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Regulation of p42/p44 MAPK and p38 MAPK by the adenosine A_1 receptor in DDT_1MF-2 cells

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Abstract

The mitogen-activated protein kinase (MAPK) family consists of the p42/p44 MAPKs and the stress-activated protein kinases, c-Jun N-terminal kinase (JNK) and p38 MAPK. We have previously reported that the human adenosine A_1 receptor stimulates p42/p44 MAPK in transfected Chinese hamster ovary cells. In this study, we have investigated whether the endogenous adenosine A_1 receptor in the smooth muscle cell line, DDT_1MF-2 activates p42/p44 MAPK, JNK and p38 MAPK. The adenosine A_1 receptor agonist N^6-cyclopentyladenosine stimulated time and concentration-dependent increases in p42/p44 MAPK and p38 MAPK phosphorylation in DDT_1MF-2 cells. No increases in JNK phosphorylation were observed following adenosine A_1 receptor activation. N^6-cyclopentyladenosine-mediated increases in p42/p44 MAPK and p38 MAPK phosphorylation were blocked by the selective adenosine A_1 receptor antagonist 1,3-dipropylcyclopentylxanthine and following pretreatment of cells with pertussis toxin. Furthermore, adenosine A_1 receptor-mediated increases in p42/p44 MAPK were sensitive to the MAPK kinase 1 inhibitor PD 98059 (2′-amino-3′-methoxyflavone), whereas p38 MAPK responses were blocked by the p38 MAPK inhibitor SB 203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole). The broad range protein tyrosine kinase inhibitors genistein and tyrphostin A47 (α-cyano-(3,4-dihydroxy)thiocinnamide) did not block adenosine A_1 receptor stimulation of p42/p44 MAPK. For comparison, insulin-mediated increases in p42/p44 MAPK were blocked by genistein and tyrphostin A47. The Src tyrosine kinase inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) and the epidermal growth factor receptor tyrosine kinase inhibitor AG1478 (4-(3-chloroanilino)-6,7-dimethoxyquinazoline) also had no effect on adenosine A_1 receptor stimulation of p42/p44 MAPK. Furthermore, the protein kinase C inhibitors Ro 31-8220 (3-{1-[3-(2-isothioureido) propyl]indol-3-yl}-4-(1-methylindol-3-yl)-3-pyrrolin-2,5-dione), chelerythrine and GF 109203X (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide) were without effect on adenosine A_1 receptor-induced p42/p44 MAPK phosphorylation. In contrast, wortmannin and LY 294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one), inhibitors of phosphatidylinositol 3-kinase, attenuated adenosine A_1 receptor stimulation of p42/p44 MAPK phosphorylation. In conclusion, the adenosine A_1 receptor stimulates p42/p44 MAPK through a pathway which appears to be independent of tyrosine kinase activation but involves phosphatidylinositol 3-kinase. Finally, adenosine A_1 receptor stimulation in DDT_1MF-2 cells also activated p38 MAPK but not JNK via a pertussis toxin-sensitive pathway.

Keywords: Adenosine A_1 receptor; MAP (mitogen-activated protein) kinase; DDT_1MF-2 cell

1. Introduction

Adenosine regulates a variety of physiological functions through the activation of specific cell surface receptors. 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 (for extensive reviews see Olah and Stiles, 1995; Ralevic and Burnstock, 1998). Adenosine A_1 receptors couple to pertussis toxin-sensitive G proteins (G_{i1}, G_{i2}, G_{i3}, and G_o) and regulate several intracellular signalling pathways including inhibition of adenylyl cyclase, opening of K^+ channels and closing of voltage-sensitive Ca^{2+} channels (Olah and Stiles, 1995). Our previous studies have investigated adenosine A_1 receptor-mediated cell signalling pathways in the hamster vas deferens derived smooth muscle cell line, DDT_1MF-2. As expected, adenosine A_1 receptor stimulation in DDT_1MF-2 cells inhibits adenylyl cyclase activity as measured by the ability of adenosine A_1 receptor agonists to inhibit forskolin-stimulated cyclic AMP accumulation (Dickenson and Hill, 1993a). In addition, to

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the predicted negative coupling to adenylyl cyclase, adenosine A_1 receptor activation in DDT_1MF-2 also stimulates pertussis toxin-sensitive increases in inositol phosphate formation and Ca^{2+} mobilisation (measures of phospholipase C activation; White et al., 1992; Dickenson and Hill, 1993a). More recently, we have shown that the adenosine A_1 receptor also activates protein kinase B in DDT_1MF-2 cells suggesting that the adenosine A_1 receptor may also regulate anti-apoptotic cell signalling pathways (Germack and Dickenson, 2000). These observations clearly demonstrate the diversity of cell signalling pathways regulated by the adenosine A_1 receptor.

Mitogen-activated protein kinases (MAPKs) are a group of serine/threonine protein kinases comprising of three well characterised subfamilies: the p42/p44 extracellular regulated kinases (ERKs), also known as p42/p44 MAPKs; the c-Jun N-terminal kinases (JNKs) which are also known as stress-activated protein kinases and the p38 MAPKs (Denhardt, 1996; Widmann et al., 1999). The p42/p44 MAPKs are primarily associated with the regulation of cell proliferation and differentiation, whereas the JNKs and p38 MAPKs are involved in apoptosis, inflammation and responses to environmental stress (Paul et al., 1997; Ono and Han, 2000). The ERK pathway can be activated by receptor tyrosine kinases (e.g., growth factor receptors), whereas p38 MAPK and JNK are activated by stimuli such as UV irradiation, osmotic stress and inflammatory cytokines (Malarkey et al., 1995; Paul et al., 1997). Recent studies indicate that a wide range of G-protein-coupled receptors also activate the p42/p44 MAPK, p38 MAPK and JNK signalling pathways (for reviews see Sugden and Clerk, 1997; Van Biesen et al., 1996).

Several recent reports have shown that members of the adenosine receptor family activate MAPK signalling pathways. For example, perfusion of isolated rat hearts with adenosine and stimulation of the adenosine A_{2B} receptor in human mast cells results in the activation of p42/p44 MAPK, p38 MAPK and JNK signalling pathways (Haq et al., 1998; Feoktistov et al., 1999). The adenosine A_{2A} receptor has also been shown to activate p42/p44 MAPK in primary human endothelial cells (Sexl et al., 1997; Seidel et al., 1999). We have previously shown that the human adenosine A_1 receptor activates p42/p44 MAPK in transfected Chinese hamster ovary (CHO) cells (Dickenson et al., 1998). In the present study, we have investigated whether the endogenous adenosine A_1 receptor regulates p42/p44 MAPK, p38 MAPK and JNK signalling pathways in DDT_1MF-2 cells.

2. Materials and methods

2.1. Cell culture

The hamster vas deferens smooth muscle cell line (DDT_1MF-2) was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). DDT_1MF-2 cells were cultured in 75-cm^2 flasks in Dulbecco's modified Eagles medium (DMEM) supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere until confluency and subcultured (1:10 split ratio) using trypsin (0.05% w/v)/EDTA (0.02% w/v). Cells for determination of p42/p44 MAPK, p38 MAPK and JNK activation were grown in 6-well cluster dishes.

2.2. Western blot analysis

DDT_1MF-2 cells were grown in 6-well plate cluster dishes and when 80–90% confluent placed in DMEM 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. Each incubation was 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 (sodium dodecyl sulphate), 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 (and discarded) by centrifugation (5 min; 12,000 g) 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.

Aliquots of the cell lysate (20 μg protein) 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 antibody (goat anti-rabbit/mouse 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).

2.3. Data analysis

Agonist p[EC_{50}] values ($-\log EC_{50}$; concentration of drug producing 50% of the maximal response) were ob-

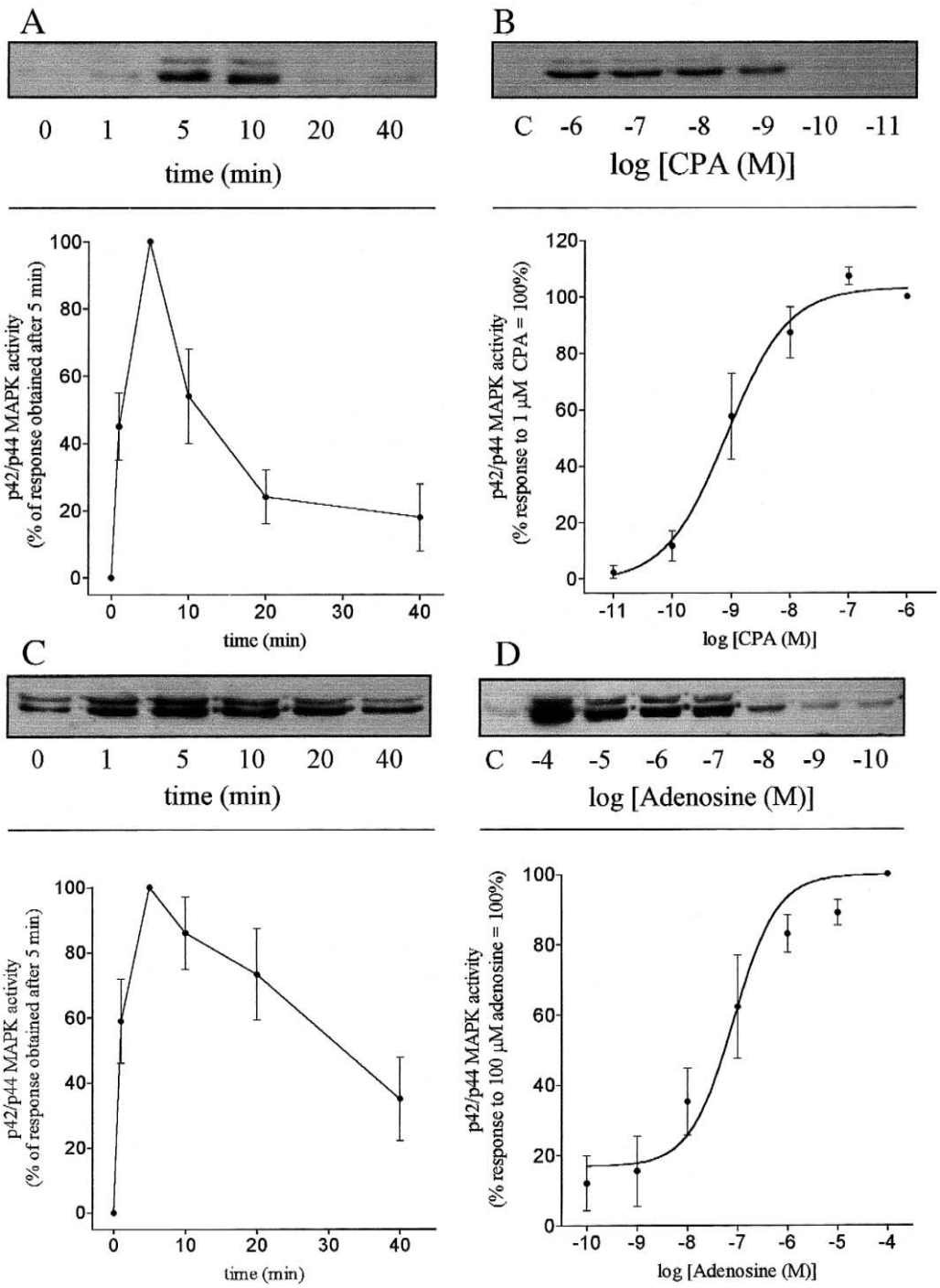

Fig. 1. Adenosine A_1 receptor stimulation of p42/p44 MAPK in DDT_1MF-2 cells. Cell lysates (20 μg) were analysed for p42/p44 MAPK phosphorylation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Representative Western blots for each experiment are shown in the upper panels. Time-course profiles for (A) CPA and (C) adenosine-induced p42/p44 MAPK phosphorylation in DDT_1MF-2 cells treated with vehicle (time 0), CPA (1 μM) or adenosine (100 μM) for the indicated periods of time. Concentration–response curves for (B) CPA and adenosine (D) in DDT_1MF-2 cells treated with vehicle (control) or the indicated concentrations of CPA and adenosine for 5 min. Combined results represent the mean ± S.E.M. from four (A), six (B), six (C) and four (D) independent experiments.

tained 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. Antagonist dissociation constants (K_D) were estimated by a modification of the method of Lazareno and Roberts (1987). A concentration–response curve to an agonist was generated and a concentration (C) of the agonist under study chosen which gave a response greater than 50% of the maximal response. The concentration of antagonist (IC_{50}) required to reduce the response of this concentration (C) of N^6-cyclopentyladenosine (CPA) by 50% was then determined. The agonist concentration–response curve was fitted to a logistic equation as described above and a concentration of the agonist identified (C^1) which yielded a response equivalent to 50% of that produced by concentration C (in the absence of antagonist). The apparent K_D was then determined from the following relationship: $C/C^1 = IC_{50}/K_D + 1$.

2.4. Materials

Aprotinin, bovine serum albumin, N^6-cyclopentyladenosine, Dulbecco's modified Eagles medium, DPCPX (1,3-dipropylcyclo-pentylxanthine), foetal calf serum, insulin, IGEPAL CA-630 (octylphenoxy)polyethoxyethanol), leupeptin and pertussis toxin were obtained from Sigma (Poole, Dorset, UK). AG 1478 (4-(3-chloroanilino)-6,7-dimethoxyquinazoline), BAPTA/AM ([1,2-bis(*o*-amino-5-bromophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetyl-methyl) ester]), chelerythrine, daidzein, epidermal growth factor, 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), Ro 31-8220 (3-{1-[3-(2-isothioureido) propyl]indol-3-yl}-4-(1-methylindol-3-yl)-3-pyrrolin-2,5-dione), SB 203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole) and tyrphostin A47 (α-cyano-(3,4-dihydroxy)thiocinnamide) were from Calbiochem (Nottingham, UK). Phospho-specific p42/p44 MAPK (Thr^{202}/Tyr^{204}) and p38 MAPK (Thr^{180}/Tyr^{182}) antibodies were purchased from Sigma. Phospho-specific JNK (Thr^{183}/Tyr^{185}) antibody was from Promega. All other chemicals were of analytical grade.

3. Results

3.1. Adenosine A_1 receptor-mediated phosphorylation of p42/p44 MAPK

The adenosine A_1 receptor selective agonist N^6-cyclopentyladenosine (CPA; 1 μM) produced a marked increase in p42 and p44 MAPK phosphorylation with dominant phosphorylation of p42 MAPK in DDT_1MF-2 cells (Fig. 1A). Maximal phosphorylation was observed after 5 min and then slowly declined towards basal. The phosphorylation of p42/p44 MAPK by CPA was concentration-dependent, producing a $p[EC_{50}]$ value of 8.98 ± 0.24 ($n = 6$; Fig. 1B). Adenosine also produced time-dependent (Fig. 1C) and concentration-dependent (Fig. 1D) increases in p42/p44 MAPK phosphorylation ($p[EC_{50}] = 7.18 \pm 0.36$; $n = 4$) in DDT_1MF-2 cells. CPA-mediated increases in p42/p44 MAPK phosphorylation were antagonised by the selective adenosine A_1 receptor antagonist DPCPX (1,3-dipropylcyclopentylxanthine) yielding an apparent K_D

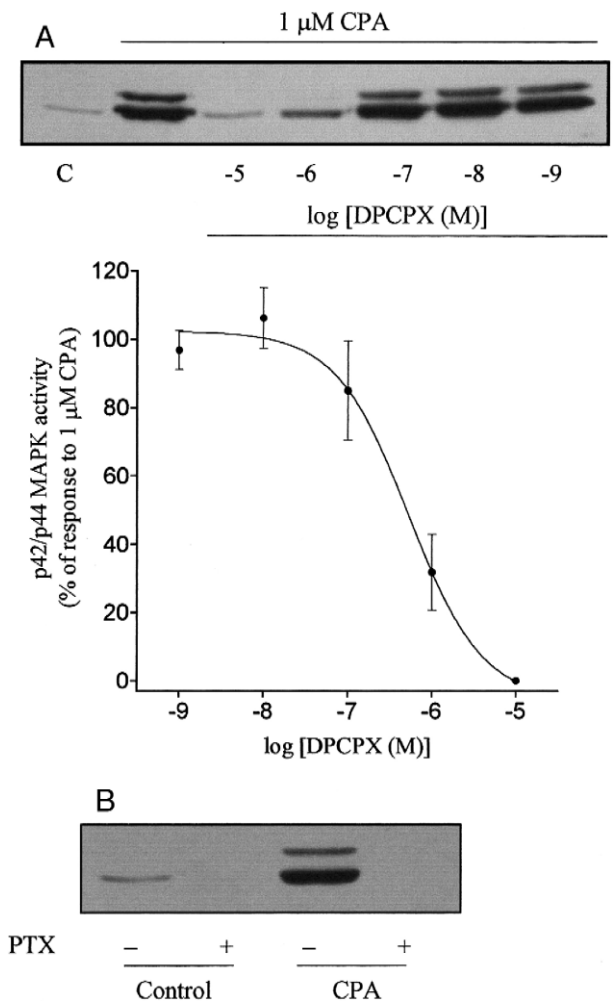

Fig. 2. Effect of the selective adenosine A_1 receptor antagonist DPCPX and pertussis toxin on CPA-induced p42/p44 MAPK phosphorylation. (A) DDT_1MF-2 cells were preincubated for 30 min with the indicated concentrations of the selective adenosine A_1 receptor antagonist DPCPX before stimulating with 1 μM CPA for 5 min. A representative immunoblot showing inhibition by DPCPX is depicted in the upper panel. Combined results obtained from five independent experiments (mean ± S.E.M.) are shown in the lower panel. Data are expressed as the percentage of the control response to 1 μM CPA (100%). (B) Control cells and cells treated with pertussis toxin (100 ng/ml for 16 h) before stimulating with CPA (1 μM) for 5 min. Similar results were obtained in at least four independent experiments.

value of 0.9 ± 0.2 nM ($n = 5$; Fig. 2A). The adenosine A_1 receptor couples to the pertussis toxin-sensitive family of G-proteins (G_{i1}, G_{i2}, G_{i3}, and G_o) (Ralevic and Burnstock, 1998). As expected, pretreatment with pertussis toxin (100 ng ml^{-1} for 16 h) completely abolished CPA-induced p42/p44 MAPK phosphorylation in DDT_1MF-2 cells (Fig. 2B). MAP kinase kinase 1 (MEK1) is responsible for the dual phosphorylation and activation of p42/p44 MAPK (Widmann et al., 1999). Pretreatment with the MEK1 inhibitor, PD 98059 (50 μM; 30 min; Dudley et al., 1995) significantly reduced CPA-induced phosphorylation of p42/p44 MAPK in DDT_1MF-2 cells (79 ± 11% inhibition; $n = 4$; $P < 0.05$; Fig. 3A). Recent studies indicate that tyrosine kinases are involved in the regulation of p42/p44 MAPKs by G_i/G_o-protein-coupled receptors (for review see Lopez-Ilasaca, 1998). We have recently shown that the broad range tyrosine kinase inhibitor, genistein blocked human adenosine A_1 receptor-mediated p42/p44 MAPK activation in transfected CHO cells (Dickenson et al., 1998). In this study, we explored the involvement of tyrosine kinases using genistein, tyrphostin A47 and the Src-family tyrosine kinase inhibitor, PP2 (Hanke et al., 1996). The effects of these tyrosine kinase inhibitors on CPA-induced p42/p44 MAPK activation were compared with responses obtained by activating the insulin receptor (protein tyrosine kinase receptor). Representative immunoblots obtained with each inhibitor are shown in Fig. 3 and the results obtained are summarised in Table 1. These data indicate that adenosine A_1 receptor coupling to p42/p44 MAPK in DDT_1MF-2 cells is independent of tyrosine kinase activation. Recent studies have also demonstrated that G-protein-coupled receptors can activate p42/p44 MAPK via transactivation (ligand-independent) of the epidermal growth factor receptor tyrosine kinase (Zwick et al., 1999). In this study, preincubation of cells with AG1478 (1 μM; 30 min), an inhibitor specific for the epidermal growth factor receptor tyrosine kinase had no significant effect on CPA (1 μM)-mediated p42/p44 MAPK phosphorylation. In contrast, epidermal growth factor (10 nM)-induced p42/p44 MAPK phosphorylation was completely inhibited (10 ± 8% of control response;

Table 1
Effect of tyrosine kinase inhibitors on adenosine A_1 receptor and insulin-mediated p42/p44 MAPK phosphorylation in DDT_1MF-2 cells

Treatment	CPA (% of control)	*n*	Insulin (% of control)	*n*
Genistein (100 μM)	94 ± 12	4	4 ± 2[a]	3
Daidzein (100 μM)	98 ± 7	3	95 ± 9	3
Tyrphostin A47 (100 μM)	103 ± 8	4	8 ± 6[a]	4
PP2 (10 μM)	95 ± 10	4	86 ± 9	4

Serum-starved DDT_1MF-2 cells were preincubated for 30 min with the various tyrosine kinase inhibitors before stimulating with CPA (1 μM) or insulin (100 nM) for 5 min. Values are expressed as a percentage of the response obtained with 1 μM CPA (100%) and 100 nM insulin (100%) in control cells (independent controls were used for each inhibitor). Control cells were pretreated for 30 min with vehicle (0.1% dimethyl sulphoxide). Values represent the mean ± S.E.M. of *n* experiments.

[a] Significantly ($P < 0.05$, Student's *t*-test) different from control responses.

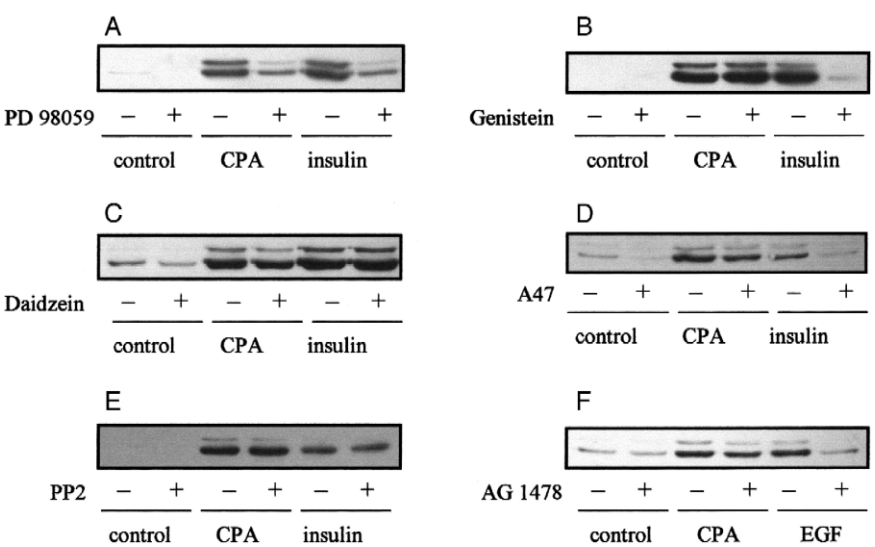

Fig. 3. Effect of MAPK kinase inhibition and various tyrosine kinase inhibitors on adenosine A_1 receptor stimulation of p42/p44 MAPK in DDT_1MF-2 cells. Representative immunoblots showing the effects of pretreating DDT_1MF-2 cells for 30 min with (A) the MAPK kinase inhibitor PD 98059 (50 μM), (B) the broad range tyrosine kinase inhibitor genistein (100 μM), (C) daidzein (100 μM) the inactive analogue of genistein, (D) the broad range tyrosine kinase inhibitor tyrphostin A47 (100 μM), (E) the Src tyrosine kinase inhibitor PP2 (10 μM) and (F) AG1478 (1 μM) the EGF receptor tyrosine kinase inhibitor. Cells were stimulated with CPA (1 μM), insulin (100 nM) or EGF (10 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. Similar results were obtained in at least three independent experiments.

Table 2
Effect of protein kinase C inhibitors and protein kinase C down-regulation on adenosine A_1 receptor and phorbol ester-mediated p42/p44 MAPK phosphorylation in DDT_1MF-2 cells

Treatment	CPA (1 μM) (% of control)	*n*	PMA (1 μM) (% of control)	*n*
Ro 31-8220 (10 μM)	99±8	8	30±5[a]	5
GF 109203X (1 μM)	107±8	6	25±9[a]	4
Chelerythrine (10 μM)	102±10	7	19±6[a]	4
Down-regulation	90±4	5	20±6[a]	4

Serum-starved DDT_1MF-2 cells were preincubated for 30 min with the various protein kinase C inhibitors before stimulating with CPA (1 μM) or phorbol 12-myristate 13-acetate (PMA, 1 μM) for 5 min. Protein kinase C down-regulation was achieved by preincubating DDT_1MF-2 cells for 36 h with phorbol 12-myristate 13-acetate (PMA, 1 μM) before stimulating with CPA (1 μM) or PMA (1 μM) for 5 min. Values are expressed as a percentage of the response obtained with 1 μM CPA (100%) and 1 μM phorbol 12-myristate 13-acetate (100%) in control cells (independent controls were used for each inhibitor). Control cells were pretreated for 30 min with vehicle (0.1% dimethyl sulphoxide). Values represent the mean±S.E.M. of *n* experiments.

[a]Significantly ($P < 0.05$, Student''s *t*-test) different from control responses.

$n = 4$; Fig. 3E) by AG1478 (1 μM). These data suggest that epidermal growth factor receptor transactivation is not involved in adenosine A_1 receptor-mediated p42/p44 MAPK activation in DDT_1MF-2 cells.

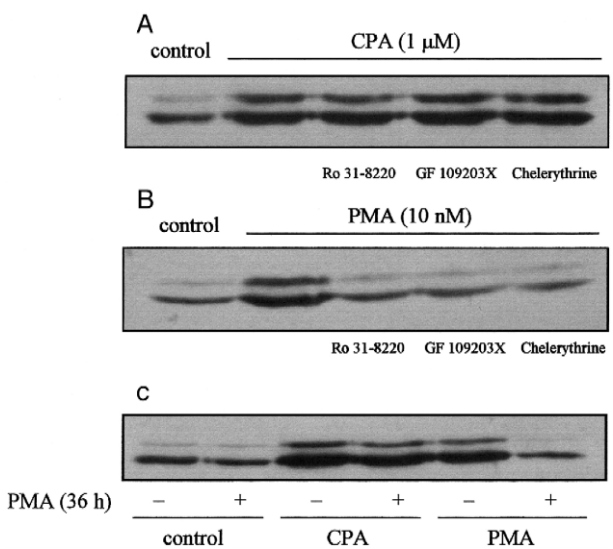

Fig. 4. Role of protein kinase C in adenosine A_1 receptor stimulation of p42/p44 MAPK in DDT_1MF-2 cells. DDT_1MF-2 cells were pretreated for 30 min with the protein kinase C inhibitors Ro 31-8220 (10 μM), GF 109203X (1 μM) and chelerythrine (10 μM) before stimulating with (A) 1 μM CPA or (B) 1 μM phorbol 12-myristate 13-acetate (PMA) for 5 min. (C) DDT_1MF-2 cells were preincubated for 36 h with phorbol 12-myristate 13-acetate (PMA, 1 μM) before stimulating with CPA (1 μM) or PMA (1 μM) 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. Similar results were obtained in at least four independent experiments.

3.2. Role of protein kinase C and Ca^{2+} in adenosine A_1 receptor-induced p42/p44 MAPK phosphorylation

Our previous studies have shown that the adenosine A_1 receptor stimulates phospholipase C in DDT_1MF-2 cells via pertussis toxin-sensitive G-proteins (White et al., 1992). Phospholipase C activation generates the second messengers inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, which mobilise intracellular Ca^{2+} and activate certain protein kinase C isoforms, respectively (Berridge, 1993). Indeed, the adenosine A_1 receptor in DDT_1MF-2 cells stimulates intracellular Ca^{2+} release, Ca^{2+} influx and increases in protein kinase C activity (Dickenson and Hill,

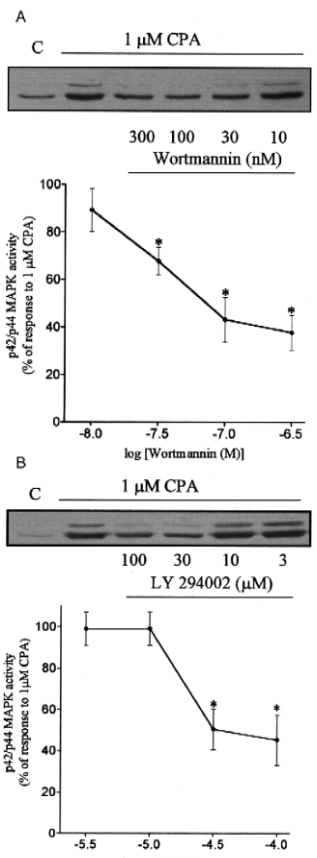

Fig. 5. Role of phosphatidylinositol 3-kinase in adenosine A_1 receptor-mediated phosphorylation of p42/p44 MAPK in DDT_1MF-2 cells. DDT_1MF-2 cells were preincubated for 30 min with the indicated concentrations of the phosphatidylinositol 3-kinase inhibitors (A) wortmannin and (B) LY 294002 before stimulating with 1 μM CPA 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. 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 1 μM CPA (100%) in the absence of PI-3K inhibitor. * Significantly different ($P < 0.05$) from the control response to 1 μM CPA.

1993b; Gerwins and Fredholm, 1995). In this study, we have examined the role of Ca^{2+} and protein kinase C in the regulation of p42/p44 MAPK by the adenosine A_1 receptor in DDT_1MF-2 cells. The role of Ca^{2+} influx was explored by measuring p42/p44 MAPK responses in the absence of extracellular Ca^{2+} (using nominally Ca^{2+}-free Hanks/HEPES buffer containing 0.1 mM EGTA). This procedure prevents adenosine A_1 receptor-mediated Ca^{2+} influx in DDT_1MF-2 cells (Dickenson and Hill, 1993b). Removal of extracellular Ca^{2+} had no significant effect on CPA (1 μM; 98 ± 9% of control response; $n = 4$) induced p42/p44 MAPK phosphorylation. The potential role of Ca^{2+} derived from intracellular stores was investigated using the Ca^{2+} chelator BAPTA (cells were preincubated for 30 min with 50 μM BAPTA/AM) in the absence of extracellular Ca^{2+}. Loading cells with BAPTA in the absence of extracellular Ca^{2+} did not inhibit CPA (94 ± 12% of control; $n = 4$)-induced p42/p44 MAPK phosphorylation. These observations demonstrate that p42/p44 MAPK activation by adenosine A_1 receptor is independent of Ca^{2+} elevation in DDT_1MF-2 cells.

The role of protein kinase C in the regulation of p42/p44 MAPK by the adenosine A_1 receptor was explored using selective inhibitors of protein kinase C, 10 μ M Ro 31-8220 (Davis et al., 1989), 1 μM GF 109203X and (Matiny-Brown et al., 1993) and 10 μM chelerythrine (Herbert et al., 1990). As summarised in Table 2, protein kinase C inhibition had no significant effect on 1 μM CPA-induced p42/p44 MAPK phosphorylation in DDT_1MF-2 cells (representative blots are shown in Fig 4A). In contrast, increases in p42/p44 MAPK phosphorylation elicited by phorbol 12-myristate 13-acetate (1 μM) were sensitive to 10 μ M Ro 31-8220, 1 μM GF 109203X 10 μM chelerythrine (summarised in Table 2 and shown in Fig. 4B). Finally, the potential involvement of protein kinase C was also determined following down-regulation

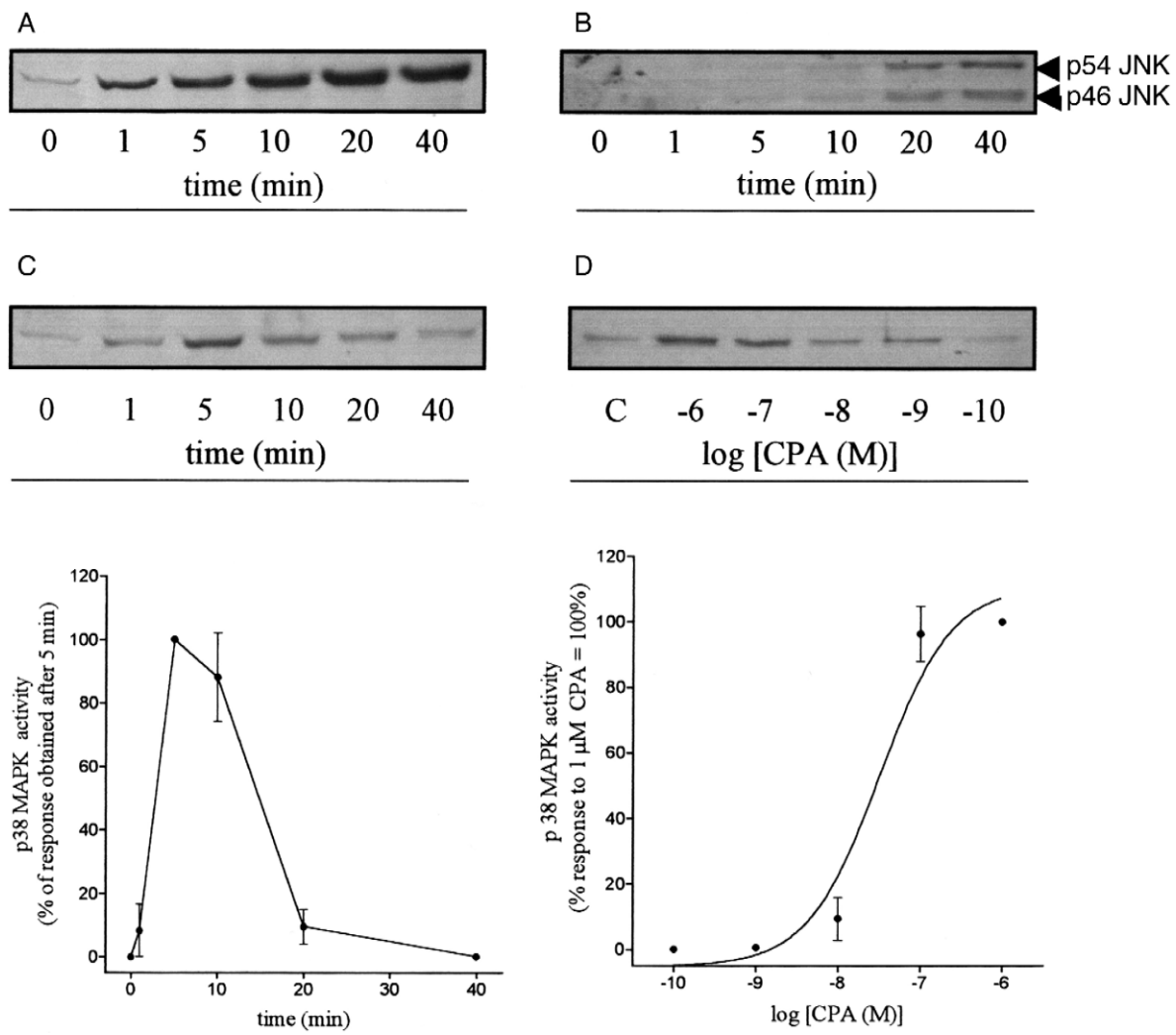

Fig. 6. Adenosine A_1 receptor stimulation of p38 MAPK in DDT_1MF-2 cells. DDT_1MF-2 cells were treated with 0.5 M sorbitol for the indicated periods of time. Cell lysates were resolved by SDS/PAGE and transferred to nitrocellulose before being probed with antisera specific for (A) phospho p38 MAPK and (B) phospho JNK. Stimulation of DDT_1MF-2 cells with CPA produced (C) time-dependent (using 1 μM CPA) and (D) concentration-dependent increases in p38 MAPK phosphorylation (5 min stimulation). Combined results obtained from four independent experiments (mean ± S.E.M.) are shown in the lower panels.

of protein kinase C by pretreating DDT_1MF-2 cells with the protein kinase C activator phorbol 12-myristate 13-acetate (1 μM; 36 h). As shown in Fig. 4C, the subsequent activation of p42/p44 MAPK by phorbol 12-myristate 13-acetate (1 μM) was abolished; however, responses to CPA (1 μM) were unaffected in protein kinase C down-regulated cells.

3.3. Effect of phosphatidylinositol 3-kinase inhibition on adenosine A_1 receptor-induced p42 / p44 MAPK phosphorylation

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). Indeed, we have previously reported that human adenosine A_1 receptor stimulation of p42/p44 MAPK in transfected CHO cells is sensitive to the phosphatidylinositol 3-kinase inhibitors wortmannin and LY 294002 (Dickenson et al., 1998). In this study, we have examined the role of phosphatidylinositol 3-kinase in the regulation of p42/p44 MAPK by the adenosine A_1 receptor in DDT_1MF-2 cells. As shown in Fig. 5, responses to CPA (1 μM) were inhibited following pretreatment (30 min) of cells with wortmannin and LY 294002 For example, wortmannin (100 nM) and LY 294002 (100 μM) inhibited CPA (1 μM)-induced p42/p44 MAPK responses by $57 \pm 8\%$ ($n = 5$) and $55 \pm 11\%$ ($n = 5$), respectively. These observations clearly demonstrate that a phosphatidylinositol 3-kinase-dependent pathway is involved in adenosine A_1 receptor-mediated p42/p44 MAPK activation in DDT_1MF-2 cells.

3.4. Effect of adenosine A_1 receptor stimulation on SAPK / JNK and p38 MAPK phosphorylation

We initially determined whether osmotic stress stimulates p38 MAPK and JNK activation in DDT_1MF-2 cells. Sorbitol (0.5 M) treatment activated p38 MAPK (Fig. 6A) and the 46 and 54 kDa isoforms of JNK (Fig. 6B) in DDT_1MF-2 cells, although with different time-course profiles. Stimulation of DDT_1MF-2 cells with CPA produced time-dependent (peak activation occurring at 5 min; Fig. 6C) and concentration-dependent ($p[EC_{50}] = 8.1 \pm 0.3$; $n = 4$; Fig. 6D) increases in p38 MAPK phosphorylation. However, CPA (1 μM) did not stimulate measurable increases in JNK phosphorylation in DDT_1MF-2 cells during time course experiments conducted up to 40 min (data not shown). CPA-mediated increases in p38 MAPK phosphorylation were antagonised by the selective adenosine A_1 receptor antagonist DPCPX yielding an apparent K_D value of 1.2 ± 0.1 nM ($n = 5$; Fig. 7A). These data indicate that CPA-stimulated increases in p38 MAPK phosphorylation are mediated through the adenosine A_1 receptor in DDT_1MF-2 cells. Finally, pretreatment of DDT_1MF-2 cells with the p38 MAPK inhibitor SB 203580 (20 μM) and

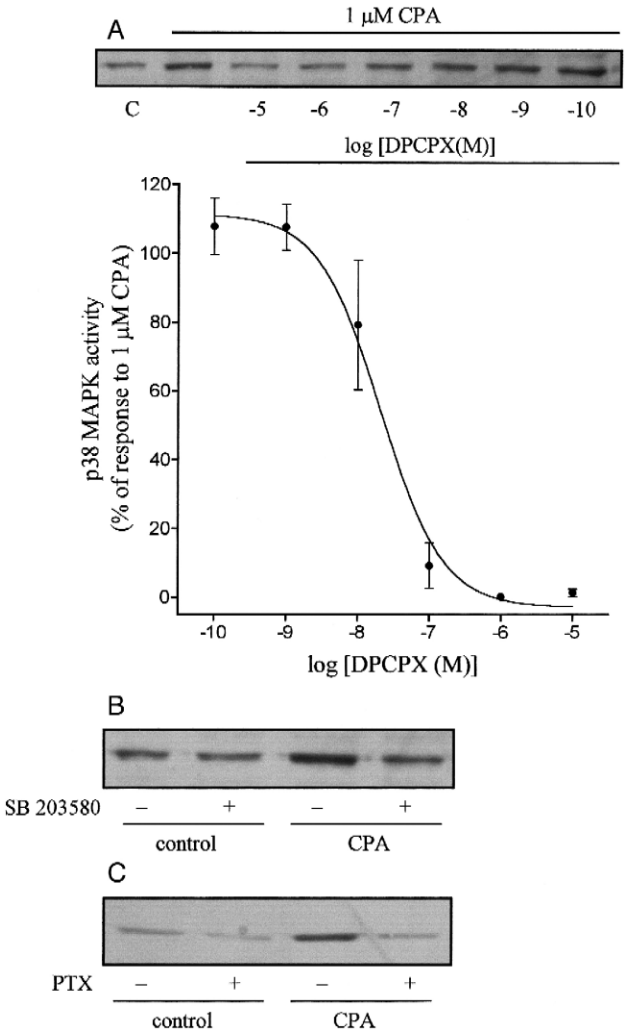

Fig. 7. Effect of the selective adenosine A_1 receptor antagonist DPCPX, pertussis toxin and SB 203580 on CPA-induced p38 MAPK phosphorylation. (A) DDT_1MF-2 cells were preincubated for 30 min with the indicated concentrations of the selective adenosine A_1 receptor antagonist DPCPX before stimulating with 1 μM CPA for 5 min. A representative immunoblot showing inhibition by DPCPX is depicted in the upper panel. Combined results obtained from five independent experiments (mean ± S.E.M.) are shown in the lower panel. Data are expressed as the percentage of the control response to 1 μM CPA (100%). (B) Control cells and cells treated with pertussis toxin (16 h, 100 ng/ml) before stimulating with CPA (1 μM) for 5 min. (C) Cells were pretreated for 30 min with the p38 MAPK inhibitor SB 203580 (20 μM) prior to stimulating with 1 μM CPA for 5 min. Similar results were obtained in at least four independent experiments.

pertussis toxin (100 ng/ml for 16 h) inhibited 1 μM CPA-induced p38 MAPK phosphorylation by $91 \pm 7\%$ ($n = 5$; Fig. 7B) and $95 \pm 7\%$ ($n = 4$; Fig. 7C), respectively.

4. Discussion

We have previously reported that the human adenosine A_1 receptor activates p42/p44 MAPK signalling in trans-

fected CHO cells (Dickenson et al., 1998). In this study, using DDT_1MF-2 smooth muscle cells, we have shown for the first time that an endogenously expressed adenosine A_1 receptor stimulates p42/p44 MAPK activation. The activation of p42/p44 MAPK by G_i/G_o-protein-coupled receptors involves G protein-derived $\beta\gamma$ 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; Igishi and Gutkind, 1998). Indeed, our previous studies have shown that human adenosine A_1 receptor coupling to p42/p44 MAPK activation in transfected CHO cells is blocked by the tyrosine kinase inhibitor genistein and by the phosphatidylinositol 3-kinase inhibitors wortmannin and LY 294002 (Dickenson et al., 1998). In this study, wortmannin and LY 294002 also blocked p42/p44 MAPK phosphorylation by the adenosine A_1 receptor in DDT_1MF-2 cells. However, it is notable that adenosine A_1 receptor-induced p42/p44 MAPK responses in DDT_1MF-2 cells are insensitive to the broad range protein tyrosine kinase inhibitors genistein and tyrphostin A47. In marked contrast, p42/p44 MAPK responses to insulin were sensitive to genistein and tyrphostin A47. In addition, p42/p44 MAPK responses mediated via the G_q-protein-coupled histamine H_1 receptor in DDT_1MF-2 cells are sensitive to genistein and tyrphostin A47 (Robinson and Dickenson, 2000; unpublished observations). These data suggest that adenosine A_1 receptor coupling to p42/p44 MAPK in DDT_1MF-2 cells may involve genistein and tyrphostin A47-insensitive tyrosine kinases or is independent of tyrosine kinase activation. Furthermore, adenosine A_1 receptor stimulation of p42/p44 MAPK was also insensitive to the selective Src tyrosine kinase inhibitor PP2 and the epidermal growth factor receptor tyrosine kinase inhibitor AG1478. These data would seem to eliminate a potential for the Src family tyrosine kinases and epidermal growth factor receptor transactivation. Genistein-insensitive p42/p44 MAPK activation has been observed with other G_i-protein-coupled receptors including 5-HT_{1A} receptors and dopamine D_3 receptors (Cowen et al., 1996; Cussac et al., 1999). Similarly, p42/p44 MAPK responses mediated via receptors coupled to G_s proteins (adenosine A_{2B} receptors) and G_q proteins ($P2Y_2$ purinoceptors) have also been shown to be insensitive to genistein (Gao et al., 1999). Recent studies have shown that Ras-dependent activation of p42/p44 MAPK can be inhibited by the GTP-binding protein Rap1 (Zwartkruis and Bos, 1999). Interestingly, Mochizuki et al. (1999) have proposed that $G_{\alpha i}$ subunits activate p42/p44 MAPK by membrane recruitment of a novel Rap1 GTPase-activating protein (GAP) called rap1GAPII. These authors also showed that activation of the G_i-coupled muscarinic m2 receptor promotes the translocation of rap1GAPII from the cytosol to the membrane and decreases the levels of GTP-bound Rap1 (Mochizuki et al., 1999). These observations raise the intriguing possibility that p42/p44 MAPK activation by the adenosine A_1 receptor in DDT_1MF-2 cells involves Rap1 inactivation via $G_{\alpha i}$ rather than $\beta\gamma$-subunits. Such a mechanism may explain why adenosine A_1 receptor coupling to p42/p44 MAPK in DDT_1MF-2 cells is tyrosine kinase-independent.

Recent studies suggest that G_q/G_{11}-protein-coupled receptors preferentially employ protein kinase C and/or Ca^{2+} signals to activate p42/p44 MAPK in a Raf-dependent but Ras-independent manner (Sugden and Clerk, 1997). Since the adenosine A_1 receptor activates phospholipase C in DDT_1MF-2 cells, we examined the role of protein kinase C and Ca^{2+} in the regulation of p42/p44 MAPK by the adenosine A_1 receptor. The data presented indicate that adenosine A_1 receptor-induced p42/p44 MAPK responses in DDT_1MF-2 cells are independent of Ca^{2+} influx and intracellular Ca^{2+} release.

There is also increasing evidence for a role of protein kinase C (which may also explain genistein insensitivity) in G_i-protein-coupled receptor-mediated p42/p44 MAPK activation. For example, Takeda et al. (1999) reported that lysophosphatidic acid activates MAPK through a Ras-independent pathway that involves phosphatidylinositol 3-kinase-dependent activation of atypical protein kinase C-ζ. Similarly, the dopamine D_3 receptor couples to p42/p44 MAPK activation via phosphatidylinositol 3-kinase and an atypical protein kinase C isoform (Cussac et al., 1999). It is notable that responses to lysophosphatidic acid and the dopamine D_3 receptor were sensitive to Ro 31-8820, which at high concentrations (>1 μM) blocks the activation of atypical protein kinase C isoforms and insensitive to Gö 6976, which inhibits conventional Ca^{2+}-dependent protein kinase C α and βI isoforms (Martiny-Brown et al., 1993). In this study, the data obtained using a range of protein kinase C inhibitors (with a broad inhibition spectrum) indicate that adenosine A_1 receptor-mediated p42/p44 MAPK phosphorylation is independent of protein kinase C activation.

In this study, we have also explored the potential regulation of p38 MAPK and JNK signalling pathways by the adenosine A_1 receptor. The p38 MAPK and JNK signalling pathways are typically activated by stimuli such as UV irradiation, osmotic stress and inflammatory cytokines, although an increasing number of G-protein-coupled receptors have been shown to activate these pathways (Paul et al., 1997; Ono and Han, 2000). The data presented in this study clearly indicate that the adenosine A_1 receptor stimulates p38 MAPK phosphorylation through a pertussis toxin-sensitive pathway, indicating a role for G_i/G_o proteins. At present, the signalling pathways that connect G-protein-coupled receptors to p38 MAPK are largely unknown. Recent studies have shown that muscarinic m2 (G_i-coupled) and β-adrenoceptor (G_s-coupled) activation of p38 MAPK is mediated via $G\beta\gamma$, whereas activation by the G_q-coupled muscarinic m1 receptor involves $G\alpha_{q/11}$ and $G\beta\gamma$ (Yamauchi et al., 1997). The EC_{50} for CPA-induced p38 MAPK activation (ca. 20 nM) is comparable to

values previously reported for adenosine A_1 receptor-mediated inositol phosphate accumulation (26 nM; White et al., 1992) and Ca^{2+} mobilisation (19 nM; Dickenson and Hill, 1993a) in DDT_1MF-2 cells. In contrast, the EC_{50} values for CPA-stimulated p42/p44 MAPK activation and inhibition of adenylyl cyclase in DDT_1MF-2 cells are 1.3 and 2.8 nM, respectively (this study, Dickenson and Hill, 1993a). Recent studies suggest that G_i-protein-coupled receptors, such as the adenosine A_1 receptor, mediate inhibition of adenylyl cyclase via $G\alpha_i$ subunits and stimulate phospholipase C via $G\beta\gamma$ subunits released from G_i proteins (Dickenson and Hill, 1998). The higher concentrations of CPA required to stimulate inositol phosphate accumulation and Ca^{2+} mobilisation compared to the inhibition of adenylyl cyclase may reflect signalling pathways involving $G\beta\gamma$ and $G\alpha_i$ subunits, respectively. Hence, the EC_{50} values for CPA-induced stimulation of p42/p44 MAPK and p38 MAPK suggest the involvement of $G\alpha_i$ and $G\beta\gamma$-dependent pathways, respectively. However, further studies are required in order to identify the molecular mechanisms mediating p38 MAPK activation by the adenosine A_1 receptor and to determine whether p38 MAPK activation by the adenosine A_1 receptor contributes to the physiological effects of adenosine.

In summary, we have shown that the adenosine A_1 receptor stimulates p42/p44 MAPK and p38 MAPK in DDT_1MF-2 cells but not JNK. It is now apparent that the adenosine A_1 receptor in DDT_1MF-2 cells regulates a wide variety of signalling pathways including inhibition of adenylyl cyclase and activation of phospholipase C (with associated increases in intracellular Ca^{2+} and protein kinase C activity), p42/p44 MAPK, p38 MAPK and protein kinase B (Dickenson and Hill, 1993a; White et al., 1992; Gerwins and Fredholm, 1995; Germack and Dickenson, 2000). In addition, the adenosine A_1 receptor can potentiate inositol phosphate and calcium responses triggered by G_q-protein-coupled receptors in DDT_1MF-2 cells (Dickenson and Hill, 1993b; Hill et al., 1994). Future studies will investigate the possibility of intracellular cross-talk occurring between adenosine A_1 receptor and G_q-protein-coupled receptor signalling at the level of p42/p44 MAPK and p38 MAPK.

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