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Histamine H_4 receptor mediates chemotaxis and calcium mobilization of mast cells

Claudia L. Hofstra[1], Pragnya J. Desai, Robin L. Thurmond and Wai-Ping Fung-Leung.

Johnson and Johnson Pharmaceutical Research and Development L.L.C, San Diego, CA, USA

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Running title: H_4 receptor mediates chemotaxis and signaling of mast cells

Address of correspondence: Wai-Ping Fung-Leung

Johnson and Johnson Pharmaceutical Research and Development,

3210 Merryfield Row

San Diego, CA 92121, USA

phone: 1-858-450-2016, fax: 1-858-450-2038

wleung@prdus.jnj.com

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H_3R-/- H_3 receptor gene knock-out

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PTX pertussis toxin

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Abstract

The diverse physiological functions of histamine are mediated through distinct histamine receptors. Mast cells are major producers of histamine, yet effects of histamine on mast cells are currently unclear. The present study shows that histamine induces chemotaxis of mouse mast cells, without influencing mast cell degranulation. The histamine H_3 and H_4 receptor antagonist thioperamide (but not H_1 or H_2 receptor antagonists) inhibited histamine-induced chemotaxis of mast cells. The chemotactic response is mediated by H_4 receptors, since chemotaxis toward histamine was absent in mast cells derived from H_4 receptor-deficient mice, but was detected from H_3 receptor-deficient mice. In addition, Northern blot analysis showed the expression of H_4 but not H_3 receptors on mast cells. Activation of H_4 receptors by histamine resulted in calcium mobilization derived from intracellular stores. Calcium mobilization and chemotaxis involve Gαi/o protein pathways and phospholipase C (PLC), since these responses were completely inhibited by pertussis toxin (PTX) and PLC inhibitor U73122. In the present study it is shown that histamine acting on mast cells through the H_4 receptor mediates signaling leading to mast cell chemotaxis. This mechanism might be responsible for mast cell accumulation in allergic tissues.

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Histamine is a biogenic amine playing an important role in the regulation of different physiological processes in the body. Histamine is synthesized from L-histidine by histidine decarboxylation in specific cell types, such as mast cells, basophils, enterochromaffin like cells and neurons. The diverse biological effects of histamine are mediated through different histamine receptors, which are all G-protein coupled receptors. Almost a century of extensive pharmacological research using specific histamine receptor agonists and antagonists has identified three histamine receptors (H_1, H_2 and H_3 receptor). Each receptor has its own expression pattern and mediates distinct effects: H_1 receptors trigger smooth muscle contractions and is generally thought to play an important role in allergy; H_2 receptors regulate gastric acid secretion in the stomach and H_3 receptors control the release of histamine and neurotransmitters by neurons (Hill et al., 1997). However, not all effects of histamine can be attributed to these three histamine receptors. Therefore, it has been suggested that another histamine receptor might exist (Raible et al., 1994). The molecular identity of this fourth human histamine receptor H_4 receptor) was revealed recently (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001), and subsequently the H_4 receptor in mouse, rat and guinea pig were cloned (Liu et al., 2001b).

The amino acid sequence of the H_4 receptor has low homology with other histamine receptors. Its closest member in the histamine receptor family is the H_3 receptor that shares only a 35% amino acid homology with the H_4 receptor, although the homology in the transmembrane region is 58%. However, the H_4 receptor expression pattern is distinct from the H_3 receptor. While the expression of the H_3 receptor is mainly restricted to cells in the central nervous system (Lovenberg et al., 2000), the H_4 receptor

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seems to be limited to cells of the haemopoietic lineage (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Zhu et al., 2001). The expression of H_4 receptors on haemopoietic cells is not unique among the histamine receptor family, since T cells and dendritic cells also express H_1 and H_2 receptors (Jutel et al., 2001; Szeberenyi et al., 2001).

Pharmacological properties of the H_4 receptor have been revealed using H_4 receptor-transfected cells (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001). It was shown that specific H_1 and H_2 receptor antagonists and agonists do not bind to the H_4 receptor. However, more typical H_3 receptor ligands (like thioperamide, clobenpropit, imetit and R-α-methylhistamine) could bind the H_4 receptor with affinities different from that of the H_3 receptor.

Similar to other G-protein coupled receptors, histamine receptors activate specific G proteins that lead to the activation of signal transduction pathways (for review see (Leurs et al., 1995). It has been shown that H_1 receptors mediate this action through Gαq proteins resulting in calcium mobilization, H_2 receptors signal through Gαs proteins and cAMP increase, while H_3 receptors signal through Gαi/o proteins and inhibition of cAMP (Lovenberg et al., 1999). In literature two signaling pathways are thought to be utilized by the H_4 receptor. Firstly, using cells transfected with the H_4 receptor and a cAMP responding reporter construct, studies have shown that histamine could inhibit forskolin-stimulated cAMP increases (Oda et al., 2000; Liu et al., 2001a; Zhu et al., 2001). However, this cAMP inhibitory effect is low in comparison with that mediated by the H_3 receptor. Secondly, one study showed that histamine could not alter cAMP levels in H_4 receptor transfected cells, but instead increased calcium mobilization if the cells were

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cotransfected with Gαq/i1/2, Gαq/i3, or Gα16 proteins (Morse et al., 2001). The same study showed that histamine induced MAP kinase phosphorylation, which was inhibited by PTX. However, signaling pathways mediated by endogenous H_4 receptors have not been studied.

Mast cells are important effector cells in allergic diseases. Mast cells bind IgE with IgE receptor and subsequent contact with antigens will trigger IgE receptor cross-linking and the release of preformed mediators, such as serotonin and histamine, and de novo produced mediators, such as prostaglandins and leukotrienes. Although mast cells are best known for their histamine releasing capacity, little is known about the effect of histamine on mast cells themselves.

In the present study, the expression pattern of mouse H_4 receptor on various purified hematopoietic cells and in various tissues was investigated. We showed that the mouse H_4 receptor was expressed specifically on eosinophils and mast cells. Bone marrow-derived mast cells were used to study the functional aspects and signaling pathways of the endogenous mouse H_4 receptor.

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Materials

Human HMC-1 and WEHI-3 cells were purchased from American Type Culture Collection (Rockville, MD). Human $CD34^+$ cord blood cells were from AllCells LLC (Berkeley, CA). Basophil enrichment kit and serum free medium were from Stem Cells Technologies (Canada). RNeasy kit was from Qiagen (Valencia, CA). RT reaction kits and ExpressHyb solution were from Clontech (Palo alto, CA). cAMP detection kit, nylon blot (Hybond) and Rediprime II kit were from Amersham Pharmacia Biotech (Piscataway, NJ).

Thapsigargin, U73122 and U73433 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Transwells were purchased from Costar (Cambridge, MA). LTB_4 and prostaglandin detection kits were from Cayman Chemical (Ann Arbor, MI). Fluo-3 was from TEF Labs (Austin, TX) and pluronic acid from Molecular Probes (Eugene, OR). Pertussis toxin and anti-DNP IgE was from ICN Pharmaceuticals (Costa Mesa, CA). All other antibodies were from Becton Dickinson Pharmingen (San Diego, CA). Polylysine-coated black wall 96-well tissue culture plates were purchased from BD Biosciences (San Jose, CA). All other reagents were purchased from Sigma (St. Louis, MO).

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Methods

Generation of H_4 receptor gene knockout mice (H_4 R-/-).

H_4R-/- mice were generated by Lexicon Genetics, Woodlands, TX. A 9 kb mouse genomic fragment containing the mouse H_4 receptor gene was obtained from the ES cell line 2G9 and was used as a template to prepare the knockout construct. A 0.5 kb region

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covering most of exon 1 and part of intron 1 of the H_4 receptor gene was deleted from this genomic fragment and replaced with a neomycin resistant gene cassette. Homologous recombination in ES cells was confirmed by Southern analysis using a 3' external probe amplified from the mouse H_4 receptor gene with oligonucleotides 5'-GAG ATG TAG ATG TGG TCG TTT G and 5'-CAT GTG CAG GCA CAC ACA TAC. The Southern blot of ES cell DNA digested with NcoI produced a 4.9 kb wild-type band and a 3.4 kb targeted band (figure 1). Chimeric mice were generated from embryos injected with embryonic stem cells. Germline mice were obtained by breeding chimeric male mice with C57BL/6 females. Germline mice heterozygous for the disrupted H_4 receptor gene were identified by PCR. Wild-type and H_4 R-/- mice were obtained from cross-breeding of heterozygous mice.

Detection of mouse H_4 receptor expression

RNA from tissues and purified cells was prepared using a RNeasy kit according to the manufacturers instructions. H_4 receptor RNA was detected by RT-PCR using specific mouse H_4 receptor primers (5'-ATG TCG GAG TCT AAC AGT ACT GG and 5'-AGA AGA TAC TGA CTG GTT CTG TGA). RT products of multiple tissues and cell types were amplified by PCR under conditions of 94ºC 45 sec, 55ºC 45 sec, and 72ºC 2 min for 35 cycles. The PCR products were run on a 1% agarose gel with ethidium bromide (10 μg/ml), and DNA was visualized with UV light. The amplified mouse H_4 receptor cDNA is 1185 bp in size.

Mouse Th1 cells, Th2 cells, Tc1 cells, Tc2 cells, B cells and macrophages were activated and total RNA was prepared as described before (Shier et al., 2000).

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Eosinophils were in vitro differentiated from C57BL/6J mouse bone marrows. Bone marrow was aseptically isolated from the femurs. The cells ($2x10^5$/ml) were cultured at 37°C with 5% CO_2 in RPMI culture medium consisting of 10% FCS, 0.1 mM non-essential amino acids, 50 µg/ml penicillin/streptomycin, 0.2 ng/ml IL-3, 0.4 ng/ml IL-5 and 0.2 ng/ml GM-CSF. After 6 days the medium was refreshed and cells were cultured for 7 more days. Cells were stained with haematoxylin-eosin dyes and > 95% of the cells displayed eosinophil phenotype. Mouse kidney, liver, thymus, spleen, lung and brain were isolated from C57BL/6J mice. RNA was isolated from the tissues as described above.

Total RNA samples (5 µg) were run on a RNA gel and then transferred overnight to a nylon blot. The blot was prehybridized with ExpressHyb solution for 30 min at 68°C. The mouse H_4 receptor cDNA clone (Liu et al., 2001a) and the purified RT-PCR product of the mouse H_3 receptor (Liu et al., 2001b) were labeled using the rediprime II kit. The blot was hybridized for 2 h at 68°C, followed by one wash (2x SSC and 0.05% SDS) of 40 min at room temperature, and a second wash (0.1 x SSC and 0.1% SDS) of 40 min at 50°C. The blots were exposed to x-ray film at –70°C with two intensifying screens for 16 hours.

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Detection of human H_4 receptor expression

Human basophils (98% purity) were isolated from human periperal blood mononuclear cells using a basophil enrichment kit.

Human mast cells were differentiated from human $CD34^+$ cells purified from cord blood, using serum free medium supplemented with hSCF (100 ng/ml) and IL-6 (50 ng/ml)

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(Dahl et al., 2002). Cells were grown for 12-14 weeks, media supplemented with cytokines was changed once a week. Cells were monitored weekly for their mast cell properties, using Geimsa, Toluidine blue, tryptase and CD117 staining. The cells showed metachromatic granule staining properties, as early as 2 –3 weeks and by week 12 the purity of these cells for mast cell properties was 95%. Human mast cell line HMC-1 was cultured in Iscove's medium containing 10% FCS, 2 mM glutamine and 1.2 mM monothioglycerol. Total RNA was extracted from human basophils, mast cells and HMC-1 cells using an RNeasy kit and 250 ng RNA was used for the RT reaction according to manufacturer's instructions. PCR using human H_4 receptor specific primers (5'-ACT AGA ATT CGC CAC CAT GCC AGA TAC TAA TAG CAC and 5'-ATG CAG GAT CCA GCA TTT GAG ACT GAC AGG TAT) was carried out as described before (Liu et al., 2001a).

Bone marrow mast cell culture

Mast cells were differentiated from bone marrows collected from H_4 receptor gene knock-out (H_4R-/-), H_3 receptor gene knockout (H_3R-/-) mice (Toyota et al., 2002), wild-type mice, BALB/c and C57BL/6J mice. Bone marrow was aseptically isolated from the femurs. The cells ($5x10^5$/ml) were cultured at 37°C with 5% CO_2 in RPMI culture medium consisting 10% FCS, 0.1 mM non-essential amino acids, 50 µg/ml penicillin/streptomycin and 20% WEHI-3 conditioned medium. WEHI-3 cells were cultured in Iscove's Dulbeccos medium with 10% FCS, 4 mM L-glutamine, 1.5 g/l sodium carbonate, 0.05 µM beta-mercaptoethanol and 50 µg/ml penicillin/streptomycin. The filtrated supernatant was used as WEHI-3 conditioned medium.

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After 16 h culture, the non-adherent bone marrow cells were transferred to a new flask for further culture. The medium was refreshed once a week. After 4 weeks, the cells were analyzed by flow cytometry for IgE receptor and CD117 (c-kit), which are expressed specifically on mast cells. IgE receptor on mast cells were detected by incubating with anti-DNP IgE or vehicle for 30 min, followed by FITC labeled anti-IgE antibody. Mast cells were incubated with FITC/PE labeled anti-CD117 antibody for 30 min on ice. The majority of the bone marrow cells was confirmed to be mast cells with >99% IgE receptor positive and >99% CD117 positive. Mast cells of 4-8 weeks were used for experiments. No difference in proliferation and in expression of IgE receptor or c-kit receptor was observed in mast cells derived from H_3R-/-, H_4R-/- and wild-type mice.

Degranulation assay

Mast cells ($5x10^5$/ml) were sensitized overnight with 2 µg/ml anti-DNP IgE. Mast cells ($2x10^5$/well) were plated out in a 96-wells plate and incubated for 15 min with 10 µM histamine or vehicle at 37°C. Degranulation was achieved by adding different concentrations of DNP-HSA for an additional 30 min. Total release of mast cell contents was achieved by adding 1% Triton X-100. The plates were spun down (1000 rpm, 5min, 5°C), and supernatants were analyzed for beta-hexosaminidase.

Beta-hexosaminidase was measured by adding 25 µl supernatant to 50 µl of 10 mM p-nitrophenyl N-acetyl-beta D glucosaminide in 0.1 M sodium citrate buffer (pH 4.5) for 2 h at 37°C. The reaction was stopped by adding 50 µl of 0.4 M glycine (pH 9). The plates were measured at wavelength 405 nm. The percentage of degranulation was calculated as

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((A-B)/(T-B) x100), where A is levels of beta-hexosaminidase released from stimulated cells, B is that released from unstimulated cells and T is total content of the cells.

To study the induction of leukotriene and prostaglandins, IgE-sensitized mast cells ($1x10^6$/well) were incubated for 15 min with 10 µM thioperamide or vehicle, followed by 30 or 210 min incubation with 10 µM histamine or 5 µg/ml compound 48/80. LTB_4 and prostaglandin levels were measured in supernatants according to the manufacturers instruction.

Chemotaxis assay

Transwells with a pore size 8 µm were coated with 100 µl of 100 ng/ml human fibronectin for 2 h at room temperature. After removal of the fibronectin, 600 µl of RPMI with 5% BSA in the presence of histamine (ranging from 1.25-20 µM) was added to the bottom chamber. Subsequently, 10 µM histamine receptor antagonists (diphenhydramine, ranitidine, thioperamide), U73122 (1.1, 3.3 and 10 µM), or U73433 (1.1, 3.3 and 10 µM), were added to the top and bottom chambers. Mast cells ($2x10^5$/well) were added to the top chamber. The plates were incubated for 3 h at 37°C. Transwells were removed and the number of cells in the bottom chamber was counted for 1 min by flow cytometer. To study PTX effects, mast cells ($1x10^6$ cells/ml) were pretreated for 16 h with 0, 0.5, 5 or 50 ng/ml PTX. Cells were washed afterwards and put in the upper chamber as described above.

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Calcium mobilization

Mast cells ($2x10^5$/well) were loaded with 4 µM calcium dye Fluo-3 (AM) in dye-loading buffer for 1 h at 37°C. The dye-loading buffer is RPMI medium without phenol red and contains 0.5% BSA, 2.5 mM probenecid and 0.08% pluronic acid. Cells were spun down and taken up in loading medium which is RPMI medium (without phenol red) containing 0.5% BSA. Cells were plated out in polylysine-coated black wall 96-well tissue culture plates. Prior to measurements, the plates were spun for 3 min at 1000 rpm at room temperature. Calcium mobilization was assayed in a fluorometric imaging plate reader 384 (Molecular Devices, Sunnyvale, CA). The fluorescence intensity was calculated as the maximum minus the minimum fluorescence over a 2 min period. All data points were done in triplicates, and experiments were repeated at least three times with different batches of mast cells.

Histamine receptor agonists and antagonists were added to the cells 10 min prior to the calcium measurements.

In calcium storage experiments, Fluo-3 loaded mast cells received a first addition of 3 mM EDTA or 10 µM thapsigargin or PBS. After stabilization of the signal, mast cells received a second addition of 10 µM histamine.

For pertussis toxin (PTX) treatment, mast cells ($1x10^6$ cells/ml) were pretreated for 16 h with 0, 0.5, 5 or 50 ng/ml PTX. Cells were washed and loaded with Fluo-3 as described above.

For PLC inhibitor treatment, mast cells were treated with 1.1, 3.3 and 10 µM U73122 or U73433 10 min prior to stimulation with 10 µM histamine.

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To detect calcium response triggered by IgE receptor cross-linking, mast cells ($5x10^5$/ml) were sensitized overnight with 2 µg/ml anti-DNP IgE. Cells were washed and loaded with Fluo-3 as described above. During the calcium measurements, 5 µM histamine was added followed 3 min later with different concentrations of DNP-HSA.

cAMP measurements

Mast cells ($1x10^6$/well) in culture medium containing 1 µM 3-isobutyl-1-methylxanthine (IBMX) were plated out in a 96 well plate. Cells were incubated for 30 min at 37°C. Histamine receptor antagonist (10 µM) was added 15 min prior to histamine and/or 100 µM forskolin addition for 30 min at 37°C. Intra-cellular cAMP levels in cell lysates were determined using the Biotrack cAMP enzyme immunoassay system according to the manufacturers instructions.

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Results

Mast cells, basophils and eosinophils express H_4 receptors.

Previously we reported that the human H_4 receptor is expressed mainly in bone marrow and eosinophils (Liu et al., 2001a). In the present study, mouse H_4 expression was determined in different tissues and purified cell types of the haemopoietic lineage. Abundant expression of mouse H_4 receptor was detected in untreated mast cells and antigen-activated IgE-primed mast cells by RT-PCR (figure 2A). In contrast, H_4 receptor was not detected in any of the tissues tested, such as lymph nodes, kidney, liver, thymus, spleen, heart, lung and brain. In addition, expression was not detected in many different immune cell types including $CD4^+$ effector Th1 and Th2 cells, $CD8^+$ effector Tc1 and Tc2 cells, resting and LPS-activated B cells, as well as macrophages.

Expression of H_4 receptor in mast cells and eosinophils was further confirmed by Northern blot analysis (figure 2B and 2C). Both H_1 and H_2 receptors were detected on mast cells (results not shown), whereas H_3 receptor was undetectable by Northern blot analysis (figure 2C, left panel) and RT-PCR (results not shown). Consistent with the expression profile in mice, H_4 receptor was detected in human cord blood-derived mast cells and in human HMC-1 mast cell line by RT-PCR (figure 2D). In addition, human H4 receptor was expressed in basophils (figure 2D) but not in neutrophils (data not shown). In summary our data shows that the H_4 receptor is expressed on mast cells, basophils and eosinophils.

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H_4 receptors mediate calcium mobilization in mast cells.

Histamine binding to its receptors activate G-proteins, which result in changes of calcium or cAMP levels. Histamine induces a concentration-dependent increase of cAMP in mast cells (figure 3). This response was unaffected by H_3/ H_4 antagonist thioperamide and H_1 antagonist diphenhydramine (results not shown), thereby excluding a role for H_1, H_3 or H_4 receptors. However, the H_2 receptor antagonist ranitidine could inhibit the histamine-induced cAMP increase. The results indicate that the histamine-induced cAMP increase in mast cells is H_2 receptor-mediated.

Calcium mobilization was observed in mast cells induced by histamine in a concentration dependent fashion (figure 4A). The response peaked at about 20 seconds after histamine addition and returned to basal levels within one minute (figure 4A insert). The ED50 of histamine-induced calcium mobilization was 3.8 μM. Neither H_1 receptor antagonists nor H_2 receptor antagonists altered the histamine-induced calcium mobilization (figure 4B). However, thioperamide (figure 4B) inhibited the histamine-induced calcium mobilization in a concentration dependent manner, with an IC_{50} of 1.00 ± 0.5 μM. This IC_{50} value is consistent with the relative binding affinities of histamine and thioperamide (Liu et al., 2001a). Taken together, the data suggest that H_3 and/or H_4 receptors are involved in calcium mobilization in mast cells.

Since mast cells do not express H_3 receptors (see figure 2C), it is likely that the calcium response is mediated by H_4 receptors. A direct proof of H_4 receptor mediated calcium mobilization was demonstrated in mast cells generated from H_4R-/- and H_3R-/- mice. In contrast to wild-type mast cells, up to 30 μM histamine stimulation in H_4R-/-

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mast cells did not result in calcium mobilization (figure 4C). Mast cells from H_4R+/- mice showed an intermediate calcium response as compared to mast cells from wild-type mice. This response is histamine-specific since H_4R-/- mast cells mediated normal calcium responses to ATP or ionomycin (results not shown). Furthermore, H_3R-/- mast cells showed a normal calcium response to histamine comparable to that in wild-type mast cells (results not shown). Therefore it can be concluded that histamine induces calcium mobilization in mast cells via the H_4 receptor.

H_4 receptors trigger calcium release from intra-cellular calcium stores.

To determine the source of calcium in histamine induced calcium mobilization, either EDTA or thapsigargin was used in experiments to deplete calcium from extra-cellular environment or intra-cellular calcium storage, respectively. Histamine induced calcium mobilization was not affected by EDTA but was completely abolished by thapsigargin (figure 5A and 5B). Thus, histamine mediates the release of calcium from intra-cellular calcium stores in mast cells.

The H_4 receptor mediates calcium mobilization through $G\alpha i/o$ proteins and PLC.

To determine the G-proteins utilized by H4 receptor in mast cells, $G\alpha i/o$ protein inhibitor PTX was used in experiments. Pretreatment of mast cells with PTX inhibited the histamine-induced calcium response completely (figure 5C), but the calcium response toward ionomycin or ATP was unaffected (results not shown), indicating that the PTX inhibitory effect is histamine-specific. Therefore it appears that $G\alpha i/o$ proteins are acting downstream of the H_4 receptor leading to calcium mobilization.

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The possible involvement of phospholipase C (PLC) in histamine induced calcium mobilization was studied using the PLC inhibitor U73122 and its inactive analog U73433(Thompson et al., 1991). U73122 inhibited the histamine-induced calcium mobilization in a concentration dependent manner with a complete inhibition at 10 μM, whereas the inactive analog U73433 (up to 10 μM) was unable to alter this response (figure 5D). These results indicate that the histamine effects on calcium mobilization involved PLC activation.

Histamine does not alter degranulation through H_4 receptors.

Effects of histamine on IgE receptor-mediated calcium response and degranulation in mast cells were investigated. IgE-primed mast cells were pretreated with histamine, followed by antigen stimulation. Histamine did not alter antigen-IgE triggered calcium mobilization (figure 6A). Antigen induced degranulation of IgE-primed mast cells from wild-type and H_4R-/- mice was also unaffected by histamine pretreatment (figure 6B). In addition, thioperamide did not have any effects on antigen-IgE mediated mast cell degranulation (results not shown).

The effects of histamine on the production of de novo synthesized mediators like prostaglandins and leukotrienes were also investigated. Compound 48/80 induced LTB_4 and prostaglandin release by mast cells, whereas histamine did not alter LTB_4 or prostaglandin levels (table 1). In summary, H_4 receptor and histamine do not seem to be involved in antigen-induced degranulation since histamine does not induce degranulation nor is it involved in the de novo production of LTB_4 and prostaglandins by mast cells.

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Histamine mediates chemotaxis through H_4 receptors.

Chemotaxis of mast cells toward histamine was investigated using a transwell system. Histamine induced mast cell migration in a concentration dependent manner (figure 7A). This observed effect was due to chemotaxis but not chemokinesis, since cell migration was abolished when histamine concentration gradient was disrupted. Thioperamide inhibited histamine-induced mast cells chemotaxis in a concentration dependent fashion, whereas neither diphenhydramine nor ranitidine had any effects (figure 7B and 7C). The IC_{50} for thioperamide is similar compared to that in the calcium mobilization assay. To distinguish between H_3 and H_4 receptor-mediated effects on chemotaxis of mast cells, the chemotaxis assay was performed using mast cells derived from H_4R-/- or H_3R-/- mice. No migration of H_4R-/- mast cells toward histamine was observed (figure 7A). In contrast, H_3R-/- mast cells responded to histamine in chemotaxis similar to that in wild-type mast cells (results not shown). Thus, histamine-induced chemotaxis of mast cells is mediated through the H_4 receptor.

H_4 receptor mediated chemotaxis involves Gαi/o proteins and PLC.

Similar to the PTX inhibitory effects on histamine-induced calcium mobilization, preincubation of mast cells with PTX caused a concentration-dependent decrease in mast cell chemotaxis toward histamine (figure 7D). A complete inhibition of histamine-induced chemotaxis was observed at 50 ng/ml, a concentration with similar effects in inhibiting calcium mobilization. PLC was also involved in the histamine-induced chemotaxis since U73122 could inhibit the chemotaxis in a concentration dependent fashion (figure 7E), while its inactive analog U73433 did not alter the chemotaxis. Taken

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together, these results suggest that the histamine-induced chemotaxis of mast cells involves Gαi/o proteins and PLC, similar to that of the calcium response.

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Discussion

In the present study we showed that the mouse H_4 receptor is expressed on mast cells, and eosinophils, but not on other haemopoietic cells including T cells, B cells or macrophages. Furthermore, human H_4 receptor is expressed on mast cells and basophils in addition to the previously reported expression on human eosinophils (Oda et al., 2000; Liu et al., 2001a; Morse et al., 2001). Interestingly, this is the first study to show that mast cells express the H_4 receptor, but not the H_3 receptor. In literature it has been unclear whether mast cells express the H_3 receptor. Most studies used thioperamide as a specific H_3 antagonist (Kohno et al., 1994; Bissonnette, 1996), but recent data indicate that both the mouse and human H_4 receptor can bind thioperamide as well (Liu et al., 2001b). It is therefore likely that the effects of histamine on mast cells that can be blocked by thioperamide are actually mediated by H_4 receptors.

The role of the H_4 receptor in some of the physiological functions of mast cells was investigated. One of the major biological functions of mast cells is to release inflammatory mediators in response to antigens. The major mechanism of such release is through IgE-mediated degranulation. The present work shows that histamine does not appear to have any effects on degranulation, either on its own or in combination with antigen-IgE complexes. In addition, histamine does not seem to alter mast cell proliferation or survival (results not shown). Similarly, H_4R-/- mast cells did not show any defects in degranulation, proliferation or survival, indicating that the H_4 receptor has no role in these processes.

Mast cell progenitor cells, which are present in the bone marrow, migrate to connective or mucosal tissue where they differentiate into the mature form. It is thought

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that chemoattractants like stem cell factor (SCF) might be important for this localization. Migration of mast cells may also play a role in allergic rhinitis and allergy where increases in mast cell number are found (Kirby et al., 1987; Crimi et al., 1991; Amin et al., 2000; Gauvreau et al., 2000; Kassel et al., 2001). In addition, it is known that in response to antigens there is a redistribution of mast cells to the epithelial lining of the nasal mucosa (Fokkens et al., 1992; Slater et al., 1996). It is possible that some of the redistribution that is seen in allergic conditions may be mediated by histamine since it would be continually produced under such circumstances. The data presented here shows that histamine is a potent chemoattractant for mast cells and that this chemotaxis is mediated via the H_4 receptor. Taken together, antagonists of the H_4 receptor may be useful in the treatment of asthma or allergic rhinitis. Currently, we are addressing these questions with in vivo models.

Using specific histamine receptor antagonists as well as mast cells derived from H_4R-/- and H_3R-/- mice, we demonstrated that histamine-induced calcium mobilization from intra-cellular stores in mast cells via the H_4 receptor. Calcium mobilization via the H_4 receptor has also been observed using cells cotransfected with both the H_4 receptor and chimeric G-proteins (Morse et al., 2001). However, other studies using human H_4 receptor transfected cells have shown that histamine activation of the cells resulted mainly in decreased cAMP levels (Oda et al., 2000; Liu et al., 2001a; Zhu et al., 2001). Since results of transfected cells depend on the endogenous machinery of the cell, different cell types can yield different results. In the present study these complications are not present, since the endogenous H_4 receptor was studied. Previously, similar calcium mobilization induced by histamine was demonstrated in human eosinophils, which have

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been shown to express the H_4 receptor (Raible et al., 1994). The calcium mobilization was inhibited by thioperamide, the dual H_3/H_4 receptor antagonist. However, R-α-methyl-histamine and N-α-methyl-histamine were less potent than histamine in inducing calcium mobilization, which is more consistent with their respective affinities for the H_4 receptor than for the H_3 receptor (Raible et al., 1994). Therefore, it is likely that this response is mediated by the H_4 receptor and not the H_3 receptor. Thus, the activation of H_4 receptor both on mast cells and eosinophils results in calcium mobilization.

The signaling pathways involved in H_4 receptor activation by histamine have also been studied. Both Gαi/o proteins and PLC are involved since PTX, which inactivates Gαi/o proteins, and the PLC inhibitor U73122, inhibited chemotaxis and calcium mobilization. Since Gαi/o proteins does not activate PLCβ, but G-protein βγ subunits can activate $PLC\beta_{2/3}$ (Exton, 1996, Clapham and Neer, 1997, Rhee, 2001). It is possible that $PLC\beta_{2/3}$ is activated by the G-protein βγ subunits that are released from the coupling with Gαi/o proteins when histamine binds to the H_4 receptor. Other G-protein coupled receptors have also been shown to signal via PLC and Gαi/o (Seebeck et al.,1998, Zussman et al., 1998, and Yang et al., 2002). The activation of PLC may lead to the release of inositol 1,4,5 triphosphate (IP_3). IP_3 can activate an IP_3 receptor in the endoplasmic reticulum, which causes the release of calcium in the cytoplasm, a mechanism that is known to occur in mast cells (Pacher et al., 2000). Compound 48/80 has been reported to elicit calcium response in mast cells through Gαi/o proteins, PI-3 kinase, Src and Syk (Shefler and Sagi-Eisenberg, 2001). SCF is also known to induce calcium mobilization in mast cells involving activation of Gαi/o proteins, PI-3 kinase, p38 MAP and MEK kinases (Dastych et al., 1998; Sundstrom et al., 2001).

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We propose the following signaling pathway involved in histamine activation of the H_4 receptor (figure 8). Histamine binds to the H_4 receptor on mast cells and eosinophils and causes the activation of PTX-sensitive Gαi/o proteins. Possibly G-protein βγ subunits dissociated from Gαi/o proteins trigger the activation of PLC. PLC hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP_2) to diacylglycerol and IP_3. IP_3 activates a calcium channel in the endoplasmic reticulum, possibly through an IP_3 receptor to release calcium. The increased calcium levels trigger currently unknown signaling pathways, which will cause mast cell chemotaxis toward histamine.

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Footnotes

Reprint requests: Wai-Ping Fung-Leung

Johnson and Johnson Pharmaceutical Research and Development,

3210 Merryfield Row

San Diego, CA 92121, USA

wleung@prdus.jnj.com

[1]Current address Claudia L. Hofstra:

N.V. Organon, Oss, the Netherlands. Claudia.Hofstra@organon.com

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Figure legends

Figure 1 Generation of H_4R-/- mice.

(A) The mouse H_4 receptor gene was disrupted by replacing exon 1 with a neomycin resistant gene cassette. (B) The disrupted H_4 receptor gene in H_4R+/- and H_4R-/- mice was confirmed by Southern blot hybridization. A 4.9 kb DNA band from the wild-type gene and a 3.4 kb DNA band from the disrupted gene were shown.

Figure 2 Mouse H_4 receptor expression is restricted to mast cells, basophils and eosinophils.

(A) cDNA of different mouse tissues and cell types were used as templates for reverse-transcription-PCR. The expected PCR product for mouse H_4 receptor is 1185 bp. Mast cells were primed with antigen-specific IgE (untreated) and activated with antigens for 30 min. B cells and macrophages were activated with LPS for 24 h and 1 h respectively. (B) Northern blot analysis of mouse mast cells and eosinophils. The blot was probed with the mouse H_4 receptor. (C) Northern blots analysis of mouse mast cells (BMMC) expressing H_3 and H_4 receptor. In the left blot mast cells, human (h) H_3 receptor transfected and untransfected SK-N-MC were blotted with a mouse H_3 receptor probe. In the right blot, mast cells, mouse H_4 receptor transfected and untransfected SK-N-MC were blotted with a mouse H_4 receptor probe. The difference in size in transfected and endogenous receptor is due to the presence of the untranslated regions in endogenous H_4 receptor RNA, which are deleted in the transfected receptor. (D) RT-PCR detection of human H_4 receptor expression in cDNA from human basophils, cord blood-derived mast cells and HMC-1

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cells. SK-N-MC cells transfected with human H_1, H_2, H_3 and H_4 receptors were used as controls. The expected size of the human H_4 receptor PCR product is 350 bp.

Figure 3 Histamine induces cyclic AMP in mast cells through H_2 receptors, but not H_4 receptors.

Mast cells of H_3R-/- mice were activated with vehicle (black bars), 10 μM ranitidine (striped bars) or 10 μM thioperamide (white bars) followed by 0.6, 2.5 or 10 μM histamine. Shown are average values +/- standard deviations of triplicate determinations. The experiment was repeated three times on different batches of cells.

Figure 4 Histamine induces calcium mobilization in mast cells through H_4 receptors.

(A) Histamine induces calcium mobilization in mast cells in a concentration dependent fashion. A typical tracing (insert) of control treatment (light gray tracing) and 20 μM histamine (black tracing) and concentration response curve are shown. The decrease in fluorescent signal upon addition of histamine was due to a disturbance of the signal by the pipetting system of the machine. When non-adherent cells such as mast cells are used, addition of reagents may slightly stir up cells and therefore decrease the flourescent signal transiently. (B) Mast cells from H_3R-/- mice were treated with vehicle (black bars), or various concentrations of diphenhydramine (H_1 receptor antagonist, vertical striped bars), ranitidine (H_2 receptor antagonist, horizontal striped bars) or thioperamide (H_3/ H_4 antagonist, white bars) followed with 10 μM histamine. (C) A concentration response curve of histamine-induced calcium mobilization using mast cells from wild-type (diamonds), H_4R+/- (squares) and H_4R-/- mice (triangles). Calcium mobilization was

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determined using fluorometric imaging plate reader, and fluorescence intensity was calculated as the maximum minus the minimum fluorescence over a 2 min period. Shown are average values +/- standard deviations of triplicate determinations. These graphs are representative of at least three similar experiments.

Figure 5 Histamine-induced calcium mobilization through the H_4 receptor is derived from intra-cellular stores and is mediated through PLC and PTX sensitive pathways.
(A/B) Mast cells were treated at t= 10 sec with 10 μM thapsigargin, 3 mM EGTA or PBS, followed by 10 μM histamine at t= 370 sec (arrow). (A) Typical tracing (light gray = thapsigargin, dark gray = PBS, black = EDTA) (B) Peak values of histamine-induced calcium mobilization after PBS, thapsigargin or EDTA pretreatment. (C) Mast cells were treated for 16 hours with PBS (black bars) or 50 ng/ml pertussis toxin (white bars), followed by a concentration response curve of histamine. (D) Mast cells were treated for 10 min with U73122 (white bars) or U73433 (black bars) prior to stimulation with 10 μM histamine. Calcium mobilization was determined using fluorometric imaging plate reader, and fluorescence intensity was calculated as the maximum minus the minimum fluorescence over a 2 min period. Shown are average values +/- standard deviations of triplicate determinations. These graphs are representative of at least three similar experiments.

Figure 6 Histamine does not induce mast cell degranulation or alter antigen-induced degranulation.

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(A) Mast cells incubated overnight with 5 μg/ml IgE and were treated with vehicle (white bars) or 5 μM histamine (black bars) followed 3 minutes later with different concentrations of the antigen DNP-HSA. Calcium mobilization was determined using a fluorometric imaging plate reader. (B) Mast cells were incubated overnight with 5 μg/ml IgE. Mast cells from H_4R-/- (diamonds) and wild-type (circles) mice were incubated for 15 min with vehicle (black) or 10 μM histamine (white) followed by 30 min incubation with DNP-HSA. In the supernatants levels of beta-hexosaminidase were determined.

Figure 7 Histamine induces chemotaxis of mast cells through H_4 receptors.
(A) Chemotaxis of wild-type (black diamonds) and H_4R-/- (white squares) mast cells to different concentrations of histamine. As a negative control wild-type mast cells were added to the upper chamber while histamine was added to both chambers (black triangles). (B) In the lower chamber 10 μM histamine was added, while in both chambers histamine receptor antagonists were added and mast cells were added in the upper chamber. (C) In the lower chamber 10 μM histamine was added, while in both chambers thioperamide were added and mast cells were added in the upper chamber. (D) Mast cells were treated for 16 hours with pertussis toxin. In the lower chamber 10 μM histamine was added, and mast cells were added to the upper chamber. (E) In the lower chamber 10 μM histamine was added, while in both chambers 10 μM U73122 (black bars) or 10 μM U73433 (white bars) were added and mast cells were added to the upper chamber. Each experiment was performed at least in triplicate.

Figure 8 Signaling pathway of H_4 receptor on mast cells.

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Histamine binds to the receptor (1), which will activate Gαi/o proteins (2). Activation of G proteins will activate PLC (3), which hydrolyses IP_3. IP_3 possible activates IP_3 receptors on the endoplasmic reticulum (ER, 4), causing the release of intra-cellular calcium (5). Via unknown pathways, this will lead to chemotaxis (6).

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Table1: Mast cell production of prostaglandin and LTB_4.

	T (min)	Maximum	Untreated	Histamine	Histamine+ thioperamide	Compound 48/80
Prostaglandin	30	810 ± 16	28 ± 3	33 ± 0.3	34 ± 3	51 ± 6
	210	789 ± 98	50 ± 13	44 ± 5	37 ± 0.3	73 ± 5
LTB_4	30	56 ± 2	5 ± 0.7	6 ± 1.6	5 ± 1.5	53 ± 14
	210	58 ± 4	10 ± 3	8 ± 2	7 ± 0.5	107 ± 37

Mast cells were incubated with anti-DNP IgE for 16 h followed by 15 min incubation with 10 μM thioperamide, 30 or 210 min incubation with 10 μM histamine or 5 μg/ml compound 48/80. Maximal was measured by lysing the cells with 1% Triton X-100. In the supernatants prostaglandin and LTB4 levels were measured.

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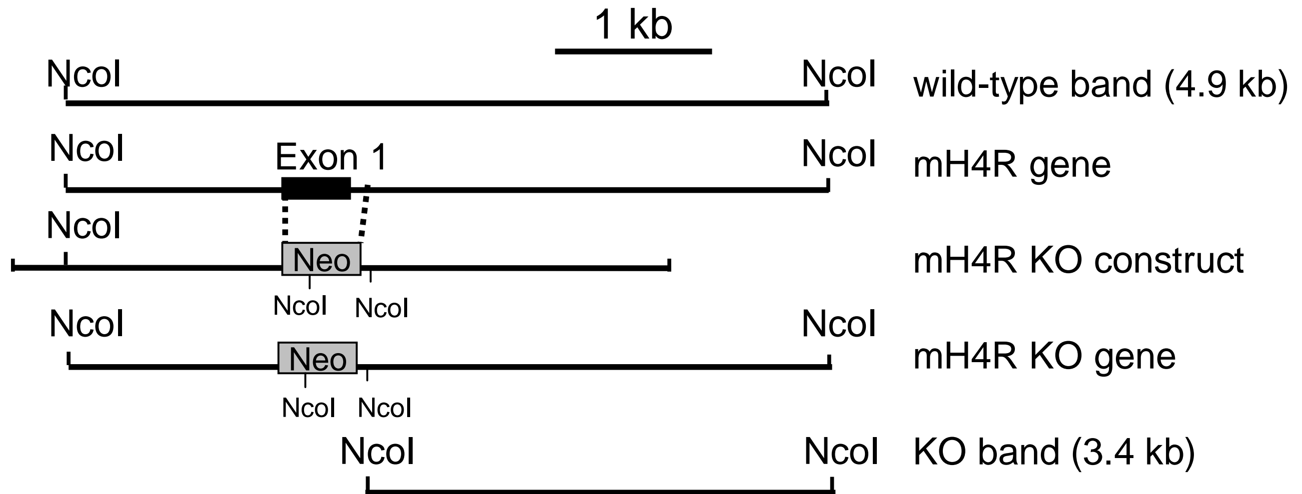
Figure 1

A



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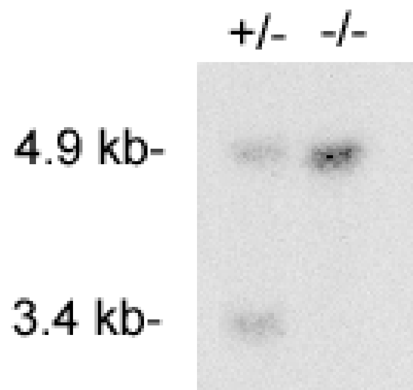

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Figure 2

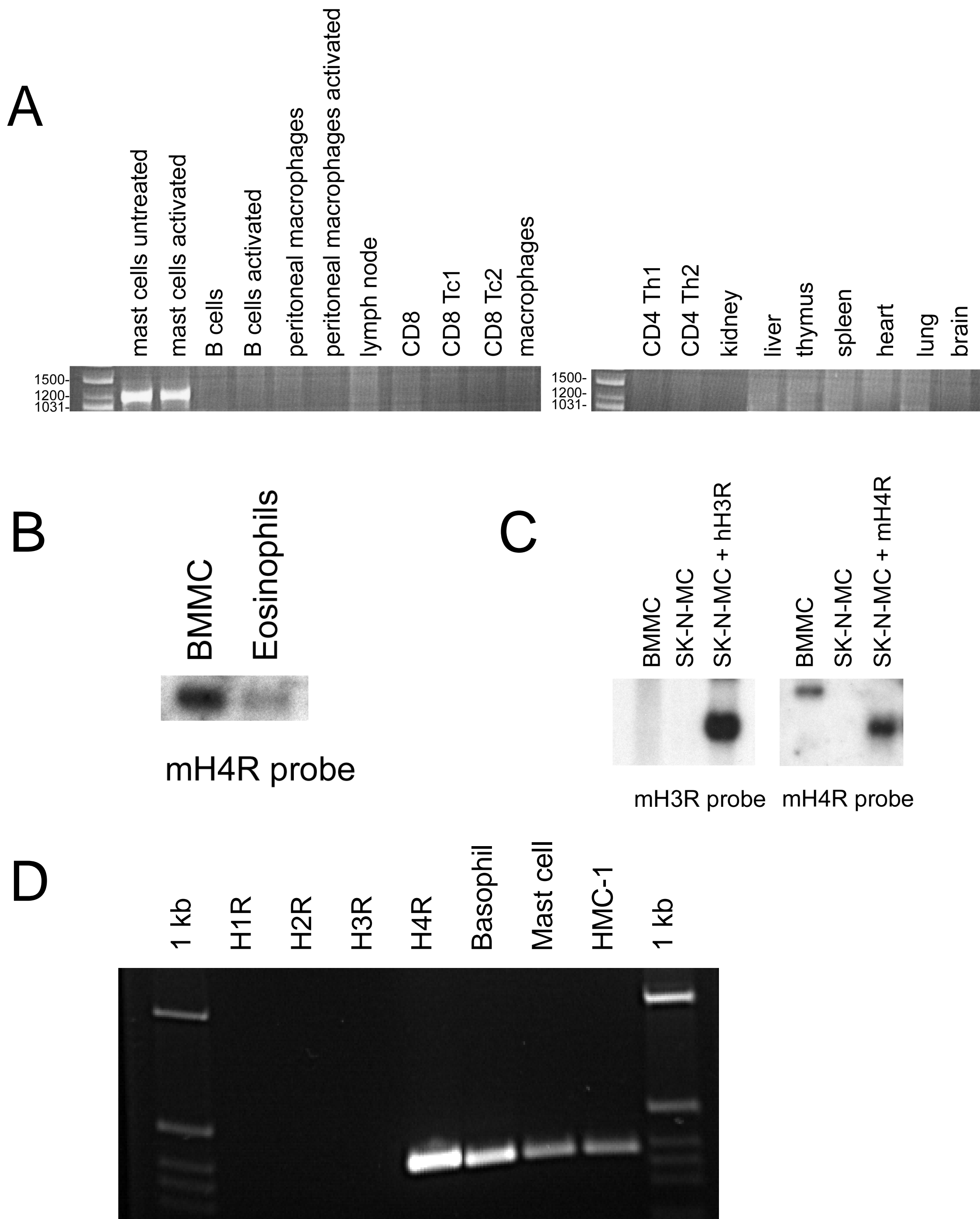

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Figure 3

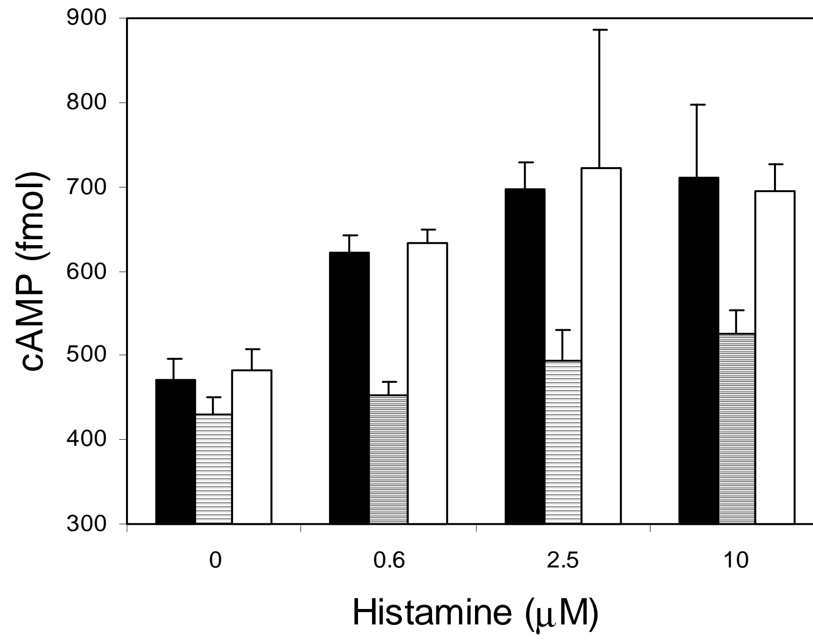

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Figure 4

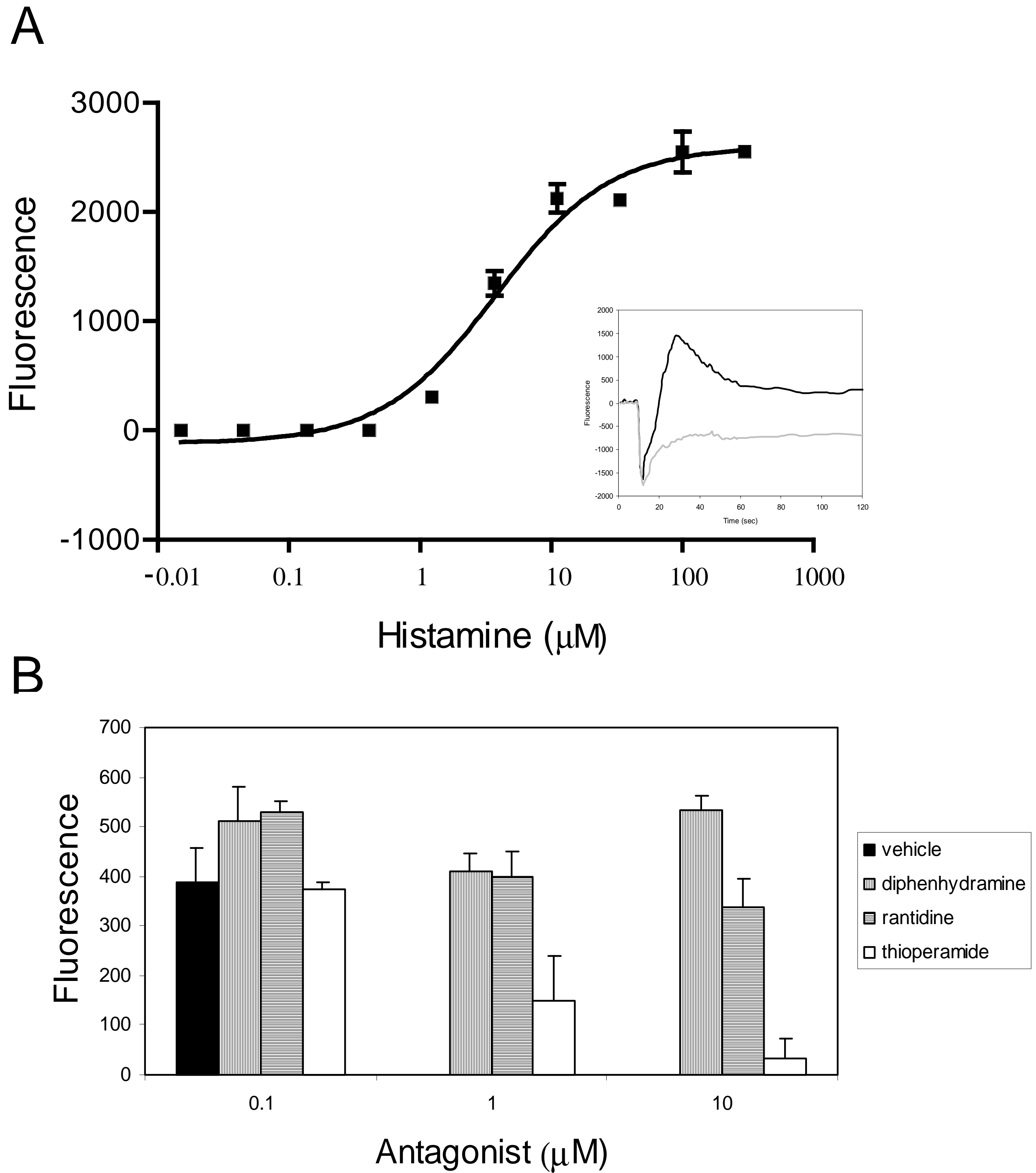

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Figure 4

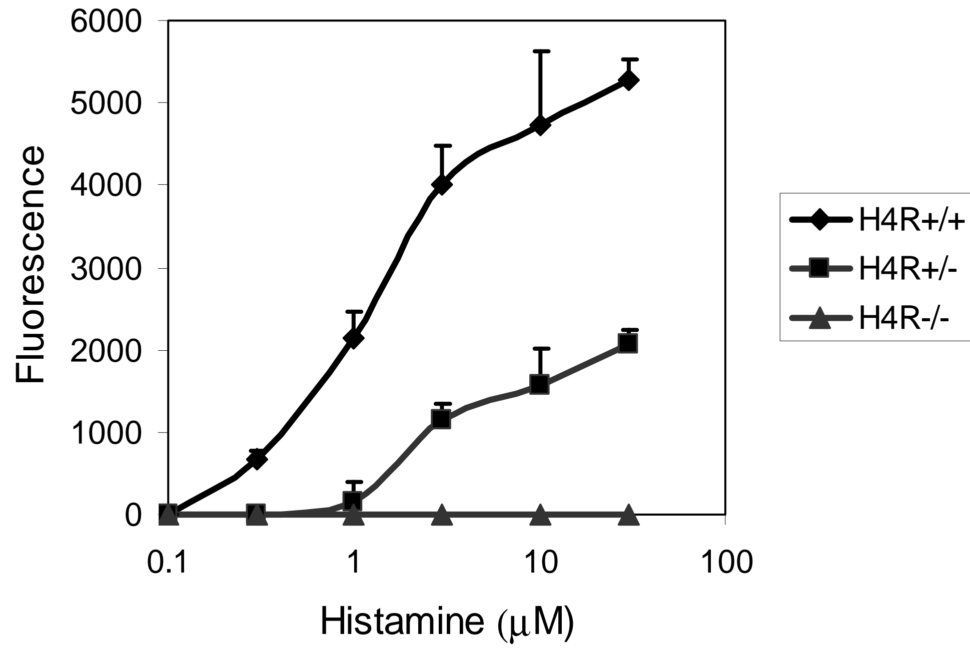

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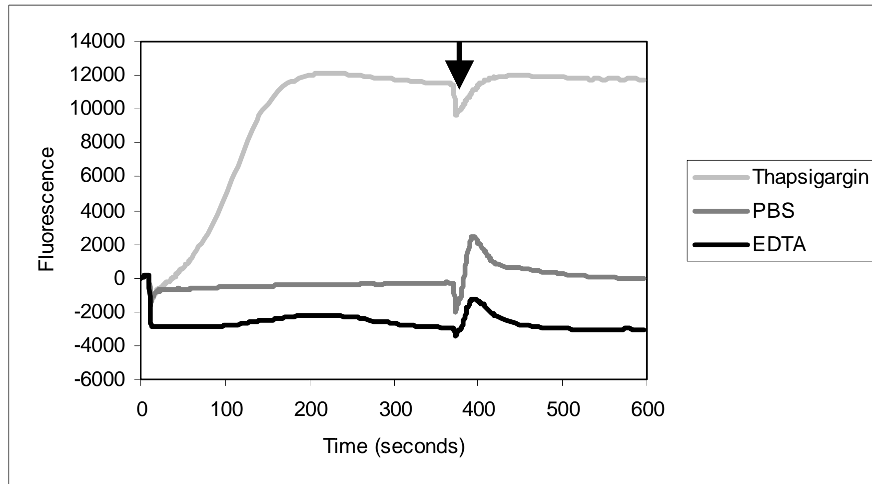
Figure 5

A



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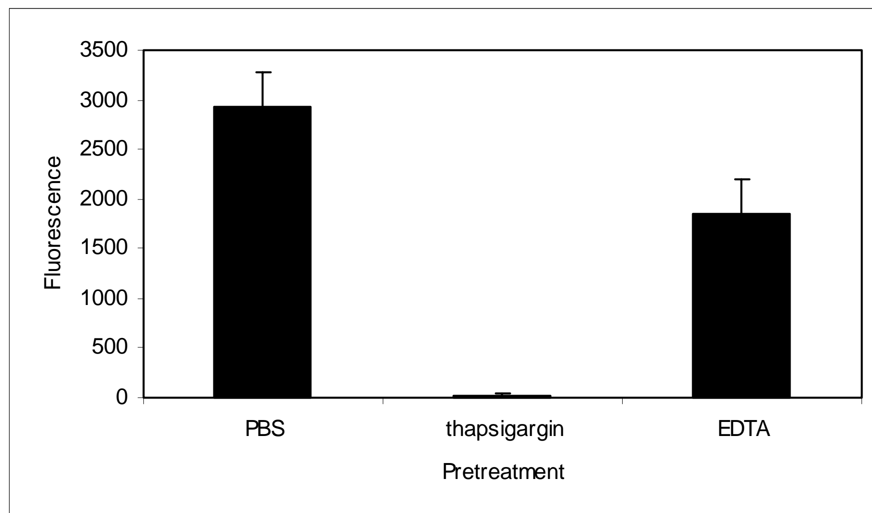

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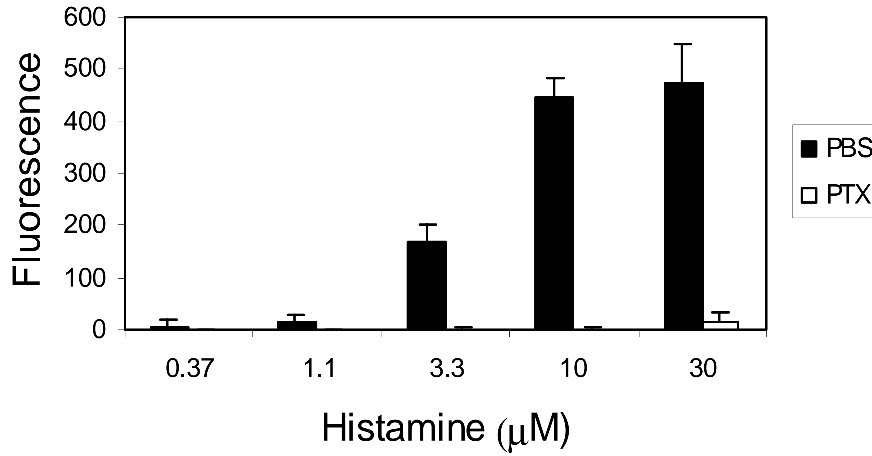
Figure 5

C



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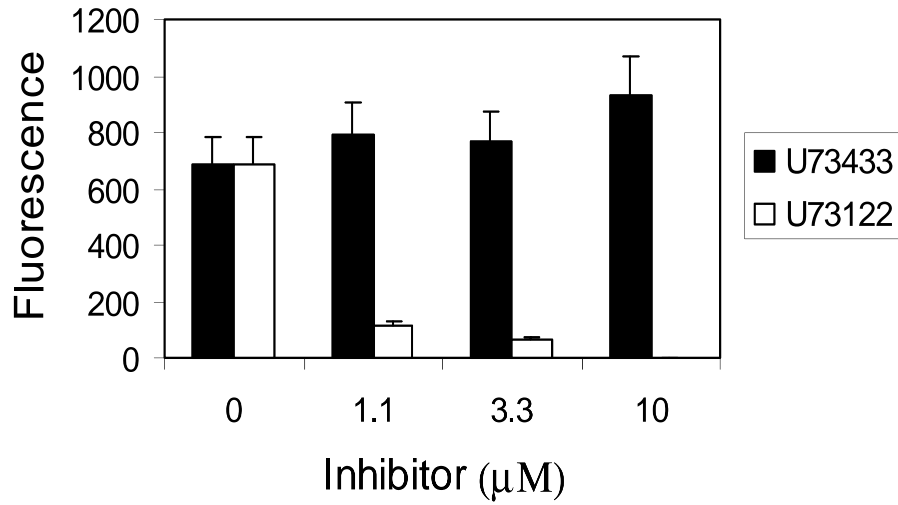

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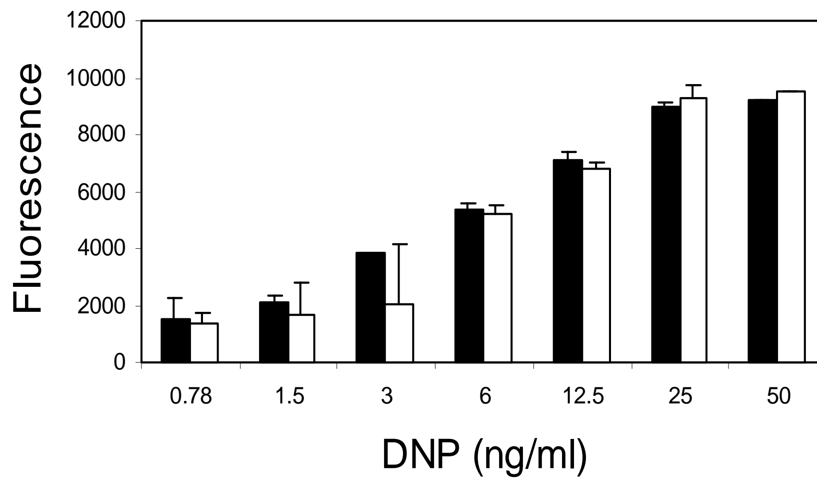
Figure 6

A



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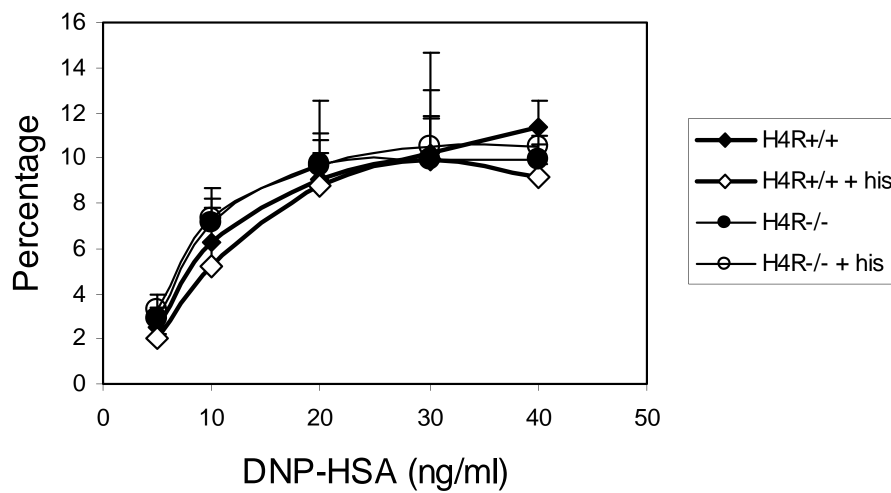

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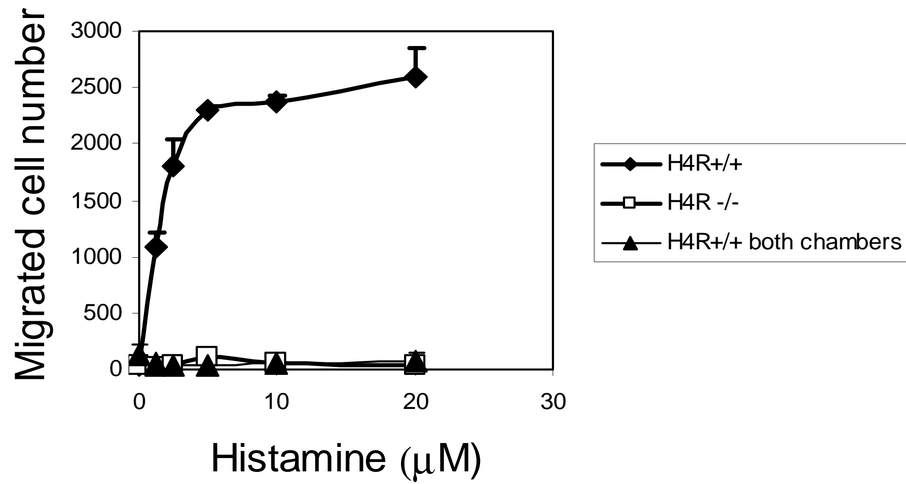
Figure 7

A



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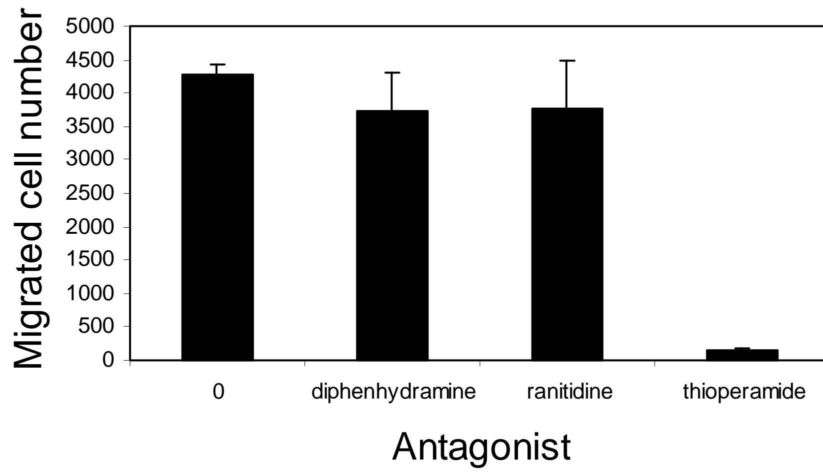

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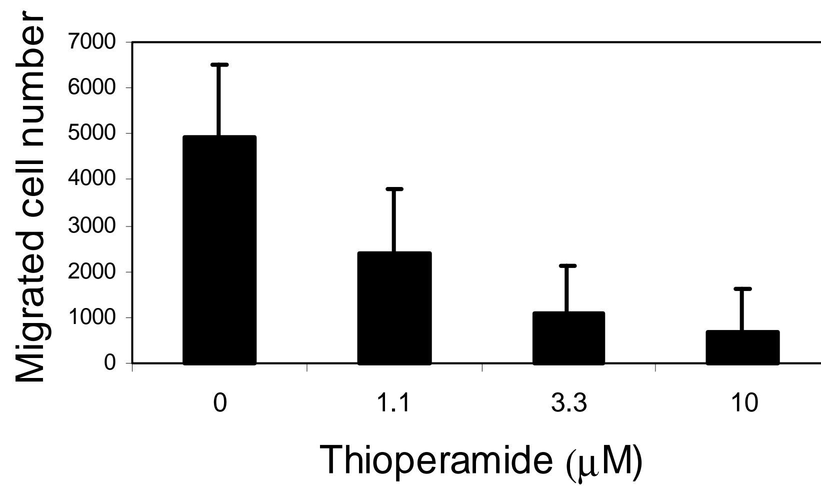
Figure 7

C



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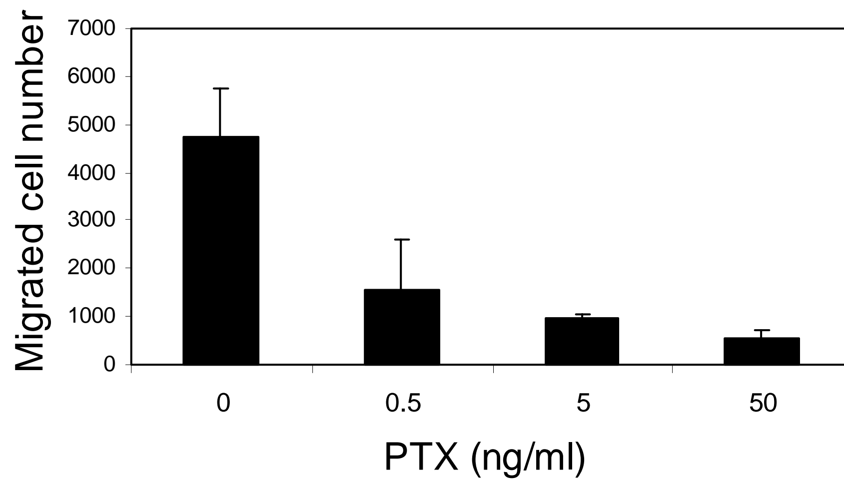

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Figure 7

E

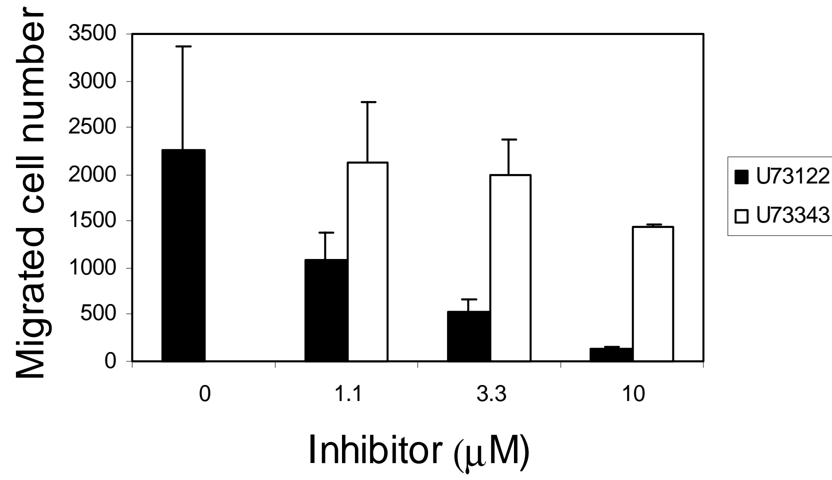

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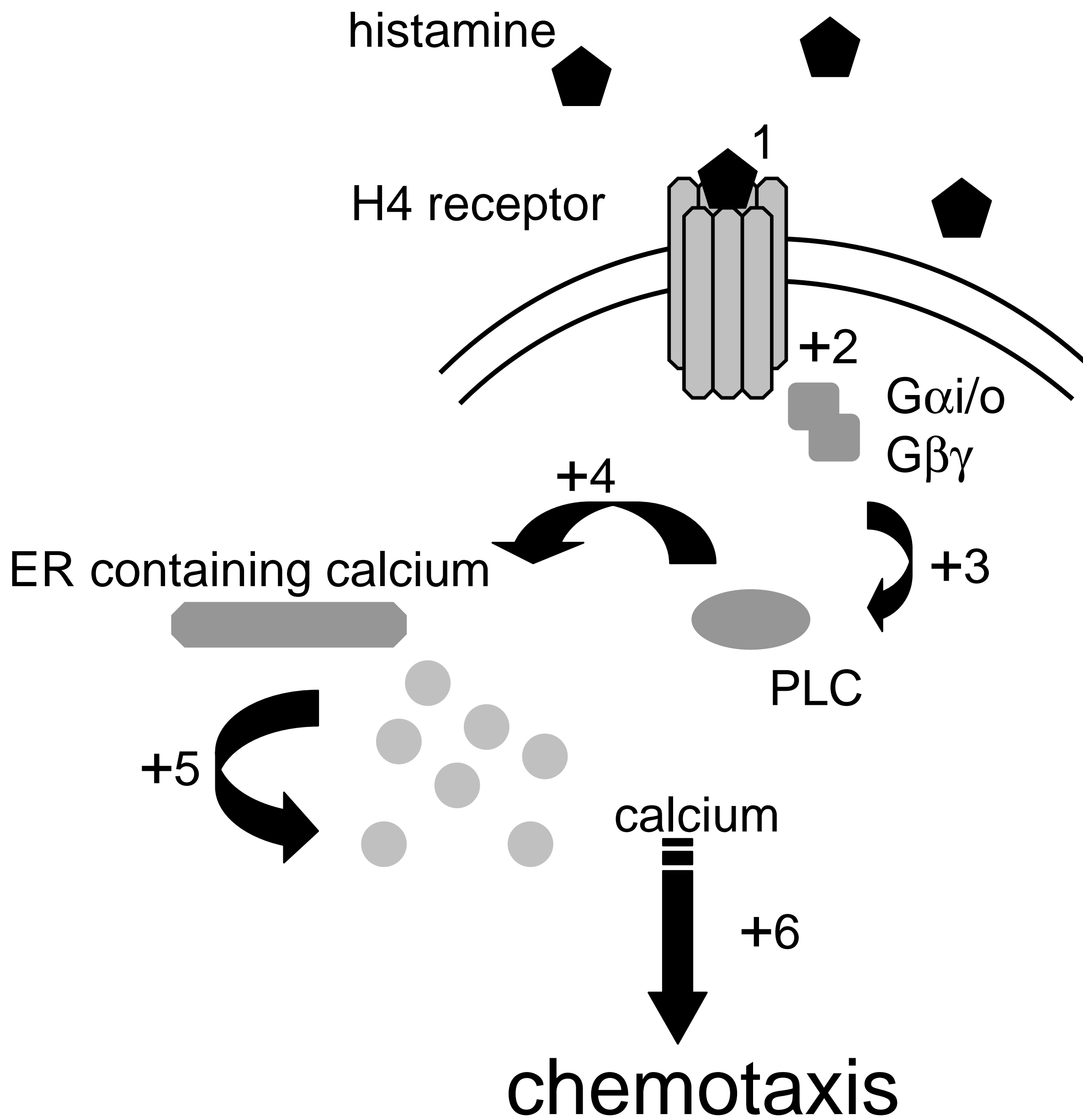
Figure 8



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