Full Paper

Identification of Amino Acid Residues Responsible for Agonist-Induced Down-Regulation of Histamine H₁ Receptors

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Abstract. The histamine H₁ receptor (H1R) level is dynamically regulated in vivo under various physiological and pathological conditions. The H1R regulation may consist of various processes, and this study focused on the process of receptor trafficking, that is, receptor internalization to endosomes and the following receptor degradation. First, we identified five possible phosphorylation residues of human H1R, Thr¹⁴⁰, Thr¹⁴², Ser³⁹⁶, Ser³⁹⁸, and Thr⁴⁷⁸, based on in vitro phosphorylation studies. Then to determine the role of these residues, we constructed a mutant H1R in which all of these five residues were substituted with alanine. Both wild-type and the mutant receptors expressed in Chinese hamster ovary (CHO) cells had similar values of K_d for [³H]mepyramine binding and K_i for histamine, and these cells showed similar levels of histamine-stimulated inositol phosphate formation. Both types of H1Rs were internalized essentially in the same way upon stimulation with histamine (100 μM) for 30 min. However, down-regulation of the mutant H1R was completely impaired, whereas that of wild-type H1R occurred by approximately 60% by the treatment with 100 μM histamine for 24 h. These results suggest that these residues are responsible for receptor down-regulation but not for receptor internalization. Possibly, phosphorylation of the residues is required for receptor transport from endosomes to lysosomes.

Keywords: desensitization, internalization, down-regulation, phosphorylation, histamine H₁ receptor

Introduction

The histamine H₁ receptor (H1R) level is dynamically regulated in vivo under various physiological and pathological conditions: up-regulation of H1Rs in the electrical foci in the temporal cortex of epileptic patients (1), up-regulation of the H1R mRNA in the nasal mucosa of patients with allergic rhinitis (2, 3), down-regulation of H1Rs in the frontal cortex of patients with chronic schizophrenia (4), down-regulation of them in the frontal and temporal areas of patients with Alzheimer's disease (5), and a decrease in the H1R level in the brain upon aging (6). These examples indicate that H1R expression is susceptible to various regulatory

changes and that the H1R level may be closely related to certain pathological conditions. Therefore, it is worthwhile to elucidate the mechanism underlying the regulation of H1R level.

The H1R regulation may consist of various processes just as indicated in the regulation of other types of G protein-coupled receptors (GPCRs); for example, modulation of receptor gene transcription (7), alteration of mRNA stability (8), and modulation of receptor degradation (9, 10). Stimulation of GPCR leads to intracellular signaling, but it generally induces desensitization or uncoupling of the receptor from the effector system, and thereafter leads to internalization of the receptor to the intracellular membrane. The internalized receptor may be recycled to the cell surface, but continued stimulation of the receptor leads to down-

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regulation or degradation of the receptor (11–13). Thus the processes of receptor internalization and down-regulation take important roles in regulating the expression level of the receptor.

Stimulation with agonist often induces phosphorylation of GPCR, and there is much evidence indicating that phosphorylation of the receptor is involved in receptor internalization and down-regulation; for example, in the β_2 -adrenergic receptor (14), muscarinic m1-receptor (15), and muscarinic m2-receptor (16). Although there have been no such convincing evidence for H1R, it is probable that phosphorylation of H1R is involved in these processes.

As the first step to clarify this point, now we explored possible phosphorylation sites of H1R based on *in vitro* phosphorylation studies. We used a series of synthetic peptides that corresponded to the intracellular domain of human H1R and identified five phosphorylation sites: Thr¹⁴⁰, Thr¹⁴², Ser³⁹⁶, Ser³⁹⁸, and Thr⁴⁷⁸. Then to determine the role of these residues, we constructed a mutant H1R in which all of these five residues were substituted with alanine residues. This mutation did not affect the histamine response of H1R nor H1R internalization, but it completely impaired H1R down-regulation. Thus these residues may be involved in H1R down-regulation and this mutant H1R will provide a useful tool for further investigating the process of H1R down-regulation.

Materials and Methods

Materials

[³H]Mepyramine ([pyridinyl-5-³H]pyrilamine, 0.74 TBq/mmol) and [γ -³²P]ATP (111 TBq/mmol) were purchased from NEN Life Science Products (Boston, MA, USA). *Myo*-[³H]inositol (3.40 TBq/mmol) was from Amersham (Buckinghamshire, UK). Restriction enzymes were from Takara Biochemicals (Kyoto). All synthetic peptides were obtained from Chiron Mimotopes Pty., Ltd. (Clayton, Victoria, Australia). Protein kinase C (PKC, mixture of α , β , and γ isoforms) was kindly donated by Dr. U. Kikkawa (Kobe University); calcium/calmodulin-dependent protein kinase II (CaMK II) was from Dr. E. Miyamoto (Kumamoto University); and G protein-coupled receptor kinase-2 (GRK2) was a generous gift of Dr. T. Haga (University of Tokyo). Cyclic AMP-dependent protein kinase catalytic subunit (PKA) and cGMP-dependent protein kinase (PKG) were obtained from Promega (San Luis Obispo, CA, USA). All other reagents, unless otherwise stated, were of analytical grade and were from Wako Pure Chemicals (Osaka) or Sigma (St. Louis, MO, USA).

In vitro phosphorylation assay of synthetic peptides

Synthetic peptides were phosphorylated by PKA (100 units enzyme) in reaction mixtures containing 25 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 0.2 mM ATP, 2 mM EGTA, and 20 μ M [γ -³²P]ATP; by PKC (0.4 ng/ml) in reaction mixtures containing 25 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 0.2 mM ATP, 0.4 mM CaCl₂, 8 μ g/ml phosphatidylserine, 0.8 μ g/ml 1,2-diolein, and 20 μ M [γ -³²P]ATP; by PKG (100 units enzyme) in reaction mixtures containing 25 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 0.2 mM ATP, 2 mM EGTA, and 20 μ M [γ -³²P]ATP; by GRK2 (20 nM) in reaction mixtures containing 25 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 10 μ M ATP, 2 mM EDTA, 0.5 mM EGTA, 6.6 nM G-protein $\beta\gamma$, and 20 μ M [γ -³²P]ATP; or phosphorylated by CaMK II (0.75 μ M) in reaction mixtures containing 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.5 mM ATP, 0.2 mM CaCl₂, and 20 μ M [γ -³²P]ATP. The reaction was initiated in a volume of 50 μ l by the addition of 1 mg of the desired peptide. After incubation for 30 min at 30°C, the reaction was terminated by the addition of Laemmli sodium dodecyl sulfate (SDS) sample buffer containing 50 mM dithiothreitol and was heated for 5 min at 90°C. The suspension was subjected to SDS-polyacrylamide gel electrophoresis using 15% to 20% acrylamide gels (Daiichi Pure Chemicals, Tokyo). The extent of phosphorylation was analyzed by autoradiography and quantitated on a BAS-2000II (Fuji Photo Film, Tokyo).

Plasmid construction

The BclI fragment (1.8 kilobase pairs) of the human H1R gene was subcloned into the M13 mp19 phage at the EcoRI site by using EcoRI-NotI-BamHI adaptors (Takara Biochemicals). Site-directed mutagenesis was performed with an Oligonucleotide-directed *In Vitro* Mutagenesis System (Amersham) and long-range polymerase chain reaction (PCR) system (17). The nucleotide sequences of the mutated H1R genes were confirmed by the dideoxynucleotide method. Two serine residues, Ser³⁹⁶ and Ser³⁹⁸, and three threonine residues, Thr¹⁴⁰, Thr¹⁴², and Thr⁴⁷⁸, were substituted with alanine residues, forming the mutant receptor designated 5MT. Each coding region of wild-type and mutant H1R genes was subcloned into a mammalian expression vector, pDKCR-dhfr, for stable expression in Chinese hamster ovary (CHO) cells.

Construction of stable CHO transformants

CHO cells that were deficient in dihydrofolate reductase were transfected with the plasmid constructs using the calcium phosphate precipitation method (18). The cells were cultured in α -minimum essential medium

without ribonucleosides and deoxyribonucleosides (Life Technologies, Rockville, MD, USA) supplemented with 10% dialyzed fetal calf serum. After 2 weeks of incubation, individual colonies were transferred to new plates, and the culturing was continued. Then, these cells were screened for expression of the H1R using a [3 H]mepyramine binding assay.

Radioligand binding assay

The [3 H]mepyramine binding assay was performed as described previously (19). A suspension of cell membranes (150–300 μ g of protein) was incubated with [3 H]mepyramine in the absence (total binding) or presence (nonspecific binding) of 10 μ M triprolidine in 50 mM sodium-potassium phosphate buffer (pH 7.4) at 25°C for 60 min in a final volume of 600 μ l. The membrane-bound radioligands were separated from free radioligands by rapid filtration through a Whatman GF/B glass fiber filter (Whatman, Maidstone, UK). The filter was placed in 10 ml of Aquasol II (Packard Instrument, Inc., Meriden, CT, USA), and then the radioactivity on the filter was counted in a liquid scintillation counter.

Measurement of inositol phosphates (IPs)

Measurement of IPs was carried out as described previously (20). CHO cells expressing wild-type or mutant H1R in 6-well culture plates were labeled with *myo*-[3 H]inositol in inositol-free Dulbecco's modified Eagle's medium (Nissui, Tokyo) supplemented with 10% dialyzed fetal calf serum for 24 h. The cells were washed twice and incubated for 10 min at 37°C with HEPES-buffered saline solution (125 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 15 mM NaHCO₃, 11 mM glucose, and 15 mM HEPES, pH 7.4) containing 10 mM LiCl. The reaction was started by the addition of histamine (0.01–100 μ M) in a final volume of 1 ml. After appropriate times of incubation at 37°C, the medium was aspirated and the reaction was terminated by the addition of 5% trichloroacetic acid. Total [3 H]IPs was isolated by anion exchange chromatography using AG1-X8, 100-200 mesh (Bio-Rad, Hercules, CA, USA), and radioactivities were determined in a liquid scintillation counter using Aquasol II. The results were presented as increase in IPs accumulation above the basal levels.

Receptor internalization assay

Agonist-induced H1R internalization was measured using the sucrose density gradient centrifugation method (21, 22). Semiconfluent CHO cells were treated with 100 μ M histamine for 0, 15, 30, or 60 min at 37°C. Then cells were washed three times with ice-cold

phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.5) and then incubated with serum-free α -minimum essential medium containing 200 μ g/ml concanavalin A (Sigma) for 20 min on ice, washed once with lysis buffer (1 mM Tris, 2 mM EDTA, pH 7.4), and hypotonically lysed by incubation in the same buffer for 20 min on ice. Then cells were scraped into a small volume of the lysis buffer (approximately 1 ml). The lysate was layered on top of a stepwise sucrose gradient consisting of a cushion of 55% sucrose (1.5 ml) and 30% sucrose (4.0 ml) buffered with 2 mM Tris-Cl (pH 8.0) and centrifuged at 150,000 $\times$ g for 90 min. Each fraction of 0.5 ml was collected from the bottom and the number of H1R was determined by radioligand binding assay with a nearly saturating concentration of [3 H]mepyramine (5 nM).

Receptor down-regulation assay

Nearly confluent cells were incubated at 37°C for the indicated periods of time in the α -minimum essential medium described above with or without histamine (100 μ M). Following the incubation, the cells were washed three times with ice-cold 50 mM sodium-potassium phosphate buffer (pH 7.4) and were then scraped into a small volume of the same buffer. The cells were next homogenized with an ultrasonic disruptor (Tomy Seiko Co., Ltd., Tokyo) and subsequently centrifuged at 50,000 $\times$ g for 30 min. The pelleted membranes were resuspended in the same buffer, and radioligand binding was performed as described above. Down-regulation was defined as the decrease in specific [3 H]mepyramine binding due to exposure of the cells to the desensitizing agent (23). Changes in H1R density were expressed as a percentage of [3 H]mepyramine binding of the histamine-treated cells to that of non-treated cells.

Results

In vitro phosphorylation of synthetic peptides corresponding to partial sequences of the intracellular domain of human H1R

As we have shown previously, human H1R can be phosphorylated in vitro by several kinases including PKA, PKC, PKG, and CaMK II (24). Threonine and serine residues that are present in the intracellular domain of human H1Rs (25–27) can be potential phosphorylation sites of these kinases. Thus to determine which amino acids can be phosphorylated, we synthesized ten peptides (P1 through P10) corresponding to partial sequences of the intracellular domain of the human H1R (Fig. 1). We then performed in vitro

[1st IL]
YAVRSERKLHTVGN 63
 P1

[2nd IL]
CIDRYRSVQQPLRYLKVRTKTRAS 145
 P2

[3rd IL]
YAKIYKAVRQHCQHRELINRSLPSFSEIKLRPENPKGDAKKPGKESPWEVLK 261
 P3 P4
RKPKDAGGGSVLKSPSQTPKEMKSPVVFSQEDDREVDKLYCFPLDIVHMQAA 313
 P5
AEGSSRDYVAVNRSHGQLKTDEQGLNTHGASEISEDQMLGDSQSFSRTDSDT 365
 P6 P7
TTETAPGKGKLRSGSNTGLDIYIKFTWKRLRSHSRQYVSGLHMNRERKAAKQLG 418
 P8 P9

[C terminus]
CNENFKKTFKRLHRS 487
 P10

Fig. 1. The synthetic peptides used in the phosphorylation study. Amino acid sequences of intracellular domain of human H₁R are shown for the first (1st IL), second (2nd IL), and third (3rd IL) intracellular loops and the carboxyl terminus (C terminus). The synthetic peptides, P1 through P10, are underlined or boxed. The amino acid numbers in the sequence are shown to the right of the sequence.

Fig. 2. Phosphorylation of the synthetic peptides. Synthetic peptides P1 through P10 were phosphorylated by the kinases, PKA, PKC, PKG, CaMK II, or GRK2 in the presence of 20 μ M [γ -³²P]ATP for 30 min at 30°C. Then the reaction products were separated by SDS-PAGE and visualized by autoradiography (numbers 1 through 10 corresponding to P1 through P10). Estimates for the extent of phosphorylation of each peptide are shown by – (not phosphorylated) or + (slightly phosphorylated) to +++ (highly phosphorylated).

phosphorylation experiments using these peptides as substrates for the kinases PKA, PKC, PKG, CaMK II, and GRK2. The results are shown in Fig. 2. The peptide

P1 was not phosphorylated at all by any of the tested kinases. P2 was phosphorylated slightly by PKC and significantly by CaMK II. P3 was phosphorylated significantly by CaMK II. The peptides P4 to P7 were not phosphorylated at all. P8 was phosphorylated slightly by PKA, PKC, PKG, CaMK II, and GRK2. P9 was phosphorylated significantly by PKA, PKC, PKG, CaMK II, and GRK2. P10 was phosphorylated significantly by PKC and slightly by CaMK II and GRK2. In summary, P2, P3, P9, and P10 were phosphorylated significantly, indicating that these peptides contained residues to be phosphorylated.

Then to determine the residues that can be phosphorylated, we synthesized mutant peptides in which Thr¹⁴⁰ and/or Thr¹⁴² of the peptide P2, Ser³⁹⁶ and/or Ser³⁹⁸ of the peptide P9, and Thr⁴⁷⁸ of the peptide P10 were substituted with alanines. The results are summarized in Table 1. Both peptides P2-T140A (Thr¹⁴⁰ to alanine) and P2-T142A (Thr¹⁴² to alanine) were phosphorylated by CaMK II, but the extent of phosphorylation was small compared to the wild-type peptide P2. The peptide P2-T140A/T142A (both Thr¹⁴⁰ and Thr¹⁴² to alanines) was phosphorylated only slightly. Thus, both Thr¹⁴⁰ and Thr¹⁴² can be phosphorylated by CaMK II. Similarly, both peptides, P9-S396A (Ser³⁹⁶ to alanine) and P9-S398A (Ser³⁹⁸ to alanine) were phosphorylated in a smaller extent compared to the wild-type peptide P9 by CaMK II and PKC. The peptide P9-S396A/S398A (both Ser³⁹⁶ and Ser³⁹⁸ to alanines) was phosphorylated only slightly or not phosphorylated by these kinases. Thus both Ser³⁹⁶ and Ser³⁹⁸ can be phosphorylated by CaMK II and PKC. The peptide P9-S396A was phosphorylated slightly and P9-S398A was not phosphorylated by PKG. The peptide P9-S396A/S398A was also not phosphorylated by PKG. Thus both Ser³⁹⁶ and Ser³⁹⁸

Table 1. Phosphorylation of the synthetic mutant peptides

Kinase	Peptide									
	P2	P2-T140A	P2-T142A	P2-T140A/T142A	P9	P9-S396A	P9-S398A	P9-S396A/S398A	P10	P10-T478A
PKA	N.D.	N.D.	N.D.	N.D.	+++	+++	–	–	N.D.	N.D.
PKC	+	+	+	+	+++	++	++	+	+++	–
PKG	N.D.	N.D.	N.D.	N.D.	+++	+	–	–	N.D.	N.D.
CaMK II	+++	++	+	+	+++	++	++	–	N.D.	N.D.
GRK2	N.D.	N.D.	N.D.	N.D.	++	++	–	–	N.D.	N.D.

Synthetic peptides were used for in vitro phosphorylation experiments (see Materials and Methods). Estimates for the extent of phosphorylation of each peptide are shown by – (not phosphorylated) or + (slightly phosphorylated) to +++ (highly phosphorylated). N.D., not determined.

Table 2. Possible phosphorylation sites identified in the intracellular domain of human H1R and the kinases involved in the phosphorylation

Phosphorylation site	Kinase			
Thr ¹⁴⁰	CaMK II			
Thr ¹⁴²	CaMK II			
Ser ³⁹⁶	CaMK II	PKC	PKG	
Ser ³⁹⁸	CaMK II	PKC	PKG	PKA
Thr ⁴⁷⁸		PKC		GRK2

Synthetic peptides were used for in vitro phosphorylation experiments (see Materials and Methods).

can be phosphorylated by PKG. In this case, the mutation of S396A or S398A might have affected phosphorylation by PKG of the other possible phosphorylation residue (S396 or S398) leading to only slight phosphorylation in peptides P9-S396A and P9-S398A. The peptide P9-S396A was phosphorylated in similar extent to P9 by PKA and GRK2, but the peptide P9-S398A was not phosphorylated by these kinases, indicating that only Ser³⁹⁸ can be phosphorylated by PKA and GRK2. The peptide P10-T478A (Thr⁴⁷⁸ to alanine) was not phosphorylated by PKC, indicating that Thr⁴⁷⁸ can be phosphorylated by PKC. We have not yet determined which residue is phosphorylated in peptide 3. In summary, these data suggest that Thr¹⁴⁰, Thr¹⁴², Ser³⁹⁶, Ser³⁹⁸, and Thr⁴⁷⁸ can be phosphorylated by the kinases described above (Table 2). Figure 3 describes the primary structure of human H1R and the possible phosphorylation sites that were identified in this study. It is to be noted that these five residues are widely conserved among other species including mouse, rat, guinea pig, and bovine (Fig. 4), indicating that these residues may take an important role in the H1R signaling pathway.

Expression of wild-type and mutant H1Rs in CHO cells

To investigate the role of these residues in agonist-induced receptor internalization and down-regulation,

we constructed a mutant H1R (5MT H1R) in which all of these residues (Thr¹⁴⁰, Thr¹⁴², Ser³⁹⁶, Ser³⁹⁸, and Thr⁴⁷⁸) were substituted with alanines. Then the pdKCR-dhfr vectors containing wild type (WT) or the mutant (5MT) H1R gene were transfected by the calcium phosphate precipitation method into dihydrofolate reductase-deficient CHO cells. Stable transfectants were selected in the medium devoid of nucleosides, and several clonal cell lines were isolated for each type of H1R. The pharmacological properties of WT and 5MT H1Rs expressed in CHO cells are shown in Table 3. The K_d value for [³H]mepyramine of 5MT H1R was comparable to that of WT H1R. The K_i value for histamine of 5MT H1R determined from histamine inhibition of [³H]mepyramine binding was also comparable to that of WT H1R. Histamine-induced inositol phosphate production of 5MT H1R was also similar to that of WT H1R. These results indicate that 5MT H1R retained the functional properties to couple to the effector system.

Histamine-induced internalization of WT and 5MT H1Rs expressed in CHO cells

Histamine-induced H1R internalization was studied using the sucrose density gradient centrifugation method (21). CHO cells expressing WT or 5MT H1R were treated 100 μ M histamine for 15, 30, or 60 min at 37°C. Then the cells were washed, incubated with

Fig. 3. Schematic representation of the primary structure of human H1R. Possible phosphorylation sites identified in this study are shown in black. All of these five residues, Thr¹⁴⁰, Thr¹⁴², Ser³⁹⁶, Ser³⁹⁸, and Thr⁴⁷⁸, were replaced by alanines in the mutant receptor designated as 5MT H1R.

[2nd IL]				
		* *		
human	CIDRYRSVQQPLRYLKYRTKTRAS	145		
mouse	CIDRYRSVQQPLRYLRYRTKTRAS	145		
rat	CIDRYRSVQQPLRYLRYRTKTRAS	145		
guinea pig	CIDRYRSVQQPLRYLRYRTKTRAS	154		
bovine	CIDRYRSVQQPLKYLRYRNKTRAS	146		
[3rd IL]				
		* *		
human	NTGLDYIKFTWKRLRSHSRQYVSGLHMRERKA	413		
mouse	NSGLDYIKFTWKRLRSHSRQYVSGLHLNRERKA	414		
rat	NSGLDYIKFTWKRLRSHSRQYVSGLHLNRERKA	412		
guinea pig	NSGLDYIKFTWRRLRSHSRQYTSGLHLNRERKA	414		
bovine	STGLEIYIKFTWKRLRSHSRQYVSGLHMRERKA	417		
[C terminus]				
		*		
human	CNENFKKTFKRLHIRS	487		
mouse	CNENFKKTFKKILHIRS	488		
rat	CNENFKKTFKKILHIRS	486		
guinea pig	CNENFRKTFKRLRIPP	488		
bovine	CNENFKKTFKKILHIRS	491		

Fig. 4. Amino acid sequences of the second and the third intracellular loops and the carboxyl terminus of human, mouse, rat, guinea pig, and bovine H1Rs. Asterisked residues indicate putative phosphorylation sites of human H1R that are identified in this study. These residues are conserved among the five species, with only one exception, bovine Asn¹⁴¹ corresponding to human Thr¹⁴⁰. The amino acid numbers in the sequence are shown to the right of the sequences.

concanavalin A for 20 min on ice, hypotonically lysed, and subjected to the centrifugation, which resulted in the separation of two fractions: a heavy membrane fraction containing cell surface membranes and a light membrane fraction containing intracellular vesicles (endosomes). As shown in Fig. 5A, WT H1Rs resided mainly in the heavy fraction at first, but many of them moved to light fraction after the treatment with histamine for 30 min. This result indicates that H1Rs were transferred from cell membranes to light vesicle fractions upon stimulation with histamine. Similarly, 5MT H1Rs moved from the heavy fraction to the light fraction by the same treatment with histamine, indicating that 5MT H1Rs can also be transferred from cell membranes to light vesicle fractions. Figure 5B shows the time course of H1R internalization upon stimulation with histamine. Receptor internalization is expressed by an increase in the portion of the receptors in the light fraction. The mutant (5MT) H1R was internalized essentially in the same time course and to the same extent with WT H1R, indicating that the mutation did not affect H1R internalization.

Table 3. Pharmacological properties of wild-type (WT) and mutant (5MT) H1R expressed in CHO cells

Receptor	[³ H]Mepyramine binding			Histamine-induced IPs accumulation	
	K _d	B _{max}	K _i	EC ₅₀	Maximum response
	nM	pmol/mg protein	μM	μM	fold
WT	1.17 ± 0.45	1.30 ± 0.15	19.7 ± 7.3	1.4 ± 0.5	5.19 ± 0.46
5MT	1.19 ± 0.39	1.71 ± 0.13	28.1 ± 6.6	1.1 ± 0.4	5.94 ± 0.25

Plasma membranes were prepared from CHO cells expressing WT or 5MT H1R, and [³H]mepyramine binding assays were performed as described in Materials and Methods. K_d and B_{max} values for [³H]mepyramine binding were obtained from Scatchard plots. K_i values for histamine were obtained from the respective IC₅₀ values. Results represent the mean ± S.E.M. of three independent experiments. The measurement of histamine-induced accumulation of IPs was performed as described in Materials and Methods. Results represent the mean ± S.E.M. of three independent experiments.

Fig. 5. Histamine-induced internalization of wild type (WT) and the mutant (5MT) human H1R expressed in CHO cells. A: CHO cells expressing WT or 5MT H1R were treated with 100 μM histamine for 30 min and washed with ice-cold phosphate-buffered saline. Washed cells were hypotonically lysed and subjected to sucrose density gradient centrifugation as described in Materials and Methods. After centrifugation, [³H]mepyramine binding assays were carried out on each fraction. Experiments were repeated three times with essentially the same results, and results of one typical experiment are shown. B: CHO cells expressing WT or 5MT H1R were treated with 100 μM histamine for 15, 30, and 60 min and then subjected to sucrose density gradient centrifugation. Percentage of the receptors in the heavy and light fractions was calculated by dividing the two fractions halfway between the two peaks. Results are shown as the means ± S.E.M. from three independent experiments. There were no significant differences between WT and 5MT H1R.

Histamine-induced down-regulation of WT and 5MT H1Rs expressed in CHO cells

Down-regulation or degradation of H1R was assessed by measuring an agonist-induced decrease in [³H]mepyramine binding sites, since the lipophilic tracer [³H]mepyramine can penetrate the cell membrane and thus label total receptors in the cell. CHO cells expressing WT or 5MT H1R were treated with 100 μ M histamine at 37°C for periods ranging from 12 to 24 h and then washed with ice-cold phosphate buffer. The cells were scraped into a small volume of the same buffer, and membrane preparations were prepared as described in Materials and Methods. Then the membrane preparations were subjected to [³H]mepyramine binding assays. As shown in Fig. 6, the [³H]mepyramine binding sites of cells expressing WT H1R decreased time dependently and by approximately 60% after 24 h-treatment with histamine. However, the [³H]mepyramine binding of cells expressing 5MT H1R was not changed at all upon 24-h treatment with histamine. Thus this mutation completely impaired histamine-induced H1R down-regulation. It is to be noted that the level of 5MT H1R expressed in CHO cells was a little higher than that of WT H1R (Table 2). This may be one of the causes of the difference found in receptor down-regulation between WT and 5MT H1Rs. Therefore to determine if

this is so, we used another clone expressing a lower level of 5MT H1R, with $B_{\max} = 0.73 \pm 0.08$ pmol/mg of protein and $K_d = 1.35 \pm 0.10$ nM ($n = 3$). The number of [³H]mepyramine binding sites of this clone was not changed after a 24-h treatment with histamine ($107.5 \pm 4.8\%$ of control, $n = 4$). Thus these results indicate that histamine-induced down-regulation was completely impaired in 5MT H1R irrespective of its expression level in CHO cells.

Discussion

To investigate the molecular mechanism underlying H1R internalization and down-regulation, we have constructed a mutant receptor, 5MT H1R, in which five possible phosphorylation residues, Thr¹⁴⁰, Thr¹⁴², Ser³⁹⁶, Ser³⁹⁸, and Thr⁴⁷⁸, were replaced with alanine. This mutant H1R had pharmacological properties similar to wild-type H1R in ligand binding and responses to histamine, indicating that it retained the ability to couple to the effector system. Moreover, 5MT H1R was internalized in the same time course and to the same extent with WT H1R, indicating that it can be internalized normally. However, 5MT H1R was not down-regulated at all by 24-h treatment with histamine, whereas WT H1R was down-regulated approximately 60% by the same treatment. These results indicate that 5MT H1R lacks some property that is indispensable for the receptor to be down-regulated. Thus this mutant receptor provides a useful tool for investigating the mechanism of H1R down-regulation.

The five possible phosphorylation residues described above were identified in this study using synthetic peptides. Although it is confirmed that some of these residues can certainly be phosphorylated in vitro using WT and 5MT H1Rs expressed in Sf9 (*Spodoptera frugiperda*) cells (24; N. Kawakami, personal communication), it is not yet clear whether these residues can be phosphorylated in vivo. Therefore, at present, it is not yet conclusive whether phosphorylation of these residues is responsible for H1R down-regulation. It is also possible that the mutation has changed the conformation of the intracellular domain of H1R and thus affected cellular signaling or interaction with other associated proteins, leading to loss of H1R down-regulation. However, since 5MT H1R did not down-regulate at all, it is certain that these residues have an important role in H1R down-regulation. Moreover, these five residues are conserved among several species as shown in Fig. 4, including rat (18), mouse (28), bovine (29), guinea pig (30), and human (25–27), supporting the view that these residues play an important role in the H1R signaling pathway.

Fig. 6. Histamine-induced down-regulation of WT and 5MT H1Rs expressed in CHO cells. Cells were treated with 100 μ M histamine at 37°C for the indicated times and washed with ice-cold phosphate buffer. Then, membrane preparations were prepared and were subjected to [³H]mepyramine binding assays. The [³H]mepyramine binding for each point is expressed as a percentage of [³H]mepyramine binding measured in non-treated cells. The binding of each cell type at $t = 0$ was 1.23 ± 0.11 pmol/mg or 1.58 ± 0.28 pmol/mg for WT or 5MT H1R, respectively. Each point is the mean $\pm$ S.E.M. of six independent experiments. *** $P < 0.001$ from WT H1R.

One possible mechanism for down-regulation of GPCR is degradation of the receptors transported to lysosomes via endosomes (11–13). The present result that the mutant (5MT) H1R was internalized normally, but was not down-regulated at all suggests that the mutant receptor lacked signals required for its transport to the lysosomes. Possibly one or some of the mutated residues are involved in marking the receptor to be transported to the lysosomes. Phosphorylation of these residues can be one possible mechanism to mark the receptor. The similar mechanism has been proposed for sorting the β_2 -adrenergic receptor to lysosomes (14, 31). However, at present, we have no data to support this idea further, and thus other possible mechanisms should also be considered. Alternatively, phosphorylation of these residues may affect ubiquitination of the receptor, which may be involved in receptor sorting to lysosomes as indicated for the β_2 -adrenergic receptor and the chemokine receptor CXCR4 (32, 33). However, there remained a possibility that H1R was down-regulated through a pathway independent of receptor internalization. H1R in the plasma membrane might have been a target for the proteolysis and were degraded there, or H1R might have been directly transported to lysosomes, as indicated for the β_2 -adrenergic receptor (10, 34), the V2 vasopressin receptor (35) and the m2-muscarinic receptor (16). The mutant H1R described here will be very useful in clarifying these points.

There have been a few studies that attempted to determine the kinases involved in H1R down-regulation. PKC may not be involved in H1R down-regulation, since activation of PKC with phorbol ester does not affect the level of H1R that is expressed in CHO cells (36). A specific PKG inhibitor inhibits H1R down-regulation in CHO cells (37). However, direct activation of PKG in these cells does not cause H1R down-regulation, indicating that more studies are required to clarify the role of PKG in H1R down-regulation. Consequently, what kinases are involved in H1R down-regulation remains to be determined.

Although there have been several studies indicating that receptor phosphorylation is involved in internalization of GPCR (14–16), mutation of the possible phosphorylation residues of H1R did not impair H1R internalization, indicating that these residues were not responsible for H1R internalization. Therefore, other possible phosphorylation residues that we have not yet determined may be involved in H1R internalization, or it may be mediated by another type of kinase such as tyrosine kinase (38). It is also possible that H1R internalization is mediated by a mechanism that does not require receptor phosphorylation, such as ubiquitination of the receptor (32).

In conclusion, we identified five possible phosphorylation residues of human H1R and suggested that phosphorylation of these residues are involved in the receptor down-regulation process. One possible mechanism is that these residues are required for receptor transport from endosomes to lysosomes. However, it is not yet conclusive that phosphorylation of these residues are involved in receptor down-regulation, and the mutant receptor 5MT H1R will provide a useful tool for further investigating the mechanism of H1R down-regulation.

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