

JPET/2002/46581

Histamine H<sub>4</sub> receptor mediates chemotaxis and calcium mobilization of mast cells

Claudia L. Hofstra<sup>1</sup>, 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

JPET/2002/46581

Running title: H<sub>4</sub> 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](mailto:wleung@prdus.jnj.com)

Number of text pages:	37
Number of tables:	1
Number of figures:	8
Number of references:	36
Number of words in abstract:	189
Number of words in introduction:	765
Number of words in discussion:	1000

Abbreviations:

H<sub>3</sub> R<sup>-/-</sup> H<sub>3</sub> receptor gene knock-out

H<sub>4</sub> R<sup>-/-</sup> H<sub>4</sub> receptor gene knock-out

PTX pertussis toxin

Section assignment: Inflammation and Immunopharmacology

JPET/2002/46581

## **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<sub>3</sub> and H<sub>4</sub> receptor antagonist thioperamide (but not H<sub>1</sub> or H<sub>2</sub> receptor antagonists) inhibited histamine-induced chemotaxis of mast cells. The chemotactic response is mediated by H<sub>4</sub> receptors, since chemotaxis toward histamine was absent in mast cells derived from H<sub>4</sub> receptor-deficient mice, but was detected from H<sub>3</sub> receptor-deficient mice. In addition, Northern blot analysis showed the expression of H<sub>4</sub> but not H<sub>3</sub> receptors on mast cells. Activation of H<sub>4</sub> receptors by histamine resulted in calcium mobilization derived from intracellular stores. Calcium mobilization and chemotaxis involve G $\alpha$ i/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<sub>4</sub> receptor mediates signaling leading to mast cell chemotaxis. This mechanism might be responsible for mast cell accumulation in allergic tissues.

JPET/2002/46581

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<sub>1</sub>, H<sub>2</sub> and H<sub>3</sub> receptor). Each receptor has its own expression pattern and mediates distinct effects: H<sub>1</sub> receptors trigger smooth muscle contractions and is generally thought to play an important role in allergy; H<sub>2</sub> receptors regulate gastric acid secretion in the stomach and H<sub>3</sub> 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<sub>4</sub> 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<sub>4</sub> receptor in mouse, rat and guinea pig were cloned (Liu et al., 2001b).

The amino acid sequence of the H<sub>4</sub> receptor has low homology with other histamine receptors. Its closest member in the histamine receptor family is the H<sub>3</sub> receptor that shares only a 35% amino acid homology with the H<sub>4</sub> receptor, although the homology in the transmembrane region is 58%. However, the H<sub>4</sub> receptor expression pattern is distinct from the H<sub>3</sub> receptor. While the expression of the H<sub>3</sub> receptor is mainly restricted to cells in the central nervous system (Lovenberg et al., 2000), the H<sub>4</sub> receptor

JPET/2002/46581

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<sub>4</sub> receptors on haemopoietic cells is not unique among the histamine receptor family, since T cells and dendritic cells also express H<sub>1</sub> and H<sub>2</sub> receptors (Jutel et al., 2001; Szeberenyi et al., 2001).

Pharmacological properties of the H<sub>4</sub> receptor have been revealed using H<sub>4</sub> 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<sub>1</sub> and H<sub>2</sub> receptor antagonists and agonists do not bind to the H<sub>4</sub> receptor. However, more typical H<sub>3</sub> receptor ligands (like thioperamide, clobenpropit, imetit and R- $\alpha$ -methylhistamine) could bind the H<sub>4</sub> receptor with affinities different from that of the H<sub>3</sub> 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<sub>1</sub> receptors mediate this action through G $\alpha_q$  proteins resulting in calcium mobilization, H<sub>2</sub> receptors signal through G $\alpha_s$  proteins and cAMP increase, while H<sub>3</sub> receptors signal through G $\alpha_i/o$  proteins and inhibition of cAMP (Lovenberg et al., 1999). In literature two signaling pathways are thought to be utilized by the H<sub>4</sub> receptor. Firstly, using cells transfected with the H<sub>4</sub> 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<sub>3</sub> receptor. Secondly, one study showed that histamine could not alter cAMP levels in H<sub>4</sub> receptor transfected cells, but instead increased calcium mobilization if the cells were

JPET/2002/46581

cotransfected with  $G\alpha q/i1/2$ ,  $G\alpha q/i3$ , or  $G\alpha 16$  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.

JPET/2002/46581

## Materials

Human HMC-1 and WEHI-3 cells were purchased from American Type Culture Collection (Rockville, MD). Human CD34<sup>+</sup> 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<sub>4</sub> 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).

## Methods

### Generation of H<sub>4</sub> receptor gene knockout mice (H<sub>4</sub>R<sup>-/-</sup>).

H<sub>4</sub>R<sup>-/-</sup> mice were generated by Lexicon Genetics, Woodlands, TX. A 9 kb mouse genomic fragment containing the mouse H<sub>4</sub> 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

JPET/2002/46581

covering most of exon 1 and part of intron 1 of the H<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> receptor gene were identified by PCR. Wild-type and H<sub>4</sub> R<sup>-/-</sup> mice were obtained from cross-breeding of heterozygous mice.

### **Detection of mouse H<sub>4</sub> receptor expression**

RNA from tissues and purified cells was prepared using a RNeasy kit according to the manufacturers instructions. H<sub>4</sub> receptor RNA was detected by RT-PCR using specific mouse H<sub>4</sub> 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 94°C 45 sec, 55°C 45 sec, and 72°C 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<sub>4</sub> 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).



JPET/2002/46581

Eosinophils were in vitro differentiated from C57BL/6J mouse bone marrows. Bone marrow was aseptically isolated from the femurs. The cells ( $2 \times 10^5$ /ml) were cultured at 37°C with 5% CO<sub>2</sub> 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<sub>4</sub> receptor cDNA clone (Liu et al., 2001a) and the purified RT-PCR product of the mouse H<sub>3</sub> 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.

### **Detection of human H<sub>4</sub> receptor expression**

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

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

JPET/2002/46581

(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<sub>4</sub> 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<sub>4</sub> receptor gene knock-out (H<sub>4</sub>R<sup>-/-</sup>), H<sub>3</sub> receptor gene knockout (H<sub>3</sub>R<sup>-/-</sup>) 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<sup>5</sup>/ml) were cultured at 37°C with 5% CO<sub>2</sub> 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.

JPET/2002/46581

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<sub>3</sub>R<sup>-/-</sup>, H<sub>4</sub>R<sup>-/-</sup> and wild-type mice.

### **Degranulation assay**

Mast cells ( $5 \times 10^5$ /ml) were sensitized overnight with 2  $\mu$ g/ml anti-DNP IgE. Mast cells ( $2 \times 10^5$ /well) were plated out in a 96-wells plate and incubated for 15 min with 10  $\mu$ M 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, 5 min, 5°C), and supernatants were analyzed for beta-hexosaminidase.

Beta-hexosaminidase was measured by adding 25  $\mu$ l supernatant to 50  $\mu$ l 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  $\mu$ l of 0.4 M glycine (pH 9). The plates were measured at wavelength 405 nm. The percentage of degranulation was calculated as

JPET/2002/46581

$((A-B)/(T-B) \times 100)$ , 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 ( $1 \times 10^6$ /well) were incubated for 15 min with 10  $\mu$ M thioperamide or vehicle, followed by 30 or 210 min incubation with 10  $\mu$ M histamine or 5  $\mu$ g/ml compound 48/80. LTB<sub>4</sub> and prostaglandin levels were measured in supernatants according to the manufacturers instruction.

### **Chemotaxis assay**

Transwells with a pore size 8  $\mu$ m were coated with 100  $\mu$ l of 100 ng/ml human fibronectin for 2 h at room temperature. After removal of the fibronectin, 600  $\mu$ l of RPMI with 5% BSA in the presence of histamine (ranging from 1.25-20  $\mu$ M) was added to the bottom chamber. Subsequently, 10  $\mu$ M histamine receptor antagonists (diphenhydramine, ranitidine, thioperamide), U73122 (1.1, 3.3 and 10  $\mu$ M), or U73433 (1.1, 3.3 and 10  $\mu$ M), were added to the top and bottom chambers. Mast cells ( $2 \times 10^5$ /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 ( $1 \times 10^6$  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.

JPET/2002/46581

### **Calcium mobilization**

Mast cells ( $2 \times 10^5$ /well) were loaded with 4  $\mu$ M 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  $\mu$ M thapsigargin or PBS. After stabilization of the signal, mast cells received a second addition of 10  $\mu$ M histamine.

For pertussis toxin (PTX) treatment, mast cells ( $1 \times 10^6$  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  $\mu$ M U73122 or U73433 10 min prior to stimulation with 10  $\mu$ M histamine.

JPET/2002/46581

To detect calcium response triggered by IgE receptor cross-linking, mast cells ( $5 \times 10^5$ /ml) were sensitized overnight with 2  $\mu$ g/ml anti-DNP IgE. Cells were washed and loaded with Fluo-3 as described above. During the calcium measurements, 5  $\mu$ M histamine was added followed 3 min later with different concentrations of DNP-HSA.

### **cAMP measurements**

Mast cells ( $1 \times 10^6$ /well) in culture medium containing 1  $\mu$ M 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  $\mu$ M) was added 15 min prior to histamine and/or 100  $\mu$ M 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.

JPET/2002/46581

## Results

### **Mast cells, basophils and eosinophils express H<sub>4</sub> receptors.**

Previously we reported that the human H<sub>4</sub> receptor is expressed mainly in bone marrow and eosinophils (Liu et al., 2001a). In the present study, mouse H<sub>4</sub> expression was determined in different tissues and purified cell types of the haemopoietic lineage. Abundant expression of mouse H<sub>4</sub> receptor was detected in untreated mast cells and antigen-activated IgE-primed mast cells by RT-PCR (figure 2A). In contrast, H<sub>4</sub> 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<sup>+</sup> effector Th1 and Th2 cells, CD8<sup>+</sup> effector Tc1 and Tc2 cells, resting and LPS-activated B cells, as well as macrophages.

Expression of H<sub>4</sub> receptor in mast cells and eosinophils was further confirmed by Northern blot analysis (figure 2B and 2C). Both H<sub>1</sub> and H<sub>2</sub> receptors were detected on mast cells (results not shown), whereas H<sub>3</sub> 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<sub>4</sub> 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 H<sub>4</sub> receptor was expressed in basophils (figure 2D) but not in neutrophils (data not shown). In summary our data shows that the H<sub>4</sub> receptor is expressed on mast cells, basophils and eosinophils.

JPET/2002/46581

### **H<sub>4</sub> 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<sub>3</sub>/ H<sub>4</sub> antagonist thioperamide and H<sub>1</sub> antagonist diphenhydramine (results not shown), thereby excluding a role for H<sub>1</sub>, H<sub>3</sub> or H<sub>4</sub> receptors. However, the H<sub>2</sub> receptor antagonist ranitidine could inhibit the histamine-induced cAMP increase. The results indicate that the histamine-induced cAMP increase in mast cells is H<sub>2</sub> 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 ED<sub>50</sub> of histamine-induced calcium mobilization was 3.8 μM. Neither H<sub>1</sub> receptor antagonists nor H<sub>2</sub> 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<sub>50</sub> of 1.00 ± 0.5 μM. This IC<sub>50</sub> value is consistent with the relative binding affinities of histamine and thioperamide (Liu et al., 2001a). Taken together, the data suggest that H<sub>3</sub> and/or H<sub>4</sub> receptors are involved in calcium mobilization in mast cells.

Since mast cells do not express H<sub>3</sub> receptors (see figure 2C), it is likely that the calcium response is mediated by H<sub>4</sub> receptors. A direct proof of H<sub>4</sub> receptor mediated calcium mobilization was demonstrated in mast cells generated from H<sub>4</sub>R<sup>-/-</sup> and H<sub>3</sub>R<sup>-/-</sup> mice. In contrast to wild-type mast cells, up to 30 μM histamine stimulation in H<sub>4</sub>R<sup>-/-</sup>



JPET/2002/46581

mast cells did not result in calcium mobilization (figure 4C). Mast cells from H<sub>4</sub>R<sup>+/-</sup> mice showed an intermediate calcium response as compared to mast cells from wild-type mice. This response is histamine-specific since H<sub>4</sub>R<sup>-/-</sup> mast cells mediated normal calcium responses to ATP or ionomycin (results not shown). Furthermore, H<sub>3</sub>R<sup>-/-</sup> 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<sub>4</sub> receptor.

#### **H<sub>4</sub> 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<sub>4</sub> receptor mediates calcium mobilization through G $\alpha$ i/o proteins and PLC.**

To determine the G-proteins utilized by H<sub>4</sub> 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<sub>4</sub> receptor leading to calcium mobilization.

JPET/2002/46581

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  $\mu$ M, whereas the inactive analog U73433 (up to 10  $\mu$ M) 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<sub>4</sub> 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<sub>4</sub>R<sup>-/-</sup> 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<sub>4</sub> and prostaglandin release by mast cells, whereas histamine did not alter LTB<sub>4</sub> or prostaglandin levels (table 1). In summary, H<sub>4</sub> 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<sub>4</sub> and prostaglandins by mast cells.

JPET/2002/46581

### **Histamine mediates chemotaxis through H<sub>4</sub> 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<sub>50</sub> for thioperamide is similar compared to that in the calcium mobilization assay. To distinguish between H<sub>3</sub> and H<sub>4</sub> receptor-mediated effects on chemotaxis of mast cells, the chemotaxis assay was performed using mast cells derived from H<sub>4</sub>R<sup>-/-</sup> or H<sub>3</sub>R<sup>-/-</sup> mice. No migration of H<sub>4</sub>R<sup>-/-</sup> mast cells toward histamine was observed (figure 7A). In contrast, H<sub>3</sub>R<sup>-/-</sup> 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<sub>4</sub> receptor.

### **H<sub>4</sub> receptor mediated chemotaxis involves G $\alpha$ i/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

JPET/2002/46581

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

JPET/2002/46581

## Discussion

In the present study we showed that the mouse H<sub>4</sub> receptor is expressed on mast cells, and eosinophils, but not on other haemopoietic cells including T cells, B cells or macrophages. Furthermore, human H<sub>4</sub> 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<sub>4</sub> receptor, but not the H<sub>3</sub> receptor. In literature it has been unclear whether mast cells express the H<sub>3</sub> receptor. Most studies used thioperamide as a specific H<sub>3</sub> antagonist (Kohno et al., 1994; Bissonnette, 1996), but recent data indicate that both the mouse and human H<sub>4</sub> 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<sub>4</sub> receptors.

The role of the H<sub>4</sub> 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<sub>4</sub>R<sup>-/-</sup> mast cells did not show any defects in degranulation, proliferation or survival, indicating that the H<sub>4</sub> 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

JPET/2002/46581

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<sub>4</sub> receptor. Taken together, antagonists of the H<sub>4</sub> 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<sub>4</sub>R<sup>-/-</sup> and H<sub>3</sub>R<sup>-/-</sup> mice, we demonstrated that histamine-induced calcium mobilization from intra-cellular stores in mast cells via the H<sub>4</sub> receptor. Calcium mobilization via the H<sub>4</sub> receptor has also been observed using cells cotransfected with both the H<sub>4</sub> receptor and chimeric G-proteins (Morse et al., 2001). However, other studies using human H<sub>4</sub> 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<sub>4</sub> receptor was studied. Previously, similar calcium mobilization induced by histamine was demonstrated in human eosinophils, which have

JPET/2002/46581

been shown to express the H<sub>4</sub> receptor (Raible et al., 1994). The calcium mobilization was inhibited by thioperamide, the dual H<sub>3</sub>/H<sub>4</sub> receptor antagonist. However, R- $\alpha$ -methyl-histamine and N- $\alpha$ -methyl-histamine were less potent than histamine in inducing calcium mobilization, which is more consistent with their respective affinities for the H<sub>4</sub> receptor than for the H<sub>3</sub> receptor (Raible et al., 1994). Therefore, it is likely that this response is mediated by the H<sub>4</sub> receptor and not the H<sub>3</sub> receptor. Thus, the activation of H<sub>4</sub> receptor both on mast cells and eosinophils results in calcium mobilization.

The signaling pathways involved in H<sub>4</sub> receptor activation by histamine have also been studied. Both G $\alpha$ i/o proteins and PLC are involved since PTX, which inactivates G $\alpha$ i/o proteins, and the PLC inhibitor U73122, inhibited chemotaxis and calcium mobilization. Since G $\alpha$ i/o proteins does not activate PLC $\beta$ , but G-protein  $\beta\gamma$  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  $\beta\gamma$  subunits that are released from the coupling with G $\alpha$ i/o proteins when histamine binds to the H<sub>4</sub> receptor. Other G-protein coupled receptors have also been shown to signal via PLC and G $\alpha$ i/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<sub>3</sub>). IP<sub>3</sub> can activate an IP<sub>3</sub> 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 G $\alpha$ i/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 G $\alpha$ i/o proteins, PI-3 kinase, p38 MAP and MEK kinases (Dastyk et al., 1998; Sundstrom et al., 2001).

JPET/2002/46581

We propose the following signaling pathway involved in histamine activation of the H<sub>4</sub> receptor (figure 8). Histamine binds to the H<sub>4</sub> receptor on mast cells and eosinophils and causes the activation of PTX-sensitive G $\alpha$ i/o proteins. Possibly G-protein  $\beta\gamma$  subunits dissociated from G $\alpha$ i/o proteins trigger the activation of PLC. PLC hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP<sub>2</sub>) to diacylglycerol and IP<sub>3</sub>. IP<sub>3</sub> activates a calcium channel in the endoplasmic reticulum, possibly through an IP<sub>3</sub> receptor to release calcium. The increased calcium levels trigger currently unknown signaling pathways, which will cause mast cell chemotaxis toward histamine.



JPET/2002/46581

### **Acknowledgements**

We thank Karen Ngo for her technical help in genotyping H<sub>4</sub> receptor deficient mice and in culturing bone marrow derived mast cells. We also thank Dr. Tim Lovenberg for providing us the human SK-N-MC cells transfected with different histamine receptors.

JPET/2002/46581

## References

- Amin K, Ludviksdottir D, Janson C, Nettelbladt O, Bjornsson E, Roomans GM, Boman G, Seveus L and Venge P (2000) Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. BHR Group. *Am J Respir Crit Care Med* **162**:2295-2301.
- Bissonnette EY (1996) Histamine inhibits tumor necrosis factor alpha release by mast cells through H2 and H3 receptors. *Am J Respir Cell Mol Biol* **14**:620-626.
- Crimi E, Chiaramondia M, Milanese M, Rossi GA and Brusasco V (1991) Increased numbers of mast cells in bronchial mucosa after the late- phase asthmatic response to allergen. *Am Rev Respir Dis* **144**:1282-1286.
- Clapham DE and Neer EJ (1997) G protein  $\beta\gamma$  subunits. *Annu Rev Pharmacol Toxicol* **37**:167-203.
- Dahl C, Saito H, Nielsen HV and Schiotz PO (2002) The establishment of a combined serum-free and serum-supplemented culture method of obtaining functional cord blood-derived human mast cells. *J Immunol Methods* **262**:137-143.
- Dastyh J, Taub D, Hardison MC and Metcalfe DD (1998) Tyrosine kinase-deficient Wv c-kit induces mast cell adhesion and chemotaxis. *Am J Physiol* **275**:C1291-1299.
- Exton JH (1996) Regulation of phosphoinositide phospholipases by hormones, neurotransmitters, and other agonists linked to G proteins. *Annu Rev Pharmacol Toxicol* **36**:481-509.
- Fokkens WJ, Godthelp T, Holm AF, Blom H, Mulder PG, Vroom TM and Rijntjes E (1992) Dynamics of mast cells in the nasal mucosa of patients with allergic rhinitis and non-allergic controls: a biopsy study. *Clin Exp Allergy* **22**:701-710.

JPET/2002/46581

Gauvreau GM, Lee JM, Watson RM, Irani AM, Schwartz LB and O'Byrne PM (2000)

Increased numbers of both airway basophils and mast cells in sputum after allergen inhalation challenge of atopic asthmatics. *Am J Respir Crit Care Med* **161**:1473-1478.

Hill SJ, Ganellin CR, Timmerman H, Schwartz JC, Shankley NP, Young JM, Schunack W, Levi R and Haas HL (1997) International Union of Pharmacology. XIII.

Classification of histamine receptors. *Pharmacol Rev* **49**:253-278.

Jutel M, Watanabe T, Klunker S, Akdis M, Thomet OA, Malolepszy J, Zak-Nejmark T,

Koga R, Kobayashi T, Blaser K and Akdis CA (2001) Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors. *Nature* **413**:420-425.

Kassel O, de Blay F, Duvernelle C, Olgart C, Israel-Biet D, Krieger P, Moreau L, Muller C, Pauli G and Frossard N (2001) Local increase in the number of mast cells and expression of nerve growth factor in the bronchus of asthmatic patients after repeated inhalation of allergen at low-dose. *Clin Exp Allergy* **31**:1432-1440.

Kirby JG, Hargreave FE, Gleich GJ and O'Byrne PM (1987) Bronchoalveolar cell profiles of asthmatic and nonasthmatic subjects. *Am Rev Respir Dis* **136**:379-383.

Kohno S, Nakao S, Ogawa K, Yamamura H, Nabe T and Ohata K (1994) Possible participation of histamine H3-receptors in the regulation of anaphylactic histamine release from isolated rat peritoneal mast cells. *Jpn J Pharmacol* **66**:173-180.

Leurs R, Smit MJ and Timmerman H (1995) Molecular pharmacological aspects of histamine receptors. *Pharmacol Ther* **66**:413-463.

JPET/2002/46581

- Liu C, Ma X, Jiang X, Wilson SJ, Hofstra CL, Blevitt J, Pyati J, Li X, Chai W, Carruthers N and Lovenberg TW (2001a) Cloning and pharmacological characterization of a fourth histamine receptor (H(4)) expressed in bone marrow. *Mol Pharmacol* **59**:420-426.
- Liu C, Wilson SJ, Kuei C and Lovenberg TW (2001b) Comparison of human, mouse, rat, and guinea pig histamine H4 receptors reveals substantial pharmacological species variation. *J Pharmacol Exp Ther* **299**:121-130.
- Lovenberg TW, Pyati J, Chang H, Wilson SJ and Erlander MG (2000) Cloning of rat histamine H(3) receptor reveals distinct species pharmacological profiles. *J Pharmacol Exp Ther* **293**:771-778.
- Lovenberg TW, Roland BL, Wilson SJ, Jiang X, Pyati J, Huvar A, Jackson MR and Erlander MG (1999) Cloning and functional expression of the human histamine H3 receptor. *Mol Pharmacol* **55**:1101-1107.
- Morse KL, Behan J, Laz TM, West RE, Jr., Greenfeder SA, Anthes JC, Umland S, Wan Y, Hipkin RW, Gonsiorek W, Shin N, Gustafson EL, Qiao X, Wang S, Hedrick JA, Greene J, Bayne M and Monsma FJ, Jr. (2001) Cloning and characterization of a novel human histamine receptor. *J Pharmacol Exp Ther* **296**:1058-1066.
- Nguyen T, Shapiro DA, George SR, Setola V, Lee DK, Cheng R, Rauser L, Lee SP, Lynch KR, Roth BL and O'Dowd BF (2001) Discovery of a novel member of the histamine receptor family. *Mol Pharmacol* **59**:427-433.
- Oda T, Morikawa N, Saito Y, Masuho Y and Matsumoto S (2000) Molecular cloning and characterization of a novel type of histamine receptor preferentially expressed in leukocytes. *J Biol Chem* **275**:36781-36786.

JPET/2002/46581

Pacher P, Csordas P, Schneider T and Hajnoczky G (2000) Quantification of calcium signal transmission from sarco-endoplasmic reticulum to the mitochondria. *J Physiol* **529 Pt 3**:553-564.

Raible DG, Lenahan T, Fayvilevich Y, Kosinski R and Schulman ES (1994) Pharmacologic characterization of a novel histamine receptor on human eosinophils. *Am J Respir Crit Care Med* **149**:1506-1511.

Rhee SG (2001) Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem* **70**:281-312.

Seeback J, Kruse ML, Schmidt-Choudhury A, Schmidt WE (1998) Pituitary adenylate cyclase activating polypeptide induces degranulation of rat peritoneal mast cells via high-affinity PACAP receptor-independent activation of G proteins. *Ann N Y Acad Sci*. **865**:141-6.

Shefler I and Sagi-Eisenberg R (2001) Gi-mediated activation of the Syk kinase by the receptor mimetic basic secretagogues of mast cells: role in mediating arachidonic acid/metabolites release. *J Immunol* **167**:475-481.

Shier P, Hofstra CL, Ma XJ, Wu Y, Ngo K and Fung-Leung WP (2000) Tbt-1, a new T-box transcription factor induced in activated Th1 and CD8+ T cells. *Immunogenetics* **51**:771-778.

Slater A, Smallman LA and Drake-Lee AB (1996) Increase in epithelial mast cell numbers in the nasal mucosa of patients with perennial allergic rhinitis. *J Laryngol Otol* **110**:929-933.

JPET/2002/46581

- Sundstrom M, Alfredsson J, Olsson N and Nilsson G (2001) Stem cell factor-induced migration of mast cells requires p38 mitogen- activated protein kinase activity. *Exp Cell Res* **267**:144-151.
- Szeberenyi JB, Pallinger E, Zsinko M, Pos Z, Rothe G, Orso E, Szeberenyi S, Schmitz G, Falus A and Laszlo V (2001) Inhibition of effects of endogenously synthesized histamine disturbs in vitro human dendritic cell differentiation. *Immunol Lett* **76**:175-182.
- Thompson AK, Mostafapour SP, Denlinger LC, Bleasdale JE and Fisher SK (1991) The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. A role for Gp in receptor compartmentation. *J Biol Chem* **266**:23856-23862.
- Toyota H, Dugovic C, Koehl M, Laposky AD, Weber C, Ngo K, Wu Y, Lee DH, Yanai K, Sakurai E, Watanabe T, Liu C, Chen J, Barbier AJ, Turek FW, Fung-Leung WP and Lovenberg TW (2002) Behavioral Characterization of Mice Lacking Histamine H3 Receptors. *Mol. Pharmacol.* **62**:389-397.
- Yang C-M, Hsiao L-D, Chien C-S, Lin C-C, Luo S-F and Wang C-C (2002) Substance P-induced activation of p42/44 mitogen-activated protein kinase associated with cell proliferation in human tracheal smooth muscle cells. *Cell Signal* **14**: 913-923.
- Zhu Y, Michalovich D, Wu H, Tan KB, Dytko GM, Mannan IJ, Boyce R, Alston J, Tierney LA, Li X, Herrity NC, Vawter L, Sarau HM, Ames RS, Davenport CM, Hieble JP, Wilson S, Bergsma DJ and Fitzgerald LR (2001) Cloning, expression, and pharmacological characterization of a novel human histamine receptor. *Mol Pharmacol* **59**:434-441.

JPET/2002/46581

Zussman A, Hermuet S and Sagi-Eisenberg R (1998) Stimulation of Ca<sup>2+</sup>-dependent exocytosis and arachidonic acid release in cultured mast cells (RBL-2H3) by a GTPase-deficient mutant of G alpha i3. *Eur J Biochem* **258**:144-149.

JPET/2002/46581

### 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](mailto:wleung@prdus.jnj.com)

<sup>1</sup>Current address Claudia L. Hofstra:

N.V. Organon, Oss, the Netherlands. [Claudia.Hofstra@organon.com](mailto:Claudia.Hofstra@organon.com)



JPET/2002/46581

## Figure legends

Figure 1 Generation of H<sub>4</sub>R<sup>-/-</sup> mice.

(A) The mouse H<sub>4</sub> receptor gene was disrupted by replacing exon 1 with a neomycin resistant gene cassette. (B) The disrupted H<sub>4</sub> receptor gene in H<sub>4</sub>R<sup>+/-</sup> and H<sub>4</sub>R<sup>-/-</sup> 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<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> receptor. (C) Northern blots analysis of mouse mast cells (BMMC) expressing H<sub>3</sub> and H<sub>4</sub> receptor. In the left blot mast cells, human (h) H<sub>3</sub> receptor transfected and untransfected SK-N-MC were blotted with a mouse H<sub>3</sub> receptor probe. In the right blot, mast cells, mouse H<sub>4</sub> receptor transfected and untransfected SK-N-MC were blotted with a mouse H<sub>4</sub> receptor probe. The difference in size in transfected and endogenous receptor is due to the presence of the untranslated regions in endogenous H<sub>4</sub> receptor RNA, which are deleted in the transfected receptor. (D) RT-PCR detection of human H<sub>4</sub> receptor expression in cDNA from human basophils, cord blood-derived mast cells and HMC-1

JPET/2002/46581

cells. SK-N-MC cells transfected with human H<sub>1</sub>, H<sub>2</sub>, H<sub>3</sub> and H<sub>4</sub> receptors were used as controls. The expected size of the human H<sub>4</sub> receptor PCR product is 350 bp.

Figure 3 Histamine induces cyclic AMP in mast cells through H<sub>2</sub> receptors, but not H<sub>4</sub> receptors.

Mast cells of H<sub>3</sub>R<sup>-/-</sup> 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<sub>4</sub> 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 fluorescent signal transiently. (B) Mast cells from H<sub>3</sub>R<sup>-/-</sup> mice were treated with vehicle (black bars), or various concentrations of diphenhydramine (H<sub>1</sub> receptor antagonist, vertical striped bars), ranitidine (H<sub>2</sub> receptor antagonist, horizontal striped bars) or thioperamide (H<sub>3</sub>/ H<sub>4</sub> 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<sub>4</sub>R<sup>+/-</sup> (squares) and H<sub>4</sub>R<sup>-/-</sup> mice (triangles). Calcium mobilization was

JPET/2002/46581

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<sub>4</sub> 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.

JPET/2002/46581

(A) Mast cells incubated overnight with 5  $\mu\text{g/ml}$  IgE and were treated with vehicle (white bars) or 5  $\mu\text{M}$  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  $\mu\text{g/ml}$  IgE. Mast cells from  $\text{H}_4\text{R}^{-/-}$  (diamonds) and wild-type (circles) mice were incubated for 15 min with vehicle (black) or 10  $\mu\text{M}$  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  $\text{H}_4$  receptors.

(A) Chemotaxis of wild-type (black diamonds) and  $\text{H}_4\text{R}^{-/-}$  (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  $\mu\text{M}$  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  $\mu\text{M}$  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  $\mu\text{M}$  histamine was added, and mast cells were added to the upper chamber. (E) In the lower chamber 10  $\mu\text{M}$  histamine was added, while in both chambers 10  $\mu\text{M}$  U73122 (black bars) or 10  $\mu\text{M}$  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  $\text{H}_4$  receptor on mast cells.

JPET/2002/46581

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

JPET/2002/46581

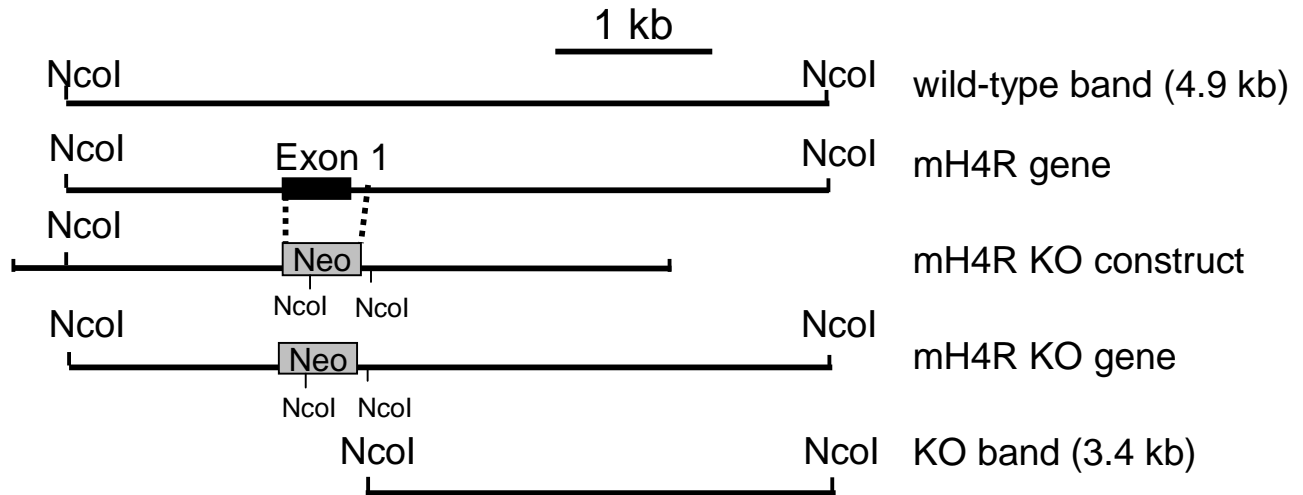
Table1: Mast cell production of prostaglandin and LTB<sub>4</sub>.

	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 <sub>4</sub>	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 LTB<sub>4</sub> levels were measured.

Figure 1

A



B

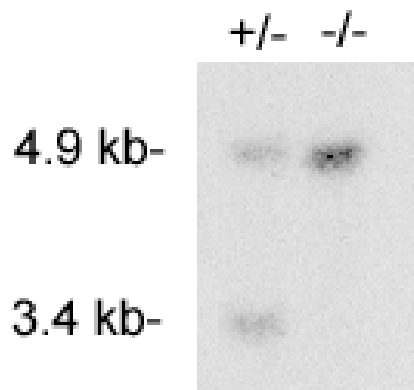


Figure 2

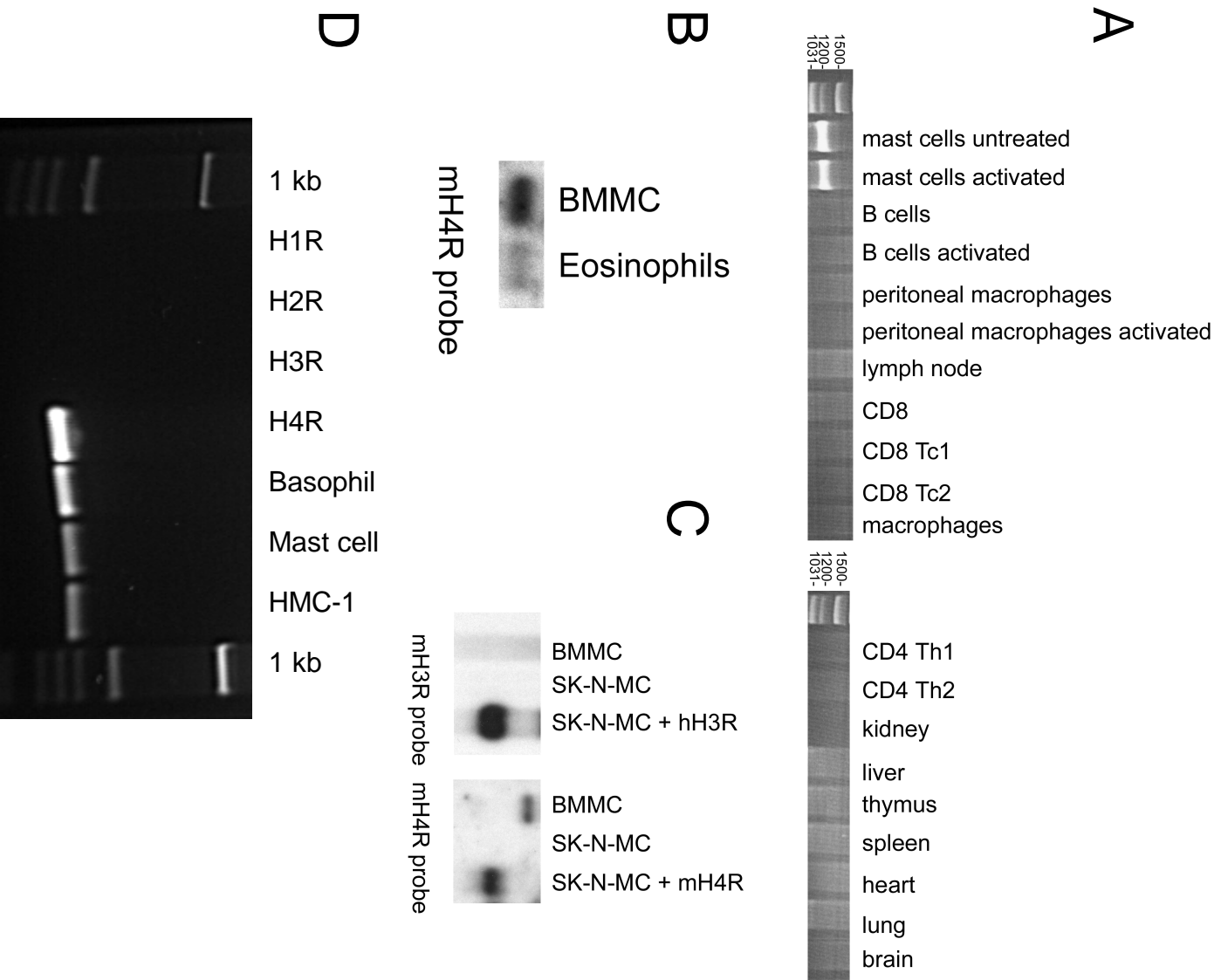




Figure 3

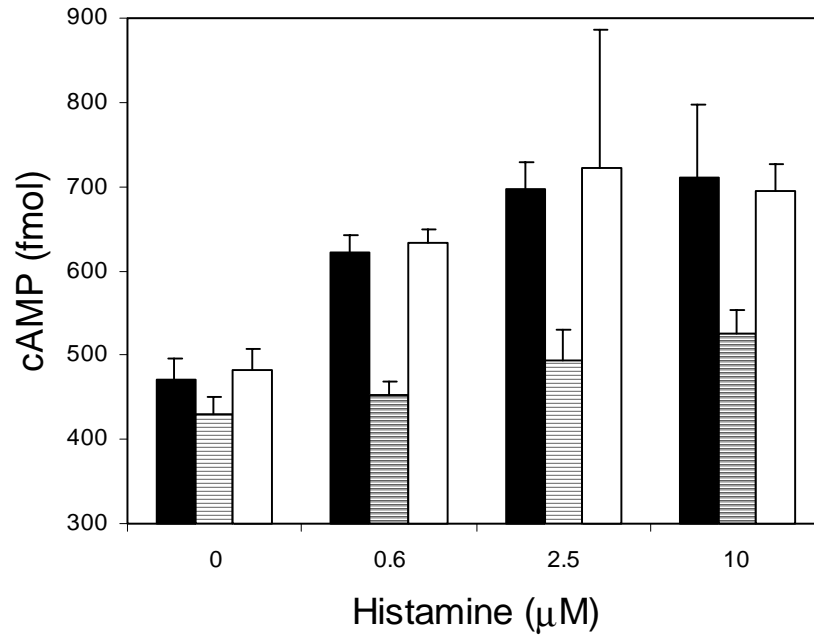


Figure 4

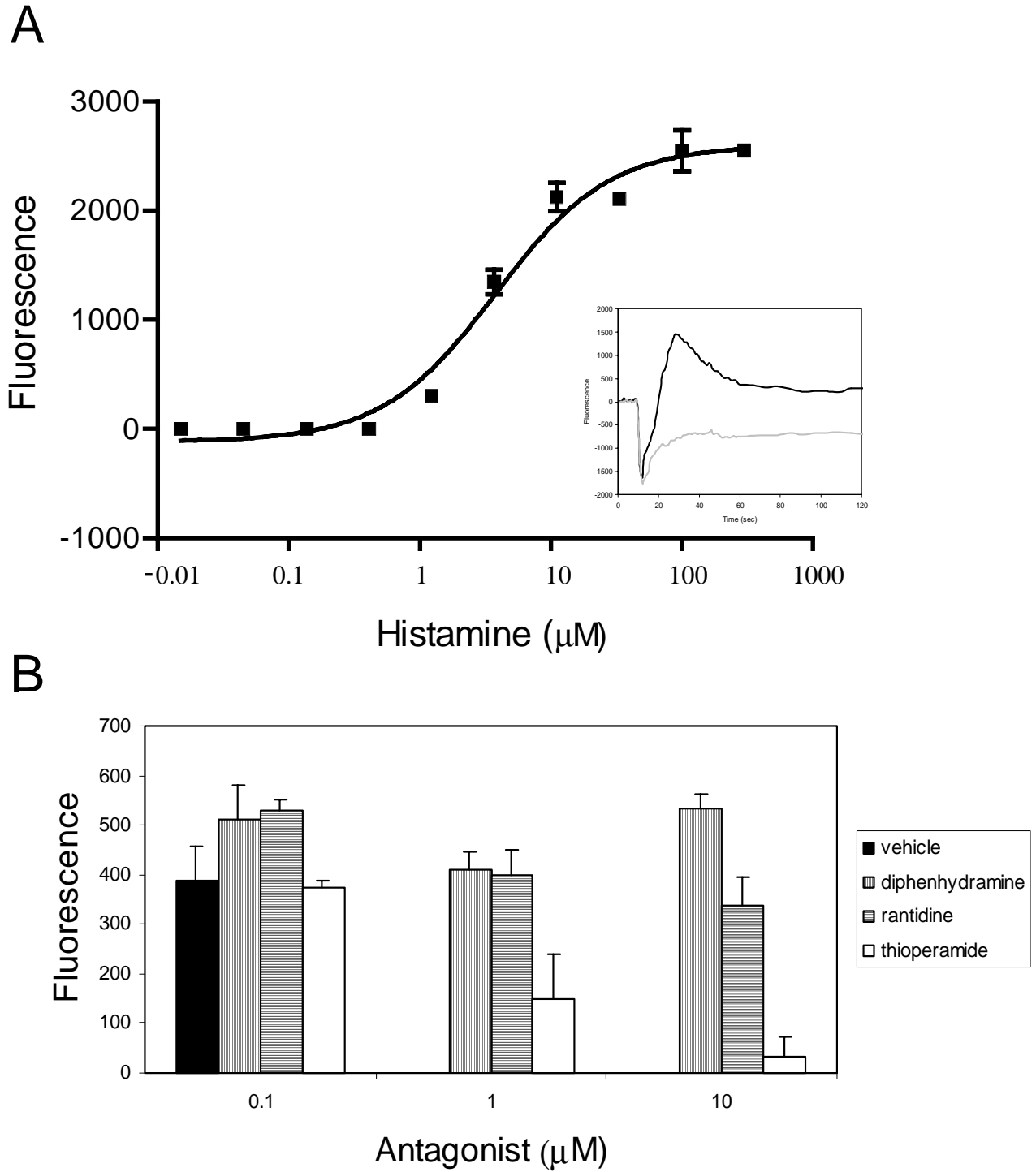


Figure 4

C

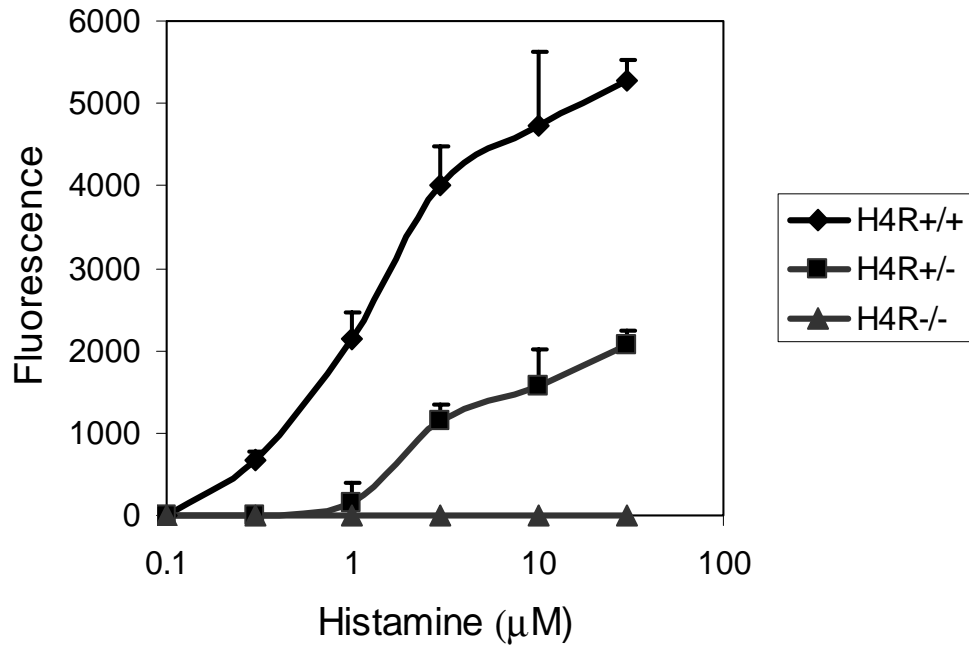
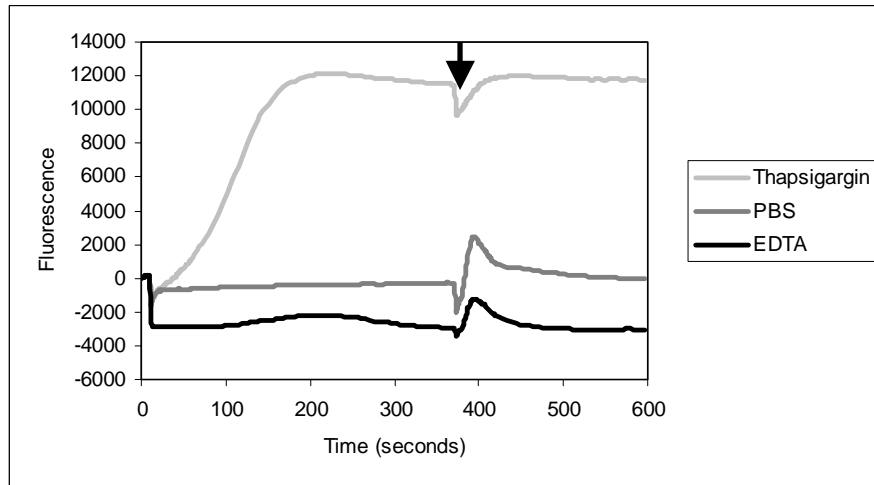


Figure 5

A



B

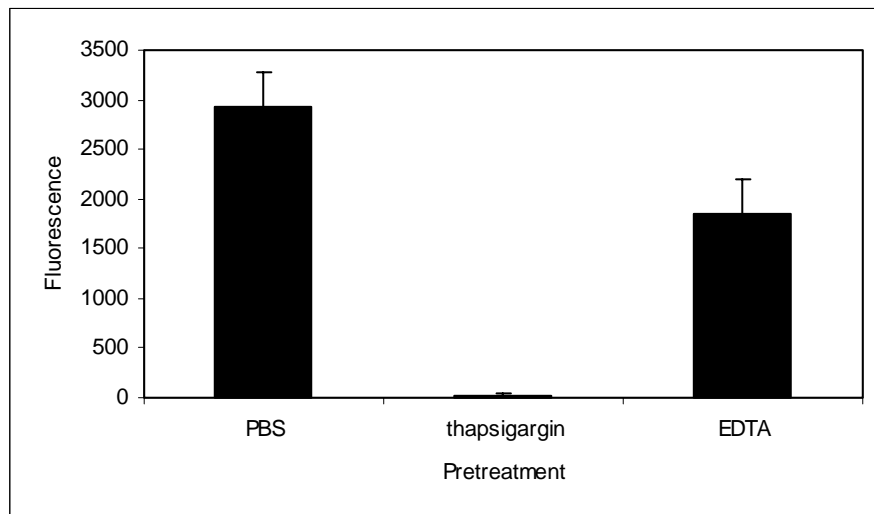
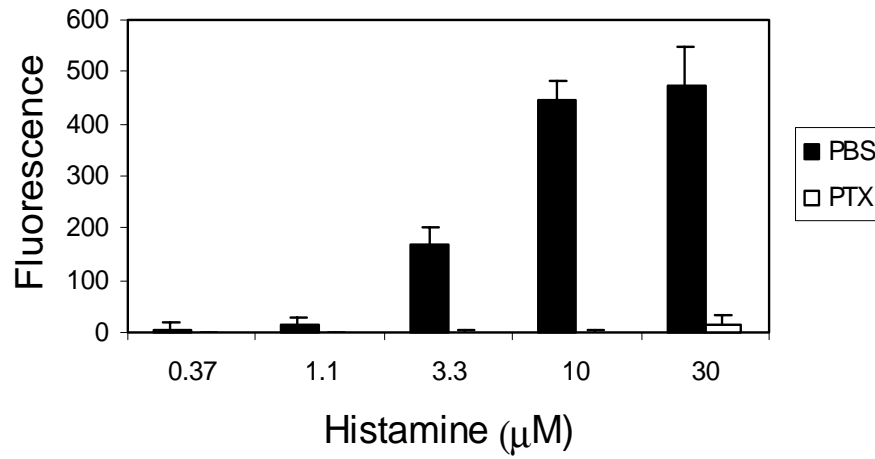


Figure 5

C



D

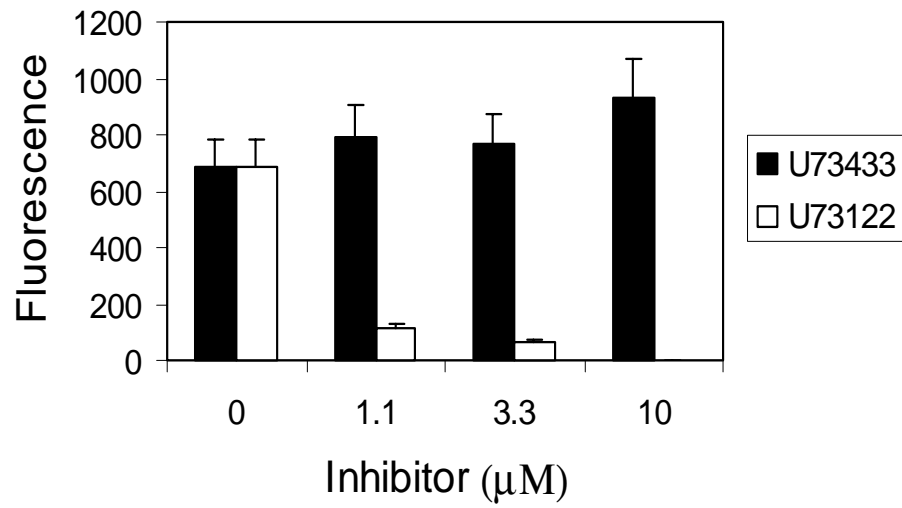
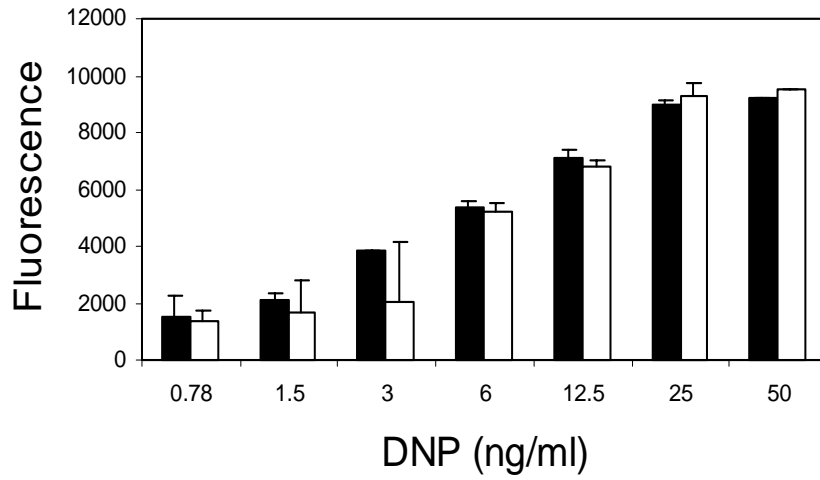


Figure 6

A



B

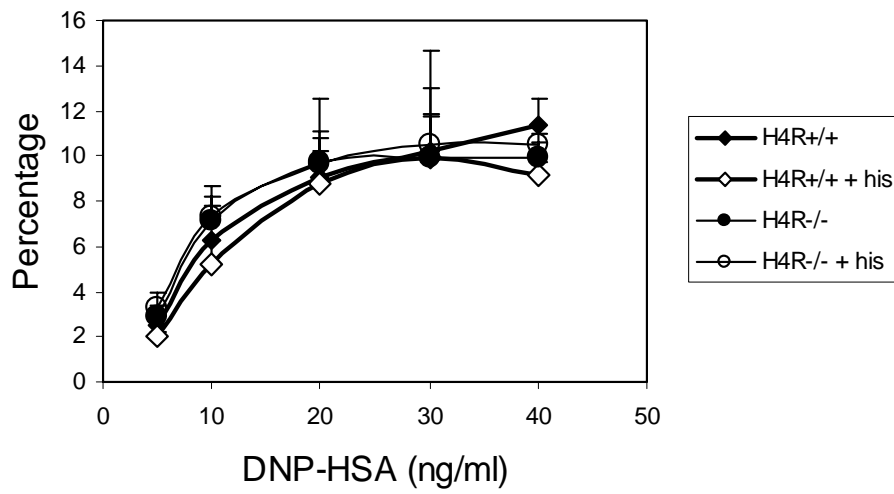
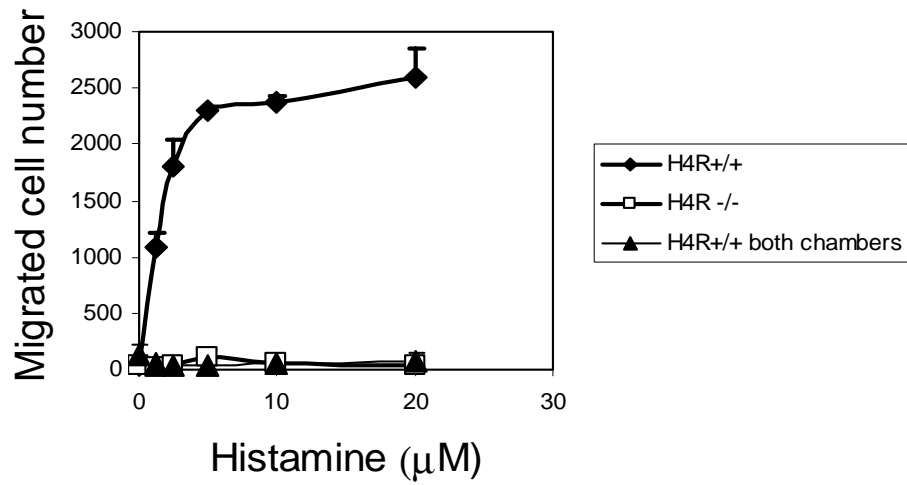


Figure 7

A



B

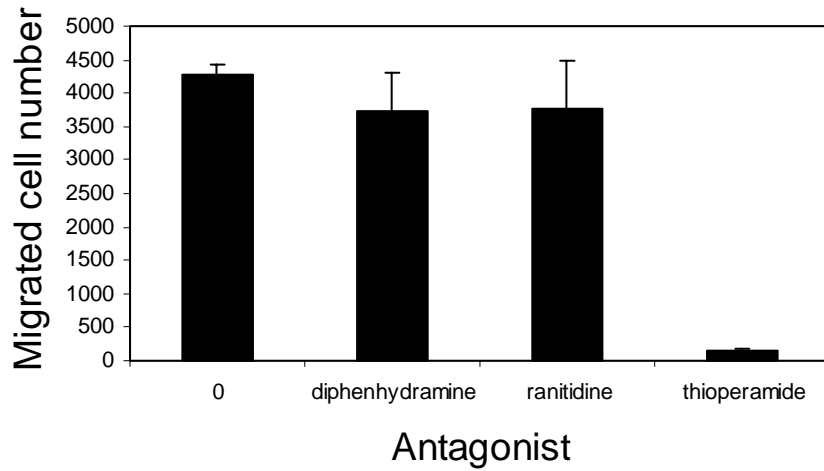
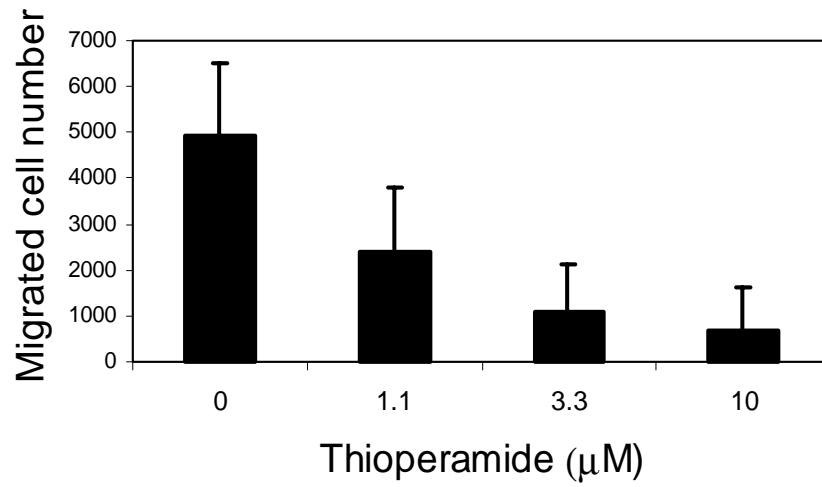


Figure 7

C



D

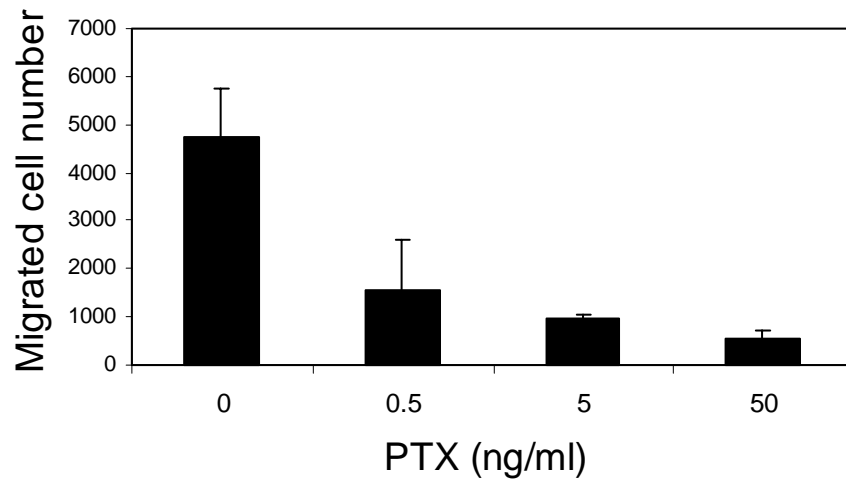




Figure 7

**E**

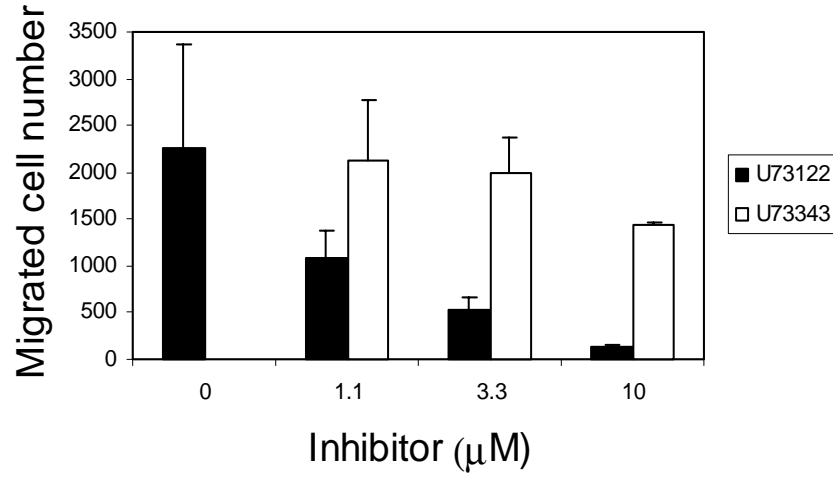


Figure 8

