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Histamine induces cytoskeletal changes in human eosinophils *via* the H_4 receptor

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1 Histamine (0.004–2 μM) induced a concentration-dependent shape change of human eosinophils, but not of neutrophils or basophils, detected as an increase in forward scatter (FSC) in the gated autofluorescence/forward scatter (GAFS) assay.

2 The histamine-induced eosinophil shape change was completely abolished by thioperamide (10 μM), an H_3/H_4 receptor antagonist, but was not inhibited by pyrilamine or cimetidine (10 μM), H_1 and H_2 receptor antagonists, respectively. The H_4 receptor agonists, clobenpropit and clozapine (0.004–2 μM), which are also H_3 receptor antagonists, both induced eosinophil shape change, which was inhibited by thioperamide (10 μM). The H_3/H_4 receptor agonists, imetit, *R*-α-methyl histamine and *N*-α-methyl histamine (0.004–2 μM) also induced eosinophil shape change.

3 Histamine induced actin polymerisation (0.015–10 μM), intracellular calcium mobilisation (10–100 μM) and a significant upregulation of expression of the cell adhesion molecule CD11b (0.004–10 μM) in eosinophils, all of which were inhibited by thioperamide (10–100 μM). In addition, the H_4 receptor agonist/H_3 receptor antagonist clozapine (20 μM) stimulated a rise in intracellular calcium in eosinophils.

4 Activation of H_4 receptors by histamine (1 μM) primed eosinophils for increased chemotactic responses to eotaxin, but histamine (0.1–10 μM) did not directly induce chemotaxis of eosinophils.

5 Pertussis toxin (1 μg ml^{-1}) inhibited shape change and actin polymerisation responses induced by histamine showing that these effects are mediated by coupling to a $G\alpha_{i/o}$ G-protein.

6 This study demonstrates that human eosinophils express functional H_4 receptors and may provide a novel target for allergic disease therapy.
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Keywords: Histamine; H_4 receptors; eosinophils; shape change; actin polymerisation

Abbreviations: BSA, bovine serum albumin; CTX, cholera toxin; DMSO, dimethylsulphoxide; FITC, fluorescein isothiocyanate; FSC, forward scatter; GAFS, gated autofluorescence/forward scatter assay; *N*-α-meH, *N*-α-methyl histamine; PMNL, polymorphonuclear leucocytes; PE, phycoerythrin; PTX, pertussis toxin; *R*-α-meH, *R*-α-methyl histamine; RT, room temperature; SSC, side scatter

Introduction

Histamine is an endogenous mediator contained in the granules of mast cells and basophils, and is released upon activation by IgE crosslinking (Kinet, 1999). The actions exerted by histamine through its classical receptors H_1 and H_2 are well characterised, as are those of the more recently cloned H_3 receptor (Ash & Schild, 1966; Arrang *et al*., 1983; Hill, 1990; Lovenberg *et al*., 1999). Histamine modulates inflammatory and allergic responses predominantly through H_1 receptors, gastric acid and mucus production *via* H_2 receptors and neurotransmitter release in the central nervous system (CNS) through H_3 receptors (Hill, 1990). Histamine was one of the first inflammatory mediators identified to have a role in the pathophysiology of asthma where it has been shown to be primarily involved in bronchoconstriction and excess mucus production seen during asthmatic episodes (White, 1990; Broide *et al*., 1991; Hart, 2001). However, the recent discovery of a fourth histamine receptor, H_4, which is preferentially expressed on leucocytes, has reignited interest in the role of histamine in inflammation (Nakamura *et al*., 2000; Oda *et al*., 2000; Morse *et al*., 2001; Nguyen *et al*., 2001; Zhu *et al*., 2001; Liu *et al*., 2001a). The human H_4 receptor has ~40% amino-acid sequence homology with human H_3 receptors, and 65–72% sequence identity with the rat, guinea-pig, murine and porcine receptor homologues, which have now been cloned (Liu *et al*., 2001b; Oda *et al*., 2002). Human H_4 receptor mRNA has been shown to be expressed in leucocytes, including human neutrophils, eosinophils, monocytes, lymphocytes, B cells, dendritic cells and also in tissues including bone marrow, spleen, thymus, colon, small intestine and stomach (Nakamura *et al*., 2000; Oda *et al*., 2000; Coge *et al*., 2001; Morse *et al*., 2001; Liu *et al*., 2001a; Zhu *et al*., 2001). In addition, H_4 mRNA expression was observed in cells from human lung and trachea including bronchial epithelial cells, bronchial smooth muscle cells, fibroblasts and microvascular endothelial cells (Gantner *et al*., 2002). The function of H_4 receptors in leucocytes or indeed in these lung cells has not yet been established.

Histamine receptors are all G-protein coupled receptors (GPCR): H_1 receptors couple to $G\alpha_{q/11}$ causing intracellular

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calcium mobilisation; H_2 receptors signal *via* $G\alpha_s$ to cause cAMP accumulation; H_3 receptors couple to pertussis toxin-sensitive $G\alpha_{i/o}$ and inhibit adenylate cyclase; H_4 receptors have been reported to couple to $G\alpha_{i/o}$ and inhibit cAMP generation (Oda *et al*., 2000; Liu *et al*., 2001a; Morse *et al*., 2001).

Eosinophils are major effector cells in the immune system with a beneficial role in host defence against nematodes and other parasites and are active participants in many immune responses (Grove *et al*., 1977; Capron *et al*., 1978; and reviewed by McEwen, 1992). However, eosinophil accumulation and inappropriate activation causes symptoms and pathology in allergic asthma (Bousquet *et al*., 1990; Gleich *et al*., 1993; Lacoste *et al*., 1993). Eosinophils can be stimulated by an extensive range of endogenous proteins such as chemokines, cytokines and lipid mediators, which cause a variety of effects such as increased adhesion, chemotaxis, activation of respiratory burst, mediator release and degranulation (Weller, 1991; reviewed by Giembycz & Lindsay, 1999). Eosinophils can themselves release an array of inflammatory mediators including cytokines, chemokines, lipid mediators, cationic proteins and reactive oxygen metabolites. Histamine is known to affect eosinophils, by stimulating superoxide ion generation (attributed to H_1 receptors) while inhibiting eosinophil peroxidase release through H_2 receptors and can modulate eosinophil chemotaxis *via* non-H_1, non-H_2 receptors (Clark *et al*., 1975; 1977; Wadee *et al*., 1980; Pincus *et al*., 1982; Ezeamuzie & Philips, 2000). The pharmacology of the H_4 receptor matches that of an atypical H_3-like receptor previously found to mediate histamine-induced calcium mobilisation in human eosinophils (Raible *et al*., 1992; 1994), particularly as expression of the H_3 receptor, cloned in 1999, is restricted to the brain (Lovenberg *et al*., 1999). Since the discovery of the H_4 receptor, there have been only three reports of its function: the partial involvement of H_4 receptors in the control of IL-16 release by human $CD8^+$ T cells, involvement of H_4 receptors in histamine-induced chemotactic responses of human eosinophils and mediating chemotaxis and intracellular calcium mobilisation in mast cells (Gantner *et al*., 2002; O'Reilly *et al*., 2002; Hofstra *et al*., 2003).

In this study, we have investigated the effects of histamine on human eosinophils and compared it to eotaxin/CCL11, a known eosinophil activator (Sabroe *et al*., 1999). We have shown that histamine stimulates actin polymerisation, shape change and upregulation of CD11b expression in these cells. The finding that clobenpropit and clozapine can also stimulate these responses, while thioperamide inhibits them, suggests that they are transduced *via* H_4 receptors. Furthermore, we have shown that H_4 receptor activation may be involved in the priming of eosinophils for increased responses to eotaxin. Importantly, our experiments have demonstrated the presence of functional H_4 receptors on human eosinophils.

Methods

Volunteer blood donors were healthy normal or atopic subjects who were not taking systemic medication. Venous blood was sampled according to local ethics committee approved protocol. Peripheral blood polymorphonuclear leucocytes (PMNL, containing eosinophils and neutrophils) were prepared as described previously (Haslett *et al*., 1985). Briefly, 35 ml of blood was taken into 4.4 ml of 3.8% tri-sodium citrate and centrifuged at $300 \times g$ for 20 min. The plasma was discarded and remaining haematocrit resuspended in 0.6% Dextran® in saline. Following erythrocyte sedimentation for 30 min, the remaining leucocyte-rich upper suspension was layered over Histopaque® and centrifuged at $300 \times g$ for 25 min.

Mononuclear leucocytes separated as a narrow band on top of the Histopaque® and were removed first, washed, stabilised for 30 min in PBS buffer and labelled with anti-HLA-DR-FITC ($0.6\,\mu g\,ml^{-1}$) and anti-CDw123-RPE ($2\,\mu g\,ml^{-1}$) for 10 min at room temperature (RT) for detection of basophils ($CDw123^{pos}HLA\text{-}DR^{neg}$) (Heinemann *et al*., 2000). The granulocyte-rich cell pellet was resuspended and the contaminating erythrocytes lysed by hypotonic shock. The cells were washed, counted and resuspended in PBS buffer (PBS without Ca^{2+} Mg^{2+}, containing 0.1% w v^{-1} BSA, 10 mM glucose, 10 mM HEPES) at 1×10^7 cells ml^{-1}.

Shape change assay (gated autofluorescence/forward scatter assay (GAFS))

Eosinophil and neutrophil shape change was assayed as previously described (Sabroe *et al*., 1999). PMNLs were prepared as described above and stabilised for 30–90 min at RT. Cells were then centrifuged and resuspended at 1×10^7 cells ml^{-1} in PBS buffer (PBS containing 0.1% w v^{-1} BSA, 10 mM glucose, 10 mM HEPES) with or without Ca^{2+} Mg^{2+} or antagonists as required and incubated for a further 15 min (receptor antagonists) or 90 min (G protein inhibitors) at RT. Cells were then stimulated with agonists diluted in PBS buffer (with or without Ca^{2+} Mg^{2+}) for 4 min at 37°C, then fixed with CellFix® at 4°C to maintain cell shape and sample fluorescence measured by flow cytometry (Becton Dickinson FACSCaliber). Eosinophils were identified and gated by their natural autofluorescence, which is greater than that of neutrophils detected in fluorescence channel FL-2. Data were acquired for 500 events within the high fluorescence gated region identified as eosinophils. Results are expressed as percentage increase in forward scatter (FSC) compared to unstimulated cells. Mononuclear leucocyte cell preparations were assayed by the same method as the PMNL with an exception of the rest period, inclusion of Ca^{2+} Mg^{2+} ions in PBS buffer and data collected for 300 $CD123^{pos}$ and $HLA\text{-}DR^{neg}$ basophil events.

CD11b expression

PMNL preparations were resuspended at 1×10^7 cells ml^{-1} in PBS buffer (PBS containing 0.1% w v^{-1} BSA, 10 mM glucose, 10 mM HEPES) without Ca^{2+} Mg^{2+}. Cells were then stimulated with agonists diluted in PBS buffer at 37°C for 30 min. Following stimulation, the cells were washed with ice-cold stain buffer (PBS without Ca^{2+} Mg^{2+} containing 0.25% w v^{-1} BSA, 10 mM glucose, 10 mM HEPES) and then resuspended in stain buffer containing 1/30 dilution of anti-CD16-FITC and 1/20 dilution of anti-CD11b-RPE antibodies. After 30 min incubation at 4°C, cells were washed again and resuspended in CellFix®. Sample fluorescence was acquired for 500 CD16-negative eosinophil events measured by flow cytometry (Becton Dickinson FACSCaliber). Results are expressed as percentage increase in fluorescence compared to unstimulated cells.

Whole-blood shape change

Eosinophil and neutrophil shape change was assayed in whole blood as described in previous work (Bryan *et al*., 2002). Briefly, citrated blood was centrifuged at $300 \times g$ for 10 min, then upper platelet-rich plasma layer discarded. Aliquots of blood were incubated for 10 min at RT with or without antagonists as required. Aliquots of blood were then added to agonist diluted in PBS buffer (PBS without Ca^{2+} Mg^{2+} containing 0.1% w v^{-1} BSA, 10 mM glucose, 10 mM HEPES) and incubated for 4 min at 37°C. Samples were then fixed with CellFix® at 4°C and contaminating erythrocytes lysed (155 mM NH_4Cl, 10 mM $KHCO_3$ in H_2O). Samples were fixed and fluorescence measured by flow cytometry (Becton Dickinson FACSCaliber). Data were acquired for 500 high-fluorescence eosinophil events. Results are expressed as percentage increase in FSC compared to unstimulated cells.

Purification of human eosinophils

Eosinophils were purified from PMNL preparations by magnetic bead separation (Hansel *et al*., 1991). Briefly, mixed granulocytes were washed with PBS buffer (PBS without Ca^{2+} Mg^{2+}, containing 0.1% w v^{-1} BSA, 10 mM glucose and 10 mM HEPES, pH 7.3–7.4) and then resuspended at 2.5×10^8 cells ml^{-1} in column buffer (PBS without Ca^{2+} Mg^{2+}, containing 0.1% w v^{-1} BSA and 10 mM HEPES, pH 7.3–7.4). The cells were then labelled with MACS CD16 MicroBeads and depleted of neutrophils by immunomagnetic separation on a MACS CS column according to the manufacturer's instructions (Miltenyi Biotech). The collected CD16 negative eosinophils were centrifuged at $300 \times g$ for 7 min, counted and re-suspended in the PBS buffer.

Actin polymerisation in human eosinophils

Isolated eosinophils were resuspended at 1×10^6 cells ml^{-1} in PBS buffer (PBS without Ca^{2+} Mg^{2+} containing 0.1%. w v^{-1} BSA, 10 mM glucose, 10 mM HEPES) with antagonists pyrilamine, cimetidine, or thioperamide (15 min), PTX or CTX (90 min) as required and incubated at RT. Dilutions of agonists in 10 μl PBS buffer were prepared in polypropylene tubes. Cell suspension and agonist dilutions were warmed to 37°C for 5 min then maintained at 37°C during stimulation. 90 μl of cell suspension was added to a tube containing agonist, vortexed gently then after 5–120 s incubation, 200 μl of fix buffer (PBS without Ca^{2+} Mg^{2+} containing 4% formaldehyde, 125 ng ml^{-1} L-α-lysophatidylcholine palmitoyl, 100 nM FITC phalloidin) was added to the sample to fix, permeabilise and label F-actin, respectively. Sample fluorescence was measured by flow cytometry (Becton Dickinson FACSCaliber). Viable eosinophils were identified and gated by their side scatter (SSC) and forward scatter (FSC) characteristics. Fluorescence data were acquired for 1000 eosinophil events. Results are expressed as percentage increase in fluorescence compared to unstimulated cells.

Intracellular calcium mobilisation in human eosinophils

Isolated eosinophils were resuspended at 1×10^7 cells ml^{-1} in PBS buffer (PBS without Ca^{2+} Mg^{2+} containing 0.1% w v^{-1} BSA, 10 mM glucose, 10 mM HEPES) and loaded with 1 μg ml^{-1} Fura-2 AM for 30 min at 37°C. Cells were washed, then resuspended at 2×10^6 cells ml^{-1} in PBS buffer and equilibrated with 1 mM $CaCl_2$ at 37°C for 5 min. Changes in fluorescence following agonist stimulation were recorded by luminescence spectrometry (Perkin-Elmer LS5OB). Fluorescence plots were created by FluorScan software (Perkin-Elmer), the data were expressed as fluorescence (arbitrary units) emitted at 510 nm after stimulation at excitation wavelength of 340 and 380 nm.

Human eosinophil chemotaxis and chemokinesis

For investigation of priming, eosinophils were preincubated at 2×10^6 cells ml^{-1} in HBSS buffer (HBSS containing 0.25% w v^{-1} BSA, 30 mM HEPES, pH 7.3–7.4) without Ca^{2+} Mg^2 and containing as required, either histamine or histamine plus thioperamide for 45–60 min, then transferred to HBSS buffer with Ca^{2+} and Mg^{2+} containing histamine and/or thioperamide. For investigation of chemokinesis, histamine was either added to the cells immediately prior to placing in the top wells or in the lower wells or added to both upper and lower wells. In all cell migration experiments, 50 μl of cells were placed in the top wells of a 48-well micro-Boyden chamber, which were separated from agonists in the bottom wells by a 5 μm pore polycarbonate filter. The chambers were incubated for 1 h at 37°C 5% CO_2 in a humidified box. After incubation, the number of cells migrating into the lower wells was counted by flow cytometry (Beckton Dickinson FACSCaliber) for 30 s per sample. Results are expressed as chemotaxis index, calculated as: (mean number of cells counted in treated sample)/(mean number of cells counted in buffer control wells). The mean number of cells was calculated from at least three replicate wells on each occasion.

Reagents

Bovine serum albumin (BSA), Histopaque®, histamine, thioperamide, anti-HLA-DR-FITC, FITC-phalloidin, L-α-lysophatidylcholine palmitoyl, pertussis toxin (PTX), cholera toxin (CTX) and all other laboratory reagents, unless otherwise specified, were purchased from Sigma (Poole, U.K.). HBSS, PBS and HEPES were from Life Technologies (Paisley, U.K.). Dextran® was from Amersham Pharmacia Biotech (Amersham, U.K.). MACS CS columns and CD16 MicroBeads were from Miltenyi Biotech (Auburn, CA, U.S.A.). Pyrilamine, cimetidine, clobenpropit, clozapine, imetit, *N*-α-methyl histamine and *R*-α-methyl histamine were from Tocris Cookson Ltd (Avonmouth, U.K.). Human eotaxin/CCL11 was from Peprotech (London, U.K.). The 48-well micro-Boyden chambers were from Neuro Probe Inc. (Gaithersburg, MD, U.S.A.) and 5 μm pore polycarbonate filters from Osmonics Inc. (Minnetonka, MN, U.S.A.). Fura-2 AM was from Molecular Probes (Leiden, Netherlands). Anti-CD16-FITC was from Dako (Ely, U.K.). Anti-CD123-RPE, FACSflow and CellFix® were from Becton Dickinson Biosciences (San Jose, CA, U.S.A.).

Statistical analysis

Experimental data are presented as mean ± s.e.m. of data from a number (n) of independent experiments, each using cells from different donors. Where appropriate, statistical significance (P) was determined using the Mann–Whitney test for

comparison of two groups of data, and Kruskal–Wallis test, with Dunn's post test, for comparison of concentration–response curve data (asterisks show statistical difference of all data points forming the concentration–response curve compared to unstimulated). LOGIT transformations were performed on data tabulated as percentage increase or chemotactic index, prior to calculation of statistics. Analysis was performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, U.S.A.).

Results

Histamine induces shape change of human eosinophils

Illustrative dot plots depicting the shape change of eosinophils in response to stimulation with histamine or eotaxin/CCL11, detected as an increase in mean FSC of eosinophils in the shape change (GAFS) assay are shown in Figure 1. Eosinophils are identified and gated by their higher natural autofluorescence measured in FL-2 fluorescence channel compared to neutrophils. A histogram of FL-2 fluorescence displays two cell populations, the region with high autofluorescence (R1) were gated as eosinophils and low autofluorescence (R2) region are neutrophils. A plot of side scatter (SSC) vs forward scatter (FSC) also shows two distinct cell populations (set by region gate in FL-2 histogram) and a measure of the mean FSC for each cell population can be recorded. Following stimulation with histamine, the mean FSC of eosinophils increased from 179 (Figure 1a) to 272 (Figure 1b), a similar increase in mean FSC to 267 was induced by eotaxin (Figure 1c). Eosinophils in the mixed PMNL cell preparation responded to histamine (0.002–2 μM) in a concentration-dependent manner with a maximal response observed at 1 μM histamine representing a 37.5±5% increase in FSC compared to unstimulated cells. In contrast, the mean FSC of the neutrophils remained unchanged (Figure 1d), although these cells were able to respond to IL-8 (data not shown). In parallel experiments, basophils also failed to respond to stimulation with histamine (0.002–8 μM) (Figure 1d). We tested three commonly available histamine preparations; histamine dihydrochloride, histamine diphosphate salt and histamine (2[4-imadazoly]ethylamine) at 0.004–2 μM; each induced eosinophil shape change with similar potency and efficacy (Figure 1e). All three histamines produced a sigmoidal concentration–response curve for eosinophil shape change with peak responses between 0.5 and 1 μM histamine. Histamine dihydrochloride was used in all subsequent experiments. When compared to eotaxin/CCL11, a well-characterised inducer of eosinophil shape change (Sabroe *et al*., 1999; Stubbs *et al*., 2002), histamine was approximately 2000 times less potent (EC_{50} values for histamine and eotaxin/CCL11 are 67 nM and 30 pM, respectively). However, histamine was as efficacious as eotaxin/CCL11, as each mediator stimulated approximately the same maximal FSC increase of 37.5±5% (1 μM) and 41.8±4% (0.5 nM) respectively (Figure 2a).

Characterisation of the receptor mediating histamine-induced eosinophil shape change

In order to determine the receptor subtype mediating the histamine-induced eosinophil shape change, we examined the effect of histamine analogues and receptor antagonists in the shape change assay. The H_3/H_4 receptor antagonist, thioperamide (10 μM), completely abolished responses to histamine, whereas the H_1 receptor antagonist, pyrilamine, and the H_2 receptor antagonist, cimetidine (10 μM), had no effect (Figure 2b). The inhibition of histamine-induced eosinophil shape change by thioperamide was concentration-dependent (Figure 2c), Schild analysis gave a pK_B value for thioperamide of 6.94 and a slope of 1.25±0.12. In addition, the H_3/H_4 receptor agonists, *R*-α-meH, *N*-α-meH and imetit, stimulated eosinophil shape change in a concentration-dependent manner, with EC_{50} values of 66, 701 and 85 nM, respectively, further suggesting H_3/H_4 receptor activation (Figure 2d). Clozapine and clobenpropit, H_4 receptor agonists/H_3 receptor antagonists, also induced eosinophil shape change, with EC_{50} values of 556 and 3 nM, respectively (Figure 2d). Thioperamide (10 μM) completely abolished the shape change induced by imetit, *R*-α-meH, *N*-α-meH and clozapine (0.004–2 μM) and caused a significant parallel shift of the concentration–response curve of clobenpropit to the right (Figure 2e). In contrast, thioperamide (1–100 μM) had no effect on eotaxin/CCL11-induced eosinophil shape change ($n=5$, data not shown).

Histamine-induced eosinophil shape change is inhibited by pertussis toxin

To further characterise the nature of the receptor mediating histamine-induced eosinophil shape change responses, experiments were carried out to examine the G-protein interaction required. Preincubation of the cells with pertussis toxin (1 μg ml^{-1}), which disrupts $G\alpha_{i/o}$ G-protein function, significantly reduced the shape change response to histamine ($P<0.01$), whereas the $G\alpha_s$ G-protein inhibitor, cholera toxin, at the same concentration (1 μg ml^{-1}), had no effect (Figure 2f).

Histamine (0.02–2 μM) also induced eosinophil shape change when stimulated in whole blood, and responses to histamine were significantly inhibited by thioperamide (10 μM; $P<0.001$) (Figure 3). However, eotaxin was more efficacious than histamine in the whole-blood assay.

Histamine induces actin polymerisation in human eosinophils

Polymerisation of G-actin to F-actin is essential for changes in cell shape and locomotion. Histamine (0.015–10 μM) induced actin polymerisation in eosinophils, measured as an increase in FITC-phalloidin labelled F-actin fluorescence compared to unstimulated cells (Figure 4a). The histamine-induced actin polymerisation was concentration-dependent, reaching a maximum of 54±6% increase in fluorescence with 10 μM histamine stimulation. In agreement with the results using the shape change assay, histamine was approximately 1000 times less potent than eotaxin/CCL11 at inducing actin polymerisation, EC_{50} values of 199 nM and 127 pM, respectively (Figure 4a). Actin polymerisation induced by histamine was rapid with the greatest responses observed after 5 s stimulation with histamine (Figure 4b).

The H_3/H_4 antagonist, thioperamide (10 μM), completely ablated actin polymerisation induced by histamine, whereas pyrilamine and cimetidine (10 μM), antagonists of H_1 and H_2 receptors, respectively, were without effect (Figure 4c).

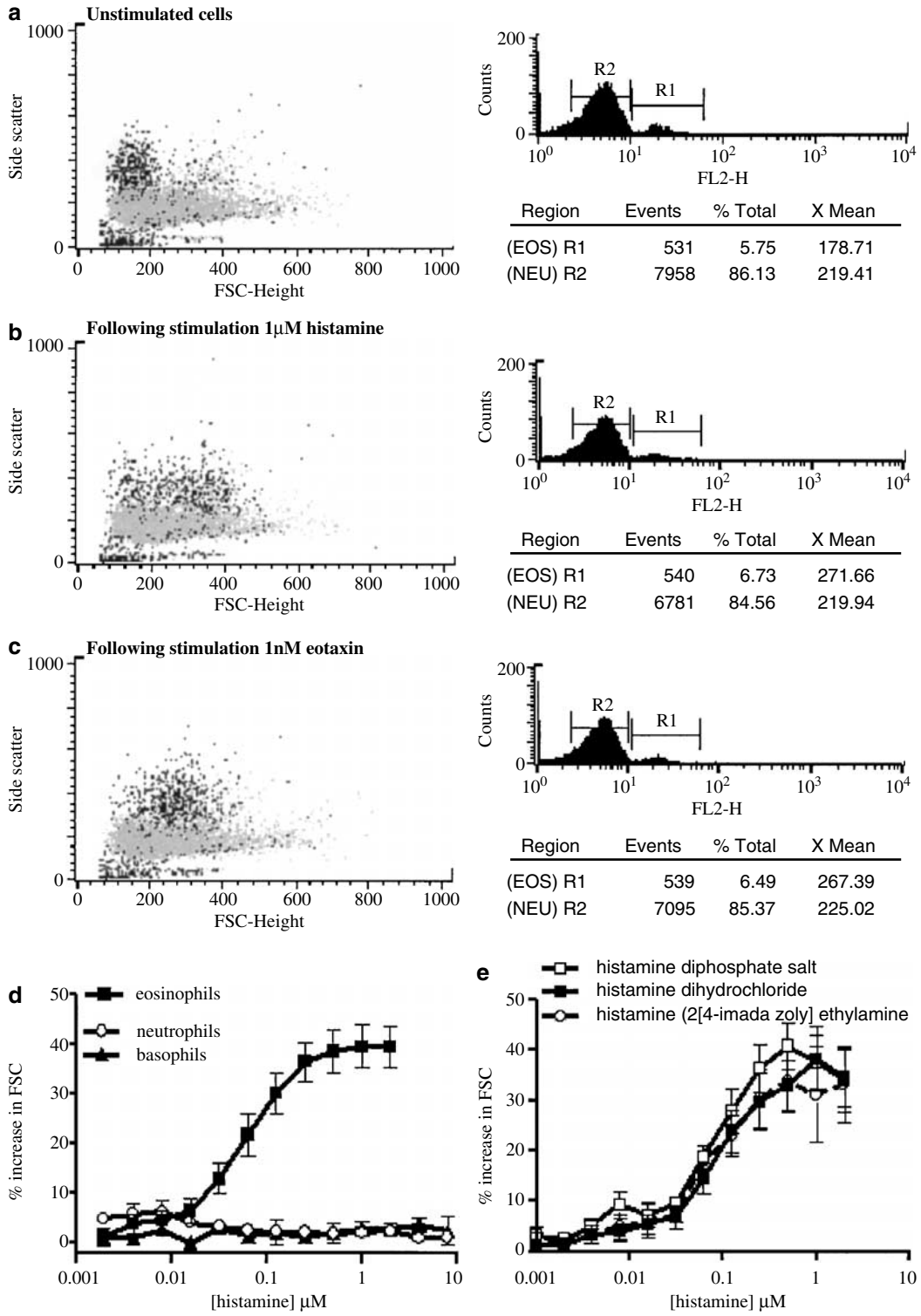
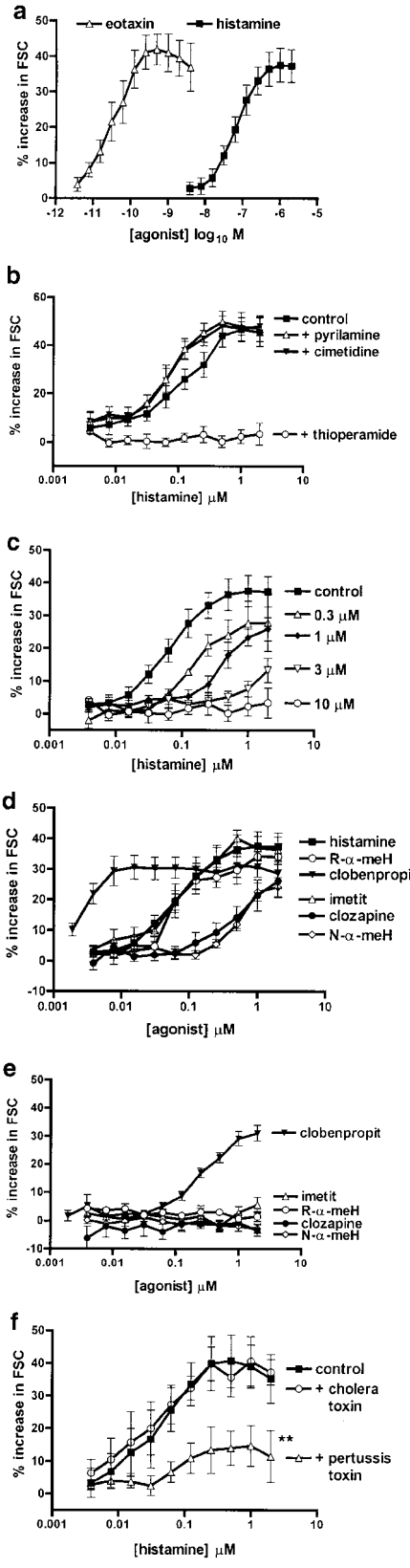


Figure 1 Histamine induced shape change of human eosinophils. Mixed granulocytes were stimulated with (a) buffer, (b) 1 μM histamine or (c) 1 nM eotaxin and their shape change measured by flow cytometry. Dot plots of the FSC vs SSC of mixed granulocytes show two separate cell populations; eosinophils in black, neutrophils in grey. Autofluorescence was measured in FL-2 fluorescence channel and displayed in histogram plots, on which regions of high (R1 = eosinophils) and low (R2 = neutrophils) autofluorescence were defined. The mean FSC of eosinophils (R1) increased from 178 (a) to 271 (b) after stimulation with histamine (1 μM) (shift to the right in dot plot) or 267 (c) after stimulation with eotaxin, while the mean FSC of neutrophils (R2) was unchanged. Basophils were detected by flow cytometry as highly positive for CD123 but negative for HLA-DR (dot plots not shown). Histamine induced eosinophil shape change in a concentration-dependent manner, which was selective for (d) eosinophils and induced by (e) all three histamine preparations. Dot plots and histograms are representative of 10 or more experiments. Concentration–response curves are mean ± s.e.m.; (d) eosinophils/neutrophils $n = 10$, basophils $n = 3$; (e) $n = 5$.

Treatment of eosinophils with pertussis toxin (1 $\mu g\,ml^{-1}$) also significantly inhibited actin polymerisation induced by histamine (0.015–10 μM; $P<0.01$), while cholera toxin (1 $\mu g\,ml^{-1}$) had no effect (Figure 4d). In addition, the H_4 agonist/H_3 antagonist, clobenpropit, induced actin polymerisation in eosinophils, which was also maximal after 5 s stimulation and thioperamide caused a parallel shift to the right of the concentration–response curve ($n=5$, data not shown).



Histamine upregulates CD11b expression and induces calcium mobilisation in human eosinophils

Histamine (0.125–10 μM) induced upregulation of the expression of the cell surface adhesion molecule, CD11b, on eosinophils, detected as an increase in fluorescence following stimulation of mixed granulocytes with histamine (Figure 5a). The histamine-induced upregulation of CD11b could be significantly inhibited by the preincubation of the cells with thioperamide (10 μM; $P<0.05$) (Figure 5a). Eotaxin/CCL11 was more potent than histamine, producing maximal increase

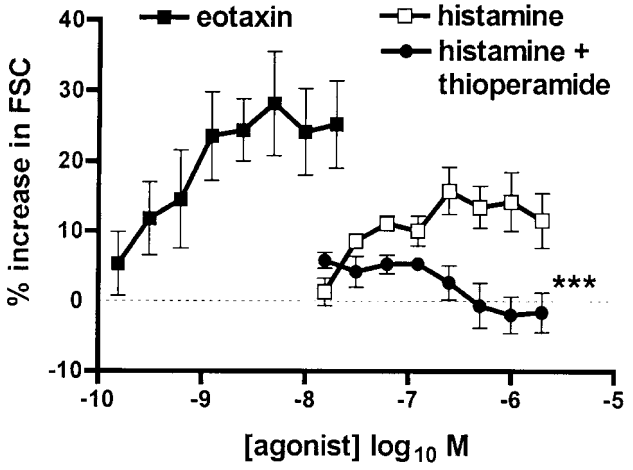


Figure 3 Histamine stimulates eosinophil shape change in whole-blood eosinophil shape change assay. Whole blood was incubated with or without thioperamide (10 μM) prior to stimulation with agonists and leucocyte shape change analysed by flow cytometry. Results are expressed as the percent increase in FSC induced by agonist compared to unstimulated cells. Data shown are mean ± s.e.m., $n=5$, statistical analysis ***$P<0.001$ comparison of entire concentration–response curve in presence of thioperamide vs control.

Figure 2 Effect of histamine and histamine analogues on human eosinophil shape change. Human eosinophils were incubated with receptor antagonists (10 min) or G-protein inhibitors (90 min) prior to stimulation with agonists and eosinophil shape change measured by flow cytometry. Results are expressed as the percent increase in FSC induced by agonist compared to unstimulated cells. (a) Comparison of histamine and eotaxin stimulation of eosinophil shape change; (b) histamine-induced eosinophil shape change is inhibited by thioperamide (10 μM) but not pyrilamine and cimetidine (10 μM). (c) Inhibition of histamine-induced shape change by thioperamide is concentration-dependent. (d) *R*-α-meH, *N*-α-meH, imetit, clozapine and clobenpropit, all induce eosinophil shape change, which can be inhibited by (e) thioperamide (10 μM). (f) Histamine-induced shape change is inhibited by pertussis toxin but not cholera toxin (1 $\mu g\,ml^{-1}$); **$P<0.01$, comparison of entire concentration–response curve in presence of inhibitor vs control. Data shown are mean ± s.e.m.: (a) $n\leqslant 10$; (b), (c) and (f) $n=5$; (d) and (e) $n=4$–11.

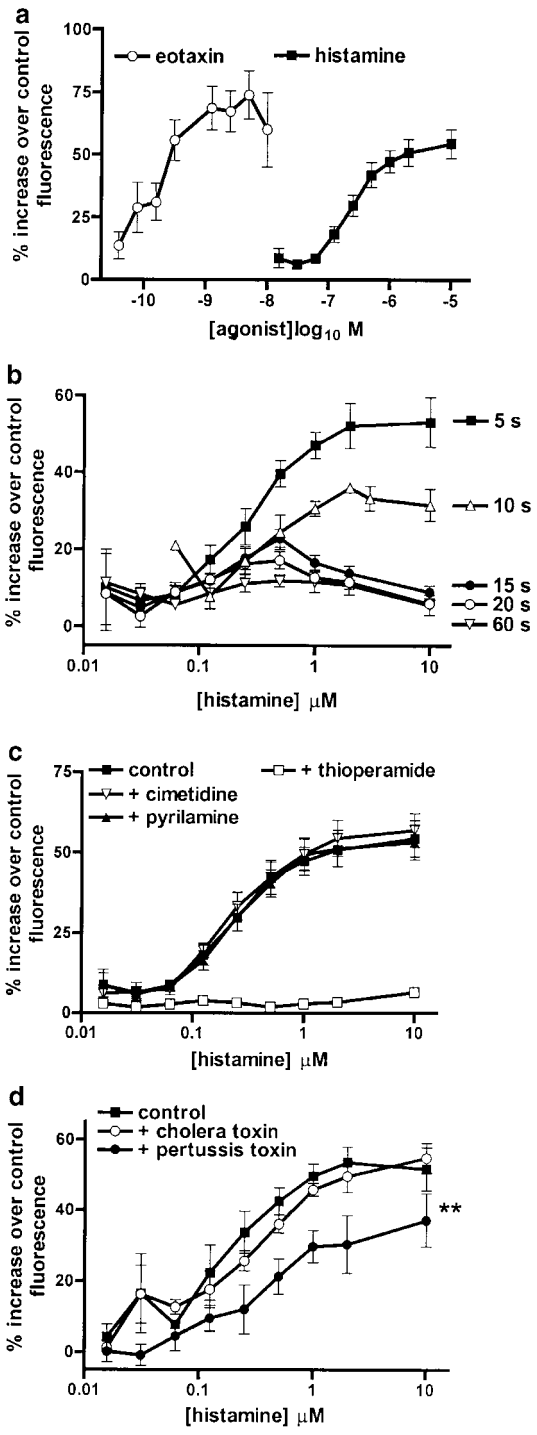


Figure 4 Effect of histamine on actin polymerisation in human eosinophils. Human eosinophils were incubated with receptor antagonists (10 min) or G-protein inhibitors (90 min) prior to stimulation with agonists. Responses were measured by flow cytometry and results are expressed as the percent increase in fluorescence induced by agonist compared to unstimulated cells. (a) Comparison of histamine and eotaxin stimulation of actin polymerisation. (b) Time course of actin polymerisation induced by histamine. (c) Histamine-induced actin polymerisation is inhibited by thioperamide (10 μM) but not pyrilamine and cimetidine (10 μM). (d) Inhibition by pertussis toxin but not cholera toxin (1 $\mu g\,ml^{-1}$) of histamine-induced actin polymerisation; $^{**}P<0.01$ comparison of entire concentration–response curve in presence of inhibitor vs control. Data shown are mean $\pm$ s.e.m.: (a) and (c) $n=5$; (b) $n=3-5$; (d) $n=3$.

in fluorescence at 3–10 nM eotaxin/CCL11 compared to 1 μM for histamine.

Histamine (10–100 μM) stimulated an increase in intracellular calcium in human eosinophils (Figure 5b), which could be inhibited by thioperamide (100 μM) (Figure 5d). Neutrophils did not respond to stimulation with histamine ($n=3$, data not shown). The maximum increase stimulated by histamine (100 μM), was of comparable size to that stimulated by eotaxin (10 nM) (Figure 5b). Clozapine (20 μM) also stimulated calcium mobilisation (Figure 5c). Both histamine and clozapine were able to desensitise the cells to subsequent stimulation with histamine (Figure 5b, c).

Human eosinophil chemotaxis and chemokinesis

Histamine (0.1–10 μM) did not induce any significant chemotaxis of human eosinophils. In contrast, eotaxin/CCL11 (1–100 nM), a potent eosinophil chemoattractant, induced a concentration-dependent chemotactic response (Figure 6a). In order to determine whether histamine induced chemokinesis (increased random movement) rather than chemotaxis (directional movement) of eosinophils, histamine was added to the upper wells, lower wells and upper and lower wells of the micro-Boyden chamber. There was no significant increase in eosinophil migration when histamine was present in the lower wells alone (chemotaxis) or in both the upper and lower wells (chemokinesis) (Figure 6b). To investigate whether histamine has a priming effect, eosinophils were preincubated with either histamine or histamine and thioperamide for 45–60 min prior to use in chemotaxis assays. Preincubation of eosinophils with histamine (1 μM) potentiated the number of cells migrating towards eotaxin/CCL11 (30 nM), inducing a significant increase in chemotactic index from 3.3 ± 0.9 to 8.7 ± 1.3 ($P<0.05$) (Figure 6c). Furthermore, this significantly enhanced migratory response to eotaxin could be significantly inhibited by thioperamide (10 μM; $P<0.05$) (Figure 6c).

Discussion and conclusion

Histamine is known to have direct effects on eosinophils, such as the stimulation of superoxide generation *via* H_1 receptors and inhibition of degranulation mediated *via* H_2 receptors (Pincus *et al*., 1982; Ezeamuzie & Philips, 2000). Histamine has also been reported to influence other eosinophil functions, such as migration and intracellular calcium mobilisation; however, the receptors involved in these responses are not clearly defined (Clark *et al*., 1975; 1977; Wadee *et al*., 1980; Raible *et al*., 1992; 1994). Indeed, experiments by Raible *et al*. (1994) studying histamine-induced calcium mobilisation in eosinophils suggested the existence of further receptors in addition to the three subtypes already described (H_1, H_2 and H_3). Recently, a fourth histamine receptor, H_4, was discovered, with an mRNA expression profile greatest in bone marrow, spleen, thymus and leucocytes, suggesting a role in the immune system (Nakamura *et al*., 2000; Oda *et al*., 2000; Liu *et al*., 2001a; Morse *et al*., 2001; Nguyen *et al*., 2001; Zhu *et al*., 2001). Since eosinophils have been shown to express high levels of H_4 receptor mRNA, the effect of histamine acting *via* H_4 receptors on these cells warranted further investigation.

In the present study, we have shown that histamine induces eosinophil shape change. Subsequently, we examined the

receptor subtype mediating this histamine-induced eosinophil shape change and have clearly shown that this response occurs *via* activation of H_4 receptors. This conclusion is based on the following findings: firstly, the ability of the H_3/H_4 antagonist, thioperamide, to inhibit the shape change response to histamine and other histamine analogues, while H_1 and H_2 receptor antagonists, pyrilamine and cimetidine, respectively, had no effect. Secondly, the H_3/H_4 agonists imetit, *R*-α-meH, *N*-α-meH, and the H_4 agonists/H_3 antagonists, clozapine and clobenpropit, also induced eosinophil shape change. Interestingly, clobenpropit was by far the most potent compound tested ($EC_{50} = 3$ nM); however, clobenpropit failed to elicit a maximal shape change response by eosinophils even at the highest concentration tested (greatest response to clobenpropit was $31 \pm 4\%$ at $2\,\mu$M). Clobenpropit acting as a partial agonist at the H_4 receptor in eosinophil shape change is in agreement with the reported partial agonist effect of clobenpropit in stimulating intracellular calcium mobilisation in H_4 receptor transfected cells (Oda *et al*., 2000; Morse *et al*., 2001) and inhibition of forskolin stimulated cAMP production (Liu *et al*., 2001a). In addition, clozapine and *N*-α-meH also appear to be acting as partial agonists on eosinophils, which is in contrast to their full-agonist action in H_4 receptor transfected cells (Oda *et al*., 2000).

The rank order of potency of the agonists in the shape change assay: clobenpropit > histamine, imetit, *R*-α-meH > clozapine, *N*-α-meH, is in good agreement with the rank order of affinity obtained in radioligand binding assays using H_4-transfected cells by Oda *et al*. (2000). In particular, the finding that the H_3 selective agonists, *R*-α-meH and *N*-α-meH, were less potent than histamine at inducing shape change shows that these compounds were not acting *via* H_3 receptors. Previous reports have shown that *R*-α-meH was several times more potent, and *N*-α-meH was equipotent to histamine in activating the H_3 receptor (Arrang *et al*., 1987; Hill, 1990; Lovenberg *et al*., 1999; Zhu *et al*., 2001). Moreover, several authors have shown by RT–PCR that H_3 mRNA is restricted to the brain, whereas H_4 mRNA expression is clearly detected in peripheral blood leucocytes and specifically eosinophils (Nakamura *et al*., 2000; Oda *et al*., 2000; Morse *et al*., 2001; Liu *et al*., 2001a).

There are some discrepancies in the rank order of agonist potency that we have described in assays of eosinophil responses compared to published data using H_4 receptor transfected cells; however, the receptor affinity measured may be dependent on the radioligand in competitive binding experiments (Nakamura *et al*., 2000; Morse *et al*., 2001; Nguyen *et al*., 2001; Shin *et al*., 2002). In addition, receptor number and distribution, as well as mechanisms of recycling, receptor redundancy and signal transduction, which can each contribute to the potency of a ligand at a receptor, may not be consistent between cells that express the native receptor and receptor transfectants. Thus, it is important to study the

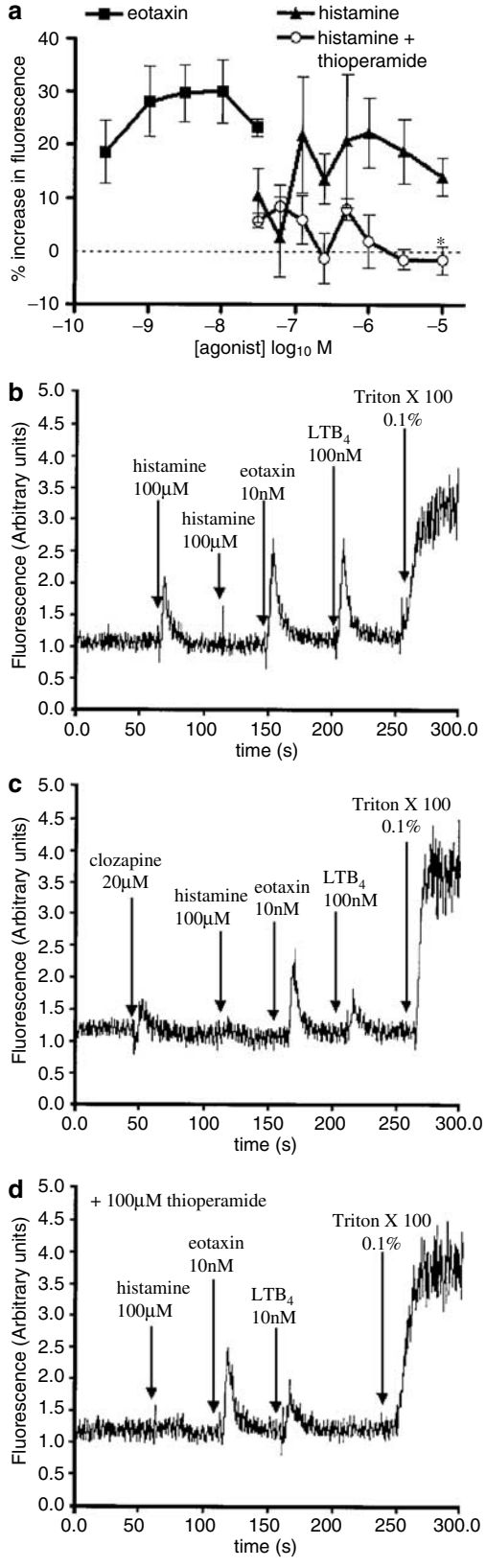


Figure 5 Histamine induced upregulation of surface CD11b expression and increase in intracellular calcium mobilisation on human eosinophils. (a) Mixed granulocytes were incubated with thioperamide (10 μM) and then stimulated with histamine or eotaxin and CD11b expression analysed by flow cytometry as described in Methods. Results are expressed as percent increase in fluorescence compared to unstimulated cells. Data shown are mean ± s.e.m., $n = 3$–7, $^*P < 0.05$ comparison of entire concentration–response curve in presence of thioperamide vs control. (b)–(d) Isolated eosinophils were loaded with Fura-2 and then stimulated with agonists. Graphs show actual recorded fluorescence output and are representative of n independent experiments; (b) $n = 2$; (c) $n = 6$; (d) $n = 4$.

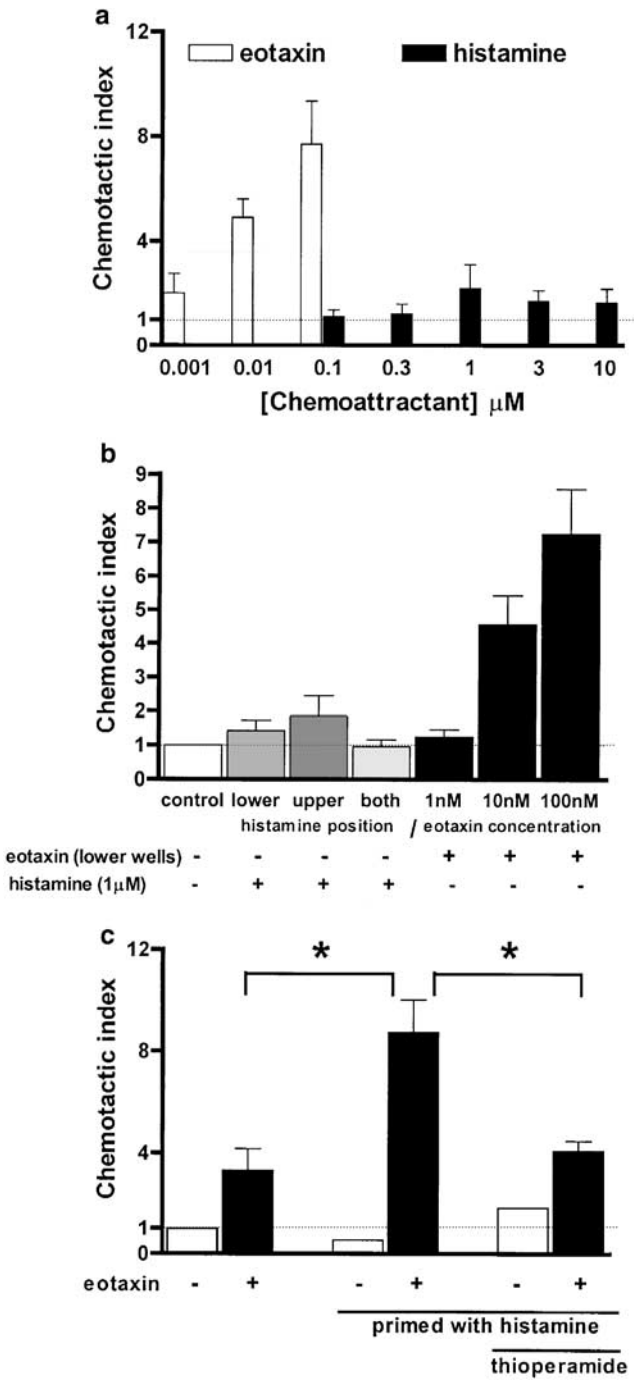


Figure 6 Human eosinophil chemotaxis and chemokinesis. (a) Human eosinophils were placed in the upper wells of a micro-Boyden chamber and either eotaxin or histamine was added to lower wells. (b) Human eosinophils were placed in the upper wells of a micro-Boyden chamber and either eotaxin added to the lower wells, or histamine added to the upper, lower or upper and lower wells of the chamber. (c) Human eosinophils were incubated with histamine (1 μM), or histamine and thioperamide (10 μM) and then placed in the upper wells of a micro-Boyden chamber and eotaxin (30 nM) was placed in the lower wells of the chamber. (a)–(c) Cells that had migrated across a polycarbonate filter after 1 h were counted by flow cytometry and expressed as chemotactic index. Data shown are mean $\pm$ s.e.m. of n experiments each using cells from a different donor and each carried out with three or more replicate wells; (a) $n=6$; (b) $n=3-5$; (c) filled bars $n=3$, open bars $n=1$. $^*P<0.05$.

response to histamine in cells, such as eosinophils, that naturally express the H_4 receptor.

In our study, we have shown that histamine-induced eosinophil shape change is pertussis toxin sensitive, and cholera toxin insensitive, demonstrating that naturally expressed H_4 receptors in eosinophils couple to $G\alpha_{i/o}$. The responses to histamine in H_4 receptor transfected 293-EBNA and HEK-293 cells were shown to be pertussis toxin sensitive, which is in agreement with our findings that the effects of activating H_4 receptor on eosinophils are $G\alpha_{i/o}$ dependent (Oda *et al*., 2000; Morse *et al*., 2001). However, assays of calcium mobilisation with the same cells cotransfected with H_4 receptors and the 'promiscuous' $G\alpha_{15}$ G-protein demonstrated that H_4 receptors could additionally signal *via* $G\alpha_{15}$ (Oda *et al*., 2000).

Despite being shown to express H_4 receptor mRNA (Oda *et al*., 2000; Morse *et al*., 2001; Zhu *et al*., 2001), in our study neutrophils and basophils failed to respond to histamine in the shape change assay. Similarly, histamine has been shown to selectively stimulate intracellular calcium mobilisation in eosinophils but not neutrophils (Raible *et al*., 1992). However, recently it has been reported that H_4 receptor mRNA is expressed by eosinophils and basophils but not neutrophils (Hofstra *et al*., 2003). Using the only commercially available anti-human H_4 receptor monoclonal antibody (Autogen Bioclear, U.K.), we have been unable to detect H_4 receptor protein expression by different leucocyte types.

Analysis of leucocyte shape change in response to agonists can also be performed in whole blood and can be a valuable assay for screening of compounds for selectivity and potential *in vivo* activity (Bryan *et al*., 2002; Zhang *et al*., 2002). We have shown that histamine can stimulate eosinophil shape change in the whole-blood assay *via* activation of H_4 receptors. However, histamine was not as efficacious as eotaxin in this assay, probably due to the rapid degradation of histamine, which is known to have a short half-life in the circulation.

We have shown that eosinophils undergo actin polymerisation when stimulated by histamine, a process that both underpins cell shape change and is prerequisite for cell migration. This histamine-stimulated actin polymerisation is inhibited by thioperamide and pertussis toxin, and is thus mediated *via* H_4 receptor activation and is dependent on $G\alpha_{i/o}$. In addition, histamine and clozapine can stimulate a rise in intracellular calcium in eosinophils and each can desensitise the receptors to subsequent stimulation by histamine. This desensitisation to histamine, but not eotaxin, suggests that histamine and clozapine are both activating the same receptor to induce calcium mobilisation. Raible *et al*. (1992) have previously shown that thioperamide inhibits histamine-induced calcium mobilisation in eosinophils. Furthermore in 1994, Raible *et al*., showed that H_3 receptor agonists, *R*-α-meH and *N*-α-meH, could both induce calcium mobilisation. Both agonists were less potent than histamine, suggesting activation of a novel receptor subtype, presumably, the newly discovered H_4 receptor. Calcium mobilisation *via* the H_4 receptor has been observed using cells cotransfected with H_4 receptor and chimeric G proteins as well as in mast cells (Oda *et al*., 2000; Morse *et al*., 2001; Zhu *et al*., 2001; Hofstra *et al*., 2003).

The lack of any significant chemotactic or chemokinetic effect of histamine in our study are in contrast to earlier studies by Clark *et al*. (1975), and a more recent study by

O'Reilly *et al*. (2002). Clark *et al*., used extremely eosinophilic donors (30–85% eosinophils of total granulocytes) compared to the normal or atopic individuals (2–20% eosinophils) we have used, thus this difference may explain the disparity.

Preincubation of eosinophils with histamine caused a significant increase in chemotaxis toward eotaxin. Furthermore, this enhanced chemotactic response of human eosinophils to eotaxin was inhibited by thioperamide and is thus mediated by the H_4 receptor. These actions of histamine are similar to those of the cytokine interleukin (IL)-5, which is chemokinetic and a weak chemoattractant but can prime eosinophils for enhanced responsiveness to other mediators such as eotaxin (Takafuji *et al*., 1991; Sehmi *et al*., 1992; Collins *et al*., 1995). Histamine has previously been shown to inhibit the chemotaxis of eosinophils to endotoxin-activated serum (EAS) *via* H_2 receptors at high concentrations (10–50 μM) yet can also enhance chemokinesis of eosinophils and neutrophils *via* H_1 receptors at lower concentrations (Hill & Quie, 1974; Clark *et al*., 1977; Wadee *et al*., 1980). Together with our evidence of the involvement of H_4 receptors in priming eosinophils for enhanced chemotaxis, these data suggest that both the differential expression of histamine receptors on eosinophils, and the concentration of histamine to which they are exposed, are important in determining the responsiveness of these cells.

The expression profile of the guinea-pig H_4 homologue corresponds with that of the human receptor, with greatest expression detected in leucocytes and bone marrow (Liu *et al*., 2001b). However, in our study, histamine failed to stimulate guinea-pig eosinophils in assays including whole-blood shape change, actin polymerisation, calcium mobilisation, chemotaxis and chemokinesis ($n=3-7$, data not shown). Comparisons of the cloned human, mouse, rat, guinea-pig and pig H_4 receptors in transfected cells have demonstrated that human and rodent as well as porcine H_4 receptors have substantial differences not only in their primary structure but also in their affinities for H_4-related ligands (Liu *et al*., 2001b; Oda *et al*., 2002). Thus, selecting an appropriate animal model will be crucial in testing of H_4 selective ligands, for elucidating the physiological role of the H_4 receptor and validating H_4 receptors as therapeutic target molecules.

There are several examples of histamine influencing the activities of leucocytes and structural cells, including inhibition of eosinophil degranulation, the release of cytokines such as IFN-γ, TNF-α, IL-1, IL-2 and IL-10, and polarisation of dendritic cells, the receptor subtype mediating these effects require re-examination in light of the discovery of H_4 receptors (Carlsson *et al*., 1985; Dohlsten *et al*., 1988; Bissonnette, 1996; Sirois *et al*., 2000; Ezeamuzie & Philips, 2000; Caron *et al*., 2001). Recently, Gantner *et al*. (2002) showed that histamine-stimulated IL-16 release by $CD8^+$ T cells is mediated by H_4 receptors in conjunction with H_2 receptors. More recently, the H_4 receptor has been shown to mediate calcium mobilisation and chemotaxis of mast cells (Hofstra *et al*., 2003).

In conclusion, using the currently available pharmacological tools, in this study we have demonstrated that the H_4 receptor is involved in eosinophil function. Histamine activates eosinophils *via* H_4, which couples to $G\alpha_{i/o}$, this stimulation induces morphological changes, enhances expression of CD11b, increases their migratory response and may also stimulate other, as yet unknown, responses of eosinophils. There is evidence that H_4 receptors mediate proinflammatory effects in other immune cells, including mast cells and $CD8^+$ T cells. Thus, in the context of an inflammatory environment such as the asthmatic lung, H_4 receptor activation could contribute significantly to the perpetuation of inflammation. Therefore, the H_4 receptor may provide a novel target for allergic disease therapy.

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