British Journal of Pharmacology (2001) 133, 1378–1386

www.nature.com/bjp

Activation of the p38 and p42/p44 mitogen-activated protein kinase families by the histamine H_1 receptor in DDT_1MF-2 cells

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1 The mitogen-activated protein kinases (MAPKs) consist of the p42/p44 MAPKs and the stress-activated protein kinases, c-Jun N-terminal kinase (JNK) and p38 MAPK. In this study we have examined the effect of histamine H_1 receptor activation on MAPK pathway activation in the smooth muscle cell line DDT_1MF-2.

2 Histamine stimulated time and concentration-dependent increases in p42/p44 MAPK activation in DDT_1MF-2 cells. Responses to histamine were inhibited by the histamine H_1 receptor antagonist mepyramine (K_D 3.5 nM) and following pre-treatment with pertussis toxin (PTX; 57% inhibition).

3 Histamine-induced increases in p42/p44 MAPK activation were blocked by inhibitors of MAPK kinase 1 (PD 98059), tyrosine kinase (genistein and tyrphostin A47), phosphatidylinositol 3-kinase (wortmannin and LY 294002) and protein kinase C (Ro 31-8220; 10 μM; 41% inhibition). Inhibitors of Src tyrosine kinase (PP2) and the epidermal growth factor tyrosine kinase (AG1478) were without effect. Removal of extracellular Ca^{2+}, chelation of intracellular Ca^{2+} with BAPTA and inhibition of focal adhesion assembly (cytochalasin D) had no significant effect on histamine-induced p42/p44 MAPK activation.

4 Histamine stimulated time and concentration-dependent increases in p38 MAPK activation in DDT_1MF-2 cells but had no effect on JNK activation. Histamine-induced p38 MAPK activation was inhibited by pertussis toxin (74% inhibition) and the p38 MAPK inhibitor SB 203580 (95% inhibition).

5 In summary, we have shown the histamine H_1 receptor activates p42/p44 MAPK and p38 MAPK signalling pathways in DDT_1MF-2 smooth muscle cells. Interestingly, signalling to both pathways appears to involve histamine H_1 receptor coupling to G_i/G_o-proteins.
British Journal of Pharmacology (2001) **133,** 1378–1386

Keywords: Histamine H_1 receptor; mitogen-activated protein kinases; DDT_1MF-2 cells

Abbreviations: DAG, diacylglycerol; GPCR, G protein-coupled receptor; IP_3, inositol 1,4,5-trisphosphate; JNK, C-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PTX, pertussis toxin

Introduction

Histamine H_1 receptors belong to the G protein-coupled receptor (GPCR) superfamily and are linked to the activation of phospholipase C (PLC)-β isoforms *via* pertussis toxin-insensitive $G_{q/11}$-proteins (for review see Hill *et al*., 1997). Stimulation of PLC triggers the hydrolysis of the plasma membrane phospholipid, phosphatidylinositol 4,5-biphosphate producing two second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates the release of Ca^{2+} from intracellular stores, whereas DAG activates specific isoforms of protein kinase C (PKC). Furthermore, histamine H_1 receptor-induced increases in intracellular Ca^{2+} are known to modulate the activity of Ca^{2+}/calmodulin-dependent enzymes such as nitric oxide synthetase, phospholipase A_2 (PLA_2) and adenylyl cyclase (for review see Leurs *et al*., 1995). In addition to the expected coupling to phospholipase C, recent studies have shown that G_q-PCRs are also capable of activating other signalling pathways including members of the mitogen-activated protein kinase (MAPK) family and protein kinase B (Sugden & Clerk, 1997; Murga *et al*., 1998).

MAPKs are a group of serine/threonine protein kinases comprising three main 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 (SAPKs) 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 & Han, 2000). The p42/p44 MAPK pathway is associated with activation 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 GPCRs, including members of the G_q-coupled family, are also involved in the regulation of p42/p44 MAPK, p38 MAPK and JNK pathways (for reviews see Sugden & Clerk, 1997; van Biesen *et al*., 1996). However, to our knowledge, there

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have been no reports describing the activation of MAPK signalling pathways by the histamine H_1 receptor.

Our previous studies have shown that the histamine H_1 receptor stimulates inositol phospholipid hydrolysis, release of Ca^{2+} from intracellular Ca^{2+} stores and Ca^{2+} influx in the hamster vas deferens derived smooth muscle cell line, DDT_1MF-2 (White *et al.*, 1993; Dickenson & Hill, 1991; 1992). In the present study we have investigated whether the histamine H_1 receptor regulates p42/p44 MAPK, p38 MAPK and JNK signalling pathways in DDT_1MF-2 cells. Preliminary data from this study has been presented to the British Pharmacological Society (Robinson & Dickenson, 2000).

Methods

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, U.K.). 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^{-1}) 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^{-1})/EDTA (0.02% w v^{-1}). Cells for determination of MAPK activation were grown in 6-well cluster dishes.

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 $well^{-1}$ 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 (mM): NaCl 150, Tris.HCl 50, EDTA 5, 1% (v v^{-1}) IGEPAL CA-630, 0.5% (w v^{-1}) sodium deoxycholate, 0.1% (w v^{-1}) SDS, Na_3VO_4 1, NaF 1, benzamidine 1, phenylmethylsulphonylfluoride 0.1, 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^{-1}) skimmed milk powder in PBS. Blots were then incubated overnight at 4°C with primary antibodies in 5% (w v^{-1}) skimmed milk powder dissolved in PBS-Tween 20 (0.5% by vol). 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 IgG coupled to horseradish peroxidase) at 1 : 1000 dilution in 5% (w v^{-1}) 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 Chemical Co.) prior to application of the primary antibody.

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, U.S.A.). Statistical significance was determined by Student's unpaired *t*-test or ANOVA followed by Dunnett's multiple comparison tests. All statistical analysis was performed using GraphPAD Software and $P<0.05$ was considered statistically significant. All data are presented as mean ± s.e.mean. 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 & 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 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: $C/C^1 = IC_{50}/K_D + 1$.

Materials

Aprotinin, bovine serum albumin, Dulbecco's modified Eagles medium, foetal calf serum, histamine, leupeptin, mepyramine, and pertussis toxin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). AG 1478 (4-(3-chloroanilino)-6,7-dimethoxyquinazoline), BAPTA/AM, cytochalasin D, daidzein, epidermal growth factor, genistein, LY 294002 (2-(4-morpholinyl)-8-phenyl-4H-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)1H-imidazole) and tyrphostin A47 (α-cyano-(3,4-dihydroxy)thiocinnamide) were from Calbiochem (Nottingham, U.K.). Tiotidine and

thioperamide were obtained from Tocris (Semat Technical (U.K.) Ltd). Phospho-specific p42/p44 MAPK and p38 MAPK antibodies were purchased from Sigma Chemical Co. Phospho-specific JNK antibody was from Promega. All other chemicals were of analytical grade.

Results

The regulation of MAPKs (p42/p44 MAPK, JNK and p38 MAPK) by the histamine H_1 receptor has been investigated in the hamster vas deferens derived smooth muscle cell line, DDT_1MF-2. The activities of MAPKs are controlled by dual phosphorylation within the amino acid sequence Thr-X-Tyr (Widmann *et al*., 1999). Increases in MAPK activation were determined by Western blot analysis using phospho-specific p42/p44 MAPK (Thr^{202}/Tyr^{204}), JNK (Thr^{183}/Tyr^{185}) and p38 MAPK (Thr^{180}/Tyr^{182}) antibodies.

Histamine H_1 receptor-mediated activation of p42/p44 MAPK

Stimulation of DDT_1MF-2 cells with histamine produced a rapid and transient increase in p42 and p44 MAPK phosphorylation with dominant activation of the p42 MAPK isoform (Figure 1a). The response to histamine was concentration-dependent ($p[EC_{50}] = 6.1 \pm 0.3$; $n = 4$; Figure 1b) and time-dependent with peak activation occurring at 5 min. p42/p44 MAPK responses to histamine (100 μM) were antagonized by the histamine H_1 receptor antagonist, mepyramine, yielding an apparent K_D value of 3.1 ± 0.8 nM ($n = 3$; Figure 1c). Furthermore, pre-treatment with tiotidine (1 μM) and thioperamide (1 μM), histamine H_2 and H_3 receptor antagonists respectively, had no effect on histamine (100 μM) induced p42/p44 MAPK activation (Figure 1d). These data indicate that increases in p42/p44 MAPK activation following stimulation of DDT_1MF-2 cells with histamine are mediated through the histamine H_1 receptor. Our previous studies have shown that histamine H_1 receptor activation in DDT_1MF-2 cells stimulates PTX-insensitive increases in inositol phosphate accumulation and calcium mobilization (White *et al*., 1993; Dickenson & Hill, 1993). These observations reflect the proposed coupling of histamine H_1 receptors to phospholipase C activation *via* G_q-proteins (Hill *et al*., 1997). However, in this study, pre-treatment with PTX (100 ng ml^{-1} for 16 h) significantly reduced histamine-induced p42/p44 MAPK activation ($57 \pm 9\%$ inhibition; $n = 7$; $P < 0.05$; Figure 2a) suggesting the involvement of G_i/G_o proteins. In addition PTX pre-treatment inhibited basal p42/p44 MAPK phosphorylation ($60 \pm 6\%$ inhibition; $n = 7$). Histamine (100 μM) induced p42/p44 MAPK activation was also inhibited by pre-treatment (30 min) of cells with the selective MAPK kinase inhibitor, PD 98059 (Dudley *et al*., 1995; 50 μM; $89 \pm 5\%$ inhibition; $n = 3$; $P < 0.05$; Figure 2b).

Tyrosine kinases belonging to the Src family are involved in the regulation of p42/p44 MAPKs by both G_q and G_i/G_o-protein-coupled receptors (Lopez-Ilasaca, 1998). In this study we explored the involvement of tyrosine kinases in histamine H_1 receptor-induced p42/p44 MAPK activation using the broad range tyrosine kinase inhibitors genistein and tyrphostin A47 and the Src-family tyrosine kinase inhibitor, PP2 (Hanke *et al*., 1996). Responses to histamine (100 μM) were significantly reduced by genistein (100 μM; $87 \pm 8\%$ inhibition; $n = 6$; $P < 0.05$; Figure 3a) and tyrphostin A47 (100 μM; $95 \pm 9\%$ inhibition; $n = 6$; $P < 0.05$; Figure 3b). Daidzein (100 μM), the inactive analogue of genistein, had no significant effect on histamine-induced p42/p44 MAPK activation ($95 \pm 8\%$ of control response; $n = 4$; $P < 0.05$; Figure 3a). Similarly, the Src-family tyrosine kinase inhibitor PP2 also had no significant effect on histamine-induced p42/p44 MAPK activation ($95 \pm 8\%$ of control response; $n = 4$; $P < 0.05$; Figure 3a). Recent studies have also demonstrated that certain GPCRs activate the p42/p44 MAPK pathway *via* transactivation (ligand-independent) of the epidermal growth factor receptor tyrosine kinase (Zwick *et al*., 1999). In this study, pre-incubation of cells with AG1478 (1 μM; 30 min), an inhibitor specific for the epidermal growth factor receptor tyrosine kinase had no significant effect on histamine (100 μM)-mediated p42/p44 MAPK activation. In contrast, epidermal growth factor (10 nM)-induced p42/p44 MAPK activation was completely inhibited ($10 \pm 8\%$ of control response; $n = 4$; Figure 3c) by AG1478 (1 μM). These data suggest that epidermal growth factor receptor transactivation is not involved in histamine H_1 receptor-mediated p42/p44 MAPK activation in DDT_1MF-2 cells.

Role of Ca^{2+}, PKC and focal adhesion kinases in histamine H_1 receptor-induced p42/p44 MAPK activation

PLC activation generates IP_3 and DAG, which mobilize intracellular Ca^{2+} and activate certain PKC isoforms, respectively (Berridge, 1993). Indeed, histamine H_1 receptor activation in DDT_1MF-2 cells stimulates intracellular Ca^{2+} release and Ca^{2+} influx (Dickenson & Hill, 1991; 1992). In this study we have examined the role of Ca^{2+} and PKC in the regulation of p42/p44 MAPK by the histamine H_1 receptor. The role 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 histamine H_1 receptor-mediated Ca^{2+} influx in DDT_1MF-2 cells (Dickenson & Hill, 1992). As shown in Figure 4a, removal of extracellular Ca^{2+} had no significant effect on histamine (100 μM; $81 \pm 9\%$ of control response; $n = 8$; $P > 0.05$) induced p42/p44 MAPK activation. The potential role of Ca^{2+} derived from intracellular stores was investigated using the Ca^{2+} chelator BAPTA (cells pre-incubated 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 histamine ($90 \pm 12\%$ of control; $n = 4$) induced p42/p44 MAPK activation (Figure 4b). These observations demonstrate that p42/p44 MAPK activation by histamine H_1 receptors is independent of Ca^{2+} elevation in DDT_1MF-2 cells.

The role of PKC in the regulation of p42/p44 MAPK by the histamine H_1 receptor was explored using the PKC inhibitor Ro 31–8220 (Davis *et al*., 1989). The PKC activator, phorbol 12-myristate 13-acetate (100 nM) induced p42/p44 MAPK activation in DDT_1MF-2 cells and as expected Ro 31–8220 (10 μM) inhibited this response ($85 \pm 5\%$ inhibition; $n = 5$). Responses to histamine (100 μM) were partially sensitive to PKC inhibition ($41 \pm 7\%$ inhibition; $n = 6$) indicating the possible involvement of a PKC-dependent pathway (Figure 4c).

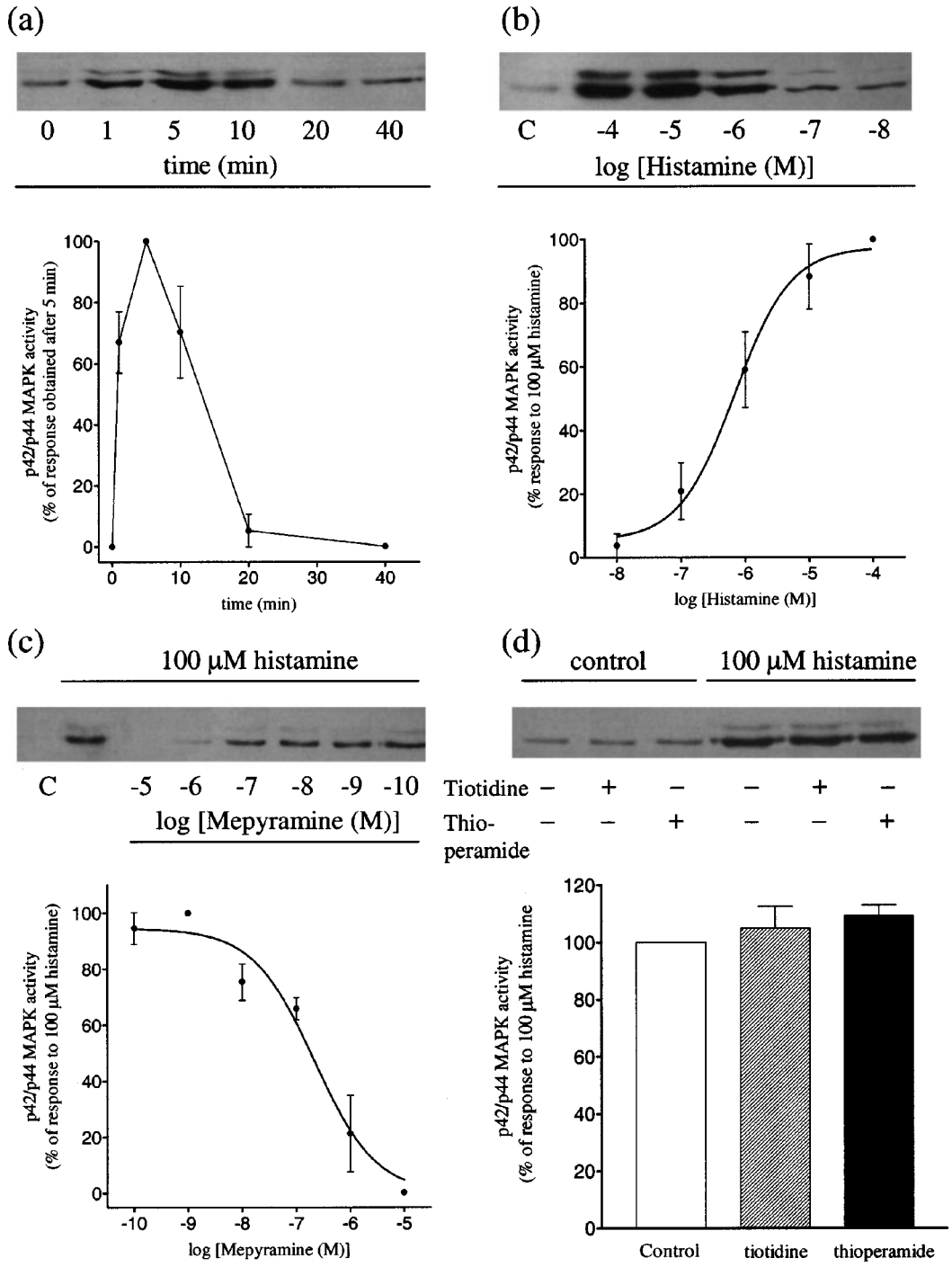

Figure 1 Histamine H_1 receptor stimulation of p42/p44 MAPK in DDT_1MF-2 cells. Cell lysates (20 μg) were analysed for p42/p44 MAPK activation by Western blotting using a phospho-specific p42/p44 MAPK antibody. Representative Western blots for each experiment are shown in the upper panels. (a) Time-course profile for histamine-induced p42/p44 MAPK activation in DDT_1MF-2 cells treated with vehicle (time zero) or histamine (100 μM) for the indicated periods of time. (b) Concentration-response curve for histamine in DDT_1MF-2 cells treated with vehicle (control; C) or the indicated concentrations of histamine for 5 min. (c) Inhibition of histamine-induced p42/p44 MAPK activation by the selective histamine H_1 receptor antagonist mepyramine. Cells were pre-incubated for 30 min with the indicated concentrations of mepyramine before stimulating with 100 μM histamine for 5 min. (d) Effect of pre-incubation (30 min) with tiotidine (1 μM; H_2 receptor antagonist) and thioperamide (1 μM; H_3 receptor antagonist) on histamine (100 μM)-induced p42/p44 MAPK activation. Combined results represent the mean$\pm$s.e.mean from three (a), four (b), three (c), and three (d) independent experiments.

Recent reports have shown that depolymerization of the actin cytoskeleton using cytochalasin D blocks GPCR-mediated p42/p44 MAPK activation suggesting the involvement of focal adhesion based signalling (Luttrell *et al.*, 1997; 1999; Della Rocca *et al.*, 1999a). However, as shown in Figure 4d, pre-treatment of DDT_1MF-2 cells with cytocha-

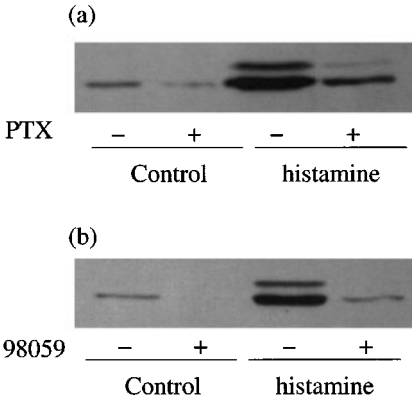

Figure 2 Effect of pertussis toxin and the MAPK kinase inhibitor PD 98059 on histamine H_1 receptor-induced p42/p44 MAPK activation in DDT_1MF-2 cells. DDT_1MF-2 cells were pre-treated with (a) pertussis toxin (PTX; 16 h 100 ng ml^{-1}) and (b) the MAPK kinase inhibitor PD 98059 (30 min; 50 μM) prior to stimulating with histamine (100 μM) for 5 min. Similar results were obtained in at least four independent experiments.

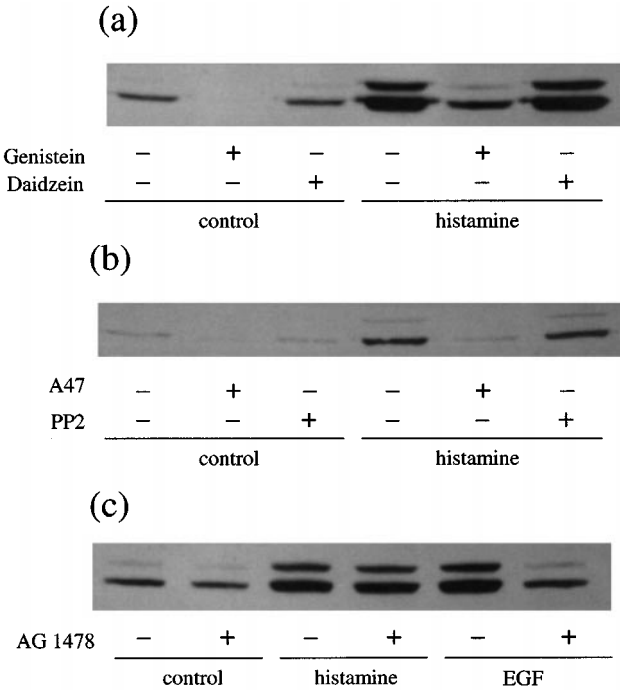

Figure 3 Effect of tyrosine kinase inhibitors on histamine H_1 receptor stimulation of p42/p44 MAPK in DDT_1MF-2 cells. Representative immunoblots showing the effects of pre-treating DDT_1MF-2 cells for 30 min with (a) the broad range tyrosine kinase inhibitor genistein (100 μM) and daidzein (100 μM), the inactive analogue of genistein, (b) the broad range tyrosine kinase inhibitor tyrphostin A47 (100 μM) and the Src tyrosine kinase inhibitor PP2 (10 μM) and (c) AG1478 (1 μM) the EGF receptor tyrosine kinase inhibitor. Cells were stimulated with histamine (100 μM) or EGF (10 nM) for 5 min. Similar results were obtained in at least three independent experiments.

lasin D (1 μM for 1 h) had no significant effect on histamine (100 μM; 95±7% of control response; $n=4$) induced p42/p44 MAPK activation.

Effect of PI-3K inhibition on histamine H_1 receptor-induced p42/p44 MAPK activation

Phosphatidylinositol 3-kinase (PI-3K) has been implicated in G_i-PCR-induced regulation of p42/p44 MAPK (Sugden & Clerk, 1997; van Biesen *et al*., 1996). In this study we have examined the role of PI-3K in the regulation of p42/p44 MAPK by the histamine H_1 receptor in DDT_1MF-2 cells. As shown in Figure 5 responses to histamine (100 μM) were inhibited following pre-treatment (30 min) with wortmannin ($p[IC_{50}]=7.6\pm0.06$; $n=4$) and LY 294002 ($p[IC_{50}]=4.8\pm0.1$; $n=4$). In these experiments, wortmannin (30 nM) and LY 294002 (30 μM) inhibited histamine (100 μM) p42/p44 MAPK responses by 64±10% ($n=4$) and 85±8% ($n=4$) respectively. These observations clearly demonstrate that a PI-3K-dependent pathway is involved in histamine H_1 receptor-mediated p42/p44 MAPK activation in DDT_1MF-2 cells.

Effect of histamine H_1 receptor activation on JNK and p38 MAPK

The p38 MAPK and JNK signalling pathways are activated by stimuli such as UV irradiation, osmotic stress and inflammatory cytokines (Paul *et al*., 1997). In our initial experiments we determined whether osmotic stress activates p38 MAPK and JNK in DDT_1MF-2 cells. Sorbitol (0.5 M) treatment activated p38 MAPK (Figure 6a) and the 46 kDa and 54 kDa isoforms of JNK (Figure 6b) in DDT_1MF-2 cells, although with different time-course profiles. Stimulation of DDT_1MF-2 cells with histamine produced concentration-dependent ($p[EC_{50}]=5.8\pm0.4$; $n=5$; Figure 6d) and time-dependent (peak activation occurring at 5 min; Figure 6c) increases in p38 MAPK activation. In contrast, histamine did not stimulate JNK phosphorylation in DDT_1MF-2 cells (data not shown). The histamine H_1 receptor antagonist mepyramine (1 μM) blocked histamine (100 μM)-induced p38 MAPK activation (95±9% inhibition; $n=4$; Figure 7a), whereas tiotidine (1 μM) and thioperamide (1 μM), histamine H_2 and H_3 receptor antagonists respectively, had no effect. These data indicate that histamine stimulated increases in p38 MAPK activation are mediated through the histamine H_1 receptor in DDT_1MF-2 cells. The p38 MAPK inhibitor SB 203580 (20 μM) blocked 100 μM histamine-induced p38 MAPK activation (95±7% inhibition; $n=4$; Figure 7b). Finally, pre-treatment with PTX (100 ng ml^{-1}) significantly reduced histamine-induced p38 MAPK activation (74±11% inhibition; $n=6$; $P<0.05$; Figure 7c).

Discussion

Histamine H_1 receptors trigger the activation of phospholipase C (PLC)-β isoforms *via* pertussis toxin-insensitive $G_{q/11}$-proteins in a variety of cell types (for review see Hill *et al*., 1997). In this study we have investigated whether the histamine H_1 receptor stimulates MAPK signalling (p42/p44 MAPK, JNK and p38 MAPK activation) in the hamster vas deferens smooth muscle cell line, DDT_1MF-2.

The data presented has shown for the first time that the histamine H_1 receptor stimulates p42/p44 MAPK activation. Recent studies suggest that G_q/G_{11}-PCRs preferentially employ PKC and/or Ca^{2+} signals to activate p42/p44 MAPK

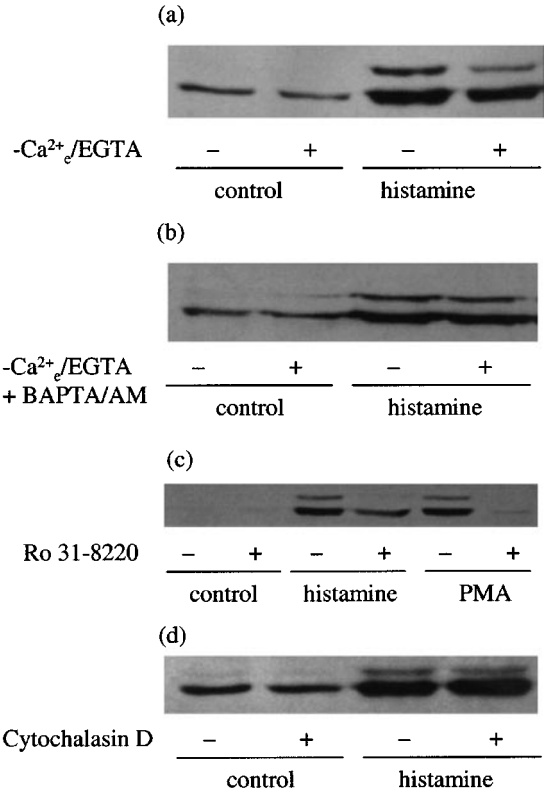

Figure 4 Role of Ca^{2+}, PKC and focal adhesion kinase in histamine H_1 receptor stimulation of p42/p44 MAPK in DDT_1MF-2 cells. Measurements of p42/p44 MAPK activation (5 min stimulation with 100 μM histamine) were made as follows: (a) in the presence (1.3 mM $CaCl_2$) and absence of extracellular Ca^{2+} (nominally Ca^{2+}-free Hanks/HEPES buffer containing 0.1 mM EGTA) to prevent Ca^{2+} influx; (b) using BAPTA (cells pre-incubated for 30 min with 50 μM BAPTA/AM) in the absence of extracellular Ca^{2+} (nominally Ca^{2+}-free Hanks/HEPES buffer containing 0.1 mM EGTA) to chelate Ca^{2+}-released from intracellular stores; (c) in cells pre-incubated with the PKC inhibitor Ro 31-8220 (10 μM) and (d) in cells pre-incubated for 1 h with cytochalasin D (1 μM) to disrupt focal adhesion-based signalling. Similar results were obtained in at least four independent experiments.

in a Raf-dependent but Ras-independent manner (Sugden & Clerk, 1997). Hence, in this study we examined the role of PKC and Ca^{2+} in the regulation of p42/p44 MAPK by the histamine H_1 receptor in DDT_1MF-2 cells. The data presented indicate that histamine H_1 receptor-induced p42/p44 MAPK responses in DDT_1MF-2 cells are independent of Ca^{2+} released from intracellular stores. However, the removal of extracellular Ca^{2+} did produce a small but non-significant reduction (*c.* 20%) in histamine-induced p42/44 MAPK activation suggesting a role for Ca^{2+} influx. In addition, p42/p44 MAPK responses to histamine were partially blocked by the selective PKC inhibitor Ro 31-8220 (*c.* 40% inhibition) indicating the involvement of a PKC-dependent pathway. Previous studies have shown that histamine H_1 receptor-mediated inositol phosphate accumulation in DDT_1MF-2 cells is reduced in the absence of extracellular Ca^{2+} (White *et al.*, 1993). Hence the small but non-significant reduction in p42/p44 MAPK activation in the absence of extracellular Ca^{2+} may be a consequence of reduced inositol phospholipid breakdown ultimately leading to a decrease in PKC activation (*via* DAG generation). This seems plausible

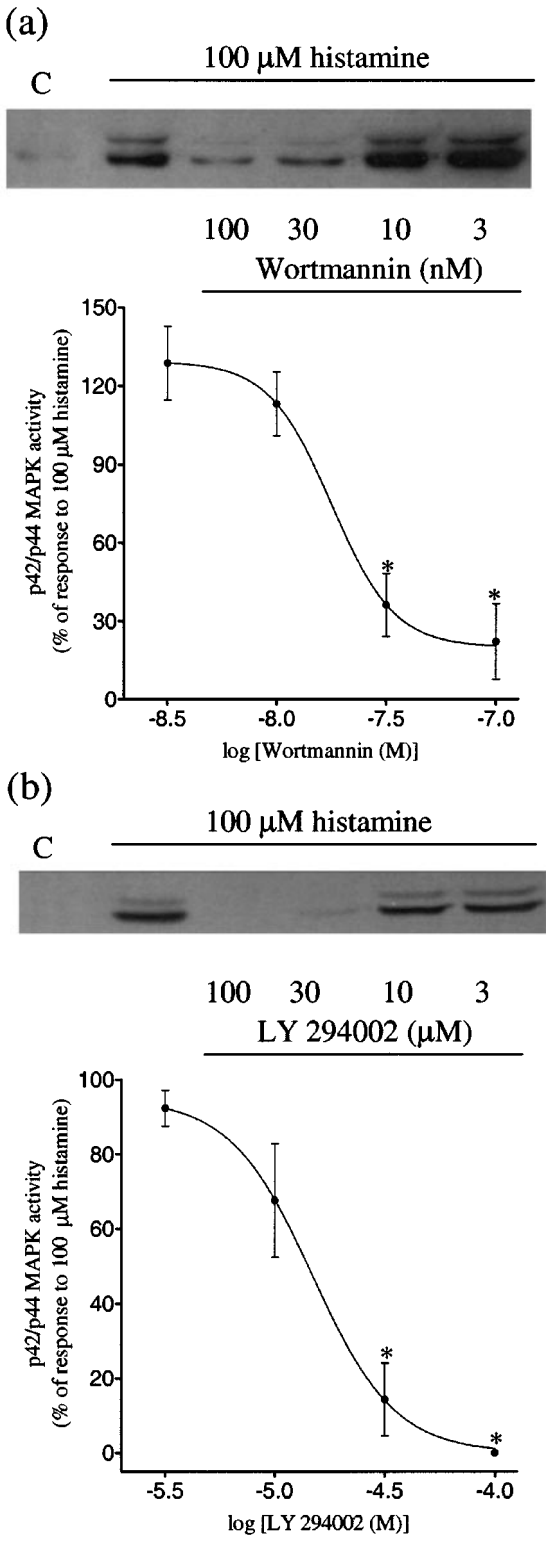

Figure 5 Role of PI-3K in histamine H_1 receptor stimulation of p42/p44 MAPK in DDT_1MF-2 cells. DDT_1MF-2 cells were pre-incubated (30 min) with the indicated concentrations of the PI-3K inhibitors (a) wortmannin and (b) LY 294002 before stimulating (5 min) with vehicle (control; C) or 100 μM histamine. Representative immunoblots for wortmannin (a) and LY 294002 (b) are shown in the upper panels. Combined results obtained from four independent experiments (mean $\pm$ s.e.mean) are shown in the lower panels. Data are presented as the percentage of the control response to 100 μM histamine (100%) in the absence of PI-3K inhibitor. *Significantly different ($P<0.05$) from the control response to 100 μM histamine.

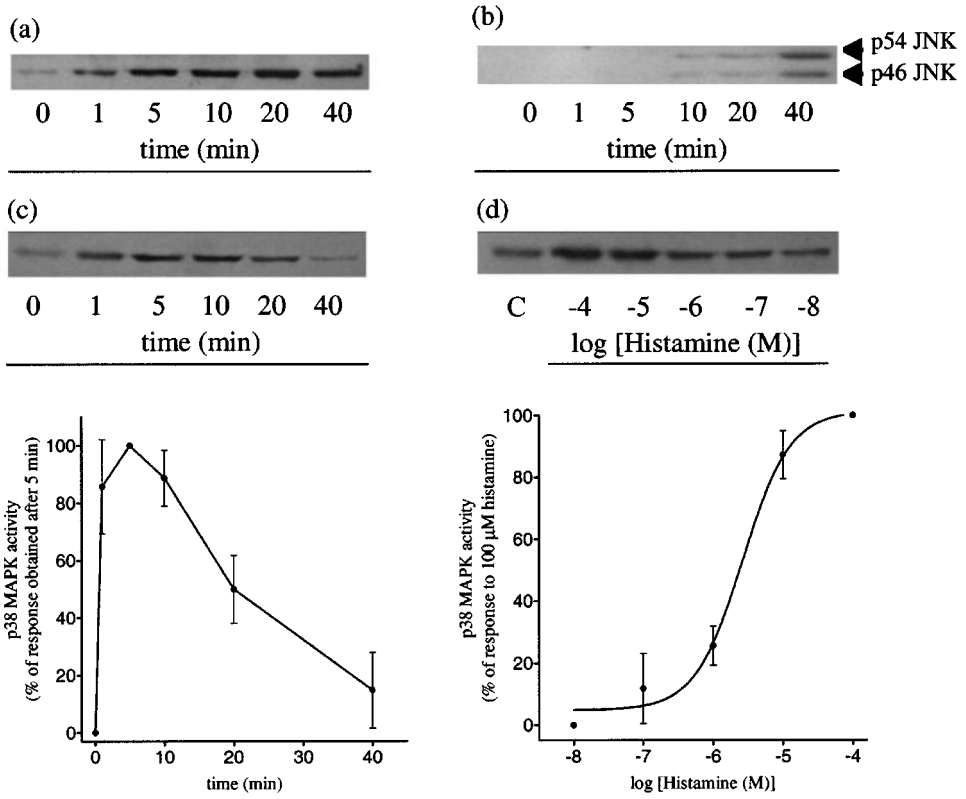

Figure 6 Histamine H_1 receptor stimulation of p38 MAPK in DDT_1MF-2 cells. DDT_1MF-2 cells were treated with 0.5 M sorbitol for the indicated periods of time. Cell lysates were resolved by SDS–PAGE and transferred to nitrocellulose before being probed with antisera specific for (a) phospho p38 MAPK and (b) phospho JNK. Stimulation of DDT_1MF-2 cells with histamine produced (c) time-dependent (using 100 μM histamine) and (d) concentration-dependent increases in p38 MAPK activation (5 min stimulation). Control cells (C) were treated with vehicle. Combined results obtained from four (c) and five (d) independent experiments (mean ± s.e.mean) are shown in the lower panels.

given the involvement of PKC in the overall p42/p44 MAPK response to histamine in DDT_1MF-2 cells.

It is notable that the response to histamine was sensitive to PTX pre-treatment (*c.* 60% inhibition) indicating the involvement of G_i/G_o proteins in coupling the histamine H_1 receptor to p42/p44 MAPK. In contrast, our previous studies have shown that the histamine H_1 receptor stimulates PTX-insensitive increases in inositol phosphate accumulation and calcium mobilization in DDT_1MF-2 cells (Dickenson & Hill, 1993; White *et al.*, 1993). Interestingly, several other studies have reported the involvement of G_i/G_o proteins in histamine H_1 receptor-mediated cell signalling. For example, the guinea-pig histamine H_1 receptor in transfected Chinese hamster ovary cells stimulates arachidonic acid (AA) release, which is partially sensitive to PTX (Leurs *et al.*, 1994). Similarly, histamine H_1 receptor activation in rabbit platelets triggers AA release, which is completely blocked by PTX (Murayama *et al.*, 1990). These observations suggest that the histamine H_1 receptor may activate different signalling pathways through coupling to various subtypes of G-protein, i.e. G_q/G_{11}-mediated PLC activation and G_i/G_o-mediated PLA_2 and p42/p44 MAPK. In view of the prominent role of PI-3K in G_i/G_o-PCR-mediated p42/p44 MAPK activation we investigated the role of PI-3K in histamine H_1 receptor-induced p42/p44 MAPK activation. The PI-3K inhibitors, wortmannin and LY 294002, significantly reduced histamine-induced p42/p44 MAPK activation suggesting a role for PI-3K activation. In summary, the data presented suggest that G_i/G_o-protein coupling and PI-3K activation are involved in histamine H_1 receptor-mediated p42/p44 MAPK activation in DDT_1MF-2 cells.

The requirement for non-receptor tyrosine kinases (Src family and the calcium-regulated focal adhesion kinase, Pyk2) in GPCR-mediated p42/p44 MAPK activation has been well-documented (Sugden & Clerk, 1997). In this study we explored the role of tyrosine kinases in histamine H_1 receptor-induced p42/p44 MAPK activation in DDT_1MF-2 cells. Responses to histamine were sensitive to the broad range tyrosine kinase inhibitors genistein and tyrphostin A47 but not to the specific Src tyrosine kinase inhibitor PP2. These data suggest that tyrosine kinases other than Src contribute to histamine H_1 receptor-mediated p42/p44 MAPK activation in DDT_1MF-2 cells. Furthermore, since histamine H_1 receptor-induced p42/p44 MAPK responses were independent of Ca^{2+} influx and intracellular Ca^{2+} release it would appear that the Ca^{2+}-activated tyrosine kinase PyK2 is not involved. Finally, the non-calcium dependent focal adhesion kinase p125FAK has been implicated in GPCR-mediated p42/p44 MAPK activation (Luttrell *et al.*, 1997; Della Rocca *et al.*, 1999a). However, this kinase appears not be involved in histamine H_1 receptor-mediated p42/p44 MAPK in DDT_1MF-2 cells since depolymerization of the actin cytoskeleton with cytochalasin D had no effect.

Transactivation of receptor tyrosine kinases such as the receptor for epidermal growth factor receptor has also been

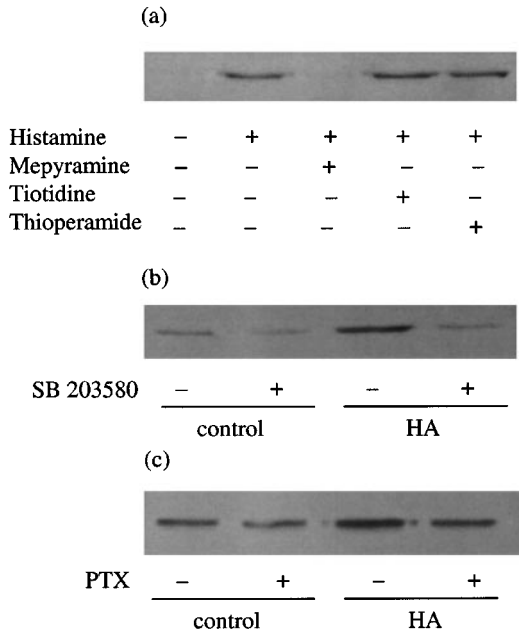

Figure 7 Effect of the histamine receptor antagonists, pertussis toxin and SB 203580 on histamine-induced p38 MAPK activation. DDT_1MF-2 cells were pre-incubated with (a) mepyramine (1 μM), tiotidine (1 μM; H_2 receptor antagonist) and thioperamide (1 μM; H_3 receptor antagonist) for 30 min, (b) the p38 MAPK inhibitor SB 203580 (20 μM) for 30 min or (c) pertussis toxin (100 ng ml^{-1} for 16 h). Cells were stimulated with 100 μM histamine for 5 min. Similar results were obtained in at least four independent experiments.

implicated in GPCR-mediated activation of p42/p44 MAPK (Zwick *et al*., 1999). However, in this study the epidermal growth factor receptor tyrosine kinase specific tyrphostin AG1478 had no significant effect on histamine-induced p42/p44 MAPK activation.

Overall the results presented in this study suggest that histamine H_1 receptor-induced p42/p44 MAPK activation in DDT_1MF-2 cells proceeds *via* two different pathways; one dependent on G_q and the other involving G_i/G_o protein coupling. The G_q protein dependent pathway presumably involves the activation of Raf *via* PKC (Ras-independent) as reported for other G_qPCRs (Sugden & Clerk, 1997). The second route appears to involve histamine H_1 receptor coupling to G_i/G_o proteins. Previous studies have shown that the activation of p42/p44 MAPK by G_i/G_o-PCRs involves G protein-derived $\beta\gamma$ subunits, genistein-sensitive *c-Src*-related protein tyrosine kinase(s) and PI-3K activation in a Ras-dependent manner (Koch *et al*., 1994; Hawes *et al*., 1995; 1996; Garnovskaya *et al*., 1996; Luttrell *et al*., 1996; Igishi & Gutkind, 1998). Our findings indicate that the G_i/G_o-protein component of the histamine H_1 receptor response signals through a similar pathway. However, further studies are required in order to firmly establish the role of G-protein $\beta\gamma$ subunits, Raf and Ras in histamine H_1 receptor-mediated p42/p44 MAPK activation in DDT_1MF-2 cells.

Recent studies have indicated a role of clathrin-mediated endocytosis in GPCR-induced p42/p44 MAPK activation (Lefkowitz, 1998). Blocking receptor endocytosis prevents p42/p44 MAPK signalling elicited by a range of receptors including the β_2 adrenergic, 5-HT_{1A} receptor, m1 muscarinic and δ-opioid receptors (Daaka *et al*., 1998; Della Rocca *et al*., 1999b; Vogler *et al*., 1999; Ignatova *et al*., 1999). Studies are currently in progress to determine whether internalization of the histamine H_1 receptor is important for histamine induced p42/p44 MAPK activation in DDT_1MF-2 cells.

In this study we have also explored the regulation of p38 MAPK and JNK signalling pathways by the histamine H_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 GPCRs have been shown to activate these pathways (Paul *et al*., 1997). The data presented in this study indicate that the histamine H_1 receptor can activate p38 MAPK signalling in DDT_1MF-2 cells. Signalling pathways linking GPCRs to p42/p44 MAPK activation have been extensively studied and characterized, in contrast the mechanisms by which GPCRs stimulate p38 MAPK are largely unknown. Recent studies have shown that m2 muscarinic (G_i-coupled) and β-adrenergic (G_s-coupled) receptor activation of p38 MAPK is mediated *via* $G\beta\gamma$, whereas activation by the G_q-coupled m1 muscarinic receptor involves $G\alpha_{q/11}$ and $G\beta\gamma$ (Yamauchi *et al*., 1997). Subsequent studies have shown that PKC and Src family kinases are involved in $G\alpha_{q/11}$ stimulated p38 MAPK activation (Nagao *et al*., 1998). In this study we have shown the activation of p38 MAPK by histamine was sensitive to PTX pre-treatment indicating the involvement of G_i/G_o proteins in coupling the histamine H_1 receptor to p38 MAPK in DDT_1MF-2 cells. However, further studies are required in order to identify the molecular mechanisms mediating p38 MAPK activation by the histamine H_1 receptor.

In summary, we have shown that the histamine H_1 receptor activates p38 MAPK and p42/p44 MAPK in the smooth muscle cell line DDT_1MF-2. Given that p38 MAPK and p42/44 MAPK regulate many cellular processes including inflammation, cell differentiation, cell growth and death (Paul *et al*., 1997; Ono & Han, 2000) future experiments will investigate the effects of histamine H_1 receptor activation on cell proliferation and apoptosis.

This work was funded by The Nottingham Trent University.

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(Received September 8, 2000
Revised March 3, 2001
Accepted June 4, 2001)