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Regulation of p42/p44 mitogen-activated protein kinase by the human adenosine A_3 receptor in transfected CHO cells

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Abstract

In this study we have investigated whether the human adenosine A_3 receptor activates p42/p44 mitogen-activated protein kinase (MAPK) in transfected Chinese hamster ovary (CHO) cells (designated CHO–A_3). The high affinity adenosine A_3 receptor agonist IB-MECA (1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide) stimulated time (peak activation occurring after 5 min) and concentration-dependent ($pEC_{50} = 9.0 \pm 0.2$) increases in p42/p44 MAPK in CHO–A_3 cells. Adenosine A_3 receptor-mediated increases in p42/p44 MAPK were sensitive to pertussis toxin and the MAPK kinase 1 inhibitor PD 98059 (2′-amino-3′-methoxyflavone). The broad range protein tyrosine kinase inhibitor genistein and the phosphatidylinositol 3-kinase inhibitors wortmannin and LY 294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one) also blocked adenosine A_3 receptor stimulation of p42/p44 MAPK. In contrast, inhibition of protein kinase C had no significant effect on adenosine A_3 receptor-induced p42/p44 MAPK activation. IB-MECA ($pEC_{50} = 10.1 \pm 0.2$) also increased the expression of luciferase in CHO–A_3 cells transiently transfected with a luciferase reporter gene containing the c-*fos* promoter. Furthermore, IB-MECA-induced increases in luciferase gene expression were sensitive to pertussis toxin, PD 98059, genistein, wortmannin and LY 294002. In conclusion, we have shown that the human adenosine A_3 receptor stimulates p42/p44 MAPK and c-*fos*-mediated luciferase gene expression in transfected CHO cells.

Keywords: Adenosine A_3 receptor; MAD (mitogen-activated protein) kinase; c-*fos* Promoter; CHO (chinese hamster ovary) cell

1. Introduction

The nucleoside adenosine regulates a number of physiological functions through the activation of specific cell surface receptors. To date, four adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3), belonging to the G protein-coupled receptor superfamily, have been cloned and pharmacologically characterised (Ralevic and Burnstock, 1998). The adenosine A_2 receptor subtypes couple to G_s proteins and elevate intracellular cyclic AMP levels, whereas the adenosine A_1 and A_3 receptors couple to members of the G_i family (Ralevic and Burnstock, 1998). To date, known intracellular signalling cascades associated with the adenosine A_3 receptor include inhibition of adenylyl cyclase, activation of phospholipase C and mobilization of intracellular Ca^{2+} (Palmer et al., 1995; Abbracchio et al., 1995; Klotz et al., 2000).

The 42- and 44-kDa mitogen-activated protein kinases (MAPKs) or extracellular signal-regulated kinases (ERK1 and ERK2) are serine/threonine protein kinases involved in the regulation of cell proliferation and differentiation in response to various growth factors (Widmann et al., 1999). The p42/p44 MAPKs phosphorylate a wide range of proteins, including phospholipase A_2 and ribosomal S6 protein kinase ($p90^{rsk}$) in the cytosol and the transcription factor Elk-1 (ets-like) in the nucleus (Karin, 1995; Lewis et al., 1998). Furthermore, activation of the p42/p44 MAPK pathway in most cell types results in the transcription of "immediate-early" genes, which are under the control of the c-*fos* promoter (Karin, 1995). It is now apparent that members of the G protein-coupled receptor family also regulate p42/p44 MAPK signalling (for reviews see Sugden and Clerk, 1997; Van Biesen et al., 1996). These observations suggest p42/p44 MAPKs con-

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tribute to the regulation of cell physiology by G protein-coupled receptors.

Several recent reports have shown that members of the adenosine receptor family can also activate p42/p44 MAPK. For example, adenosine A_{2A} and A_{2B} receptors activate p42/p44 MAPK in primary human endothelial cells and human mast cells, respectively (Feoktistov et al., 1999; Sexl et al., 1997; Seidel et al., 1999). Our own studies have shown that the adenosine A_1 receptor activates p42/p44 MAPK in Chinese hamster ovary (CHO) cells and in DDT_1MF-2 smooth muscle cells (Dickenson et al., 1998; Robinson and Dickenson, 2001). In the present study, we have investigated whether the transfected human A_3 adenosine receptor regulates p42/p44 MAPK and c-*fos* mediated luciferase gene expression in CHO cells.

2. Materials and methods

2.1. Cell culture and cDNA transfection

Chinese hamster ovary cells transfected with the human adenosine A_3 receptor (CHO–A_3 cells) were a generous gift from Professors K. Klotz and M. Lohse, Institute for Pharmacology and Toxicology, University of Würzburg, Germany (Klotz et al., 1998). CHO–A_3 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM)/nutrient F12 (1:1) supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere and subcultured using trypsin (0.05% w/v)/EDTA (0.02% w/v). CHO cells were seeded at 2.6×10^6 cells per 75-cm^2 flask 24 h prior to transient transfection using Lipofectamine (Life Technologies) according to the manufacturer's instructions. Cells were washed once with Opti-MEM (Life Technologies) and then incubated for 5 h at 37°C in 1.2 ml of Opti-MEM containing a total of 40 μg of plasmid DNA and 50 μl of Lipofectamine. After 5 h the transfection mixture was replaced with 20 ml of normal growth medium and cells cultured for a further 24 h.

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

Confluent CHO–A_3 cell monolayers 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 the 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

CHO–A_3 cells were grown in six-well plate cluster dishes and when 80–90% confluent placed in DMEM/F-12 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. Where appropriate kinase inhibitors were added during this incubation period. Agonists were subsequently added in 500 μl of medium and the incubation continued for 5 min (unless otherwise stated) at 37°C. Stimulation's 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 and samples stored at −20°C until required.

Protein samples (20 μg) were separated by Sodium Dodecyl Sulphate/Polyacrylamide Gel Electrophoresis (SDS/PAGE; 10% acrylamide gel) using a Bio-Rad Mini-Protean II 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 phosphate-buffered saline (PBS) and blocked for 1 h at room temperature with 5% (w/v) skimmed milk powder in PBS. Blots were then incubated overnight at 4°C with primary antibodies in 5% (w/v) skimmed milk powder dissolved in PBS–Tween 20 (0.5% by volume). Primary antibodies were removed and the blot extensively washed with PBS–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 PBS–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. Luciferase measurements

Luciferase assays were performed using CHO–A_3 cells transiently transfected with a plasmid containing the full

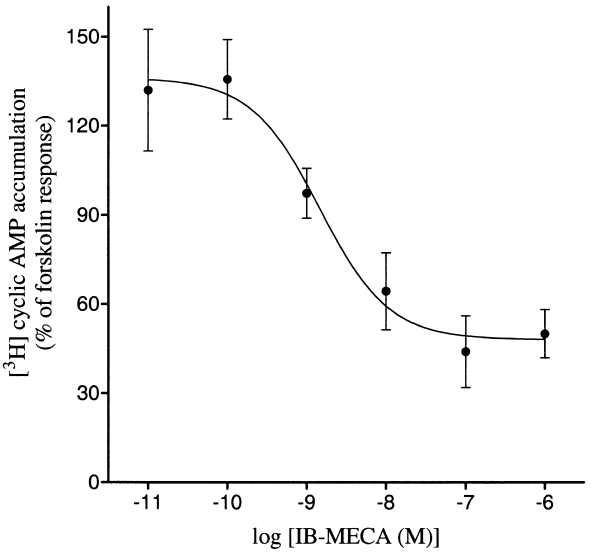

Fig. 1. Adenosine A_3 receptor-mediated inhibition of forskolin-stimulated cyclic AMP accumulation in CHO–A_3 cells. Cells were pre-stimulated for 5 min with the indicated concentrations of IB-MECA prior to stimulation with 3 μM forskolin for 10 min in the continued presence of IB-MECA. Data are expressed as the % of the forskolin response (in the absence of agonist = 100%) and represent the mean ± S.E.M. from four independent experiments each performed in triplicate.

human c-*fos* promoter (−711 to +42) ligated into the *Xho*I site of basic pGL3 luciferase plasmid (Promega; pFosLuc3; generous gift from Professor P. Shaw, School of Biomedical Sciences, University of Nottingham). Cells were grown in 24-well plate cluster dishes and when 80–90% was confluent washed once with serum-free DMEM/F-12 medium, they are incubated for 24 h in 1 ml/well serum-free DMEM/F-12. Serum-starved cells were stimulated with appropriate agonists for 6 h and then washed twice with PBS 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, vortexed and centrifuged (5 min; 12,000 × *g*). Cell lysates (20 μl) were then transferred to opaque 96-well plates and luminescence measured using a Lucy Microtiter Plate Luminometer. The luminometer injects 100 μl of luciferase assay substrate (Promega) into each sample and determines luminescence over 10 s.

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).

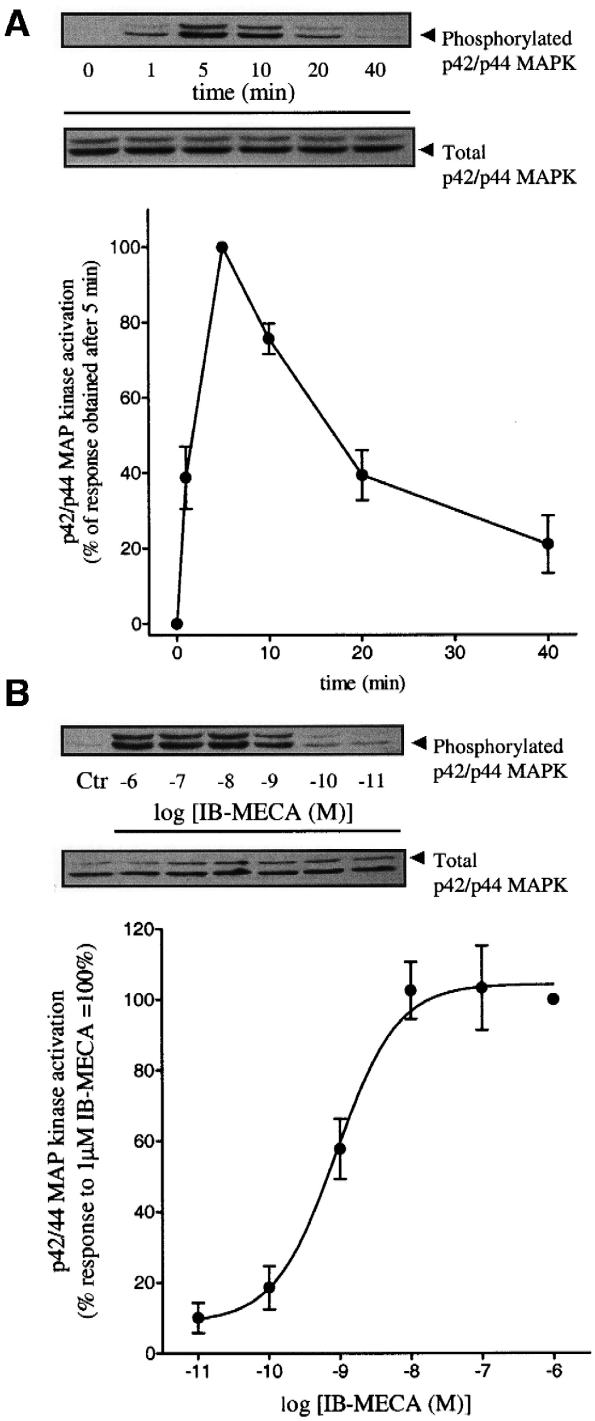

Fig. 2. Adenosine A_3 receptor-mediated activation of p42/p44 MAPK in CHO–A_3 cells. Cell lysates (20 μg) were analysed for p42/p44 MAPK activation by Western blotting using a phospho-specific p42/p44 MAPK antibody. The same samples were subsequently analysed on a separate blot using an antibody that recognises unphosphorylated (total) p42/p44 MAPK to confirm equal loading on each lane. Representative Western blots for each experiment are shown in the upper panels. (A) Time-course profile for IB-MECA-induced p42/p44 MAPK phosphorylation in CHO-A_3 cells treated with vehicle (time zero) or IB-MECA (100 nM) for the indicated periods of time. (B) Concentration–response curve for IB-MECA in CHO-A_3 cells treated with vehicle (control) or the indicated concentrations of IB-MECA for 5 min. Combined results represent the mean ± S.E.M. from six independent experiments.

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, Dulbecco's modified Eagles medium/Nutrient Mix F-12 (1:1), foetal calf serum, forskolin, leupeptin and pertussis toxin were obtained from Sigma (Poole, Dorset, UK). IB-MECA(1-deoxy-1-[6-[[3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide) was from Tocris (Semat Technical, UK). Chelerythrine, Daidzein, genistein, GF 109203X (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide), LY 294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one), PD 98059 (2′-amino-3′-methoxyflavone), PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) and Ro 31-8220 (3-{1-[3-(2-isothioureido) propyl]indol-3-yl}-4-(1-methylindol-3-yl)-3-pyrrolin-2,5-dione), rolipram and wortmannin were from Calbiochem (Nottingham, UK). Phospho-specific p42/p44 MAPK (Thr^{202}/Tyr^{204}) and total (unphosphorylated) p42/p44 MAPK antibodies were purchased from Sigma and New England Biolabs, respectively. All other chemicals were of analytical grade.

3. Results

3.1. Adenosine A_3 receptor-mediated inhibition of cyclic AMP accumulation

In agreement with previous studies using CHO cells transfected with the human adenosine A_3 receptor (Palmer et al., 1997) the high affinity adenosine A_3 receptor agonist IB-MECA elicited a concentration-dependent inhibition of forskolin-stimulated [^{3}H] cyclic AMP accumulation in CHO–A_3 cells (p[EC_{50}] = 9.2 ± 0.3; $n = 4$; Fig. 1). IB-MECA (1 μM) inhibited 50 ± 5% ($n = 4$) of the cyclic AMP accumulation induced by 3 μM forskolin.

3.2. Adenosine A_3 receptor-mediated p42/p44 MAPK activation

The high affinity adenosine A_3 receptor agonist IB-MECA (100 nM) produced a marked increase in the phosphorylation of 42 and 44 kDa MAPK in CHO–A_3 cells (Fig. 2A). Maximal activation was observed after 5 min and then the activity slowly declined towards basal levels. The activation of p42/p44 MAPK by IB-MECA was concentration-dependent, producing a p[EC_{50}] value of 9.0 ± 0.2 ($n = 6$; Fig. 2B). Pre-treatment with pertussis toxin (100 ng/ml for 16 h) completely abolished IB-MECA-induced p42/p44 MAPK activation (Fig. 3A), indicative of adenosine A_3 receptor coupling to the pertussis toxin-sensitive family of G_i-proteins (Ralevic and Burnstock, 1998). In addition, pre-treatment with the MEK1 inhibitor, PD 98059 (30 min; Dudley et al., 1995) inhibited IB-MECA induced activation of p42/p44 MAPK in CHO–A_3 cells in a concentration-dependent manner (Fig. 3B). Recent studies indicate that tyrosine kinases are involved in the regulation of p42/p44 MAPKs by G_i/G_o-protein-coupled receptors (Lopez-Ilasaca, 1998). Treat-

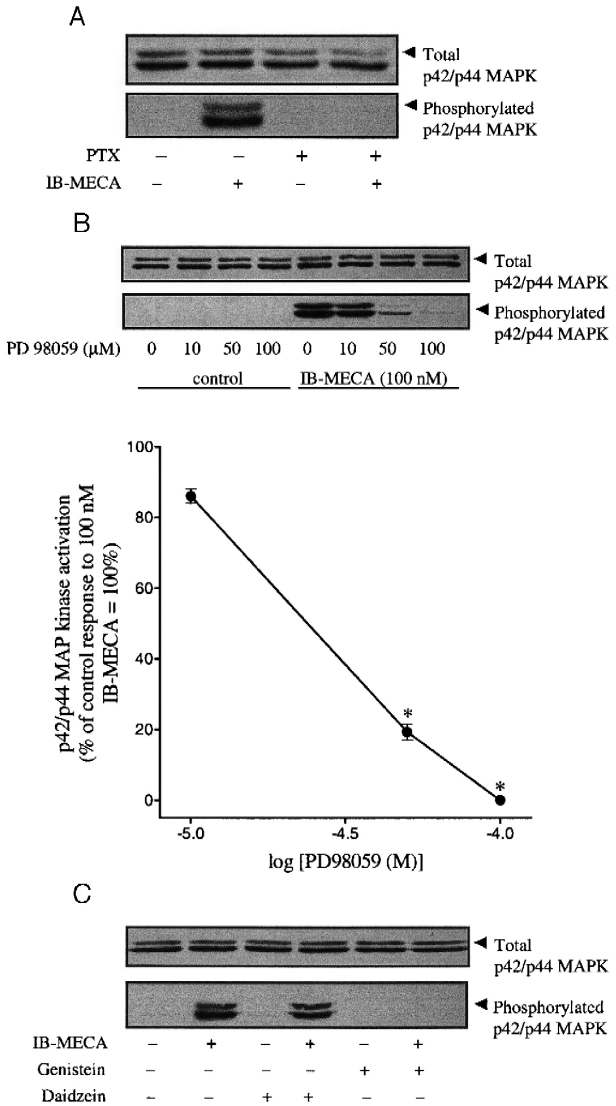

Fig. 3. Effect of MAPK kinase inhibition, pertussis toxin and tyrosine kinase inhibition on adenosine A_3 receptor stimulation of p42/p44 MAPK in CHO–A_3 cells. Representative immunoblots showing the effects of pre-treating CHO–A_3 cells with (A) pertussis toxin (100 ng/ml) for 16; (B) various concentrations of the MAPK kinase inhibitor PD 98059 (30 min) and (C) the broad range tyrosine kinase inhibitor genistein (100 μM; 30 min) and daidzein (100 μM; 30 min) the inactive analogue of genistein. Cells were stimulated with IB-MECA (100 nM) for 5 min. Cell lysates (20 μg) were resolved by SDS-PAGE and transferred to nitrocellulose before being probed with antisera specific for phosphorylated p42/p44 MAPK. The same samples were subsequently analysed on a separate blot using an antibody that recognises unphosphorylated (total) p42/p44 MAPK to confirm equal loading on each lane. Similar results were obtained in at least three independent experiments. * Significantly different ($P < 0.05$) from the control response to 100 nM IB-MECA.

ment of CHO–A_3 cells with the tyrosine kinase inhibitor, genistein (100 μM; 30 min; Akiyama et al., 1987) virtually abolished IB-MECA-induced p42/p44 MAPK activity (92 ± 4% inhibition; $n = 4$; Fig. 3C). In marked contrast, daidzein (100 μM), the inactive analogue of genistein, had no significant effect on IB-MECA-mediated p42/p44 MAPK activity (94 ± 8% of control; $n = 4$; Fig. 3C). These data suggest that adenosine A_3 receptor-mediated MAPK activation in CHO–A_3 cells involves a tyrosine kinase-dependent pathway. There is also increasing evidence for a role of protein kinase C in G_i-protein coupled receptor-mediated p42/p44 MAPK activation (Cussac et al., 1999; Takeda et al., 1999). We therefore determined whether protein kinase C is involved in adenosine A_3 receptor-mediated p42/p44 MAPK activation in CHO–A_3 cells. Pre-treatment of cells with selective inhibitors of protein kinase C, 10 μ M Ro 31-8220 (Davis et al., 1989), 2 μM GF 109203X (Matiny-Brown et al., 1993) and 10 μM chelerythrine (Herbert et al., 1990) had no significant effect on IB-MECA-induced p42/p44 MAPK activation (data not shown).

Phosphatidylinositol 3-kinase has been implicated in G_i protein-coupled receptor-induced activation of p42/p44 MAPK (Sugden and Clerk, 1997; Van Biesen et al., 1996). We have examined the role of phosphatidylinositol 3-kinase

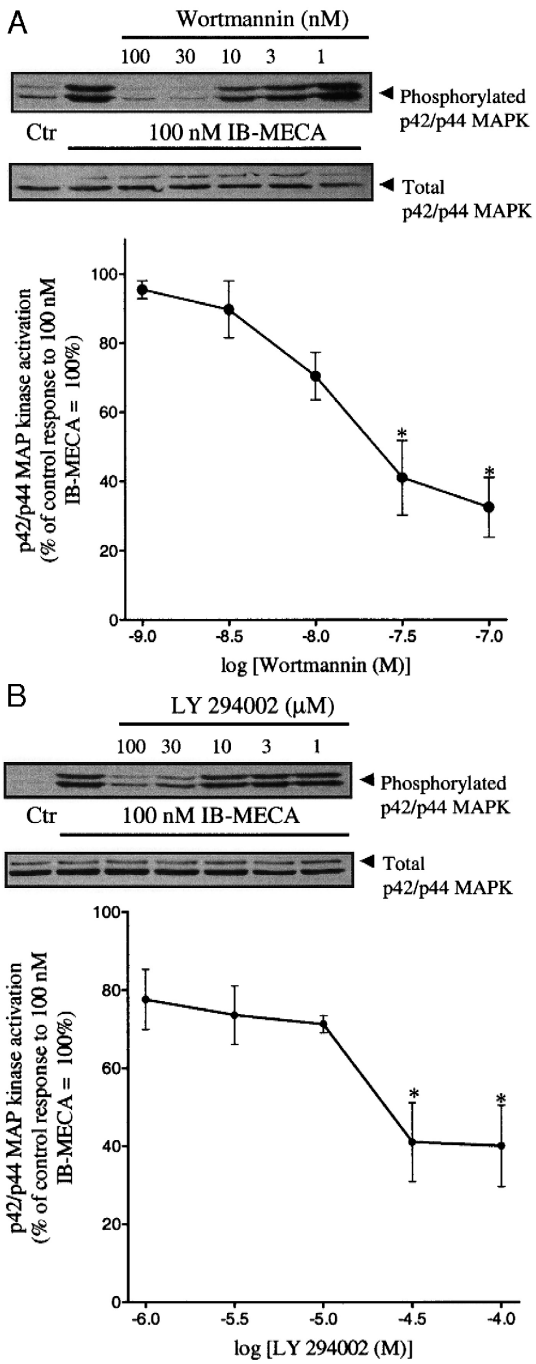

Fig. 4. Role of phosphatidylinositol 3-kinase in adenosine A_3 receptor-mediated activation of p42/p44 MAPK in CHO–A_3 cells. CHO–A_3 cells were pre-incubated (30 min) with the indicated concentrations of the phosphatidylinositol 3-kinase inhibitors (A) wortmannin and (B) LY 294002 before stimulating with 100 nM IB-MECA for 5 min. Cell lysates (20 μg) were analysed for p42/p44 MAPK activation by Western blotting using a phospho-specific p42/p44 MAPK antibody. The same samples were subsequently analysed on a separate blot using an antibody that recognises unphosphorylated (total) p42/p44 MAPK to confirm equal loading on each lane. Representative immunoblots for wortmannin (A) and LY 294002 (B) are shown in the upper panels. Combined results obtained from five independent experiments (mean ± S.E.M.) are shown in the lower panels. Data are presented as the percentage of the control response to 100 nM IB-MECA (100%) in the absence of the phosphatidylinositol 3-kinase inhibitor. * Significantly different ($P < 0.05$) from the control response to 100 nM IB-MECA.

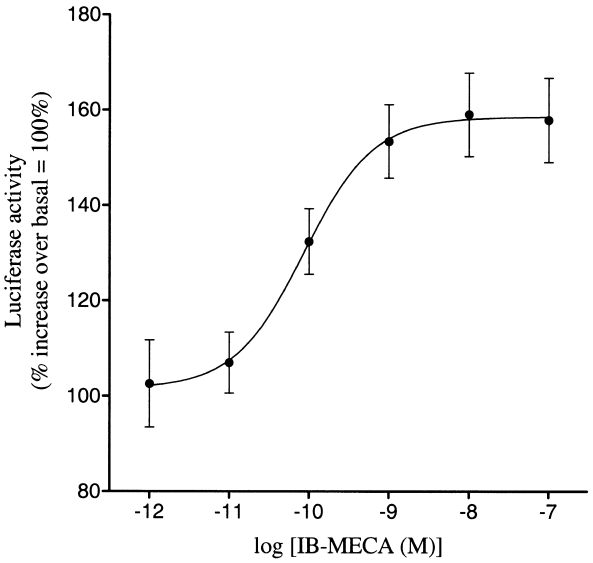

Fig. 5. Adenosine A_3 receptor-mediated stimulation of c-*fos* induced luciferase expression in CHO-A_3 cells. Concentration–response for IB-MECA-induced luciferase expression in transient pFosLuc3 transfectants of CHO-A_3 cells. Data are expressed as the percentage increase above basal luciferase activity (100%) and represent the combined results (mean ± S.E.M.) from five independent experiments each performed in triplicate. For comparison 10% foetal calf serum increased luciferase activity by 271 ± 22% ($n = 5$) above basal levels.

in the regulation of p42/p44 MAPK by the adenosine A_3 receptor in CHO–A_3 cells using the phosphatidylinositol 3-kinase inhibitors wortmannin and LY 294002. As shown in Fig. 4 responses to IB-MECA (100 nM) were inhibited in a concentration-dependent manner following pre-treatment (30 min) of cells with wortmannin and LY 294002. For example, wortmannin (30 nM) and LY 294002 (30 μM) inhibited IB-MECA (100 nM)-induced p42/p44 MAPK responses by $56 \pm 10\%$ ($n = 5$) and $78 \pm 12\%$ ($n = 5$), respectively. These observations indicate that phosphatidylinositol 3-kinase is involved in adenosine A_3 receptor-mediated p42/p44 MAPK activation in CHO–A_3 cells

3.3. Adenosine A_3 receptor-mediated luciferase reporter gene expression

Activation of the p42/p44 MAPK pathway increases the transcription of "immediate-early" genes through activation of the c-*fos* promoter (Karin, 1995). In this study we investigated whether the adenosine A_3 receptor would stimulate gene expression by transiently transfecting CHO–A_3 cells with a luciferase reporter gene plasmid under the transcriptional control of the full human c-*fos* promoter. As clearly shown in Fig. 5, treatment of transient pFosLuc3 transfectants of CHO–A_3 cells with IB-MECA produced a concentration-dependent increase in luciferase expression ($p[EC_{50}] = 10.1 \pm 0.2$; $n = 5$). Furthermore (see Fig. 6), adenosine A_3 receptor-mediated increases in luciferase activity were blocked by pertussis toxin, genistein (100 μM), wortmannin (100 nM) and LY 294002 (30 μM). Interestingly, PD 98059 (50 μM; MEK1 inhibitor) only partially blocked IB-MECA induced luciferase expression. However, 100 μM PD 98059 completely blocked IB-MECA-induced luciferase expression.

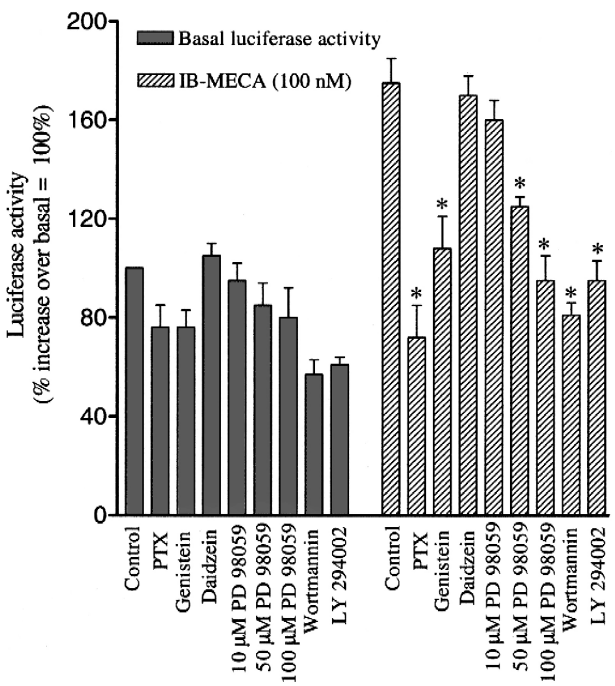

Fig. 6. Effect of pertussis toxin and various kinase inhibitors on adenosine A_3 receptor-mediated luciferase activity in transient pFosLuc3 transfectants of CHO-A_3 cells. Serum-starved transient pFosLuc3 transfectants of CHO-A_3 cells were pre-treated with pertussis toxin (100 ng/ml) for 16 h or for 30 min with genistein (100 μM), daidzein (100 μM), PD 98059 (10, 50 and 100 μM), wortmannin (100 nM) and LY 294002 (30 μM) before stimulating with IB-MECA (100 nM) for 6 h. Data are expressed as the percentage increase above basal luciferase activity (100%). Values represent the mean ± S.E.M. of five independent experiments each measured in triplicate. * Significantly ($P < 0.05$, Student's *t*-test) different from control responses.

4. Discussion

Previous studies have shown that the adenosine A_3 receptor couples to both pertussis toxin-sensitive (G_i2 and G_i3) and insensitive (G_q/G_{11}) G-proteins in transfected CHO cells (Palmer et al., 1995). Furthermore, adenosine A_3 receptor activation in CHO cells leads to the inhibition of adenylyl cyclase and mobilization of intracellular Ca^{2+} (Palmer et al., 1997; Klotz et al., 2000). Interestingly, pertussis toxin treatment blocked both responses suggesting the involvement of G_i/G_o proteins in both the inhibition of adenylyl cyclase (as would be expected) and mobilization of intracellular Ca^{2+}. The adenosine A_3 receptor has also been shown to activate phospholipase C and D in rat brain and rat mast cells, respectively (Ali et al., 1996; Abbracchio et al., 1995). In the present study we have investigated further adenosine A_3 receptor signalling in transfected CHO cells by exploring whether the adenosine A_3 receptor stimulates p42/p44 MAPK and c-*fos* mediated luciferase gene expression.

The data presented clearly indicate that the human adenosine A_3 receptor couples to the p42/p44 MAPK signalling pathway in transfected CHO cells. During the preparation of this manuscript Schulte and Fredholm (2000) also reported adenosine A_3 receptor coupling to p42/p44 MAPK in transfected CHO cells. However, in this study we have extended these findings and have shown that the adenosine A_3 receptor activates p42/p44 MAPK in CHO cells by a pathway involving coupling to pertussis toxin-sensitive G_i/G_o proteins, phosphatidylinositol 3-kinase and tyrosine kinase activation. Previous studies have reported that the activation of p42/p44 MAPK by G_i/G_o protein-coupled receptors involves G protein-derived βγ subunits, genistein-sensitive *c-Src*-related protein tyrosine kinase(s) and phosphatidylinositol 3-kinase activation in a Ras-dependent manner (Koch et al., 1994; Hawes et al., 1995, 1996; Garnovskaya et al., 1996; Luttrell et al., 1996). Our observations suggest that the adenosine A_3 receptor activates p42/p44 MAPK using mechanisms as described for other members of the G_i/G_o-protein coupled receptor family.

In addition to its role in p42/p44 MAPK signalling it is also well established that phosphatidylinositol 3-kinase plays an important role in the regulation of a diverse range of other serine/threonine protein kinases (for a review see Toker, 2000). Kinases activated downstream of phosphatidylinositol 3-kinase include protein kinase B, p70S6 kinase and ribosomal S6 kinase. We are currently investigating whether the adenosine A_3 receptor activates these protein kinases in transfected CHO cells.

There is increasing evidence for a role of protein kinase C in G_i-protein coupled receptor-mediated p42/p44 MAPK activation. For example, Takeda et al. (1999) have shown that lysophosphatidic acid activates MAPK through a Ras-independent pathway that involves phosphatidylinositol 3-kinase-dependent activation of atypical protein kinase C-ζ in African green monkey kidney COS-7 cells. Similarly, the dopamine D_3 receptor couples to p42/p44 MAPK activation via phosphatidylinositol 3-kinase and an atypical protein kinase C isoform in CHO cells (Cussac et al., 1999). In this study the data obtained using a range of protein kinase C inhibitors (with a broad inhibition spectrum including Ro 31-8820, which at high concentrations (>1 μM) blocks the activation of atypical protein kinase C isoforms) indicate that adenosine A_3 receptor-mediated p42/p44 MAPK phosphorylation in CHO cells is independent of protein kinase C activation.

A recent report by Klotz et al. (2000) as shown that the adenosine A_3 receptor mobilizes intracellular Ca^{2+} in CHO cells. Therefore, in this study we investigated the potential role of Ca^{2+} in adenosine A_3 receptor-mediated p42/p44 MAPK. However, removal of extracellular Ca^{2+} or chelation of intracellular Ca^{2+} using the Ca^{2+} chelator BAPTA ([1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) had no significant effect on adenosine A_3 receptor-induced p42/p44 MAPK activation (data not shown).

Known physiological roles of the adenosine A_3 receptor include, hypotension (Fozard and Carruthers, 1993), ischaemic preconditioning of the heart (Lui et al., 1994) and release of inflammatory mediators from mast cells (Ramkumar et al., 1993). However, the signal transduction mechanisms underlying these physiological roles of the adenosine A_3 receptor are largely unknown. Interestingly, recent studies have suggested that p42/p44 MAPK signalling protects cardiac myocytes from oxidative stress (Punn et al., 2000). Therefore, the cardioprotective effects of the adenosine A_3 receptor may involve p42/p44 MAPK activation. Our own preliminary experiments indicate that the adenosine A_3 receptor does activate p42/p44 MAPK signalling in isolated rat cardiac myocytes (Dickenson and Germack; unpublished observations).

In this study we also investigated whether adenosine A_3 receptor-mediated increases in p42/p44 MAPK are capable of triggering gene expression through the c-*fos* promoter. These experiments were conducted using a luciferase reporter gene construct under the transcriptional control of the full human c-*fos* promoter. The c-*fos* promoter can be activated by several different *cis* elements, which include the serum response element (SRE) and the cAMP response element (CRE) (Karin, 1995). Transcription factors that interact with the SRE are phosphorylated by p42/p44 MAPK, whereas protein kinase A and calmodulin-dependent protein kinase, which are activated following increases in intracellular cAMP and Ca^{2+} levels, respectively, phosphorylate transcription factors that interact with the CRE. As depicted in Fig. 5 IB-MECA produced a concentration-dependent increase in luciferase activity. Adenosine A_3 receptor-mediated increases in luciferase gene expression were blocked by pertussis toxin, genistein and the phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002. The MEK1 inhibitor PD 98059 also attenuated IB-MECA-induced increases in luciferase expression in a concentration-dependent manner. It is important to note that the IC_{50} for PD 98059 mediated inhibition of MEK2 (50 μM) is 10-fold higher than that of MEK1 (4 μM; Dudley et al., 1995). This difference in sensitivity of MEK1 and MEK2 to PD 98059 presumably explains the partial inhibition of IB-MECA-induced luciferase expression and p42/p44 MAPK activation observed using 50 μM PD 98059 whereas complete inhibition was obtained using 100 μM PD 98059.

In summary, we have shown that the human adenosine A_3 receptor phosphorylates p42/p44 MAPK and increases c-*fos* mediated gene expression in transfected CHO cells. Both responses were completely blocked by pertussis toxin (indicating the involvement of G_i/G_o proteins) and inhibitors of phosphatidylinositol 3-kinase and tyrosine kinase. Future studies will aim to determine the physiological role of adenosine A_3 receptor-mediated p42/p44 MAPK activation.

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