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Stimulation of protein kinase B and p70 S6 kinase by the histamine H_1 receptor in DDT_1MF-2 smooth muscle cells

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1 Previous studies have shown that the histamine H_1 receptor activates p42/p44 mitogen-activated protein kinases (MAPK) in DDT_1MF-2 smooth muscle cells *via* a phosphatidylinositol 3-kinase (PI-3K)-dependent pathway. In this study the effect of histamine H_1 receptor stimulation on protein kinase B (PKB) and p70 S6 kinase, both of which are downstream targets of PI-3K, has been investigated. Increases in PKB and p70 S6 kinase activation were monitored by Western blotting using phospho-specific PKB (Ser^{473}) and p70 S6 kinase (Thr^{421}/Ser^{424}) antibodies.

2 Histamine stimulated time and concentration-dependent increases in the phosphorylation of PKB and p70 S6 kinase in DDT_1MF-2 cells. Both responses were completely inhibited by the histamine H_1 receptor antagonist mepyramine and following pre-treatment with pertussis toxin, to block G_i/G_o protein dependent pathways.

3 The PI-3K inhibitors wortmannin (IC_{50} 5.9 ± 0.5 nM) and LY 294002 (IC_{50} 6.9 ± 0.8 μM) attenuated the increase in PKB phosphorylation induced by histamine (100 μM) in a concentration-dependent manner.

4 Histamine-induced increases in p70 S6 kinase phosphorylation were partially sensitive to rapamycin (20 nM; 68% inhibition) but completely blocked by wortmannin (100 nM), LY 294002 (30 μM) and the MAPK kinase inhibitor PD 98059 (50 μM).

5 In summary, these data demonstrate that the histamine H_1 receptor stimulates PKB and p70 S6 kinase phosphorylation in DDT_1MF-2 smooth muscle cells. However, functional studies revealed that histamine does not stimulate DDT_1MF-2 cell proliferation or attenuate staurosporine-induced caspase-3 activity. The challenge for future research will be to link the stimulation of these kinase pathways with the physiological and pathophysiological roles of the histamine H_1 receptor.
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Keywords: Histamine H_1 receptor; protein kinase B; p70 S6 kinase; DDT_1MF-2 cells

Abbreviations: GPCR, G protein-coupled receptor; JNK, C-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; PI-3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PTX, pertussis toxin

Introduction

The histamine H_1 receptor is a member of the G protein-coupled receptor (GPCR) superfamily which when activated stimulates phospholipase C-β *via* pertussis toxin-insensitive $G_{q/11}$-proteins (Hill *et al*., 1997). The stimulation of phospholipase C-β catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate generating inositol-1,4,5-trisphosphate and diacylglycerol (Rebecchi & Pentyala, 2000). Inositol-1,4,5-trisphosphate triggers the release of calcium from intracellular stores, whereas diacylglycerol activates specific isoforms of protein kinase C. Downstream signalling events attributed to histamine H_1 receptor-mediated increases in intracellular calcium include the modulation of calcium/calmodulin-dependent enzymes such as nitric oxide synthetase, phospholiapse A_2 and adenylyl cyclase (Leurs *et al*., 1995). In contrast, protein kinase C is known to be involved in histamine H_1 receptor stimulation of the p42/p44 mitogen-activated protein kinase (MAPK) pathway in DDT_1MF-2 cells and activation of the c-*fos* promoter in transfected CHO cells (Megson *et al*., 2001; Robinson & Dickenson, 2001).

Our previous work has shown that inhibitors of phosphatidylinositol 3-kinase (PI-3K), wortmannin (Ui *et al*., 1995) and LY 294002 (Vlahos *et al*., 1994), markedly reduced p42/p44 MAPK activation by histamine in DDT_1MF-2 cells, suggesting that the histamine H_1 receptor activates PI-3K in these cells (Robinson & Dickenson, 2001). PI-3K is now known to regulate a variety of cellular events which involve the downstream activation of serine/threonine kinases such as protein kinase C isoforms, p70 S6 kinase and protein kinase B/Akt (Duronio *et al*., 1998; Toker, 2000). The present study has therefore investigated whether the histamine H_1 receptor activates additional signalling pathways downstream of PI-3K and p42/p44 MAPK. The results presented show that the histamine H_1-receptor activates p70 S6 kinase and PKB in the smooth muscle cell line, DDT_1MF-2.

Methods

Cell culture

The hamster *vas deferens* smooth muscle cell line (DDT_1MF-2) was obtained from the European Collection of Animal Cell

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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 PKB and p70 S6 kinase 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. Incubations were terminated by aspiration of the medium and the addition of 300 μl of SDS–PAGE sample buffer. 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 III 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 antibody 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. In addition, replicate samples from each experiment were analysed on separate blots using an antibody that recognizes unphosphorylated (total) PKB to confirm equal loading of protein on each lane. Unfortunately, the commercially available antibody that recognises total p70 S6 kinase (obtained from New England Biolabs) did not detect p70 S6 kinase in hamster DDT_1MF-2 cells. In these experiments the uniformity of protein loading was confirmed by measuring total PKB (data omitted from the appropriate Figures for clarity).

Cell proliferation assay

The effects of histamine and EGF on cell proliferation were determined using the MTT assay (Denizot & Lang, 1986), which measures the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bomide (MTT) to a coloured water-insoluble formazan salt by mitochondrial dehydrogenases. DDT_1MF-2 cells were seeded at 10,000 cells per well in 96-well plates and cultured in serum-free DMEM for 24 h to arrest cell growth. Cells were subsequently incubated for 72 h with the indicated concentrations of histamine and EGF after which MTT (0.5 mg ml^{-1} final concentration) was added. After incubation of the plates at 37°C for 1 h the cells were then washed with phosphate buffered saline and the formazan salts dissolved in 200 μl of dimethyl sulphoxide with gentle shaking for 10 min at room temperature. The plates were read at 570 nm using a Tecan Spectra Fluor plate reader.

Caspase-3 activity assay

DDT_1MF-2 cells were grown in 25 cm^2 flasks and when 80–90% confluent placed in DMEM medium containing 0.1% bovine serum albumin for 16 h. Serum-starved cells were then treated with staurosporine (1 μM) for 4 h. Caspase-3 activity was subsequently measured using the colourimetric CaspA-CETM assay system (Promega) in accordance with the protocol supplied by the manufacturer. Cell lysates were incubated with 200 μM of the colourimetric substrate *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline for 4 h and release of the chromophore p-NA quantified spectrophotometrically at a wavelength of 405 nm using a Tecan Spectra Fluor plate reader.

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 ($P<0.05$ was considered statistically significant). All data are presented as mean $\pm$ s.e.mean. The *n* in the text refers to the number of separate experiments.

Materials

Bovine serum albumin, Dulbecco's modified Eagles medium, foetal calf serum, mepyramine, pertussis toxin, staurosporine and 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bomide (MTT) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Epidermal growth factor, LY 294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), PD 98059 (2′-amino-3′-methoxyflavone), rapamycin, and wortmannin were from Calbiochem (Nottingham, U.K.). Tiotidine and thioperamide were obtained from Tocris (Semat Technical (U.K.) Ltd). Phospho-specific PKB (Ser^{473}) and p70 S6 kinase (Thr^{421}/Ser^{424}) antibodies was purchased from New England Biolabs. Phospho-specific p42/p44 MAPK (Thr^{202}/Tyr^{204}) antibody was from the Sigma Chemical Co. All other chemicals were of analytical grade.

Results

We have recently reported that the PI-3K inhibitors, wortmannin and LY 294002, attenuated histamine H_1

receptor-induced p42/p44 MAPK activation in the smooth muscle cell line DDT_1MF-2 (Robinson & Dickenson, 2001). The present study has therefore investigated whether the histamine H_1 receptor stimulates protein kinase B and p70 S6 kinase, both of which are downstream targets of PI-3K (Duronio *et al*., 1998; Toker, 2000). PKB activation requires phosphorylation of amino acid residues Thr308 and Ser473 and therefore PKB activation in DDT_1MF-2 cells was detected by Western blotting using an anti-phospho-PKB (Ser^{473}) antibody that detects PKB only when phosphorylated (and therefore activated) at Ser473 (Chan *et al*., 1999). By comparison, the activation of p70 S6 kinase is complex and involves the phosphorylation of at least eight interdependent sites (Pullen & Thomas, 1997; Dufner & Thomas, 1999). The activation of p70 S6 kinase was monitored using a phospho-p70 S6 kinase (Thr^{421}/Ser^{424}) antibody that specifically detects p70 S6 kinase only when phosphorylated at Thr^{421}/Ser^{424}. The phosphorylation of amino acid residues Thr^{421} and Ser^{424}, which are located in the C-terminus autoinhibitory domain of the enzyme, represents an essential early event in p70 S6 kinase activation (Pullen & Thomas, 1997; Dufner & Thomas, 1999).

Histamine H_1 receptor-mediated increases in protein kinase B phosphorylation

Our previous studies have shown that insulin and the adenosine A_1 receptor activate PKB in DDT_1MF-2 smooth muscle cells (Germack & Dickenson, 2000). In this study, stimulation of DDT_1MF-2 cells with histamine produced time-dependent (the increase above basal PKB phosphorylation after 5 min being $199 \pm 11\%$; $n=5$; Figure 1a) and concentration-dependent ($p[EC_{50}] = 6.4 \pm 0.14$; $n=5$; Figure 1b) increases in PKB phosphorylation. The activation of PKB in response to histamine (circa 200% above basal) is comparable to increases in PKB phosphorylation reported previously for insulin and adenosine A_1 receptor stimulation in DDT_1MF-2 cells (Germack & Dickenson, 2000). In contrast, epidermal growth factor (EGF; 10 nM) elicited increases in PKB phosphorylation of $373 \pm 27\%$ ($n=5$; $P<0.05$) above basal with maximal activation occurring after 10 min (Figure 2). The histamine H_1 receptor antagonist mepyramine (1 μM) blocked histamine (100 μM)-induced PKB phosphorylation ($97 \pm 5\%$ inhibition; $n=4$; Figure 3a), 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 PKB phosphorylation are mediated through the histamine H_1 receptor in DDT_1MF-2 cells.

Previous studies have shown that histamine H_1 receptor activation in DDT_1MF-2 cells stimulates pertussis toxin-insensitive increases in inositol phosphate accumulation and calcium mobilization (White *et al*., 1993; Dickenson & Hill, 1993). These observations reflect the accepted coupling of the histamine H_1 receptor to phospholipase C activation *via* $G_{q/11}$-proteins (Hill *et al*., 1997). However, we have recently shown that histamine H_1 receptor-induced increases in p42/p44 MAPK and p38 MAPK in DDT_1MF-2 cells were partially sensitive to pertussis toxin suggesting coupling to members of the G_i/G_o-protein family (Robinson & Dickenson, 2001). Hence, in this study the role of G_i/G_o-proteins in histamine H_1 receptor-induced phosphorylation of PKB

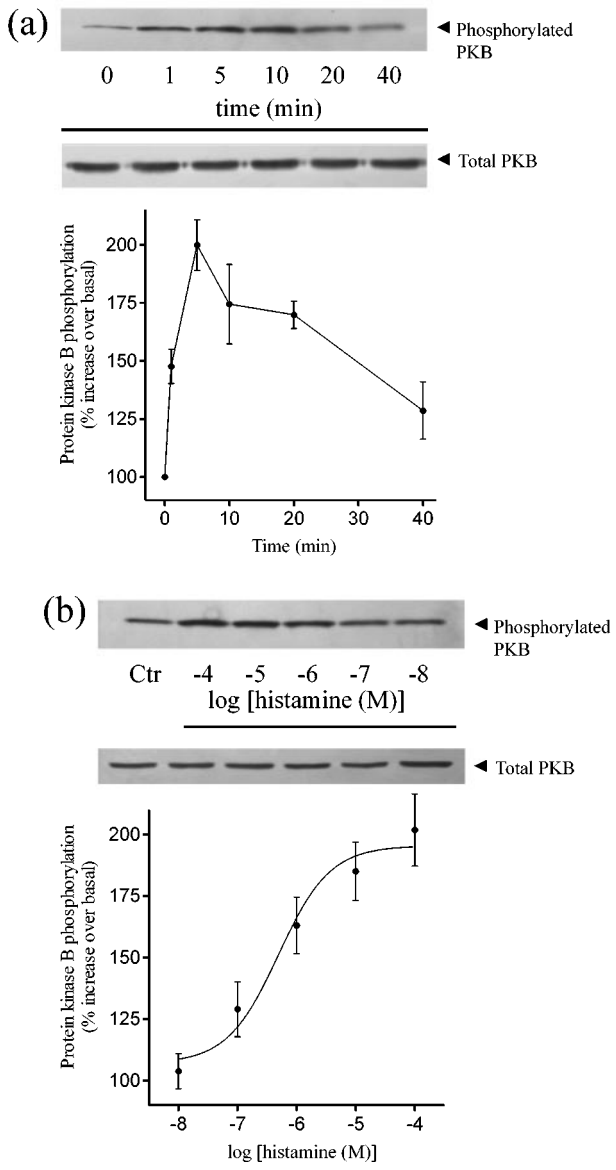

Figure 1 Histamine H_1 receptor-mediated increases in PKB phosphorylation in DDT_1MF-2 cells. Increases in PKB phosphorylation were determined by Western blotting using a phospho-specific PKB (Ser^{473}) antibody. The same samples were subsequently analysed on a separate blot using an antibody that recognizes unphosphorylated (total) PKB to confirm equal loading on each lane. Representative Western blots for each experiment are shown in the upper panels. (a) Time-course profile for histamine-induced PKB phosphorylation 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) or the indicated concentrations for 5 min. Combined results represent the mean $\pm$ s.e.mean from five independent experiments.

was determined in cells exposed to pertussis toxin (100 ng ml^{-1}) for 16 h. As shown in Figure 3b, pertussis toxin pre-treatment virtually abolished histamine-induced increases in PKB phosphorylation. In these experiments responses to 100 μM histamine obtained in control and pertussis toxin-treated cells were $215 \pm 9\%$ ($n=5$) and $109 \pm 14\%$ ($n=5$) above basal, respectively. In contrast, responses to EGF (10 nM) were insensitive to pertussis toxin

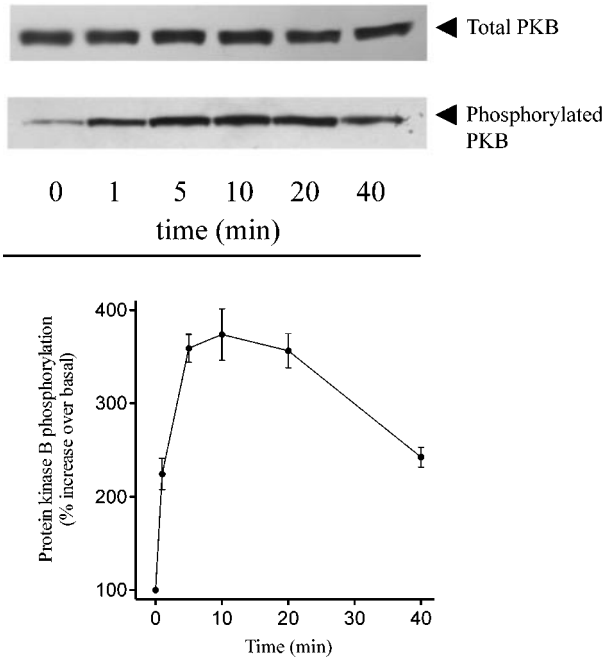

Figure 2 Epidermal growth factor-mediated increases in PKB phosphorylation in DDT_1MF-2 cells. Increases in PKB phosphorylation were determined by Western blotting using a phospho-specific PKB (Ser^{473}) antibody. The same samples were subsequently analysed on a separate blot using an antibody that recognizes unphosphorylated (total) PKB to confirm equal loading on each lane. DDT_1MF-2 cells treated with vehicle (time zero) or epidermal growth factor (10 nM) for the indicated periods of time. A representative Western blot is shown in the upper panel. The combined results represent the mean ± s.e.mean from five independent experiments.

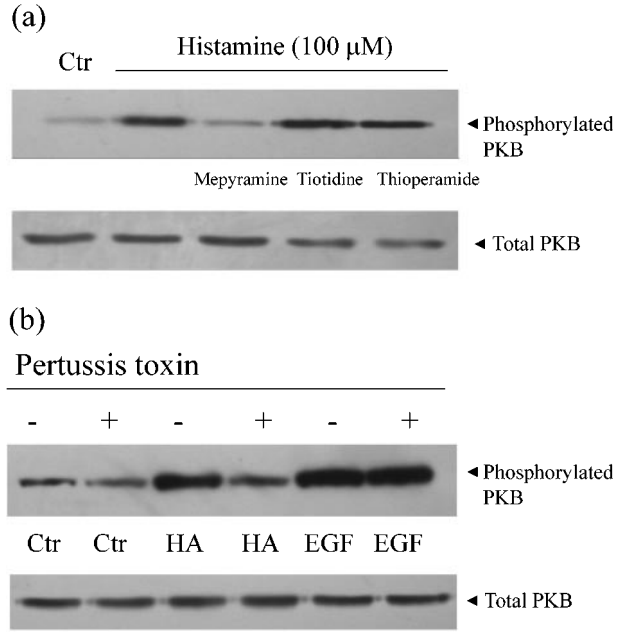

Figure 3 The role of G_i/G_o-proteins in histamine H_1 receptor-mediated increases in PKB phosphorylation in DDT_1MF-2 cells. (a) DDT_1MF-2 cells were pre-incubated (30 min) with mepyramine (1 μM; H_1 receptor antagonist), tiotidine (1 μM; H_2 receptor antagonist) and thioperamide (1 μM H_3 receptor antagonist) before stimulating with histamine (100 μM) for 5 min. (b) DDT_1MF-2 cells were pre-treated for 16 h with pertussis toxin (100 ng ml^{-1}) to block G_i/G_o-protein dependent pathways before stimulating with 100 μM histamine or 10 nM EGF for 5 min. Increases in PKB phosphorylation were determined by Western blotting using a phospho-specific PKB (Ser^{473}) antibody. The same samples were subsequently analysed on a separate blot using an antibody that recognizes unphosphorylated (total) PKB to confirm equal loading on each lane. Similar results were obtained in at least three other independent experiments.

since PKB phosphorylation increased 375 ± 19% ($n=5$) above basal in control cells and 382 ± 12% ($n=5$) above basal in cells pre-treated with pertussis toxin. These data indicate that the histamine H_1 receptor-mediated increases in PKB phosphorylation involve coupling to pertussis toxin-sensitive G_i/G_o proteins.

PI-3K plays a central role in the activation of PKB in response to tyrosine kinase growth factor receptors and G protein-coupled receptors (Chan *et al.*, 1999). Hence, the potential involvement of PI-3K in histamine H_1 receptor-induced increases in PKB phosphorylation was investigated. As clearly shown in Figure 4, responses to histamine (100 μM) were attenuated following pre-treatment (30 min) of DDT_1MF-2 cells with the PI-3K inhibitors wortmannin ($p[IC_{50}] = 8.2 \pm 0.04$; $n=4$) and LY 294002 ($p[IC_{50}] = 5.1 \pm 0.06$; $n=4$). These observations demonstrate that a PI-3K-dependent pathway mediates histamine H_1 receptor-induced increases in PKB phosphorylation in DDT_1MF-2 cells.

Histamine H_1 receptor-mediated increases in p70 S6 kinase phosphorylation

As would be predicted treatment of DDT_1MF-2 cells with EGF (10 nM) produced a robust and time-dependent increase in p70 S6 kinase phosphorylation (856 ± 31% increase above basal after 10 min; $n=5$; $P<0.05$; Figure 5). Histamine (100 μM) also produced a time-dependent increase in p70 S6 kinase phosphorylation with maximal activation (450 ± 34% increase above basal; $n=5$; $P<0.05$) occurring after 10 min (Figure 6a). The response to histamine was concentration-dependent, yielding a $p[EC_{50}]$ of 5.88 ± 0.05 ($n=5$;Figure 6b). The histamine H_1 receptor antagonist mepyramine (1 μM) blocked histamine (100 μM)-induced p70 S6 kinase phosphorylation (95 ± 8% 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. In addition, pre-treatment with pertussis toxin (100 ng ml^{-1} for 16 h) completely abolished histamine-induced increases in p70 S6 kinase phosphorylation (see Figure 7b). Responses to 100 μM histamine obtained in control and pertussis toxin-treated cells were 375 ± 25% ($n=5$) and 110 ± 10% ($n=5$) above basal, respectively. For comparison, responses to EGF (10 nM) were insensitive to pertussis toxin since PKB phosphorylation increased 880 ± 35% ($n=5$) above basal in control cells and 895 ± 29% ($n=5$) above basal in cells pre-treated with pertussis toxin. The signal transduction pathways involved in the phosphorylation of p70 S6 kinase at Thr^{421}/Ser^{424} were explored using the selective kinase inhibitors PD 98059 (MEK1 and MEK2), rapamycin (mTOR), wortmannin and LY 294002. As shown in Figure 8a, histamine-induced increases in p70 S6 kinase phosphorylation at Thr^{421}/Ser^{424} were abolished by PD 98059 (50 μM), wortmannin (100 nM) and LY 294002 (30 μM). EGF (10 nM)

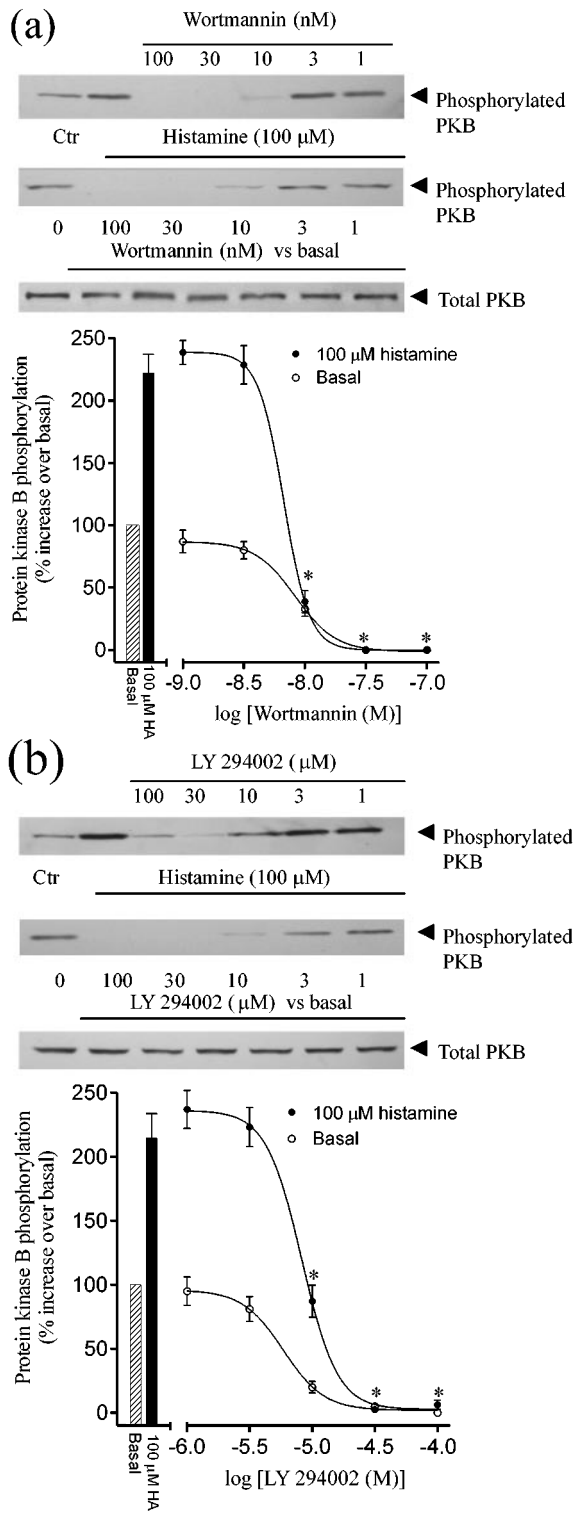

Figure 4 Role of phosphatidylinositol 3-kinase in histamine H_1 receptor stimulated increases in PKB phosphorylation in DDT_1MF-2 cells. DDT_1MF-2 cells were pre-incubated (30 min) with the indicated concentrations of the phosphatidylinositol 3-kinase inhibitors (a) wortmannin and (b) LY 294002 before stimulating with 100 μM histamine for 5 min. Cell lysates (20 μg) were analysed for PKB phosphorylation by Western blotting using a phospho-specific PKB (Ser^{473}) antibody. The same samples were subsequently analysed on a separate blot using an antibody that recognizes unphosphorylated (total) PKB to confirm equal loading on each lane. Representative immunoblots showing the inhibition by wortmannin (a) and LY 294002 (b) of the basal and histamine-induced levels of PKB phosphorylation are shown in the upper two panels. Combined results obtained from four independent experiments (mean$\pm$s.e.mean) are shown. Data are presented as the percentage of the control response to unstimulated cells (100%) in the absence of the phosphatidylinositol 3-kinase inhibitor. *Significantly different ($P<0.05$) from the control response to 100 μM histamine.

mediated increases in p70 S6 kinase phosphorylation at Thr^{421}/Ser^{424} were also sensitive to PD 98059 (85$\pm$4% inhibition; $n=5$), wortmannin (36$\pm$5% inhibition; $n=5$) and LY 294002 (30$\pm$2% inhibition; $n=5$). Pre-treatment of cells with rapamycin (20 nM) inhibited histamine and EGF-induced p70 S6 kinase phosphorylation by 68$\pm$7% ($n=5$) and 31$\pm$4% ($n=5$), respectively. The differing effect of wortmannin and LY 294002 on histamine H_1 receptor and EGF receptor-induced p70 S6 kinase phosphorylation (at Thr^{421}/Ser^{424}) may reflect the role of PI-3K in the activation of p42/p44 MAPK by these two receptor types. Our previous studies have shown that stimulation of p42/p44 MAPK by the histamine H_1 receptor is dependent upon PI-3K activation in DDT_1MF-2 cells (Robinson & Dickenson, 2001). In contrast, EGF triggers p42/p44 MAPK activation *via* a PI-3K-independent pathway (Hawes *et al*., 1996). In agreement with our previous work, histamine-induced increases in p42/p44 MAPK (measured after 10 min stimulation to correlate with the peak activation of p70 S6 kinase) were virtually abolished by wortmannin and LY 294002 (Figure 9a). In contrast, wortmannin and LY 294002 had no significant effect on EGF-mediated p42/p44 MAPK activation in DDT_1MF-2 cells (see Figure 9b). Finally, histamine

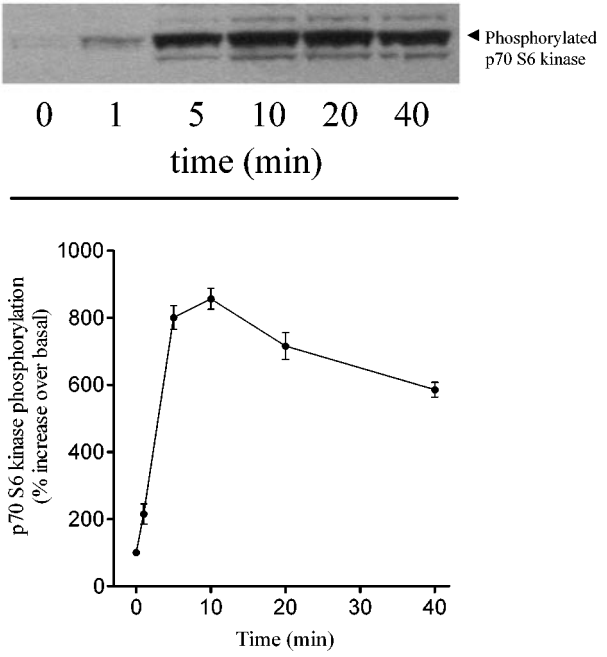

Figure 5 Epidermal growth factor-mediated increases in p70 S6 kinase phosphorylation in DDT_1MF-2 cells. Increases in p70 S6 kinase phosphorylation were determined by Western blotting using a phospho-specific p70 S6 kinase (Thr^{421}/Ser^{424}) antibody. DDT_1MF-2 cells treated with vehicle (time zero) or epidermal growth factor (10 nM) for the indicated periods of time. A representative Western blot is shown in the upper panel. The combined results represent the mean$\pm$s.e.mean from five independent experiments.

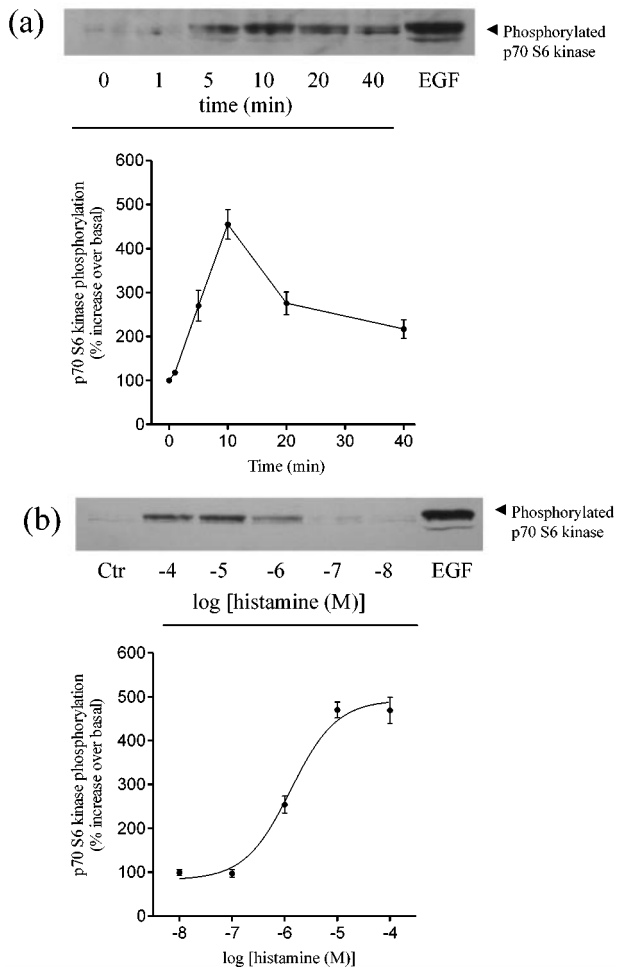

Figure 6 Histamine H_1 receptor-mediated increases in p70 S6 kinase phosphorylation in DDT_1MF-2 cells. Increases in p70 S6 kinase phosphorylation were determined by Western blotting using a phospho-specific p70 S6 kinase (Thr^{421}/Ser^{424}) antibody. Representative Western blots for each experiment are shown in the upper panels. (a) Time-course profile for histamine-induced p70 S6 kinase phosphorylation 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) or the indicated concentrations for 10 min. The increase in p70 S6 kinase phosphorylation in response to 10 nM EGF (10 min) is shown for comparison. Combined results represent the mean$\pm$s.e.mean from five independent experiments.

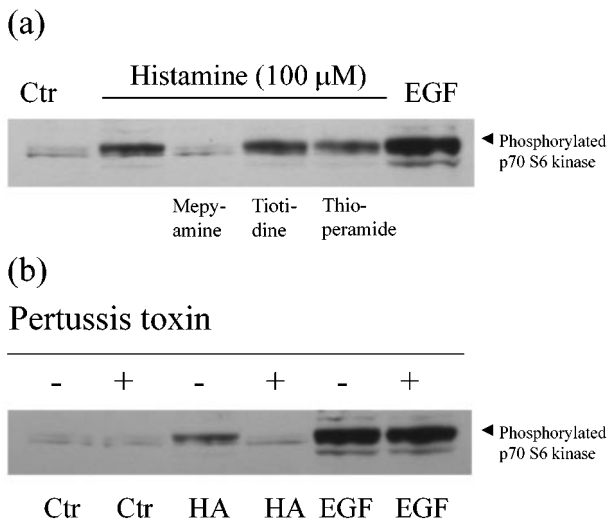

Figure 7 The role of G_i/G_o-proteins in histamine H_1 receptor-mediated increases in p70 S6 kinase phosphorylation in DDT_1MF-2 cells. (a) DDT_1MF-2 cells were pre-incubated (30 min) with mepyramine (1 μM; H_1 receptor antagonist), tiotidine (1 μM; H_2 receptor antagonist) and thioperamide (1 μM; H_3 receptor antagonist) before stimulating with histamine (100 μM) for 10 min. (b) DDT_1MF-2 cells were pre-treated for 16 h with pertussis toxin (100 ng ml^{-1}) to block G_i/G_o-protein dependent pathways before stimulating with 100 μM histamine or 10 nM EGF for 10 min. Increases in p70 S6 kinase phosphorylation were determined by Western blotting using a phospho-specific p70 S6 kinase (Thr^{421}/Ser^{424}) antibody. Similar results were obtained in four other independent experiments.

and EGF-induced increases in p42/p44 MAPK phosphorylation were insensitive to rapamycin but blocked by PD 98059 (Figure 9). It is important to note that the IC_{50} for PD 98059 mediated inhibition of MEK2 (50 μM) is 10-fold higher than that of MEK1 (4 μM; Dudley *et al*., 1995). This difference in sensitivity of MEK1 and MEK2 to PD 98059 presumably explains the partial inhibition of EGF-induced p42/p44 MAPK activation observed using 50 μM PD 98059 whereas complete inhibition was achieved using 100 μM PD 98059.

Effect of histamine and EGF on cell proliferation and staurosporine-induced caspase-3 activity

The potential physiological role(s) of histamine H_1 receptor-mediated activation of p70 S6 kinase and PKB in DDT_1MF-2 cells were explored by investigating whether histamine stimulates cell proliferation or attenuates the activation of caspase-3 induced by staurosporine. As clearly shown in Figure 10a, histamine did not stimulate DDT_1MF-2 smooth muscle cell proliferation. In contrast, EGF induced a concentration-dependent increase in the proliferation of DDT_1MF-2 cells (Figure 10b), which was not altered by co-application of histamine (100 μM). Treatment of DDT_1MF-2 cells with the protein kinase inhibitor staurosporine (1 μM for 4 h) induced a robust increase in the activity of caspase-3 (Figure 10c). Pre-treatment of cells with EGF (10 nM for 15 min) prior to the application of staurosporine resulted in a significant reduction in caspase-3 activity (53 ± 11% of control response; $n=4$; $P<0.05$), whereas histamine (100 μM) had no effect (Figure 10c). These results clearly indicate that histamine H_1 receptor-induced increases in p70 S6 kinase, PKB and p42/p44 MAPK activation (Robinson & Dickenson, 2001) are not linked to cell proliferation or inhibition of caspase-3 activity in DDT_1MF-2 cells.

Discussion

Recent studies from this laboratory have shown that the histamine H_1 receptor activates p42/p44 MAPK in the smooth muscle cell line, DDT_1MF-2 (Robinson & Dickenson, 2001). Furthermore, the PI-3K inhibitors, wortmannin and LY 294002, reduced p42/p44 MAPK activation by histamine, suggesting that the histamine H_1 receptor activates PI-3K in DDT_1MF-2 cells (Robinson & Dickenson, 2001). Given that a variety of kinases including PKB and p70 S6 kinase are regulated downstream of PI-3K and p42/p44

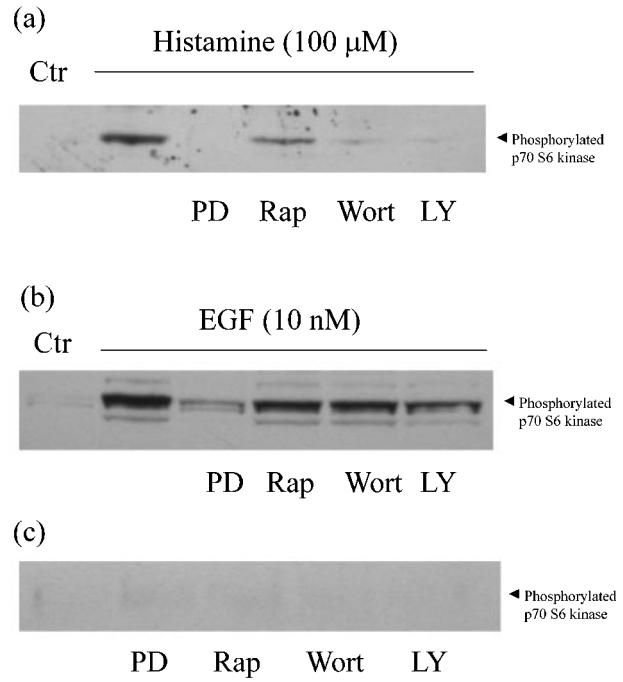

Figure 8 The effect of mTOR, MAPK kinase and PI-3K inhibitors on histamine H_1 receptor and EGF mediated increases in p70 S6 kinase phosphorylation in DDT_1MF-2 cells. DDT_1MF-2 cells were pretreated for 30 min with PD 98059 (50 μM), rapamycin (20 nM), wortmannin (100 nM) and LY 294002 (30 μM) before stimulating with (a) histamine (100 μM) or (b) EGF (10 nM) for 10 min. The effects of the various kinase inhibitors on the basal level of p70 S6 kinase phosphorylation are shown in panel (c). Increases in p70 S6 kinase phosphorylation were determined by Western blotting using a phospho-specific p70 S6 kinase (Thr^{421}/Ser^{424}) antibody. Similar results were obtained in four other independent experiments.

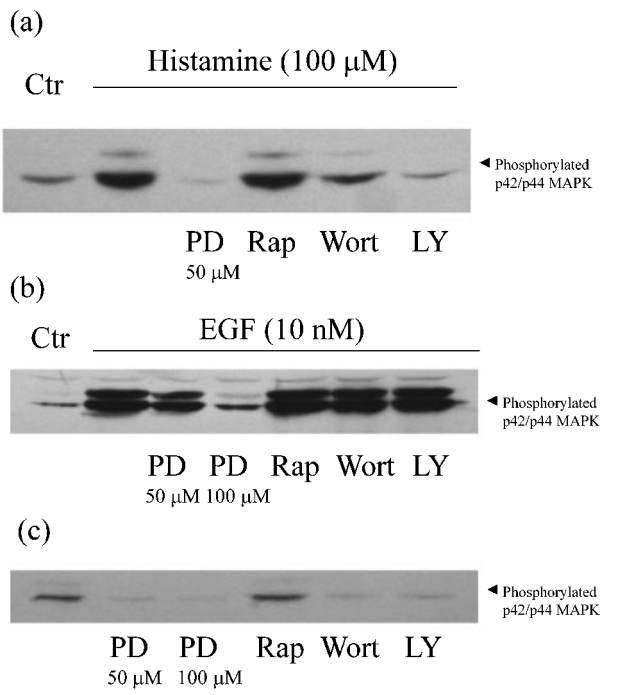

Figure 9 The effect of mTOR, MAPK kinase and PI-3K inhibitors on histamine H_1 receptor and EGF mediated increases in p42/p44 MAPK phosphorylation in DDT_1MF-2 cells. DDT_1MF-2 cells were pretreated for 30 min with PD 98059 (50 μM or 100 μM), rapamycin (20 nM), wortmannin (100 nM) and LY 294002 (30 μM) before stimulating with (a) histamine (100 μM) or (b) EGF (10 nM) for 10 min. The effects of the various kinase inhibitors on the basal level of p42/p44 MAPK phosphorylation are shown in panel (c). Increases in p42/p44 MAPK phosphorylation were determined by Western blotting using a phospho-specific p42/p44 MAPK (Thr^{202}/Tyr^{204}) antibody. Similar results were obtained in four other independent experiments.

MAPK, the present study was performed to determine whether the histamine H_1 receptor activates p70 S6 kinase and PKB in DDT_1MF-2 cells. The data presented has shown for the first time that the histamine H_1 receptor signals to PKB and p70 S6 kinase activation in smooth muscle cells.

Histamine H_1 receptor-mediated increases in PKB phosphorylation were blocked by both wortmannin and LY 294002, suggesting that PI-3K is involved in coupling the histamine H_1 receptor to PKB in DDT_1MF-2 cells. However, it is important to note that PKB can also be activated independent of PI-3K and phosphorylation on Ser^{473} and therefore the histamine H_1 receptor may also activate PKB in DDT_1MF-2 cells *via* pathways independent of Ser^{473} phosphorylation (monitored in this study) and PI-3K (Konishi *et al*., 1999; Virdee *et al*., 1999; Filippa *et al*., 1999). In addition, histamine-induced increases in PKB phosphorylation were sensitive to pertussis toxin pretreatment suggesting the involvement of G_i/G_o-proteins in coupling the histamine H_1 receptor to PKB in DDT_1MF-2 cells. It is notable that our previous studies have shown that histamine H_1 receptor-induced increases in p42/p44 MAPK and p38 MAPK in DDT_1MF-2 cells were also partially sensitive to pertussis toxin (Robinson & Dickenson, 2001). Furthermore, histamine H_1 receptor evoked release of arachidonic acid from transfected CHO cells and rabbit platelets is sensitive to pertussis toxin (Leurs *et al*., 1994; Murayama *et al*., 1990). Hence, the observations presented in this study contribute to the growing list of examples that indicate a prominent role for G_i/G_o proteins in histamine H_1 receptor-mediated signal transduction.

An increasing number of other studies have also reported that members of the GPCR superfamily activate PKB, including m1 and m2 muscarinic receptors, adenosine A_1 and A_3 receptors, β_2 and β_3 adrenoceptors and the CB_1 cannabinoid receptor (Murga *et al*., 1998; Gerhardt *et al*., 1999; Chesley *et al*., 2000; Germack & Dickenson, 2000; Gómez *et al*., 2000; Gao *et al*., 2001). It is interesting to note that β_2 and β_3 adrenoceptor-mediated activation of PKB was inhibited by pretreatment with pertussis toxin and PI-3K inhibitors. These observations, together with the data presented in this study, showing the involvement of G_i/G_o proteins and PI-3K in coupling the histamine H_1 receptor to PKB, suggests that GPCRs (including those classically referred to as being G_s-linked as in the case of β_2 and β_3-adrenoceptors or G_q-linked as in the case of the histamine H_1 receptor) employ a common pathway to activate PKB which involves G_i/G_o-protein coupling and PI-3K activation. More recent findings suggest that GPCRs stimulate PKB by a pathway involving the activation by PI-3Kβ by G-protein $\beta\gamma$ dimers (Murga *et al*., 2000). Hence, further studies are required in order to establish whether stimulation of PI-3Kβ

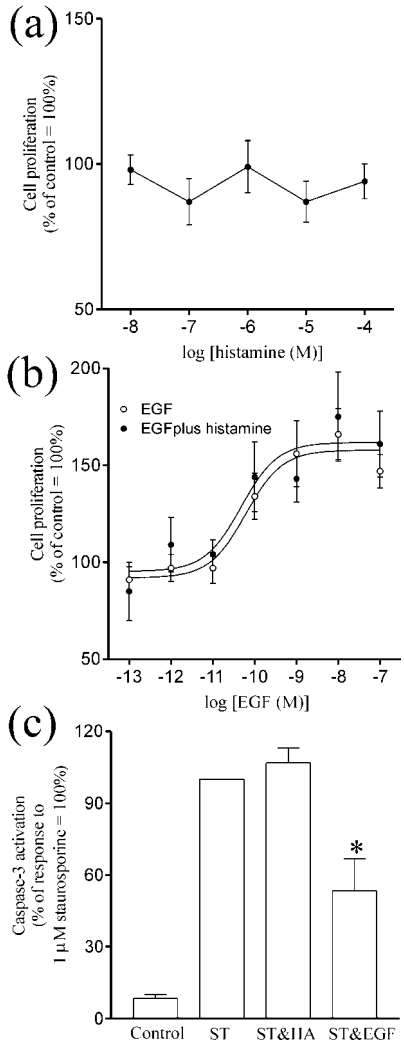

Figure 10 Effect of histamine and EGF on DDT_1MF-2 cell proliferation and caspase-3 activity. Serum-starved DDT_1MF-2 cells were exposed to increasing concentrations of histamine (a) and EGF alone or in the presence of 100 μM histamine (b) for 72 h after which the number of viable cells was determined using the MTT assay. The data shown represent the mean$\pm$s.e.mean of five independent experiments each done in triplicate and are expressed as percentage increase above untreated control cells (=100%). (c) DDT_1MF-2 cells were treated with 1 μM staurosporine for 4 h in the absence or presence of EGF (10 nM) or histamine (100 μM) and the activity of caspase-3 determined colourimetrically. Values represent the mean$\pm$ s.e.mean of four independent experiments each done in triplicate and are expressed as percentage of caspase-3 activity in the cells treated with staurosporine alone (=100%). * Significantly different ($P<0.05$) from the control response to staurosporine alone.

by G-protein $\beta\gamma$ dimers is involved in PKB activation by the histamine H_1 receptor.

The data presented in this study has also shown that the histamine H_1 receptor stimulates the phosphorylation of p70 S6 kinase in DDT_1MF-2 cells. The activation of p70 S6 kinase is complex and involves hierarchical phosphorylation of at least eight sites (Dufner & Thomas, 1999). Kinases implicated in the phosphorylation and activation of p70 S6 kinase include mTOR (mammalian Target of Rapamycin) and PDK-1, which is responsible for phosphorylation of Thr229 (Dufner & Thomas, 1999). Recent studies have also indicated a role for p42/p44 MAPK, p38 MAPK and JNK in the phosphorylation and activation of p70 S6 kinase (Zhang *et al*., 2001). In addition, Zhang *et al*. (2001) reported that UVA-induced phosphorylation of Thr^{421}/Ser^{424} is sensitive to rapamycin (mTOR inhibitor) and PD 98059 (MAPK kinase inhibitor) but not to wortmannin. Furthermore, *in vitro* phosphorylation assays indicate that Thr^{421}/Ser^{424} is phosphorylated by p42 MAPK but not by p44 MAPK, p38 MAPK or JNKs (Zhang *et al*., 2001). In this study, histamine-induced increases in p70 S6 kinase phosphorylation at Thr^{421}/Ser^{424} were completely blocked by PD 98059, wortmannin and LY 294002 but only partially sensitive to the mTOR inhibitor rapamycin. The complete inhibition of histamine-induced increases in Thr^{421}/Ser^{424} phosphorylation by wortmannin and LY 294002 (compared to EGF) presumably reflects the upstream involvement of PI-3K in histamine H_1 receptor coupling to p42/p44 MAPK (Robinson & Dickenson, 2001). EGF mediated increases in p42/p44 MAPK in COS-7 cells (Hawes *et al*., 1996) and DDT_1MF-2 cells (this study) are independent of PI-3K activation. The role of mTOR in the regulation of p70 S6 kinase is complex and not fully understood (Dufner & Thomas, 1999). Studies have shown that the rapamycin-sensitive phosphorylation sites located in the autoinhibitory domain (including Thr^{421}/Ser^{424}) do not serve as a substrate for mTOR (Burnett *et al*., 1998; Dufner & Thomas, 1999). Hence, it has been suggested that inhibition of p70 S6 kinase phosphorylation at Thr^{421}/Ser^{424} by rapamycin may occur *via* rapamycin-induced protein phosphatase 2A activation (Zhang *et al*., 2001). These findings may explain the sensitivity of histamine and EGF-induced p70 S6 kinase phosphorylation at Thr^{421}/Ser^{424} to rapamycin. In summary, it appears that the histamine H_1 receptor stimulates p70 S6 kinase phosphorylation at Thr^{421}/Ser^{424} *via* the p42/p44 MAPK dependent pathway.

At present the physiological roles of histamine H_1 receptor-induced activation of PKB and p70 S6 kinase in DDT_1MF-2 smooth muscle cells are unknown. PKB phosphorylates a wide variety of substrates involved in the regulation of numerous physiological processes such as cell growth, cell survival, protein synthesis, glucose metabolism and cell-cycle regulation (Coffer *et al*., 1998; Downward, 1998; Brunet *et al*., 2001). Similarly, p70 S6 kinase is also known to regulate cell growth and cell cycle progression through its phosphorylation of the 40S ribosomal protein subunit S6 (Pullen & Thomas, 1997). The S6 subunit is involved in the translation of 5′-oligopyrimidine tract mRNAs, which encode for many of the components of the protein synthetic apparatus. In view of the above roles for PKB and p70 S6 kinase there is clearly the potential for the histamine H_1 receptor to be involved in the regulation of a wide range of cellular processes. In this study the effects of histamine H_1 receptor activation on cell proliferation and anti-apoptotic signalling in DDT_1MF-2 cells were investigated.

Previous studies have indicated that activation of the histamine H_1 receptor stimulates proliferation and increases protein synthesis in cultured airway smooth muscle cells (Panettieri *et al*., 1990). The ability of histamine to stimulate the proliferation of DDT_1MF-2 cells was explored using the MTT assay. The data obtained from these experiments revealed that although EGF-induced a concentration-dependent increase in DDT_1MF-2 cell proliferation no increases in cell proliferation were observed following histamine treat-

ment alone. In addition, since some GPCRs appear to interact synergistically with growth factors to augment cell growth (Selbie & Hill, 1998) the possibility that histamine may modulate EGF-induced proliferation was also explored. Co-stimulation of DDT_1MF-2 cells with histamine did not enhance (or indeed inhibit) EGF-induced proliferation in DDT_1MF-2 cells. These observations clearly indicate that histamine H_1 receptor activation in DDT_1MF-2 cells does not influence cell proliferation.

As mentioned above PI-3K/PKB activation is associated with signalling pathways that promote cell survival (anti-apoptotic). For example, PKB-mediated phosphorylation of the pro-apoptotic Bcl-2 family member BAD prevents cell death by promoting the binding of BAD to the adaptor protein 14-3-3, thus preventing BAD from sequestering the survival proteins Bcl-2 or Bcl-X_L (Peso *et al*., 1997; Datta *et al*., 1997). Phosphorylation of BAD ultimately suppresses the release of cytochrome *c* from the mitochondria thus preventing caspase-9 activation and in due course the cleavage of pro-caspase-3 into active caspase-3 (Downward, 1999). With this in mind experiments were performed to determine whether histamine H_1 receptor-induced increases in PKB activation in DDT_1MF-2 cells are sufficient to inhibit staurosporine-induced caspase-3 activaton. Previous studies by Lan & Wong (1999) using CNE-2 epithelial cells have shown that EGF inhibits staurosporine-induced caspase-3 *via* a PI-3K dependent pathway (presumably involving PKB). In this study, EGF (10 nM) significantly reduced staurosporine induced capase-3 activation (*circa* 50% inhibition) whereas histamine had no significant effect. These observations raise the important question of what are the downstream targets of histamine H_1 receptor-induced PKB activation? PKB is known to phosphorylate a wide variety of number of substrates including I-κB kinase α leading to enhanced transcriptional activity of NF-κB and endothelial nitric oxide synthase (eNOS) resulting in increased production of NO (Brazil & Hemmings, 2001). Interestingly, Bakker *et al*. (2001) have shown that the histamine H_1 receptor mediates NF-κB activation in COS-7 cells. Clearly, since PKB phosphorylates proteins involved in the regulation of other varied cell signalling pathways further studies are required in order to establish the role PKB plays in regulating the physiological functions of the histamine H_1 receptor not only in DDT_1MF-2 cells but in other physiologically relevant cell types.

In conclusion, this study has shown that the histamine H_1 receptor activates PKB and p70 S6 kinase in the smooth muscle cell line DDT_1MF-2. It is now apparent that the histamine H_1 receptor in DDT_1MF-2 cells regulates a wide variety of signalling pathways including activation of phospholipase C (with associated increases in intracellular Ca^{2+} and protein kinase C activity), p42/p44 MAPK, p38 MAPK, PKB and p70 S6 kinase (Dickenson & Hill, 1992; White *et al*., 1993; Robinson & Dickenson, 2001). Future experiments will now focus on identifying the role of p42/p44 MAPK, p38 MAPK, PKB and p70 S6 kinase in mediating the physiological functions of the histamine H_1 receptor. In DDT_1MF-2 cells at least histamine did not stimulate cell proliferation or attenuate caspase-3 activity triggered by staurosporine suggesting alternative roles for histamine H_1 receptor-induced activation of p42/p44 MAPK, PKB and p70 S6 kinase in DDT_1MF-2 cells.

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