A_{2B} receptor in CHO cells to increases in intracellular cAMP (Klotz et al 1998). In contrast, activation of the G_i protein-coupled adenosine A_1 and A_3 receptors did not inhibit (or potentiate) NF-κB activity in CHO cells. It is interesting to note that Schwaninger et al (1997) reported that the adenosine A_{2B} receptor increased IL-6 gene expression via NF-κB activation in primary astrocytes. The ability of the adenosine A_{2B} receptor to activate NF-κB in astrocytes (compared with the inhibition observed in this study using CHO cells) may reflect cell-specific differences in adenosine A_{2B} receptor-mediated cell signalling. The adenosine A_{2B} receptor has been reported to activate phospholipase C (indicative of coupling to G_q protein) in rat astrocytes, and this may explain NF-κB activation by the adenosine A_{2B} receptor in astrocytes (Pilitsis & Kimelberg 1998). However, it is not known whether the adenosine A_{2B} receptor couples to phospholipase C in transfected CHO cells.

In conclusion, this study has shown that transfected human A_1, A_{2B} and A_3 receptors do not directly activate NF-κB in CHO cells. We have shown for the first time that stimulation of the adenosine A_{2B} receptor inhibits NF-κB-mediated gene transcription (adenosine A_1 and A_3 receptors had no effect), presumably via a cAMP-dependent pathway in CHO cells. However, further studies are required to investigate the potential regulation of NF-κB by adenosine receptor subtypes in physiological relevant cell types.

References

Barnes, P. J., Karin, M. (1997) Mechanisms of disease – nuclear factor kappaB – a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* **336**: 1066–1071

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

Cowen, D. S., Molinoff, P. B., Manning, D. R. (1997) 5-Hydroxytryptamine$_{1A}$ receptor-mediated increases in receptor expression and activation of nuclear factor-κB in transfected Chinese hamster ovary cells. *Mol. Pharmacol.* **52**: 221–226

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

Di Virgilio, F., Ferrari, D., Chiozzi, P., Falzoni, S., Ssanz 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

Haraguchi, S., Good, R. A., Day, N. K. (1995) Immunosuppressive retroviral peptides: cAMP and cytokine patterns. *Immunol. Today* **16**: 595–603

Kashiwada, M., Shirakata, Y., Inoue, J.-I., Nakano, H., Okazaki, K., Okumura, K., Yamamoto, T., Nagaoka, H., Takemori, T. (1998) Tumor necrosis factor receptor-associated factor 6 (TRAF6) stimulates extracellular signal-regulated kinase (ERK) activity in CD40 signaling along a ras-independent pathway. *J. Exp. Med.* **187**: 237–244

Klotz, K. N., Hessling, J., Hegler, J., Owman, C., Kull, B., Fredholm, B. B., Lohse, M. J. (1998) Comparative pharmacology of human adenosine receptor subtypes – characterisation of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch. Pharmacol.* **357**: 1–9

Kranzhöfer, R., Browatzki, M., Schmidt, J., Kubler W. (1999) Angiotensin II activates the proinflammatory transcription nuclear factor-κB in human monocytes. *Biochem. Biophys. Res. Commun.* **257**: 826–828

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

Minneman, K. P., Lee, D., Zhong, H., Berts, A., Abbott, K. L., Murphy, T. J. (2000) Transcriptional responses to growth factor and G-protein coupled receptors in PC12 cells: comparison of α_1-adrenergic subtypes. *J. Neurochem.* **74**: 2392–2400

Pilitsis, J. G., Kimelberg, H. K. (1998) Adenosine receptor mediated stimulation of intracellular calcium in acutely isolated astrocytes. *Brain Res.* **798**: 294–303

Ralevic, V., Burnstock, G. (1998) Receptors for purines and pyrimidines. *Pharmacol. Rev.* **50**: 413–492

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 A_2 receptors on rat Kupffer cells. *Hepatology* **19**: 1445–1452

Schwaninger, M., Neher, M., Viegas, E., Schneider, A., Spranger, M. (1997) Stimulation of interleukin-6 secretion and gene transcription in primary astrocytes by adenosine. *J. Neurochem.* **69**: 1145–1150

Todisco, A., Ramamoorthy, S., Pausawasdi, N., Tacey, K. (1999) Carbachol activates IκB kinase in isolated canine gastric parietal cells. *Biochem. Biophys. Res. Commun.* **261**: 877–884

Xie, P., Browning, D. D., Hay, N., Mackman, Ye, R. D. (2000) Activation of NF-κB by bradykinin through a Gα_q- and G$\beta\gamma$-dependent pathway that involves phosphoinositide 3-kinase and Akt. *J. Biol. Chem.* **275**: 24 907–24 914