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Clathrin-independent internalization of the human histamine H_1-receptor in CHO-K1 cells

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1 The aim of the present study was to investigate the cellular pathway involved in histamine-stimulated internalization of the human H_1-receptor in CHO-K1 cells expressing N-terminal myc-tagged H_1-receptor (Myc-H_1) or N-terminal myc-C-terminal green fluorescent protein (Myc-GFP H_1) versions of the receptor.

2 Studies of ^{3}H-mepyramine binding and histamine-stimulated ^{3}H-inositol phosphate accumulation in these cells showed that the Myc-H_1 and Myc-GFP H_1-receptors had identical pharmacology to the wild-type H_1-receptor.

3 The Myc-H_1-receptor was rapidly internalized in CHO-K1 cells following stimulation with histamine (0.1 mM). This response occurred within 15 min, and could be prevented by the quaternary H_1-receptor antagonist α-pirdonium. Similar data were obtained with the Myc-GFP H_1-receptors.

4 Internalization of the Myc-GFP H_1-receptor was maintained in the absence of extracellular calcium and was not inhibited by the CAM kinase II inhibitor KN-62 (10 μM).

5 Phorbol dibutyrate, an activator of protein kinase C, was also able to stimulate internalization of the H_1-receptor. However, inhibition or downregulation of protein kinase C (which significantly modified histamine-stimulated inositol phosphate responses) was without effect on the internalization of the H_1-receptor stimulated by histamine.

6 Hypertonic sucrose did not prevent histamine-induced internalization of the Myc-GFP H_1-receptor, but was able to attenuate internalization of transferrin *via* clathrin-mediated endocytosis in the same cells. In contrast, preincubation of cells with filipin or nystatin, which disrupts caveolae and lipid rafts, completely inhibited the histamine-induced internalization of the Myc-GFP H_1-receptor, but was without effect on the sequestration of transferrin.

7 The H_1-receptor and cholera toxin subunit B were colocalized under resting conditions at the cell surface. Immunohistochemical studies with an antibody to caveolin-1 confirmed that this protein was also localized predominantly to the plasma membrane. However, following stimulation of CHO-Myc-GFP H_1 cells with histamine, there was no evidence for internalization of caveolin-1 in parallel with the H_1-receptor.

8 These data provide strong evidence that the H_1-receptor is internalized *via* a clathrin-independent mechanism and most likely involves lipid rafts.
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Abbreviations: BSA, bovine serum albumin; CAM kinase II, calcium-calmodulin-sensitive protein kinase II; CHO, Chinese hamster ovary; CTB, cholera toxin subunit B; DAG, 1,2-diacylglycerol; DMEM-F-12, Dulbecco's modification of Eagle's medium-Ham's F-12 media; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; $InsP_3$, inositol 1,4,5 triphosphate; Myc-H_1-receptor, N-terminal myc-tagged H_1-receptor; Myc-GFP-H_1-receptor, N-terminal Myc C-terminal green fluorescent protein-tagged H_1-receptor; ORF, open reading frame; PBS, phosphate-buffered saline; PDBu, phorbol dibutyrate; PKC, protein kinase C

Introduction

The histamine H_1-receptor belongs to the large family of G-protein-coupled receptors (GPCRs) and is an important mediator of allergy and inflammation (Hill, 1990; Hill *et al*., 1997; Bakkers *et al*., 2002). H_1-receptor antagonists are highly effective in the treatment of allergic rhinitis (Leurs *et al*., 2002) and an increase in histamine H_1-receptor mRNA has been observed in the nasal epithelia of patients with allergic rhinitis (Iriyoshi *et al*., 1996; Hamano *et al*., 1998). Agonist activation of the H_1-receptor leads to the hydrolysis of phosphatidylinositol 4,5-biphosphate resulting in the generation of inositol 1,4,5 triphosphate ($InsP_3$) and 1,2-diacylglycerol (DAG) (Hill, 1990; Hill *et al*., 1997). $InsP_3$ is responsible for the release of Ca^{2+} ions from intracellular stores, whereas DAG activates protein kinase C (PKC) (Hill, 1990). Calcium and inositol phosphate responses to histamine H_1-receptor stimulation can, however, be attenuated by short-term prior exposure to histamine (Smit *et al*., 1992; Dickenson & Hill, 1993; Zamani *et al*., 1995; Pype *et al*., 1998a, b). Furthermore, direct activation of PKC by phorbol esters can also induce a

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desensitization of H_1-receptor responses in these cells (Smit *et al*., 1992; Dickenson & Hill, 1993; Zamani *et al*., 1995; Pype *et al*., 1998a).

Desensitization of GPCRs in response to receptor-specific agonists (homologous desensitization), or in response to agonists of other GPCRs (heterologous desensitization), is a common property of this family of seven-transmembrane spanning proteins (Lohse *et al*., 1990; Roth *et al*., 1991; Pitcher *et al*., 1998; Luttrell & Lefkowitz, 2002). The phenomenon has been extensively investigated for the β2-adrenoceptor. Homologous desensitization of the β2-adrenoceptor by β2-agonists is triggered by phosphorylation of serine residues in the C-terminus of the β2-adrenoceptor by G-protein-coupled receptor kinases (GRKs) (Pitcher *et al*., 1998; Seibold *et al*., 2000; Luttrell & Lefkowitz, 2002). This leads in turn to the binding of β-arrestins to this region of the receptor, which then interfere with the binding of G-proteins (Lohse *et al*., 1990; Pitcher *et al*., 1998; Luttrell & Lefkowitz, 2002). Heterologous desensitization of the β2-adrenoceptor is mediated *via* the action of second-messenger-stimulated protein kinases (A and C), which phosphorylate the receptor on serines in the third intracellular loop and proximal C-terminus and impair the interaction between receptor and G-protein (Seibold *et al*., 2000; Luttrell & Lefkowitz, 2002). An important consequence of GRK-mediated phosphorylation, however, is that β-arrestin targets the β2-adrenoceptor to clathrin-coated pits and the endocytic machinery resulting in receptor internalization (Krupnick & Benovic, 1998; Tsao *et al*., 2001; Luttrell & Lefkowitz 2002; von Zastrow, 2003; Ahn *et al*., 2003).

Evidence is accumulating, however, that some GPCRs can be internalized *via* caveolae and lipid rafts (Haasemann *et al*., 1998; Gines *et al*., 2001; Mueller *et al*., 2002). Caveolae and lipid rafts are small clathrin-free microdomains of the plasma membrane that are enriched in cholesterol and sphingolipids and are involved in the internalization of small molecules (Anderson *et al*., 1992). Caveolae are characterized by the additional presence of members of the caveolin family of scaffolding proteins (Razani & Lisanti, 2001). Both clathrin-coated pits and caveolae act as microdomains in the plasma membrane to concentrate receptors, G-proteins and various effector proteins in order to coordinate intracellular signalling pathways (Smart *et al*., 1999; Anderson & Jakobson, 2002; Hall & Lefkowitz, 2002). More recently, it has become clear that it is possible for a receptor to be targeted to a particular membrane microdomain by phosphorylation *via* a specific protein kinase (e.g. GRK or protein kinase A) (Rapacciuolo *et al*., 2003).

The histamine H_1-receptor can be phosphorylated by a number of kinases *in vitro* including protein kinase A, PKC and calcium-calmodulin-sensitive protein kinase II (CAM kinase II) (Kawakami *et al*., 2003). However, the mechanisms underlying homologous and heterologous desensitization of the H_1-receptor have not been fully elucidated. The H_1-receptor has an intracellular PKC phosphorylation site (Fujimoto *et al*., 1999; Kawakami *et al*., 2003); however, despite PKC activation causing desensitization of the H_1-receptor, PKC inhibitors do not prevent the homologous desensitization of the H_1-receptor by histamine in GT1-7 cells (Zamani *et al*., 1995), bovine trachea (Pype *et al*., 1998b), human umbilical vein endothelial cells (McCreath *et al*., 1994) or DDT_1MF-2 cells (Dickenson & Hill, 1993). Homologous desensitization of the H_1-receptor has been reported to be inhibited by both an inhibitor of CAM kinase II (KN-62) and the absence of extracellular calcium in GT1-7 cells, but not in other cell types (McCreath *et al*., 1994; Zamani & Bristow, 1996; Pype *et al*., 1998b). The role of H_1-receptor internalization and the microdomains involved are, however, unknown. The aim of the present study was to investigate the extent and cellular pathway involved in histamine-stimulated H_1-receptor internalization.

Methods

Materials

Rabbit polyclonal anti-GFP and mouse monoclonal anti-caveolin-1 were purchased from Abcam (Cambridge, U.K.) and BD Transduction Laboratories (Oxford, U.K.), respectively. Goat anti-mouse Rhodamine Red X and goat anti-rabbit Alexa-Fluor 488 were from Molecular Probes (Leiden, The Netherlands). The mouse monoclonal anti-myc was from an in-house mouse hybridoma. Filipin complex, Nystatin, sucrose, phorbol dibutyrate (PdBu) and histamine were purchased from Sigma (Poole, Dorset, U.K.). Go 6976 and KN-62 were purchased from Calbiochem (Nottingham, U.K.). Transferrin Alexa-Fluor 633, cholera toxin subunit B Alexa-Fluor 647 and BODIPY TR C_5-ceramide BSA were purchased from Molecular Probes. α-Pirdonium was a kind gift from R. Leurs, Amsterdam, The Netherlands. ^{3}H-Mepyramine was purchased from Amersham Bioscience (Bucks, U.K.).

Generation of CHO-K1 cell lines expressing the wild-type, 5′-myc- and 5′myc-3′-GFP-tagged human H_1-receptor

The wild-type histamine H_1-receptor (WT-H_1) was generated by PCR amplification of the full-length H_1-receptor from human genomic DNA using GCCACTCATCACCCAAGT CTCTGACCTTAC and TGCCTGGCAACACACAGGCCT ACGTCCTCT as forward and reverse primers, respectively. These primers anneal in the immediate 5′ and 3′ noncoding regions of the human H_1-receptor gene. The PCR product was then *t*-tailed cloned into pCR3.1 (Invitrogen, Groeningen, The Netherlands) to form pCR3.1HH1R. The 5′-myc-tagged human H_1-receptor was obtained by PCR amplification of a 256 bp fragment of the front end of the H_1-receptor in pCR3.1HH1R using the primers: GCCGCCACCATGGAGC AGAAACTGATCTCCGAGGAGGACCTGCTGAGCCTC CCCAATTCCTCCTGCCTC (forward) and ATGACGACG GCACCCACGATCAAGTCCG (reverse). The PCR product was then *t*-tailed cloned into pCR3.1 to form pCR3.myc. The unique 5′ *Dra*III sites within the pCR3.1 vector and the H_1-receptor sequence were then used to substitute the 5′-myc-tagged H_1-receptor sequence (from pCR3.1myc) for the 5′-end of the WT-H_1 sequence (in pCR3.1HH1R) to form the full-length 5′-tagged H_1-receptor construct pCR3.1myc-HH1R.

The fluorescent protein open reading frame (ORF) of the Topaz variant of GFP in pGFPtpz-basic (Packard Bioscience, Pangbourne, Berks, U.K.) was subcloned by PCR using the primers, CTCGAGCCTGGTGAGCAAGGGCGAG (forward) and GACTTCTAGAAGCCCGGGTAACTTGTACA GCTCGTC (reverse) to produce a fragment containing the ORF of the Topaz gene with the start codon replaced with CTG and an upstream *Xho*I site. This was then *t*-tail cloned

into pCR3.1 (Invitrogen, Groeningen, The Netherlands). The 3′-end of the histamine H_1-receptor ORF (bases 984–1635) of pCR3.1HH1R was subcloned by PCR using the primers, CTCTACTGCTTTCCACTTG (forward) and TCTAGACCC GGGAGCTTCTCGAGGAGAGAATATGCAGAA (reverse) to produce a fragment containing the 3′-end of the H_1-receptor ORF with the stop codon replaced with a *Xho*I site and an additional 3′ *Xba*I site, which was then cloned into pCR3.1. The 3′-end of this receptor sequence (bases 1275–1635; with the stop codon replaced with a *Xho*I site) was excised with *Bsm*I and *Xba*I and inserted into the equivalent sites of the 5′-myc-H_1-receptor sequence in pCR3.1myc-HH1R to form pCR3.1myc-HH1R-XhoI. The Topaz ORF was excised using the enzymes *Xho*I and *Xba*I and band purified. The pCR3.1myc-HH1R-XhoI plasmid was also cut with *Xho*I and *Xba*I and the linking DNA removed by gel purification. The Topaz fragment was then ligated in frame into pCR3.1myc-HH1R-XhoI to generate the plasmid pCR3.1myc-HH1R-Tpz.

All receptor constructs were sequenced on both strands using automated fluorescent sequencing (University of Nottingham, Nottingham, U.K.).

Cell transfection was carried out using Lipofectamine (Gibco BRL, Paisley, U.K.) according to the manufacturer's instructions. Briefly, CHO cells were grown to 80% confluency in 75 cm^2 flasks in Dulbecco's modification of Eagle's medium-Ham's F-12 media (DMEM-F-12) containing 10% ($v\,v^{-1}$) fetal calf serum (FCS) and 2 mM glutamine. Transfection was carried out in Optimem media (Gibco) using 15 μg of plasmid DNA for 16 h at 37°C. Positive transfects were selected using 200 $\mu g\,ml^{-1}$ geneticin in DMEM-F-12 media containing 10% ($v\,v^{-1}$) FCS, then dilution cloned prior to screening for functional receptor expression using inositol phosphate accumulation assays and confocal microscopy. Clonal cell lines were routinely cultured in DMEM-F-12 media (Sigma RBI) containing 10% ($v\,v^{-1}$) FCS and 2 mM glutamine.

^{3}H-Mepyramine binding

Membranes were prepared from cells as described previously (Cordeaux *et al*., 2000). Protein concentration of membranes was determined according to the method described by Lowry *et al*. (1951). Saturation binding experiments were performed in triplicate in Tris-HCl buffer (50 mM; pH = 7.5) in a final volume of 1 ml. Membranes (100–200 μg protein ml^{-1}) were incubated for 90 min at 37°C with increasing concentrations of ^{3}H-mepyramine (0.1–20 nM). Nonspecific binding was determined in the presence of promethazine (2 μM). The incubation was terminated by rapid filtration through GF-B Whatman filters, presoaked in polyethylenimine (0.3%, 90 min), using a Brandel MR24 cell harvester. The filters were washed three times (approximate total volume 10 ml) with ice-cold Tris-HCl buffer (50 mM) before being transferred to scintillation vials. Emulsifier-Safe scintillant (3 ml) was added to each tube for 12 h before being counted in a liquid scintillation counter.

Measurement of ^{3}H-inositol phosphate accumulation

^{3}H-inositol phosphate accumulation was measured based on the method described by Cordeaux *et al*. (2000). Following 24 h prelabelling with ^{3}H-myoinositol (37 kBq $well^{-1}$) in 24-well cluster dishes, cells were washed (1 ml $well^{-1}$) with Hanks HEPES-buffered saline (HBS) and incubated with LiCl (20 mM, 30 min, 37°C) before the addition of histamine (10 nM–1 mM, 30 min, 37°C). Where appropriate, mepyramine (100 nM), filipin (1–5 $\mu g\,ml^{-1}$) or PdBu (1 μM) was added 30 min prior to agonist addition.

Incubations were terminated by the aspiration of media and addition of cold (−20°C) methanol: 0.12 M HCl (1 : 1 $v\,v^{-1}$). Cells were then left a minimum of 2 h at −20°C before isolation of total inositol phosphates by anion chromatography (Cordeaux *et al*., 2000).

Confocal microscopy

Confocal microscopy was performed using a Zeiss LSM 510 laser scanning microscope with either a Zeiss Plan-Apo chromat. 63 × 1.4 NA or Zeiss Plan-Neofluar 40 × 1.3 NA oil-immersion objective. The GFP was excited using a 488 nm argon laser and detected with a 505 nm long-pass filter. The myc-tagged receptors were visualized by exciting the Rhodamine Red X secondary antibody with a 543 nm HeNe laser and a 560 nm long-pass filter. Transferrin Alexa-Fluor 633 and cholera toxin subunit B (CTB)-Alexa-Fluor 647 were excited using a 633 nm HeNe laser and detected with a 650 nm long-pass filter. Colocalization images were captured using the Zeiss multi-track system with BP505-530 (GFP) and LP560 (Rhodamine) or LP650 (transferrin) filters. Colocalized pixel images were generated using the Zeiss LSM software.

Immunohistochemistry

For immunocytochemistry, cells were grown to approximately 70% confluency on glass coverslips in six-well plates containing 3 ml DMEM-F12 media, 10% FCS and 2 mM glutamine. Where appropriate, cells were incubated with antagonists for 30 min prior to histamine addition (0.1 mM for 30 min). Following agonist stimulation, cells were fixed with 2% paraformaldehyde–PBS for 20 min. Cells were then washed with PBS (3 × 5 min, 1 ml) before being blocked with BSA–glycine–PBS (3% BSA, 1% glycine) followed by goat serum (10% in PBS). Cells were then labelled with primary antibody (diluted in horse serum; 10% in PBS); mouse monoclonal anti-myc (5 $\mu g\,ml^{-1}$), rabbit polyclonal anti-GFP (0.25 $\mu g\,ml^{-1}$) and mouse monoclonal anti-caveolin-1 (0.25 $\mu g\,ml^{-1}$) overnight at 4°C. Cells were finally washed three times for 5 min in PBS before being labelled with secondary antibody (1 h at room temperature in the dark) (goat anti-mouse Rhodamine Red X or goat anti-rabbit Alexa-Fluor 488, 4 $\mu g\,ml^{-1}$). After a further three washes (5 min), coverslips were mounted on glass slides. For experiments to investigate intracellular localization of proteins, cells were treated with Igepal (0.5% in PBS at 4°C for 5 min) following fixation of the cells.

Live cell imaging

CHO cells expressing the human H_1-receptor tagged with GFP were grown to 70% confluency in 35 mm glass-bottomed culture dishes (MatTek Corporation, Ashland, MA, U.S.A.). Cells were washed twice with sterile HBS (HEPES 25 mM, glucose 10 mM, NaCl 146 mM, KCl 5 mM, $MgSO_4$ 1 mM, Na pyruvate 2 mM, $CaCl_2$ 1.3 mM) in the presence or absence of Ca^{2+} (1.3 mM) and imaged at 37°C on a heated microscope stage with an objective heater using a Zeiss LSM 510

microscope. Where appropriate, cells were incubated with α-pirdonium (100 nM) or Go 6976 (3 μM) for 30 min prior to agonist addition (0.1 mM for 30 min). Cells were imaged in the continued presence of agonist (1024 × 1024 pixels; averaging of four frames) using the 488 nm Argon laser line and a 505 nm long-pass filter. In some experiments, cells were pretreated with PDBu (1 μM) for 30 min or 24 h prior to experimentation. For the Golgi marker experiments, cells were incubated on ice for 30 min with BODIPY TR C_5-ceramide BSA (5 μM) to load the cell surface. The cells were then washed in ice-cold HBS and then incubated for 30 min at 37°C. Where appropriate, histamine (0.1 mM) was added at the beginning of this 30 min period at 37°C. For studies of the colocalization of the histamine receptor (Myc-GFP hH_1R) with the lipid raft marker CTB, CHO cells expressing the Myc-GFP hH_1R were incubated with CTB-Alexa-Fluor 647 nm (10 μg ml^{-1}) on ice for 15 min in HBS, washed briefly and imaged at room temperature. Confocal microscopy was then performed using multitracking with 488 nm excitation line of an argon laser and a 505–530 nm band-pass filter (for the Myc-GFP hH_1R) and 633 nm excitation of an HeNe laser and a 650 nm long-pass filter (for CTB).

Data analysis

Agonist concentration–response curves were fitted using the program Prism 2 to the equation:

$$\text{Response} = E_{MAX} \times [A]^n / ([A]^n + EC_{50}^n)$$

where E_{MAX} is the maximal response, [A] is the agonist concentration, EC_{50} is the concentration of agonist producing 50% of the maximal response and n is the Hill coefficient. Mepyramine dissociation constants (K_b) were determined by observing the shift in the histamine concentration–response curve produced by 100 nM mepyramine using the equation: $DR = 1 + [A]/K_b$, where DR (dose ratio) is the ratio of the concentration of histamine required to produce an identical response in the presence and absence of mepyramine. $pK_b = -\log K_b$. Curves of specific binding (SB) of ^{3}H-mepyramine at different concentrations of the ^{3}H-ligand were fitted using Prism 2 to the equation:

$$SB = (L \times B_{BAX})/(L + K_d)$$

where L is the concentration of ^{3}H-mepyramine, K_d is the dissociation constant and B_{MAX} is the maximum SB capacity. All data are presented as mean ± s.e.m. of at least three separate experiments. The n in the text refers to the number of separate experiments.

Results

Characterization of the cell lines

^{3}H-Mepyramine binding was used to determine the expression level of a series of clonal cell lines stably expressing the WT-H_1, an H_1-receptor variant with an N-terminal myc-tagged H_1-receptor (Myc-H_1) or one with both an N-terminal Myc tag and a C-terminal green fluorescent protein tag (Myc-GFP H_1). Clones that expressed the receptors at a level between 0.35 and 1.49 pmol mg protein^{-1} were selected for further study (Table 1.) The affinity of ^{3}H-mepyramine for the H_1-receptor was unaffected by the addition of the two tags (K_d WT-H_1 4.4 ± 0.9 nM, Myc-H_1 3.2 ± 0.3 nM, Myc-GFP-H_1 2.6 ± 0.4 nM; Table 1). Histamine induced a concentration-dependent increase in ^{3}H-inositol phosphate accumulation in all three cell lines (Table 1). The maximum response to histamine was dependent on the level of receptor expression with the highest expressing cell line (Myc-H_1) having the greatest maximum response (Table 1). Preincubation of the cells with mepyramine (100 nM, 30 min) caused a parallel shift to the right of the concentration–response curves to histamine in each cell line. There were no significant differences in the affinity constants determined for mepyramine from antagonism of histamine-induced inositol phosphate accumulation in each cell line (Table 1).

Agonist-stimulated H_1-receptor internalization

Agonist-induced receptor internalization was investigated using both Myc-H_1 and Myc-GFP-H_1 cell lines. Unfortunately, the currently available commercial antibodies for the H_1-receptor were not able to identify selectively the wild-type receptor expressed in CHO-K1 cells. Immunohistochemical detection of cell surface histamine H_1-receptors using the c-myc antibody in nonpermeant cells indicated that both Myc-H_1 (Figure 1a and b) and Myc-GFP-H_1 (Figure 1c and d) receptors were internalized (i.e. lost from the cell surface) following treatment with histamine (0.1 mM; 30 min). This could be prevented by pretreatment of cells with the quaternary H_1-receptor antagonist α-pirdonium (100 nM; Figure 1e and f). Using real-time live cell imaging of the GFP attached to the C-terminus of the Myc-GFP H_1-receptor, the Myc-GFP H_1-receptor was shown to translocate from the cell surface to large intracellular perinuclear focal spots in response to histamine (0.1 mM; 30 min; Figure 1j). In the absence of histamine, the receptor was maintained at the cell surface (Figure 1g and h). Incubation (30 min) with α-pirdonium prevented the stimulation by histamine of the formation of intracellular focal spots containing the Myc-GFP H_1-receptor protein (Figure 1k and l). Internalization of Myc-GFP H_1-receptors into intracellular

Table 1 Ligand binding and functional properties of wild-type, Myc- and Myc-GFP-tagged human histamine H_1-receptor cell lines

	^{3}H-mepyramine binding			*Histamine-stimulated ^{3}H-inositol phosphate accumulation*			
H_1-CHO cell clone	B_{max} (pmol mg protein^{-1})	K_d (nM)	n	*Log EC_{50}*	*E_{MAX} (fold over basal)*	*pK_b (mepyramine)*	n
Wild-type H_1	0.35 ± 0.04	4.4 ± 0.9	3	−5.6 ± 0.2	5.5 ± 1.0	7.9 ± 0.1	3
Myc-H_1	1.49 ± 0.11	3.2 ± 0.3	6	−6.2 ± 0.1	19.5 ± 2.4	8.2 ± 0.1	5
Myc-GFP H_1	0.84 ± 0.06	2.6 ± 0.4	6	−5.4 ± 0.1	7.7 ± 0.9	8.2 ± 0.2	5

Values are mean ± s.e.m. and n refers to the number of separate experiments.

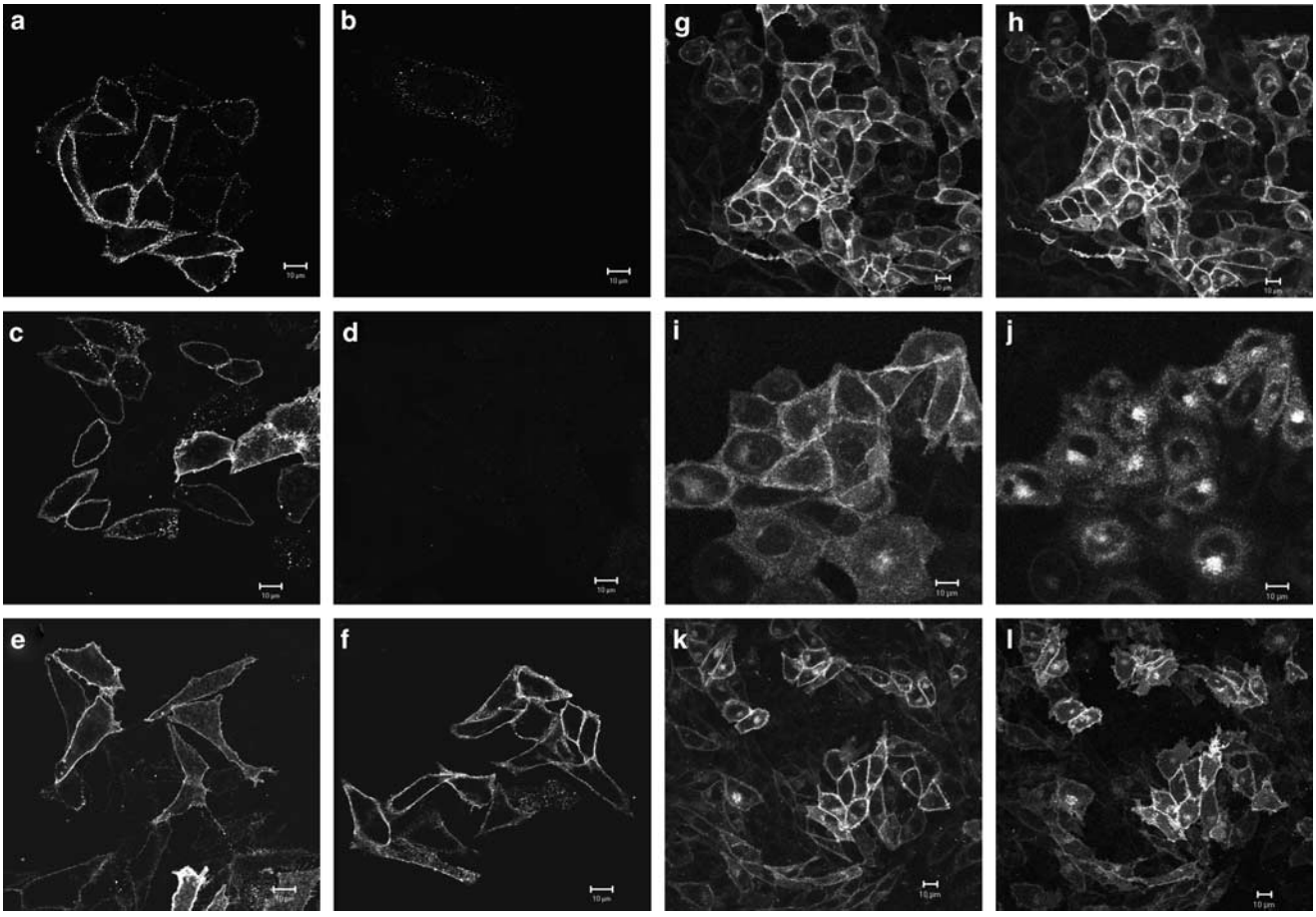

Figure 1 (a–f) Immunocytochemical location of the Myc-tagged H_1R (a, b) or the myc-tagged H_1-receptor-GFP fusion (c, d) expressed in CHO cells under basal conditions (a, c) and following 30 min stimulation with histamine (0.1 mM; b, d). In (e, f), myc-H_1-receptor cells were pretreated with 0.1 μM α-pirdonium for 30 min prior to the addition of histamine (f; 0.1 mM final concentration) or vehicle control (e). Cells were fixed in 2% paraformaldehyde and processed for immmunocytochemistry as described in Methods. Cells were then labelled with mouse monoclonal anti-myc followed by goat anti-mouse Rhodamine Red X antibodies without permeabilization. Confocal microscopy was performed using 543 nm excitation with the HeNe laser and a 560 nm long-pass emission filter. Similar data were obtained in four (a, b), six (c), five (d) and three (e, f) separate experiments. Scale bars 10 μm. (g–l) Live cell imaging of the GFP fluorescence in CHO cells expressing the Myc-GFP H_1-receptor under basal conditions (g, i) and after a further 30 min of real-time imaging under the same conditions (h) or following 30 min stimulation with 0.1 mM histamine (j). In (k, l), cells were preincubated with the H_1-receptor antagonist α-pirdonium (0.1 μM) for 30 min (k) prior to the addition of histamine (final concentration of 0.1 mM) for a further 30 min (l). Confocal microscopy was performed at 37°C using the 488 nm excitation line of an argon laser and a 505 nm long-pass emission filter. Similar data were obtained in eight (g, i, h), 11 (j) and three (k, l) separate experiments. Scale bars 10 μm.

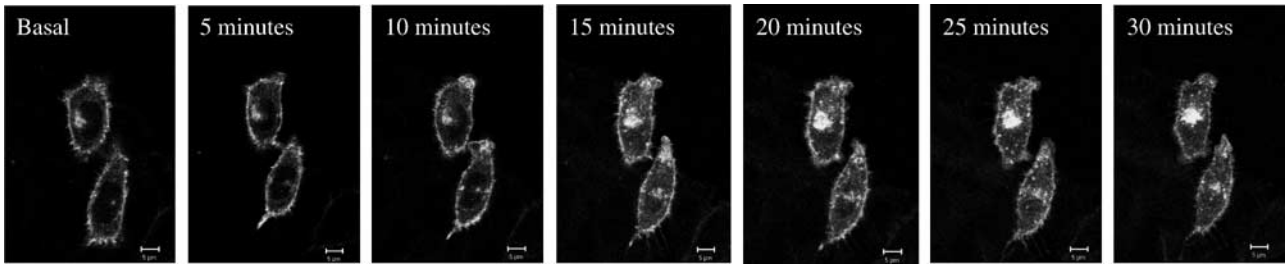

Figure 2 Time course of histamine-induced internalization of the Myc-GFP H_1-receptor in CHO-K1 cells in calcium-free HBS. Real-time confocal imaging showing the translocation of the Myc-GFP H_1-receptor from the cell surface to cytoplasmic focal spots after 30 min histamine (0.1 mM) stimulation. Confocal microscopy was performed at 37°C using the 488 nm excitation line of an argon laser and a 505 nm long-pass emission filter. Similar data were obtained in eight separate experiments. Scale bars 5 μm.

focal spots was still apparent when cells were pretreated with cycloheximide to prevent formation of newly synthesized receptor (100 μg ml^{-1}, 4 h; $n=2$; data not shown).

Histamine-induced desensitization of neuronal mouse H_1-receptors has previously been reported to be dependent on extracellular calcium and mediated through activation of

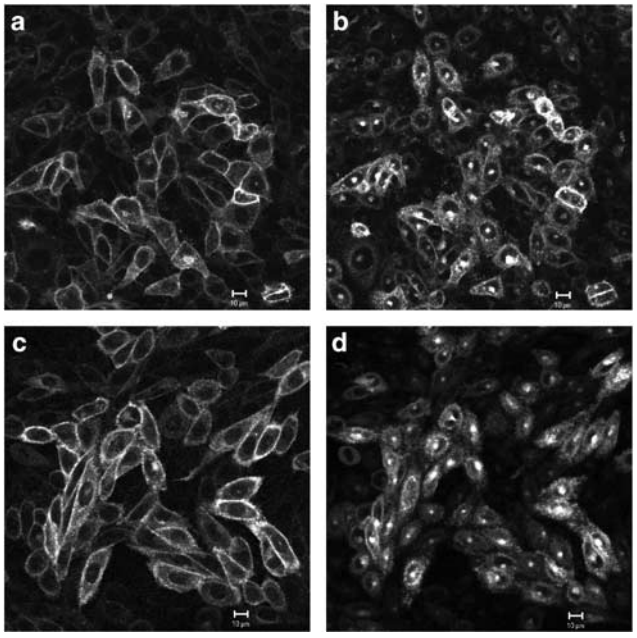

Figure 3 Influence of KN62 (a, b) and calcium-free conditions (c, d) on agonist-induced internalization of the Myc-GFP hH_1R. Myc-GFP hH_1R cells were incubated with the CAM kinase inhibitor KN62 (10 μM) for 30 min (a) and then stimulated for 30 min with histamine (0.1 mM) (b). In (c, d), Myc-GFP hH_1R cells were washed in HBS without added calcium and then imaged in the HBS containing 0.05 mM EGTA under basal conditions (c) and after 30 min histamine (0.1 mM) stimulation (d). Confocal microscopy was performed at 37°C using the 488 nm excitation line of an argon laser and a 505 nm long-pass emission filter. (a, b) and (c, d) are each representative of three separate experiments. Scale bars 10 μm.

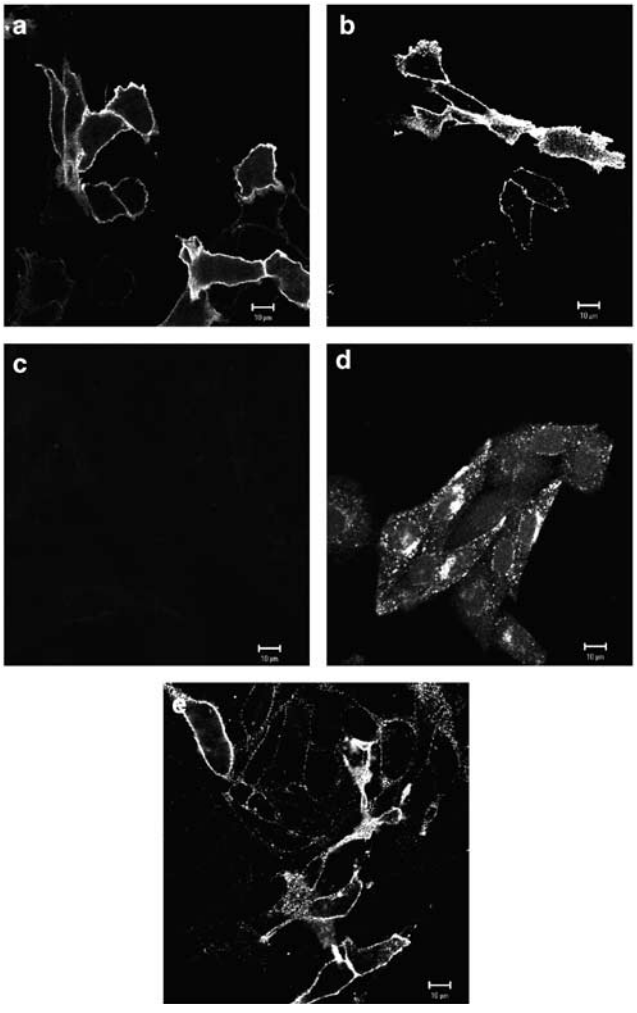

Figure 4 Internalization of anti-myc primary antibody-labelled H_1-receptor induced by histamine (0.1 mM) in living cells. Cells expressing the myc-tagged H_1-receptor were labelled with mouse anti-myc primary antibody for 1 h at 37°C. Cells were then fixed with 1% paraformaldehyde immediately (a) or fixed and then permeabilized with IGEPAL (0.5% in PBS) (b). In (c, d), cells were stimulated with histamine (0.1 mM) for 30 min (after anti-myc antibody labelling) prior to fixation (unpermeabilized; c) or fixation followed by permeabilization (d). After 30 min histamine (0.1 mM) stimulation, some cells were then washed (30 min) in HBS and then fixed (nonpermeabilized; e). After fixation, all cells were then labelled with goat anti-mouse Rhodamine Red X secondary antibody. Confocal microscopy was performed using 543 nm excitation with the HeNe laser and a 560 nm long-pass emission filter. Similar data were obtained in five separate experiments. Scale bars 10 μm.

CAM kinase II (Zamani & Bristow, 1996). However, in the present study, internalization of the Myc-GFP H_1-receptor was maintained in the absence of extracellular calcium (Figures 2 and 3c, d). In nominally Ca^{2+}-free media H_1-receptor internalization was apparent following incubation with histamine (0.1 mM) for 15 min and was maximal after 30 min (Figure 2). Furthermore, pretreatment of cells with KN-62 (an inhibitor of CAM kinase II) (10 μM, 30 min) did not inhibit internalization of GFP-Myc H_1R (Figure 3a and b).

In order to confirm that the appearance of H_1-receptor protein in intracellular perinuclear focal points had derived originally from the cell surface, the cell surface Myc-H_1-receptor population was tagged with the anti-myc primary mouse antibody at the beginning of the experiment (Figure 4a and b). Following stimulation with histamine (0.1 mM; 30 min), cells were fixed and probed with the secondary fluorophore-containing anti-mouse antibody. In Figure 4c and d, it is clear that the H_1-receptor has moved from the cell surface to intracellular sites, which include bright perinuclear focal points. After washout of histamine, cell surface-derived Myc-H_1-receptors returned to the cell surface (Figure 4e). The identity of the perinuclear location of the internalized H_1-receptor remains to be confirmed, but colocalization studies with BODIPY ceramide suggest that it is likely to be the Golgi apparatus (Figure 5).

Role of PKC

In the absence of a role for CAM kinase II in the internalization mechanism for the histamine H_1-receptor, an obvious candidate for a mediator of internalization is PKCα, which is translocated to the plasma membrane of CHO cells following H_1-receptor stimulation (Megson *et al*., 2001). Pretreatment of CHO cells expressing the Myc-GFP H_1-receptor with PDBu (1 μM, 30 min) caused a decrease in the maximum ^{3}H-inositol phosphate response to histamine ($70.1 \pm 2.3\%$ of control; mean $\pm$ s.e.m. of three separate experiments) and a small increase in the histamine EC_{50} value (control $\log EC_{50}$ -5.11 ± 0.06; $\log EC_{50}$ [+PDBu] -4.85 ± 0.04; mean $\pm$ s.e.m. of three separate experiments) (Figure 6a). Pretreatment (24 h) with PDBu (24 h; 1 μM),

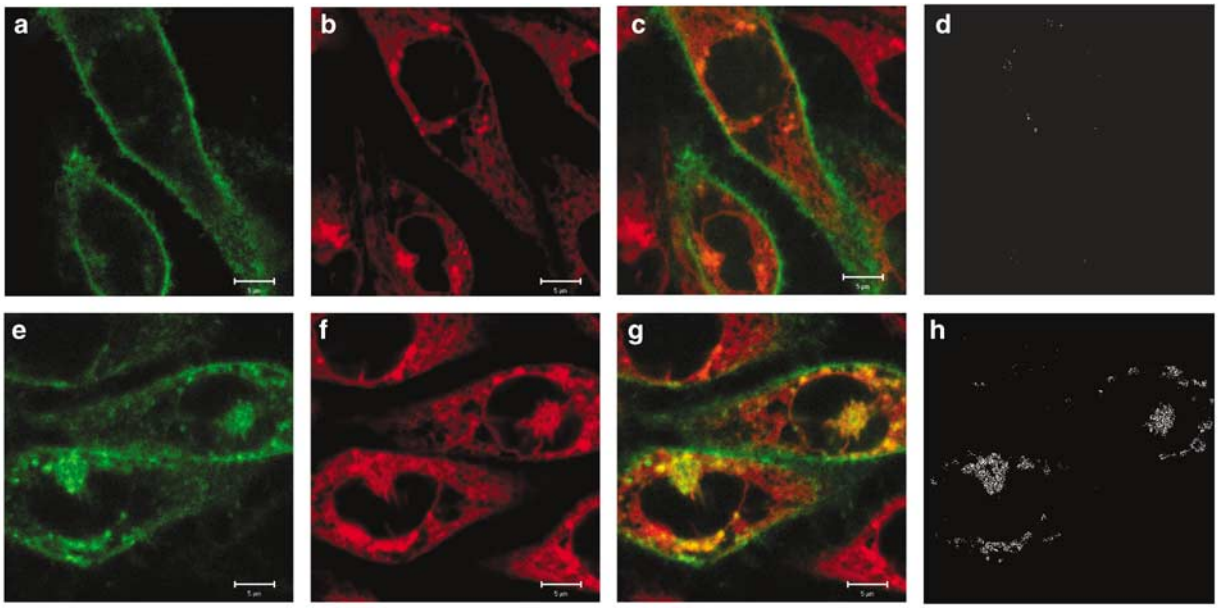

Figure 5 Internalization of the histamine H_1-receptor (Myc-GFP H_1) to the Golgi apparatus. Live cell imaging of Myc-GFP H_1 cells incubated with BODIPY TR C_5-ceramide BSA (5 μM; 4°C 30 min, prior to confocal microscopy at 37°C) under basal conditions (a–d) and after stimulation (e–h) with histamine (100 μM; 30 min). The green channel (a, e) shows location of GFP and the red channel (b, f) shows the location of BODIPY TR. The overlay (c, g) of the two images and the colocalized pixels (d, h) are also shown. Confocal microscopy was performed at 37°C using multitracking with the 488 nm excitation line of an argon laser and a 505–530 band-pass filter and 543 nm excitation of an HeNe laser with a 560 nm long-pass filter. Colocalization was determined using the Zeiss LSM510 software. Similar data were obtained in four separate experiments. Scale bars 5 μm.

which causes a downregulation of PKCα, PKCδ and PKCε in CHO cells (Megson *et al*., 2001), markedly increased the size of responses to histamine (298.5 ± 108.5% of control; mean ± s.e.m. of three separate experiments; Figure 6b). These data suggest that activation of PKC is intimately involved in desensitization of H_1-receptor-mediated ^{3}H-inositol phosphate responses. Furthermore, 30 min stimulation with PDBu (1 μM) induced an internalization of the human histamine H_1-receptor (Figures 7a, b and 8). However, 24 h treatment with 1 μM PDBu did not attenuate to any great extent the internalization of the H_1-receptor induced by histamine (0.1 mM; 30 min; Figure 7c and d). Furthermore, incubation of Myc-GFP CHO-H_1 cells with the PKCα inhibitor Go 6976 (3 μM) was also without effect on histamine-induced H_1-receptor internalization (Figure 8).

Effect of hypertonic sucrose and filipin

In order to shed light on the pathway used for Myc-GFP H_1-receptor internalization, the effect of hypertonic sucrose (which inhibits clathrin-mediated internalization; Heuser & Anderson, 1989) and filipin (which inhibits lipid raft- and caveolae-mediated internalization; Orlandi & Fishman, 1998) on histamine-induced H_1-receptor internalization was investigated. Filipin has been reported to be toxic to some cell lines at concentrations commonly used to inhibit caveolae-mediated internalization (Gines *et al*., 2001). However, the concentration of filipin (1 μg ml^{-1}) used in the present study did not significantly effect histamine-stimulated inositol phosphate accumulation (Figure 9; similar data obtained in three separate experiments).

In the present study, preincubations of cells with sucrose (0.32 M) did not prevent histamine-induced internalization of Myc-GFP H_1-receptor (Figure 10a and b). In contrast, preincubation of cells with filipin (1 μg ml^{-1}, 30 min) completely inhibited the histamine-induced internalization of the Myc-GFP H_1-receptor (Figure 10c and d). To confirm that filipin was acting to disrupt lipid rafts and caveolae and not inhibiting internalization *per se*, we studied the effect of filipin pretreatment on the internalization of transferrin, which is known to be *via* clathrin-mediated endocytosis (Kallal *et al*., 1998). As a control, we also investigated the effect of various treatments on the internalization of CTB, which is also known to enter cells *via* lipid rafts (Orlandi & Fishman, 1998). Pretreatment of cells with filipin prevented the entry of CTB labelled with Alexa-Fluor 647 (1 μg ml^{-1}, 40 min), while cells pretreated with sucrose (0.32 M) did not ($n = 4$; data not shown). Transferrin-labelled with Alexa-Fluor 633 (200 μg ml^{-1}) was internalized following 30 min incubation at 37°C in cells expressing Myc-GFP hH_1R (Figure 10e and f). In contrast to the results with histamine, pretreatment of cells with sucrose (0.32 M) did prevent transferrin Alexa-Fluor 633 transferrin (200 μg ml^{-1}) internalization, whereas filipin (1 μg ml^{-1}, 30 min) did not (Figure 10g and h). Finally, cell viability following filipin treatment was further confirmed by preincubating cells with filipin (1 μg ml^{-1}) and sucrose (0.32 M) and then adding transferrin Alexa-Fluor 633 (200 μg ml^{-1}). Under these conditions, transferrin Alexa-Fluor 633 (200 μg ml^{-1}) internalization was inhibited (Figure 10i and j). Pretreatment of cells with nystatin (50 μg ml^{-1}; 30 min), which also disrupts lipid rafts (Ushio-Fukai *et al*., 2001), also prevented the internalization of Myc-GFP H_1-receptors (Figure 11b). Finally, filipin (1 μg ml^{-1}) prevented internaliza-

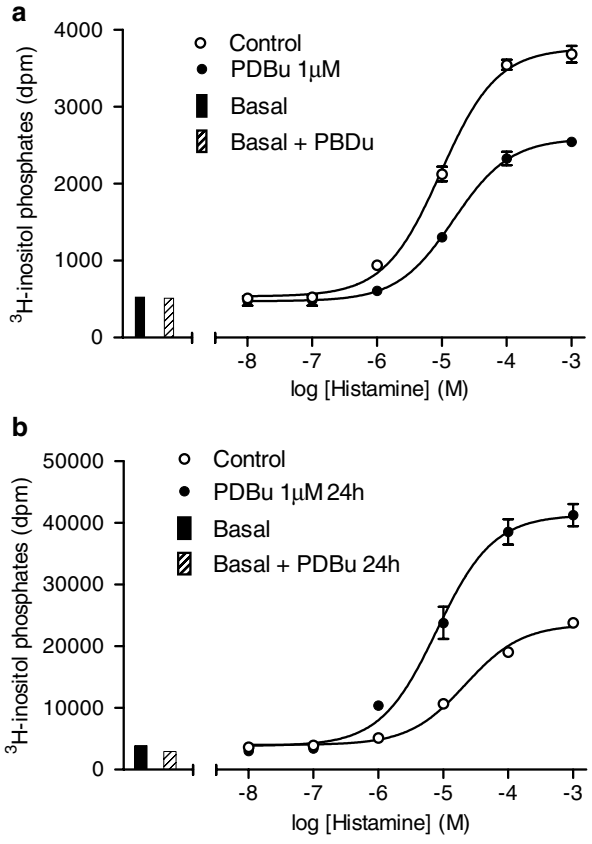

Figure 6 Influence of (a) PKC activation (1 μM PDBu added 30 min prior to histamine) and (b) PKC downregulation (1 μM PDBu; 24 h pretreatment) on histamine-stimulated ^{3}H-inositol phosphate accumulation. Values are mean ± s.e.m. of triplicate determinations in a single experiment. Similar data were obtained in two further experiments for both (a) and (b). Mean data from all three experiments (a, b) are provided in the text.

tion of cell surface Myc-H_1-receptors (Figure 11a). However, it is noticeable that the images in the presence of filipin are not as clear and this probably reflects difficulties in fixing cells in the presence of this cholesterol binding agent.

Interaction between the Myc-GFP H_1-receptor and caveolin I

To investigate further the role of caveolae in trafficking of the Myc-GFP H_1R, we monitored the cellular location of caveolin 1 prior to, and following, histamine-induced internalization of the human histamine H_1-receptor. Some colocalization between caveolin I and the Myc-GFP H_1-receptor was apparent on the cell surface under basal conditions. However, following histamine stimulation (0.1 mM, 30 min; leading to internalization of the histamine H_1-receptor), no colocalization of caveolin I and the Myc-GFP H_1R was observed in intracellular perinuclear focal spots (Figure 12a). Colocalization of the Myc-GFP H_1R with the lipid raft marker CTB was also apparent under basal conditions when cells were incubated with CTB on ice for 15 min and then imaged at room temperature (Figure 12b). High magnification showed good colocalization between the Myc-GFP H_1R and CTB (Figure 12b).

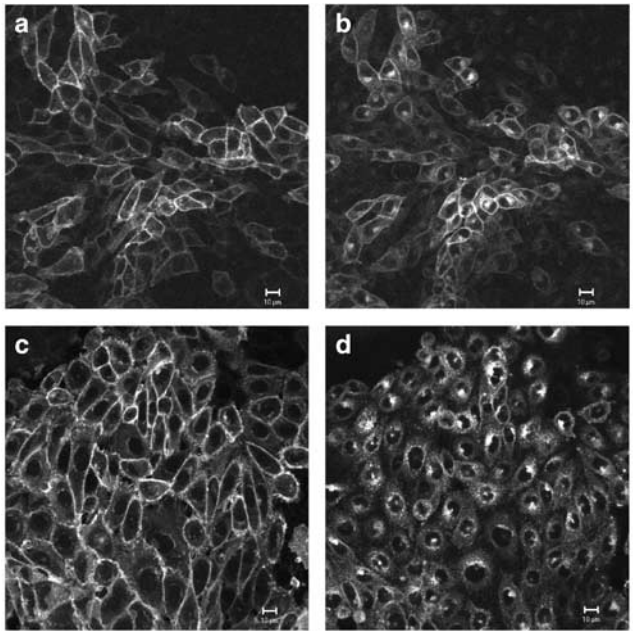

Figure 7 Effect of PdBu (1 μM) on basal and histamine-induced internalization of the histamine H_1-receptor. Live cell imaging of Myc-GFP hH_1R cells under basal conditions (a) and after 30 min incubation with 1 μM PdBu (b). In (c, d), cells were pretreated with PdBu (1 μM) for 24 h prior to monitoring basal (c) and histamine-stimulated (0.1 mM; 30 min; d) internalization of the Myc-GFP hH_1R. Confocal microscopy was performed at 37°C using the 488 nm excitation line of an argon laser and a 505 nm long-pass emission filter. Similar results were obtained in five separate experiments. Scale bars 10 μm.

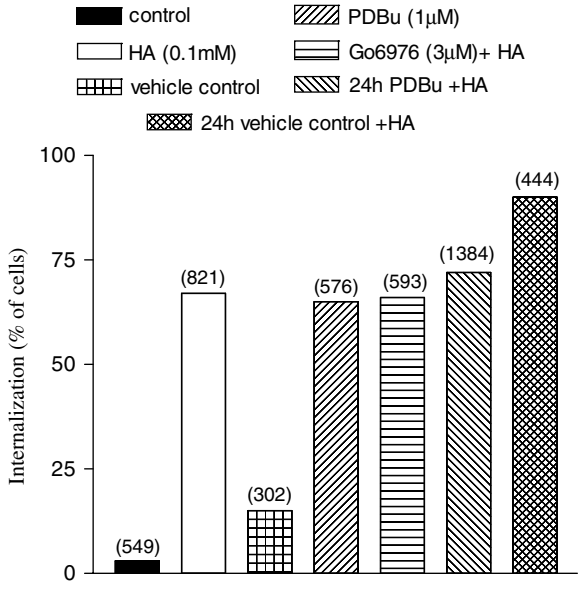

Figure 8 Quantitation of the percentage of cells in the field of view showing evidence of H_1-receptor internalization following different treatments. All evaluations of the fields of view were carried out blind. The total number of cells evaluated for each condition is shown in parenthesis above each bar. Vehicle controls represent 0.01% DMSO and are equivalent to the DMSO concentration present in the 1 μM PDBu samples. For each condition, the number of separate experiments performed and the number of separate coverslips analysed were: control (4, 6); HA (3, 3); vehicle control (5, 5); PDBu (4, 6); Go 6976 + HA (2, 3); 24 h PdBu + HA (4, 6) and 24 h vehicle control + HA (2, 3), respectively.

Discussion

In the present study, we have shown that the human histamine H_1-receptor can be internalized rapidly in CHO-K1 cells following stimulation with histamine. This response occurred within 15 min, and could be prevented by pretreatment with the quaternary H_1-receptor antagonist α-pirdonium.

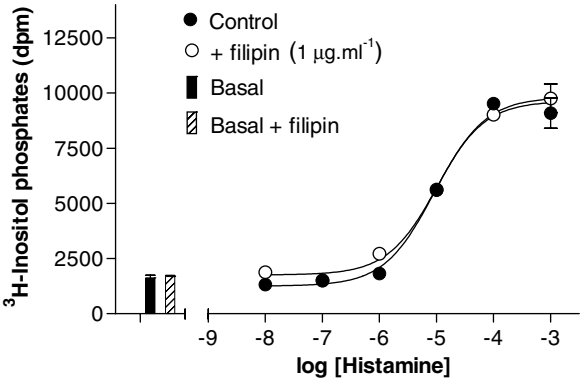

Figure 9 Influence of filipin (1 μg ml^{-1}) on histamine-stimulated ^{3}H-inositol phosphate accumulation. Concentration–response curves to histamine in the presence or absence of 1 μg ml^{-1} filipin (added 30 min prior to histamine). Values are mean ± s.e.m. of triplicate determinations in a single experiment. Similar data were obtained in three further experiments.

Similar data were obtained whether a histamine H_1-receptor containing a 5′ Myc tag or an H_1-receptor with a GFP molecule attached to the C-terminus of the 5′ Myc tag H_1-receptor variant was used. These data suggest that the C-terminal GFP moiety does not interfere with the internalization process. Similarly, the pharmacological properties of the two tagged H_1-receptor constructs were very similar to those of the wild-type receptor in terms of ^{3}H-mepyramine binding and histamine-stimulated ^{3}H-inositol phosphate responses.

Using real-time live cell imaging of the GFP attached to the C-terminus of the Myc-GFP H_1-receptor, the Myc-GFP H_1-receptor was shown to translocate from the cell surface to large intracellular perinuclear focal spots in response to histamine. The appearance of Myc-GFP H_1-receptors in these intracellular focal spots was still apparent when cells were pretreated with cycloheximide, indicating that this was not a process that was dependent upon *de novo* synthesis of the H_1-receptor. The H_1-receptor was able to colocalise to these perinuclear focal spots along with BODIPY ceramide (when applied simultaneously with histamine), which suggests that the receptor was targeted to the Golgi apparatus (Pagano *et al*., 1991). To ensure that the H_1-receptor present in the Golgi after treatment with histamine had originated from the plasma membrane, we made use of the N-terminal Myc tag on the receptor to attach a primary antibody to cell surface H_1-receptors in living cells prior to histamine administration. Following histamine stimulation, H_1-receptors were lost from

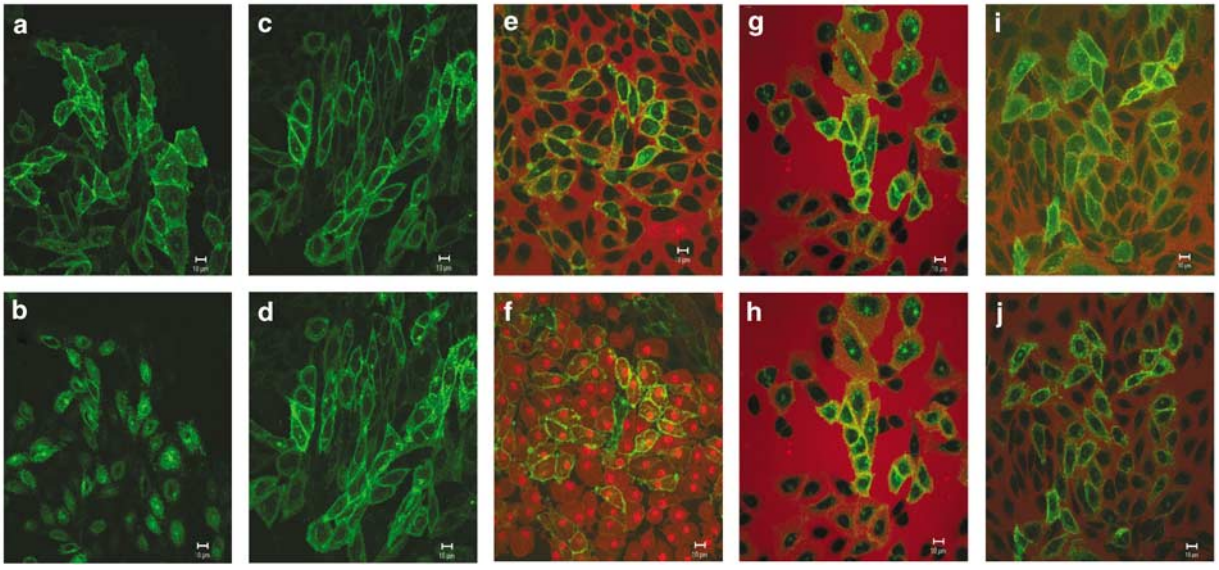

Figure 10 Influence of hypertonic sucrose and filipin on histamine-stimulated internalization of the Myc-GFP hH_1R. (a, b) Myc-GFP hH_1R cells were preincubated with 0.32 M sucrose (for 30 min to prevent clathrin-mediated endocytosis of the hH1R) prior to measurement of (a) basal (at time 0 min) or (b) histamine-stimulated (0.1 mM; 30 min) hH_1R internalization. (c, d) Myc-GFP hH_1R cells were pretreated with the sterol binding agent filipin (1 μg ml^{-1}; 30 min) to inhibit caveolae-lipid raft-mediated endocytosis prior to measurement of (c) basal (at time 0 min) or (d) histamine-stimulated (0.1 mM; 30 min) hH_1R internalization. In (e, f), Myc-GFP hH_1R cells were preincubated with filipin (1 μg ml^{-1}; 30 min) prior to the addition of transferrin Alexa-Fluor 633 nm (200 μg ml^{-1}) and images captured immediately (e) or following 40 min in the continued presence of transferrin (f). In (g, h), cells were treated with 0.32 M sucrose for 30 min prior to the addition of transferrin Alexa-Fluor 633 nm (200 μg ml^{-1}) and images taken immediately (g) or 40 min later (h). In (i, j), cells were treated with both 0.32 M sucrose and filipin (1 μg ml^{-1}) for 30 min prior to the addition of transferrin Alexa-Fluor 633 nm (200 μg ml^{-1}) and images taken immediately (i) or 40 min later (j). In (a–d), confocal microscopy was performed at 37°C using the 488 nm excitation line of an argon laser and a 505 nm long-pass emission filter. In (e–j), cells were imaged at 37°C with both 488 nm (green; band-pass emission filter 505–530 nm) and 633 nm (red; 650 long-pass emission filter) Argon and HeNe lasers using multitracking. Similar data were obtained in three (a–d, i, j) and four (e–h) separate experiments. Scale bars 10 μm.

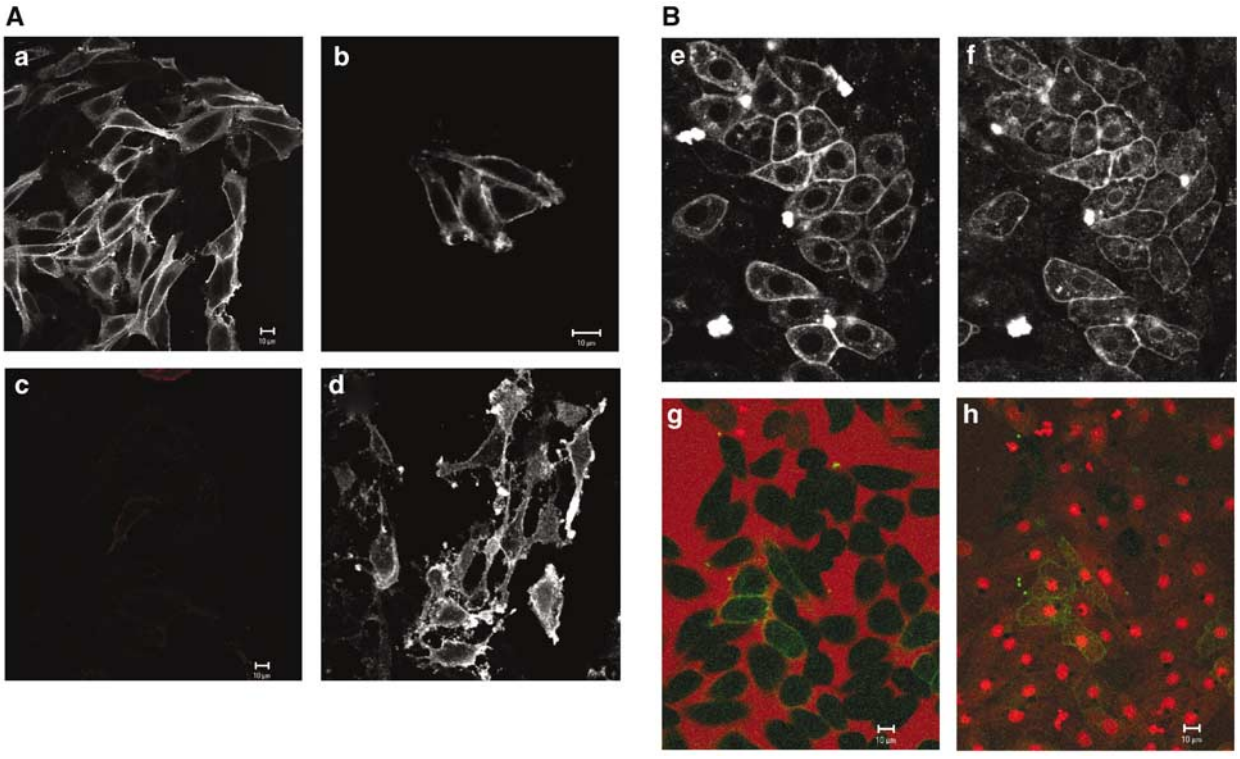

Figure 11 Influence of filipin and nystatin on histamine-induced internalization of the H_1-receptor. (A) Effect of filipin (1 μg ml^{-1} 30 min pretreatment at 37°C) on the internalization of Myc-hH_1Rs. Immunocytochemical location of the myc-tagged hH_1R expressed in nonpermeabilized CHO cells under basal conditions (a, b) and (c, d) following 30 min stimulation with histamine (0.1 mM). In (b, d), the cells were pretreated with Filipin (1 μg ml^{-1}) 30 min prior to the addition of histamine. Cells were fixed in 2% paraformaldehyde and processed for immmunocytochemistry as described in Methods. Cells were then labelled with mouse monoclonal anti-myc antibodies followed by goat anti-mouse Rhodamine Red X antibodies. Confocal microscopy was performed using 543 nm excitation with the HeNe laser and a 560 nm long-pass emission filter. Similar data were obtained in three separate experiments. Scale bars 10 μm. (B) Effect of nystatin (50 μg ml^{-1}; 30 min pretreatment) on the internalization of the Myc-GFP hH_1R. Image e shows control cells at time zero and image f the control response to histamine (0.1 mM; 30 min) following pretreatment with nystatin. In (g, h), Myc-GFP hH_1R cells were preincubated with nystatin (50 μg ml^{-1}; 30 min) prior to the addition of transferrin Alexa-Fluor 633 nm (200 μg ml^{-1}) and images captured immediately (g) or following 40 min in the continued presence of transferrin (h). In (e, f), confocal microscopy was performed at 37°C using the 488 nm excitation line of an argon laser and a 505 nm long-pass emission filter. In (g, h), cells were imaged at 37°C with both 488 nm (green; band-pass emission filter 505–530 nm) and 633 nm (red; 650 long-pass emission filter) argon and HeNe lasers using multitracking. Similar data were obtained in three separate experiments. Scale bars 10 μm.

the cell surface and localized to focal perinuclear spots inside the cells. Interestingly, when the histamine was washed from the cells, the internalized receptor reappeared on the cell membrane within 30 min. These data suggest that the H_1-receptor can cycle rapidly between the cell surface and the Golgi apparatus.

GPCRs can be internalized by one of two major mechanisms: One is triggered by phosphorylation of the receptor by GRKs and the subsequent binding of β-arrestins (Lohse *et al*., 1990; Pitcher *et al*., 1998; Seibold *et al*., 2000; Luttrell & Lefkowitz 2002). These then target the receptor to clathrin-coated pits for subsequent internalization (Krupnick & Benovic, 1998; Tsao *et al*., 2001; Luttrell & Lefkowitz 2002; Ahn *et al*., 2003; von Zastrow, 2003). Alternatively, some GPCRs can be internalized *via* caveolae (Haasemann *et al*., 1998; Gines *et al*., 2001; Mueller *et al*., 2002). The mechanisms underlying this latter process are largely unknown, but it may be that phosphorylation by other protein kinases provide the molecular address for targeting the receptors for internalization *via* caveolae (Rapacciuolo *et al*., 2003). For example, in the case of the β_1-adrenoceptor, phosphorylation by protein kinase A can lead to receptor internalization *via* caveolae (Rapacciuolo *et al*., 2003).

In the present study, strong evidence is provided for a role for lipid rafts or caveolae in the internalization of the histamine H_1-receptor. Hypertonic sucrose did not prevent histamine-induced internalization of the Myc-GFP H_1-receptor, but was able to attenuate internalization of transferrin, which involves clathrin-mediated endocytosis (Kallal *et al*., 1998). In contrast, preincubation of cells with filipin, which disrupts caveolae and lipid rafts (Orlandi & Fishman, 1998), completely inhibited the histamine-induced internalization of the Myc-GFP H_1-receptor, but was without effect on the sequestration of transferrin. These data provide strong evidence that the H_1-receptor is internalized *via* a clathrin-independent mechanism and most likely involves lipid rafts or caveolae. In keeping with this hypothesis, the internalization of CTB in these CHO cells, which is known to enter cells *via* lipid rafts (Orlandi & Fishman, 1998), was similarly affected by filipin. Furthermore, the H_1-receptor and

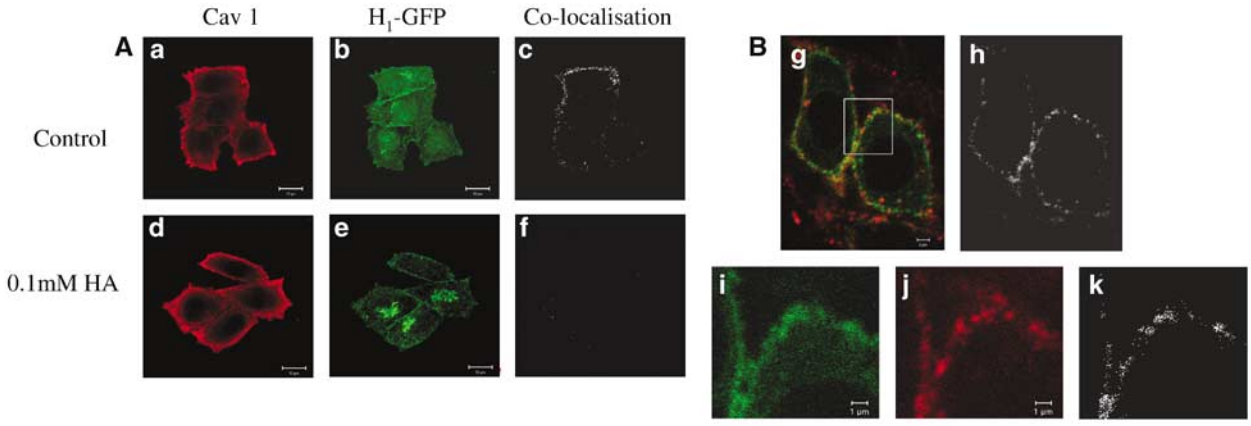

Figure 12 Colocalization of the histamine H_1-receptor (Myc-GFP hH_1R) with (A) caveolin-1 or (B) the lipid raft marker CTB. (A) Localization of caveolin-1 (Cav-1) (a, d) and Myc-GFP hH_1R (b, e) under basal conditions (a, b) and following 30 min stimulation with histamine (0.1 mM; d, e). Colocalized pixels are detailed in (c, f). Permeabilized cells were labelled with mouse anti-caveolin-1 and rabbit anti-GFP primary antibodies and then goat anti-mouse Rhodamine Red X and goat anti-rabbit Alexa-Fluor 488 nm secondary antibodies. Confocal microscopy was performed using multitracking with 488 nm excitation line of an argon laser and a 505–530 nm band-pass filter and 543 nm excitation of an HeNe laser and a 560 nm long-pass filter. Similar data were obtained in four further experiments. Scale bars 10 μm. (B) Overlay of the lipid raft marker CTB-Alexa Fluor 647 (10 $\mu g\,ml^{-1}$; 15 min) (red) and Myc-GFP hH_1R (green) within the cell membrane of living cells under control conditions (g). The colocalized pixels are shown in panel h. i, j and k show the show higher magnification images of the region identified by the white box in g for the green (i; Myc-GFP hH_1R) and red (j; CTB) channels and the colocalised pixels (k). The data shown are representative of three separate experiments. Scale bars are 2 μm (g, h) and 1 μm (i, j, k). Confocal microscopy was performed using multitracking with 488 nm excitation line of an argon laser and a 505–530 nm band-pass filter (for the Myc-GFP hH_1R) and 633 nm excitation of an HeNe laser and a 650 nm long-pass filter (for CTB).

CTB were colocalized at the cell surface. Immunohistochemical studies with an antibody to caveolin-1 confirmed that this protein was also localized predominantly to the plasma membrane. However, following stimulation of CHO-Myc-GFP H_1 cells with histamine, there was no evidence for internalization of caveolin-1 in parallel with the H_1-receptor. These data suggest that the H_1-receptor is internalized *via* lipid rafts rather than caveolae.

The exact stimulus required to initiate H_1-receptor internalization remains to be established. Histamine-induced desensitization of neuronal mouse H_1-receptors has previously been reported to be dependent on extracellular calcium and mediated through activation of CAM kinase II (Zamani & Bristow, 1996). However, in the present study, internalization of the Myc-GFP H_1-receptor was maintained in the absence of extracellular calcium and was not inhibited by the CAM kinase II inhibitor KN-62. Activation of PKC has been previously shown to cause a desensitization of H_1-receptors (Smit *et al*., 1992; Dickenson & Hill, 1993; McCreath *et al*., 1994), and PKC phosphorylation sites have been identified within the H_1-receptor sequence (Fujimoto *et al*., 1999). In addition, we have previously shown that histamine can stimulate translocation of PKCα to the plasma membrane in CHO-K1 cells expressing the H_1-receptor (Megson *et al*., 2001). In keeping with this, PDBu was able to produce a rapid internalization of the Myc-GFP histamine H_1-receptor in CHO cells and to attenuate agonist-stimulated ^{3}H-inositol phosphate accumulation. However, the PKC inhibitor Go 6976 was not able to attenuate the histamine-stimulated H_1-receptor internalization. Furthermore, 24 h treatment with PDBu, which causes a marked downregulation of PKCα, δ and ε in CHO cells (Megson *et al*., 2001), was similarly unable to influence the histamine-stimulated internalization process despite the fact that agonist-induced ^{3}H-inositol phosphate responses was markedly enhanced. This latter effect is presumably due to downregulation of a PKC-mediated negative feedback as a consequence of diacyglycerol generation in response to histamine-stimulated phospholipase C activity. Thus, removal of this negative PKC-mediated regulation of the histamine response leads to a markedly enhanced agonist response after downregulation of PKCα, δ and ε. Taken together, these data suggest that the H_1-receptor can be internalized and desensitized as a consequence of PKC activation. However, the fact that histamine-stimulated H_1-receptor internalization can still take place following downregulation of PKC α, δ and ε or in the presence of PKC inhibitors strongly suggests that other molecular mechanisms must be involved.

In conclusion, the present study has shown that histamine can stimulate a rapid internalization of the human H_1-receptor that appears to be independent of clathrin-mediated endocytosis. Desensitization of histamine H_1-receptor-mediated ^{3}H-inositol phosphate responses following agonist stimulation appears to involve, at least in part, activation of PKC. However, the internalization of the H_1-receptor is not mediated *via* this protein kinase. It remains to be established whether this internalization mechanism depends upon receptor phosphorylation and, if so, which protein kinase is involved.

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