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Human adenosine A_1 receptor and $P2Y_2$-purinoceptor-mediated activation of the mitogen-activated protein kinase cascade in transfected CHO cells

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1 The mitogen-activated protein (MAP) kinase signalling pathway can be activated by a variety of heterotrimeric G_i/G_o protein-coupled and G_q/G_{11} protein-coupled receptors. The aims of the current study were: (i) to investigate whether the G_i/G_o protein-coupled adenosine A_1 receptor activates the MAP kinase pathway in transfected Chinese hamster ovary cells (CHO-A1) and (ii) to determine whether adenosine A_1 receptor activation would modulate the MAP kinase response elicited by the endogenous $P2Y_2$ purinoceptor.

2 The selective adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA) stimulated time and concentration-dependent increases in MAP kinase activity in CHO-A1 cells (EC_{50} 7.1 ± 0.4 nM). CPA-mediated increases in MAP kinase activity were blocked by PD 98059 (50 μM; 89 ± 4% inhibition), an inhibitor of MAP kinase kinase 1 (MEKI) activation, and by pre-treating cells with pertussis toxin (to block G_i/G_o-dependent pathways).

3 Adenosine A_1 receptor-mediated activation of MAP kinase was abolished by pre-treatment with the protein tyrosine inhibitor, genistein (100 μM; 6 ± 10% of control). In contrast, daidzein (100 μM), the inactive analogue of genistein had no significant effect (96 ± 12 of control). MAP kinase responses to CPA (1 μM) were also sensitive to the phosphatidylinositol 3-kinase inhibitors wortmannin (100 nM; 55 ± 8% inhibition) and LY 294002 (30 μM; 40 ± 5% inhibition) but not to the protein kinase C (PKC) inhibitor Ro 31-8220 (10 μM).

4 Activation of the endogenous $P2Y_2$ purinoceptor with UTP also stimulated time and concentration-dependent increases in MAP kinase activity in CHO-A1 cells ($EC_{50} = 1.6 \pm 0.3$ μM). The MAP kinase response to UTP was partially blocked by pertussis toxin (67 ± 3% inhibition) and by the PKC inhibitor Ro 31-8220 (10 μM; 45 ± 5% inhibition), indicating the possible involvement of both G_i/G_o protein and G_q protein-dependent pathways in the overall response to UTP.

5 CPA and UTP stimulated concentration-dependent increases in the phosphorylation state of the 42 kDa and 44 kDa forms of MAP kinase as demonstrated by Western blotting.

6 Co-activation of CHO-A1 cells with CPA (10 nM) and UTP (1 μM) produced synergistic increases in MAP kinase activity which were not blocked by the PKC inhibitor Ro 31-8220 (10 μM).

7 Adenosine A_1 and $P2Y_2$ purinoceptor activation increased the expression of luciferase in CHO cells transfected with a luciferase reporter gene containing the c-*fos* promoter. However, co-activating these two receptors produced only additive increases in luciferase expression.

8 In conclusion, our studies have shown that the transfected adenosine A_1 receptor and the endogenous $P2Y_2$ purinoceptor couple to the MAP kinase signalling pathway in CHO-A1 cells. Furthermore, co-stimulation of the adenosine A_1 receptor and the $P2Y_2$ purinoceptor produced synergistic increases in MAP kinase activity but not c-*fos* mediated luciferase expression.

Keywords: CHO cells; adenosine A_1 receptor; MAP kinase; $P2Y_2$-purinoceptors

Introduction

Mitogen-activated protein kinases (MAP kinases) or extracellular regulated kinases (ERK1 and ERK2) are involved in the regulation of cell growth and differentiation in response to various growth factors. The signal transduction cascade involved in the activation of MAP kinase(s) by mitogenic growth factors, for example the epidermal growth factor (EGF) has recently been elucidated (for extensive reviews see Guan, 1994; Malarkey *et al*., 1995; Seger & Kreb, 1995; Denhardt, 1996). MAP kinase phosphorylates various proteins, including phospholipase A_2 and ribosomal S6 protein kinase ($p90^{rsk}$) in the cytosol and transcription factors in the nucleus (Blenis, 1993; Nemenoff *et al*., 1993; Edwards 1994; Karin, 1995).

The MAP kinase signalling pathway can also be activated by a variety of G protein-coupled receptor agonists. Studies investigating the mechanisms involved in MAP kinase activation by receptors coupled to pertussis toxin-insensitive G_qG_{11} proteins (e.g. muscarinic M_1 receptor) suggest the involvement of PKC in a Raf-dependent but Ras-independent manner (Hawes *et al*., 1995). In contrast, activation of the MAP kinase pathway by pertussis toxin-sensitive G_iG_o protein-coupled receptors, such as α_{2A}-adrenoceptors and 5-HT_{1A} receptors, involves G$\beta\gamma$ subunits in a Ras-dependent pathway (Koch *et al*., 1994; Hawes *et al*., 1995; Garnovskaya *et al*., 1996). In addition, the G$\beta\gamma$ pathway appears to involve c-*Src* tyrosine kinase-

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mediated phosphorylation of the adapter protein Shc, followed by the formation of the Shc/Grb2 complex and Sos activation (Touhara *et al*., 1995; Luttrell *et al*., 1996). The mechanism(s) or pathway responsible for G$\beta\gamma$-mediated activation of *c-Src* tyrosine kinase remains unclear. However, recent studies have implicated that phosphatidylinositol 3-kinase may be involved upstream in the activation of tyrosine kinase(s) by G_i/G_o protein-coupled receptors (Hawes *et al*., 1996).

The adenosine A_1 receptor couples to the pertussis toxin-sensitive family of inhibitory G proteins (G_{i1}, G_{i2}, G_{i3}, and G_o) (Olah & Stiles, 1995). We have previously investigated adenosine A_1 receptor-mediated second messenger signalling in CHO cells transfected with the human adenosine A_1 receptor (Dickenson & Hill, 1996; 1997; Megson *et al*., 1995). These studies have revealed that adenosine A_1 receptor-activation can: (i) inhibit adenylyl cyclase activity as measured by the ability of adenosine A_1 receptor agonists to attenuate forskolin-stimulated cyclic AMP production; (ii) stimulate pertussis toxin-sensitive increases inositol phosphate formation and calcium mobilisation (measures of phospholipase C activation) and (iii) augment the accumulation of inositol phosphates and release of arachidonic acid (measure of phospholipase A_2 activity) elicited by several endogenous G_q/G_{11} protein-coupled receptors (namely CCK_A receptors, $P2Y_2$-purinoceptors and thrombin receptors). In the present paper we have extended our studies on adenosine A_1 receptor mediated cell signalling and now report that adenosine A_1 receptor activation in CHO cells stimulates the MAP kinase pathway. Furthermore, we have also investigated whether co-activation of the $P2Y_2$-purinoceptor and the adenosine A_1 receptor (G_i/G_o protein-coupled receptor) produces synergistic increases in MAP kinase activity and luciferase reporter gene expression in CHO cells. Preliminary data from part of this study has been presented to the British Pharmacological Society (Dickenson & Hill, 1998).

Methods

Cell culture

Chinese hamster ovary cells (CHO-K1) transfected with the human brain adenosine A_1 receptor sequence (CHO-Al) were a generous gift from Dr Andrea Townsend-Nicholson and Professor John Shine, Garvan Institute, Sydney, Australia. CHO-A1 cells were cultured in 75 cm^2 flasks (Costar) in Dulbeco's modified Eagles Medium (DMEM/Nutrient F12 (1 : 1) supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum. Cells were maintained at 37°C in a humidified 10% CO_2 atmosphere until confluency and were subcultured (1 : 5 split ratio) using trypsin (0.05% w/v)/EDTA (0.02% w/v). Cells for the determination of mitogen-activated protein kinase activity were grown in 6 well cluster dishes (Costar). Luciferase measurements were performed using CHO-K1 cells (European Collection of Animal Cultures, Porton Down, Salisbury, U.K.) transfected with the human adenosine A_1 receptor (in pcDNA3) and the full human c-*fos* promoter (−711 to +42) ligated into the *Xho*I site of basic pGL3 luciferase plasmid (Promega; pFosLuc3). The human adenosine A_1 receptor cDNA (in pSVL) was obtained from the American Type Culture Collection and subcloned into the *Not*I/*Apa*I site of pcDNA3. CHO-K1 cells were initially transfected with pcDNA3A_1R using Lipofectamine (Life Technologies) and selected using 500 μg/ml geneticin (G418; Gibco). A clone expressing 277 fmol/mg of protein of adenosine A_1 receptor (similar to the level of A_1 receptor expression in CHO-A1 cells) was selected and then co-transfected with pFosLuc3 and pZEOSV (Invitrogen). Stably transfected cells were then selected and maintained using 500 μg/ml G418 and 250 μg/ml zeocin (Invitrogen).

Mitogen-activated protein kinase assay

MAP kinase activity in CHO cells was measured using a modification of the protocol described previously (Mitchell *et al*., 1995; Sugawara *et al*., 1996). CHO-A1 cells were grown in 6-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. Washing the cells and incubating for 30 min prior to stimulation had no significant effect on the basal level of MAP kinase activity (data not shown). 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 500 μl of ice-cold lysis buffer [70 mM β-glycerophosphate, 1 mM EGTA, 1 mM DTT, 2 mM $MgCl_2$, 20 μg/ml aprotinin, 5 μg/ml leupeptin and 0.5% Triton-X-100]. 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 400 μl of the cell lysate removed and stored at −20°C until required. MAP kinase was then partially purified from the cell lysates using anion-exchange chromatography. Bio-Rad chromatography columns containing 0.5 ml of Q-Sepharose were pre-equilibrated by washing once with 2 ml of distilled water and twice with 5 ml of column buffer [70 mM β-glycerophosphate, 1 mM EGTA, 100 μM Na_3V0_4 and 1 mM DTT, pH 7.2]. Cell lysates (350 μl) were applied to the pre-equilibrated columns and washed three times with 1 ml of column buffer, after which MAP kinase was eluted with 1 ml of column buffer containing 1.0 M NaCl. MAP kinase activity was determined using 20 μl of the partially purified sample and 20 μl of reaction mixture containing 50 mM β-glycerophosphate, 100 μM Na_3V0_4, 20 mM $MgCl_2$, 20 μM ATP, 1 mM EGTA, [γ-^{32}P]-ATP (0.074 MBq/assay tube) and 200 μM of the synthetic peptide NH_2-KRELVEPLTPAGEAPNQALLR-COOH. This peptide is based on the sequence surrounding the MAP kinase phosphorylation site in the epidemal growth factor receptor (Takishima *et al*., 1991). After incubation at 37°C for 20 min the reaction was terminated by the addition of 10 μl of 25% trichloroacetic acid and 45 μl of the reaction mixture was then applied onto Whatman P-81 chromatography paper (2.5 cm^2). Filters were washed three times in 0.5% phosphoric acid, washed once with acetone and then placed into scintillation vial inserts and ^{32}P radioactivity measured by Cerenkov counting.

Western blot analysis

CHO-A1 cell lysates were prepared as described for the MAP kinase assay except that the lysis buffer contained 1 mM Na_3V0_4 and 1 mM NaF. The protein concentration of the lysate was subsequently determined using the method of Lowry (1951) using bovine serum albumin as the standard. MAP kinase activation was determined using antiserum specific for the phosphorylated forms of 42 kDa and 44 kDa MAP kinase. Protein samples (20 μg) were separated by SDS–PAGE (10% acrylamide gel) using 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 vol). The primary antibody was removed and the blot extensively washed with PBS/Tween 20. Blots were then incubated for 2 h at room temperature with the secondary antibody (swine anti-rabbit IgG 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).

Luciferase measurements

Luciferase assays were performed using CHO cells co-transfected with the human adenosine A_1 receptor and a luciferase reporter plasmid containing the c-*fos* promoter. Cells were grown in 24-well plate cluster dishes and when 80–90% confluent washed once with serum-free DMEM/F-12 medium before incubating 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 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 of luciferase assay substrate (Promega) into each sample and determines luminescence over 10 s.

Data analysis

Agonist EC_{50} values (concentration of agonist producing 50% of the maximal stimulation) were obtained by computer assisted curve fitting by use of the computer programme InPlot (GraphPAD, California, U.S.A.). 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.

Methods

[γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham International (Amersham U.K.) N^6-cyclopentyladenosine (CPA), bovine serum albumin, leupeptin and aprotinin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Q Sepharose was from Pharmacia Biotech. Pertussis toxin was obtained from Porton Products Ltd. Ro 31-8220 (3-{1-[3-(2-isothioureido) propyl]indol-3-yl}-4-(1-methylindol-3-yl)-3-pyrrolin-2,5-dione), PD 98059 (2′-amino-3′methoxyflavone), LY 294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), genistein, daidzein and wortmannin were from Calbiochem (Nottingham, U.K). Phospho-specific p44/42 MAP kinase antibody was obtained from New England Biolabs. Dulbecco's modified Eagles Medium/Nutrient Mix F-12 (1:1) and foetal calf serum were from Sigma Chemical Co. (Poole, Dorset, U.K.). All other chemicals were of analytical grade.

Results

Adenosine A_1 receptor-mediated MAP kinase activation

Stimulation of CHO-A1 cells with the adenosine A_1 receptor selective agonist N^6-cyclopentyladenosine (CPA; 1 μM) produced a marked increase in MAP kinase activity (Figure 1a). Maximal activation of MAP kinase was observed after 5 min and then slowly declined towards basal. The activation of MAP kinase by CPA was concentration-dependent, with an EC_{50} value of 7.1 ± 0.4 nM ($n=6$; Figure 1b). The measurements of MAP kinase activity described in this paper should be sensitive to the selective MEK1 inhibitor, PD 98059 (Dudley *et*

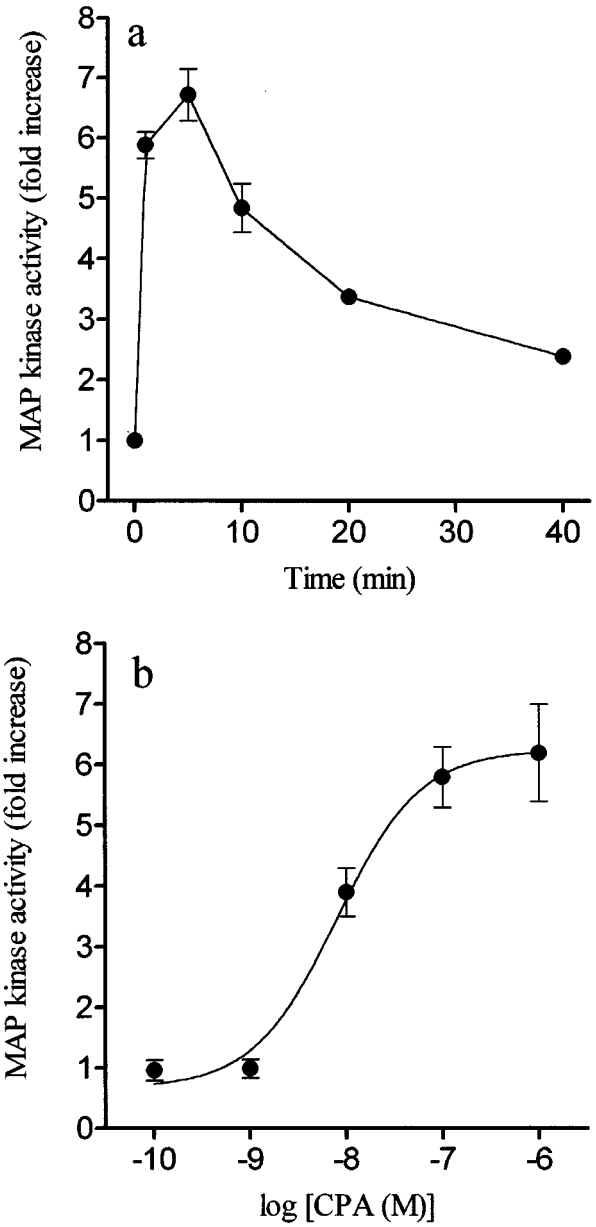

Figure 1 Adenosine A_1 receptor-mediated stimulation of MAP kinase in CHO-A1 cells. (a) Time course of adenosine A_1 receptor-mediated MAP kinase activity in CHO-A1 cells. Cells were stimulated with CPA (1 μM) for the indicated periods of time. Each time point is the mean ± s.e.m. of three independent experiments assayed in duplicate. (b) Concentration-response curve for CPA-induced MAP kinase activation. Each data point is the mean ± s.e.m. of six independent experiments assayed in duplicate.

al., 1995). Pre-incubation of CHO-A1 cells with PD 98059 (50 μM; 30 min) significantly reduced CPA-induced activation of MAP kinase (Table 1). The adenosine A_1 receptor couples to the pertussis toxin-sensitive family of inhibitory G proteins (G_{i1}, G_{i2}, G_{i3}, and G_o) (Olah & Stiles, 1995). Pre-treatment of CHO-A1 cells with pertussis toxin (100 ng/ml for 16 h) completely inhibited MAP kinase activation in response to CPA (Table 1). The sensitivity to pertussis toxin suggests that G_i/G_o proteins are involved in adenosine A_1 receptor stimulation of MAP kinase. Adenosine A_1 receptor-mediated activation of MAP kinase in CHO-A1 cells was also investigated by Western blotting using an antibody that detects $p42^{MAPK}$ (ERK1) and $p44^{MAPK}$ (ERK2) only when they are catalytically activated by phosphorylation at Thr^{202} and Tyr^{204}. As shown in Figure 2a, CPA stimulated concentration dependent increases in 42 kDa and 44 kDa MAP kinase phosphorylation. Furthermore, CPA (1 μM) mediated increases 42/44 kDa MAP kinase phosphorylation were attenuated by pertussis toxin pre-treatment (100 ng/ml for 16 h) and PD 98059 (50 μM; Figure 2a).

Table 1 The effects of various treatments on CPA-mediated MAP kinase activation in CHO-A1 cells

Treatment	*Percentage of control*	n
PD 98059 (50 μM)	11±4*	3
Pertussis toxin	5±3*	3
Genistein (100 μM)	6±10*	4
Daidzein (100 μM)	96±12	4
Ro 31-8220 (10 μM)	98±5	3
Wortmannin (100 nM)	45±8*	4
Wortmannin (1 μM)	19±9*	3
LY 294002 (30 μM)	60±5*	6
LY 294002 (100 μM)	12±9*	3

Pertussis toxin (100 ng/ml) pre-treatment was for 16 h in DMEM/F-12 medium containing 0.1% bovine serum albumin. Quiescent CHO-A1 cells were pre-incubated for 30 min with the various kinase inhibitors before stimulating with 1 μM CPA for 5 min. Values are expressed as the percentage of the response obtained with 1 μM CPA (100%) in control cells (independent controls were used for each inhibitor). Control cells were pre-treated for 30 min with vehicle (0.1% dimethyl sulphoxide). Pre-treatment with pertussis toxin or the various kinase inhibitors had no significant effect on basal MAP kinase activity. Values represent the means±s.e.m. of (n) experiments. *Statistical significant ($P<0.05$) from the control response to CPA (Student's t test).

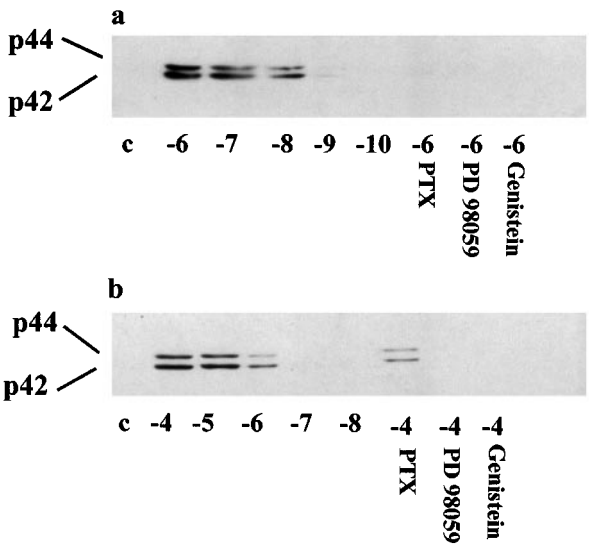

Figure 2 Western blot analysis of CHO-A1 cell lysates using phospho-p44/42 MAP kinase antibody. Quiescent CHO-A1 cells were treated with vehicle (control) or the indicated concentrations of (a) CPA or (b) UTP for 5 min. Cell lysates (20 μg) were resolved by SDS–PAGE and transferred to nitrocellulose membranes before being probed with antisera specific for phosphorylated 42 kDa and 44 kDa forms of MAP kinase (1:1000 dilution). Specific bands representing phosphorylated 42 kDa MAP kinase and 44 kDa MAP kinase were obtained following stimulation of cells with increasing concentrations of CPA and UTP. The effect of pertussis toxin (100 ng/ml for 16 h), PD 98059 (50 μM) and genistein (100 μM) on the responses to 1 μM CPA (a) and 100 μM (b) are also shown. Similar results were obtained in three independent experiments.

Recent studies have indicated that tyrosine kinase(s) are also involved in the activation of MAP kinase by G_i/G_o protein-coupled receptors (Touhara *et al*., 1995). Treatment of CHO-A1 cells with the tyrosine kinase inhibitor, genistein (100 μM; 30 min; Akiyama *et al*., 1987) virtually abolished CPA-induced MAP kinase activity (Table 1). In marked contrast, daidzein (100 μM), the inactive analogue of genistein, had no significant effect on CPA mediated MAP kinase activity (Table 1). These data suggest that adenosine A_1 receptor-mediated MAP kinase activation in CHO-A1 cells involves a tyrosine kinase-dependent pathway. We also determined whether PKC is involved in adenosine A_1 receptor-mediated MAP kinase activation. Pre-treatment of cells with the selective PKC inhibitor Ro 31-8220 (10 μM; 30 min; Davis *et al*., 1989) had no significant effect on CPA-induced MAP kinase activation (Table 1). However, this concentration of Ro 31-8220 is sufficient to inhibit the activation of MAP kinase induced by the PKC activator phorbol 12-myristate 13-acetate (PMA; 85±8% ($n=3$) inhibition of the control response to 1 μM PMA).

It has been reported that phosphatidylinositol 3-kinase (PtdIns 3-kinase) may also play a role in the activation of the MAP kinase pathway by G_i/G_o protein-coupled receptors (Hawes *et al*., 1996). The possible role for PtdIns 3-kinase in the activation of MAP kinase by the adenosine A_1 receptor in CHO-A1 cells was assessed using two inhibitors of PtdIns 3-kinase, LY 294002 (Vlahos *et al*., 1994) and wortmannin (Ui *et al*., 1995). Pre-treatment of CHO-A1 cells for 30 min with selective concentrations of LY 294002 (30 μM) or wortmannin (100 nM) inhibited CPA-induced MAP kinase activation by 40±5% ($n=6$; $P<0.05$) and 55±8% ($n=4$; $P<0.05$) respectively. Similar concentrations of LY 294002 (30 μM) and wortmannin (100 nM) have been shown to inhibit G_i-protein mediated MAP kinase activation in CHO and COS-7 cells (Garnovskaya *et al*., 1996; Hawes *et al*., 1996). The MAP kinase response to CPA (1 μM) was inhibited further in the presence of 100 μM LY 29400 and 1 μM wortmannin (Table 1). These results indicate that PtdIns 3-kinase is involved in the signalling pathway that couples the adenosine A_1 receptor to MAP kinase in CHO-A1 cells.

$P2Y_2$-purinoceptor-mediated MAP kinase activation

CHO cells express an endogenous $P2Y_2$ purinoceptor which couples to phospholipase C and hence inositol phospholipid hydrolysis and calcium mobilisation (Iredale & Hill, 1993; Megson *et al*., 1995). Our previous studies have shown that [^{3}H]-inositol phosphate responses mediated by ATP and UTP are partially sensitive to pertussis toxin (*circa* 30% inhibition) suggesting some involvement of G_i/G_o proteins in $P2Y_2$-purinoceptor coupling to phospholipase C (Megson *et al*.,

1995). Stimulation of CHO-A1 cells with UTP induced a rapid increase in MAP kinase activity, as shown in Figure 3a. The response to UTP was maximal after 1–5 min and returned to basal level within 20 min. Furthermore, activation of MAP kinase by UTP was concentration-dependent with an EC_{50} of 1.6 ± 0.3 μM ($n=6$; Figure 3b). The increases in MAP kinase activity observed with 100 μM UTP were also sensitive to the MEK1 inhibitor PD 98059 (Table 2). Pertussis toxin pre-treatment (100 ng/ml for 16 h) of CHO-A1 cells partially inhibited UTP-induced MAP kinase activation (Table 2). The sensitivity to pertussis toxin suggests that G_i/G_o proteins are also involved in UTP-induced MAP kinase activation. Western blot analysis revealed that UTP stimulated increases in 42 kDa and 44 kDa MAP kinase phosphorylation in a concentration-dependent manner (Figure 2b). Furthermore, UTP (100 μM) mediated increases 42/44 MAP kinase phosphorylation were sensitive to pertussis toxin (100 ng/ml for 16 h) and PD 98059 (50 μM; Figure 2b).

The activation of MAP kinase cascade by G_q/G_{11}, protein-coupled receptors predominantly involves a PKC-dependent pathway (Hawes *et al*., 1995). Pre-treatment of CHO-A1 cells with the selective PKC inhibitor Ro 31-8220 (10 μM) partially inhibited UTP-induced MAP kinase activation indicating the involvement of a PKC-dependent pathway (Table 2). These observations suggest that the $P2Y_2$-purinoceptor expressed in CHO cells stimulates MAP kinase via pertussis toxin-insensitive (G_q/G_{11} mediated) and pertussis toxin-sensitive (G_i/G_o mediated) pathways. It would be predicted that the pertussis toxin-resistant component of the UTP response (*circa* 40% and possibly representing the G_q/G_{11} protein-dependent pathway) should be sensitive to the PKC inhibitor, Ro 31-8220. However, the pertussis toxin-insensitive component of the UTP response was not blocked by Ro 31-8220 (10 μM). In cells exposed to both pertussis toxin and Ro 31-8220 the UTP-induced MAP kinase activation was $39 \pm 7\%$ ($n=4$) of control responses. Recently, Wan *et al*. (1996) reported that the non-receptor tyrosine kinases Lyn and Syk are involved in G_q-mediated (via muscarinic M_1 receptor) MAP kinase activation. As shown in Table 2, genistein (100 μM) pre-treatment completely abolished UTP-induced MAP kinase activation suggesting that tyrosine kinase(s) are also involved in the $P2Y_2$-purinoceptor-mediated signalling pathway.

Table 2 The effects of various treatments on UTP-mediated MAP kinase activation in CHO-A1 cells

Treatment	*Percentage of control*	(n)
PD 98056 (50 μM)	10 ± 6*	3
Pertussis toxin	33 ± 4*	7
Ro 31-8220 (10 μM)	55 ± 5*	6
Pertussis toxin + Ro 31-8220 (10 μM)	39 ± 7*	4
Genistein (100 μM)	5 ± 2*	3

Perussis toxin (100 ng/ml) pre-treatment was for 16 h in DMEM/F-12 medium containing 0.1% bovine serum albumin. Quiescent CHO-A1 cells were pre-incubated for 30 min with the various kinase inhibitors before stimulating with 100 μM UTP for 5 min. Values are expressed as the percentage of the response obtained with 100 μM UTP (100%) in control cells (independent controls were used for each inhibitor). Control cells were pre-treated for 30 min with vehicle (0.1% dimethyl sulphoxide). Pre-treatment with pertussis toxin or the various kinase inhibitors had no significant effect on basal MAP kinase activity. Values represent the means ± s.e.m. of (n) experiments. *Statistical significant ($P<0.05$) from the control response to UTP (Student's t test).

Interactions between adenosine A_1 receptor and $P2Y_2$-purinoceptor-mediated MAP kinase responses

In our previous studies we have shown that co-activation of the human transfected adenosine A_1 receptor in CHO cells synergistically enhances G_q/G_{11} protein coupled receptor (CCK_A receptor, $P2Y_2$ purinoceptors and thrombin receptor) stimulated phospholipase C (measured as total [^{3}H]-inositol phosphate accumulation) and phospholipase A_2 (measured as [^{3}H]-arachidonic acid release) responses (Dickenson & Hill, 1996; 1997; Megson *et al*., 1995). Therefore, in this paper we investigated whether adenosine A_1 receptors and $P2Y_2$-purinoceptors interact synergistically to activate MAP kinase in CHO-A1 cells. These experiments were conducted using concentrations of CPA (10 nM) and UTP (1 μM) which on their own stimulate sub-maximal increases in MAP kinase (see Figures 1b and 3b). As shown in Table 3, co-stimulation of CHO-A1 cells with 10 nM CPA and 1 μM UTP produced synergistic increases in MAP kinase activity. Synergistic increases in MAP kinase activity were also observed following stimulation of CHO-A1 cells with maximal-effective concentrations of CPA (100 nM) and UTP (10 μM; see Table 3). One notable difference between the activation of MAP kinase elicited by the adenosine A_1 receptor and the $P2Y_2$-purinoceptor is the sensitivity of the UTP response to the PKC inhibitor Ro 31-8220 (Table 2). Therefore, we investigated whether the PKC-dependent component of the UTP response plays a role in mediating the synergistic

Table 3 Effect of co-stimulating the adenosine A_1 receptor and the $P2Y_2$-purinoceptor on MAP kinase activity in CHO-A1 cells

(a)

	Control	(n)	*Ro 31-8220*	(n)
10 nM CPA	100	6	100	5
1 μM UTP	34 ± 3	6	15 ± 3	5
10 nM CPA and 1 μM UTP	194 ± 9* (134 ± 3)	6	214 ± 15* (115 ± 3)	5

(b)

	Control	(n)	*Ro 31-8220*	(n)
100 nM CPA	100	5	100	5
10 μM UTP	50 ± 6	5	20 ± 5	5
100 nM CPA and 10 μM UTP	250 ± 25* (150 ± 6)	5	230 ± 17* (120 ± 5)	5

MAP kinase activity (5 min stimulation) was measured in response to 10 nM or 100 nM CPA alone; 1 μM or 10 μM UTP alone or a combination of 10 nM CPA and 1 μM UTP or 100 nM CPA and 10 μM UTP in the absence or presence of Ro 31-8220 (10 μM; 30 min). Control cells were pre-incubated with vehicle (0.1% dimethyl sulphoxide). In (a) and (b) the MAP kinase responses to UTP alone and the combination of CPA and UTP are expressed as a percentage of the response to either 10 nM or 100 nM CPA alone (100%) measured in both control and Ro 31-8220 treated cells. The response to 10 nM CPA was 3139 ± 291 c.p.m. (basal = 1054 ± 98 c.p.m.) and 3374 ± 341 c.p.m. (basal = 1572 ± 88 c.p.m.) in control and Ro 31-8220 treated cells, respectively. Similarly, Ro 3-8220 (10 μM) had no significant effect on the MAP kinase responses elicited by 100 nM CPA. Results represent the mean ± s.e.m of (n) independent experiments each assayed in duplicate. The values in parentheses represent the predicted additive responses to CPA and UTP and were calculated by adding the response obtained with CPA alone to that obtained with UTP alone.* Significantly ($P<0.05$; Student's t test) different from the predicted additive responses to CPA and UTP obtained in control and Ro 31-8220 treated cells.

increases in MAP kinase activation observed in CHO-A1 cells. However, as clearly shown in Table 3, pre-treatment of CHO-A1 cells with the PKC inhibitor Ro 31-8220 (10 μM) had no significant effect on the synergistic activation of MAP kinase produced by 10 nM CPA and 1 μM UTP or 100 nM CPA and 10 μM UTP.

Effect of adenosine A_1 receptor and $P2Y_2$-purinoceptor-activation on luciferase reporter gene expression

Activation of the MAP kinase signalling 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). Hence, we wondered whether co-stimulating CHO cells with adenosine A_1 and $P2Y_2$-purinoceptor agonists would produce synergistic increases in c-*fos* regulated gene expression. These experiments were conducted using CHO cells stably co-transfected with the adenosine A_1 receptor and a plasmid containing the luciferase gene under the transcriptional control of the c-*fos* promoter. As shown in Figure 4, treatment of CHO cells with the adenosine A_1 receptor agonist CPA produced concentration-dependent increases in luciferase expression ($EC_{50} = 16.5 \pm 2.0$ nM; $n = 6$). Activation of the

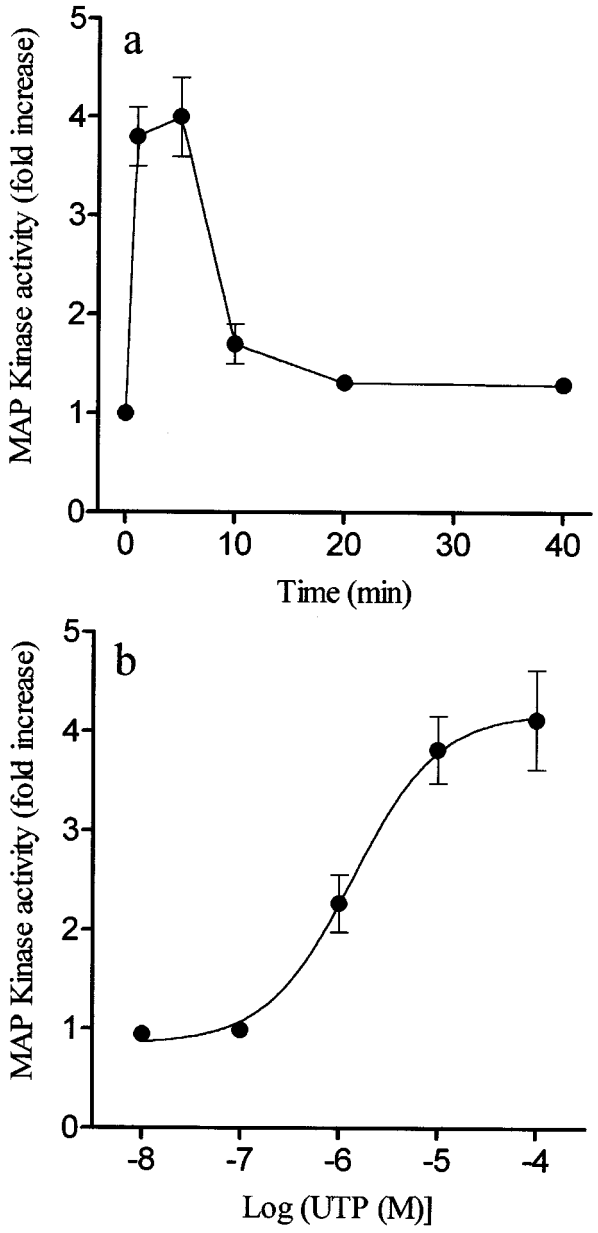

Figure 3 $P2Y_2$-purinoceptor-mediated stimulation of MAP kinase in CHO-A1 cells. (a) Time course of $P2Y_2$-purinoceptor-mediated MAP kinase activity in CHO-A1 cells. Cells were stimulated with UTP (100 μM) for the indicated periods of time. Each time point is the mean ± s.e.m. of six independent experiments assayed in duplicate. (b) Concentration-response curve for UTP induced MAP kinase activation. Each data point is the mean ± s.e.m. of six independent experiments assayed in duplicate.

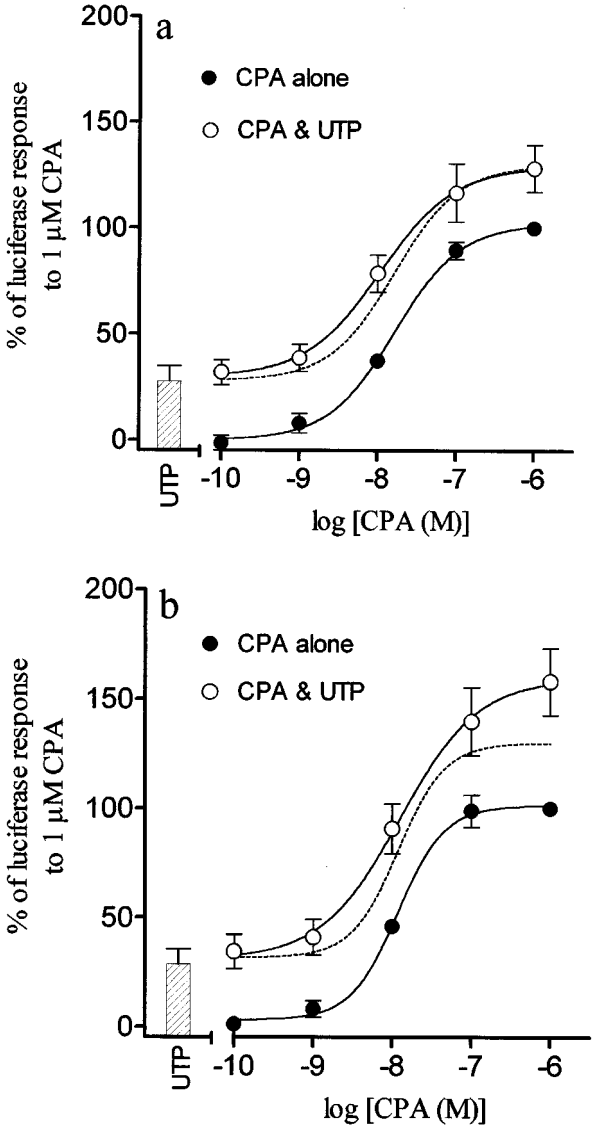

Figure 4 Adenosine A_1 receptor and $P2Y_2$-purinoceptor mediated stimulation of c-*fos*-induced luciferase expression in CHO cells. (a) Concentration-response curves for CPA-mediated luciferase expression in the absence or presence of 10 μM UTP. The histogram represents the response obtained with 10 μM UTP alone. The dotted line represents the predicted additive response to CPA and UTP and was calculated by adding the response obtained with the fixed concentration of UTP (10 μM) to that obtained with each concentration of CPA in the concentration-response curve. (b) Concentration-response curves for CPA-mediated luciferase expression in the absence and presence of 1 μM UTP. The histogram represents the response obtained with 1 μM UTP alone. Data are expressed as a percentage of the luciferase response obtained with 1 μM CPA alone (after subtracting basal luciferase expression; 100%) and represent the mean ± s.e.m. of six independent experiments, each measured in duplicate.

endogenous $P2Y_2$-purinoceptor with UTP (using 1 μM and 10 μM) also increased luciferase expression in CHO cells, although to a lesser extent (*circa* 30% of the maximum response obtained with 1 μM CPA). However, and in contrast to the synergistic increases in MAP kinase activity, co-activation of CHO cells CPA and UTP produced additive increases in c-*fos*-mediated luciferase expression (Figure 4).

Discussion

In the present study we have shown that the G_i/G_o protein-coupled human adenosine A_1 receptor stimulates MAP kinase in transfected Chinese hamster ovary cells. Recent studies have indicated that the signalling pathway associated with G_i/G_o protein-mediated activation of the MAP kinase signalling cascade involves the following components: (i) pertussis toxin-sensitive G protein-derived $\beta\gamma$ subunits (Koch *et al*., 1994; Hawes *et al*., 1995; Garnovskaya *et al*., 1996); (ii) a genistein-sensitive c-*Src*-related protein tyrosine kinase (Luttrell *et al*., 1996); (iii) phosphatidylinositol 3-kinase activation (Hawes *et al*., 1996); (iv) tyrosine phosphorylation of the adapter protein Shc (Touhara *et al*., 1995) and (v) formation of the Shc/Grb2 complex and subsequent activation of Sos and Ras (Touhara *et al*., 1995; Luttrell *et al*., 1996). To date little is known about the signalling pathway associated with adenosine A_1 receptor-mediated activation of MAP kinase. The adenosine A_1 receptor has previously been shown to activate the MAP kinase pathway via $\beta\gamma$ subunits in transfected COS-7 cells (Faure *et al*., 1994). Our current study has further characterised the signalling pathway involved in adenosine A_1 receptor-induced MAP kinase activation. We have shown that pertussis toxin completely abolished CPA-mediated responses thus indicating that G_i/G_o proteins also couple the adenosine A_1 receptor to the MAP kinase pathway. Furthermore, the results obtained with the various kinase inhibitors suggest that the adenosine A_1 receptor stimulates MAP kinase activity in CHO cells by a pathway independent of PKC but involving tyrosine kinase, PtdIns 3-kinase and MEK1 activation. These observations are consistent with the known mechanisms for G_i/G_o protein-coupled receptor-mediated activation of MAP kinase.

The nucleotides, ATP and UTP, are also capable of activating the MAP kinase pathway in several different cell types (Huwiler and Pfeilschifter, 1994; Graham *et al*., 1996 Patel *et al*., 1996). In this current study we have shown that UTP stimulates increases in MAP kinase activity in CHO-A1 cells, presumably through activation of the endogenous $P2Y_2$-purinoceptor (Iredale & Hill, 1993; Megson *et al*., 1995) The responses to UTP were sensitive to pertussis toxin pre-treatment *(circa* 70% inhibition) indicating the involvement of G_i/G_o proteins. Interestingly, ATP and UTP-mediated [^{3}H]-inositol phosphate responses in CHO cells are also sensitive to pertussis toxin, although to a lesser extent *(circa* 30% inhibition; Megson *et* al., 1995). The pertussis toxin-resistant component of the UTP-induced MAP kinase response presumably represents G_q/G_{11} protein-mediated activation. It is known that G_q/G_{11} protein-coupled receptors activate MAP kinase via a pathway that is dependent on the activity of PKC (Hawes *et al*., 1995). Indeed, UTP-induced MAP kinase responses were partially blocked by the selective PKC inhibitor Ro 31-8220 (*circa* 45% inhibition). However, in pertussis toxin-treated cells (to remove the G_i/G_o component) UTP-induced MAP kinase activity was not blocked further by the PKC inhibitor, Ro 31-8220. Hence, it is conceivable that the pertussis toxin-sensitive component is also PKC-dependent and that the pertussis toxin-insensitive response is G_q/G_{11}-mediated but PKC-independent. Interestingly, in CHO cells, the muscarinic M_1 receptor and the platelet-activating factor receptor couple to G_o to activate MAP kinase via a signalling pathway that is dependent on the activity of PKC (van Biesen *et al*., 1996). It should be noted that not all G_q protein-coupled receptors activate MAP kinase via a PKC-dependent pathway. For example, in rat vascular smooth muscle cells, the G_q protein-coupled angiotensin type 1 receptor stimulates MAP kinase via the release of calcium from intracellular stores activating a Ca^{2+}/calmodulin-dependent tyrosine kinase (Eguchi *et al*., 1996). Therefore, a Ca^{2+} and tyrosine kinase-dependent but PKC-independent mechanism may underlie the pertussis toxin-resistant (and Ro 31-8220 insensitive) component of the UTP-mediated MAP kinase response. Support for this theory is provided by the observation that the tyrosine inhibitor, genistein, completely blocked UTP-induced activation of MAP kinase. We are currently investigating the role Ca^{2+} (both Ca^{2+} influx and intracellular Ca^{2+} release) may play in $P2Y_2$-purinoceptor-mediated activation of MAP kinase in CHO cells. One of the aims of the current study was to investigate whether co-activation of the $P2Y_2$-purinoceptor and the adenosine A_1 receptor would activate MAP kinase synergistically. There have been several reports in the literature describing the potentiation of growth factor-mediated mitogenic responses by agonists for G protein-coupled receptors (Huang *et al*., 1989; Kusuhara *et al*., 1992; Neary *et al*., 1994). Interestingly, extracellular ATP (via purinoceptors) interacts synergistically with growth factors to promote DNA synthesis and cell growth in several different cell types (Huang *et al*., 1989; Neary *et al*., 1994; Neary, 1996). In the current study, we have shown that co-activation of the G_i/G_o protein-coupled adenosine A_1 receptor and the $P2Y_2$ purinoceptor resulted in the synergistic activation of MAP kinase.

The location at which the adenosine A_1 and $P2Y_2$-purinoceptor signalling pathways converge to produce a synergistic increase in MAP kinase activity is not clear. The mechanism may involve 'cross-talk' between other kinase signalling pathways, which may be activated by the adenosine A_1 and/or the $P2Y_2$-purinoceptor in CHO cells, in particular components of the stress-activated protein kinases (SAP kinases) and p38 MAP kinase signalling cascades (Guan, 1994; Denhardt, 1996).

The ability of adenosine A_1 receptor and $P2Y_2$-purinoceptor agonists to elicit synergistic increases in MAP kinase activity may influence cell signalling events downstream of MAP kinase (e.g. phospholipase A_2 (PLA_2) activity and gene transcription). Indeed, we have previously shown that adenosine A_1 receptor activation augments $P2Y_2$-purinoceptormediated activation of PLA_2 activity in CHO cells (Selbie *et al*., 1997). However, in this study, we have shown that co-activating adenosine A_1 and $P2Y_2$-purinoceptors produced only additive increases in c-*fos*-mediated luciferase gene transcription. 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). ERK1 and ERK2 phosphorylate transcription factors that interact with the SRE. In contrast, 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. Whether adenosine A_1 receptor and $P2Y_2$-purinoceptor-mediated increases in luciferase expression occur exclusively via the SRE remains to be determined. Adenosine A_1 receptor and $P2Y_2$-purinoceptor activation in CHO cells may also influence luciferase expression via the CRE since

both receptors increase intracellular Ca^{2+} in CHO cells and the A_1 receptor inhibits intracellular cAMP levels (Iredale & Hilt 1993; Iredale *et al*., 1994; Megson *et al*., 1995). Furthermore, Xing *et al*. (1996) reported that the Ras-MAP kinase pathway is also linked to CRE by the kinase RSK2. Therefore, the individual and combined responses for adenosine A_1 and $P2Y_2$-purinoceptor-mediated increases in luciferase expression may involve complex interactions (possibly both inhibitory and stimulatory) between the SRE and CRE within the c-*fos* promoter and may account for the absence of synergy at the level of gene expression.

In summary, this study has shown that in transfected CHO cells, the G_i/G_o protein-coupled adenosine A_1 receptor activates MAP kinase via a pathway which is independent of PKC but involves tyrosine kinase, PtdIns 3-kinase and MEK1 activation. We have also shown for the first time, that co-activation of two different G-protein coupled receptors (in this case the adenosine A_1 receptor and the $P2Y_2$-purinoceptor) produces synergistic increases in MAP kinase activity. However, identifying the mechanism(s) underlying the synergistic increases in MAP kinase activity will require further research.

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