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Two threonine residues and two serine residues in the second and third intracellular loops are both involved in histamine H_1 receptor downregulation

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Abstract Human histamine H_1 receptor (H_1R) contains five possible phosphorylation residues (Thr^{140}, Thr^{142}, Ser^{396}, Ser^{398} and Thr^{478}) and the substitution of all these five residues to alanine completely impairs agonist-induced receptor downregulation. In the present study, to determine which residue(s) are responsible for receptor downregulation, we used mutant H_1Rs in which single or multiple residues were substituted with alanine. The results suggested that two groups, i.e., residues Thr^{140} and Thr^{142}, and residues Ser^{396} and Ser^{398}, independently contributed to H_1R downregulation. Thr^{140} and Ser^{398} mainly contributed to downregulation, and Thr^{142} or Ser^{396} had a slight inhibitory effect on Thr^{140}- or Ser^{398}-mediated process, respectively.

Keywords: Desensitization; Downregulation; Histamine H_1 receptor; Phosphorylation; Site-directed mutagenesis; G protein-coupled receptor

1. Introduction

Histamine H_1 receptor (H_1R) is expressed in both central and peripheral tissues [1], and its expression level is dynamically regulated under various physiological and pathological conditions: upregulation of H_1Rs or H_1R mRNA in the electrical foci in the temporal cortex of epileptic patients [2] and in the nasal mucosa of patients with allergic rhinitis [3,4], downregulation of H_1Rs in the frontal cortex of patients with chronic schizophrenia [5] and in the frontal and temporal areas of patients with Alzheimer's disease [6]. The H_1R level may be regulated under these conditions by various processes as indicated for other types of G protein-coupled receptors (GPCRs), including modulation of receptor gene transcription [7], mRNA stability [8] and receptor degradation [9], and agonist-induced receptor degradation or receptor downregulation seems to play an important role among these processes.

Stimulation of GPCR leads to intracellular signaling, but chronic stimulation generally induces desensitization or uncoupling of the receptor from the effector system, and thereafter leads to internalization of the receptor to the intracellular membranes. The internalized receptor may be recycled to the cell surface, but continued stimulation of the receptor leads to degradation or downregulation of the receptor [9–11]. Although precise mechanism for such receptor downregulation has not been clarified yet, it is probable that certain amino acid residues in the receptor can be a signal that should be degraded, and in fact, residues responsible for receptor downregulation have been identified in several GPCRs [12–16]. These residues may be involved in the process of sorting of the internalized receptor either to the cell surface for recycling or to lysosomes for proteolysis. Thus, in a previous study, we explored residues of human H_1R that were responsible for agonist-induced receptor downregulation, and identified five residues possibly involved in the process, i.e., Thr^{140}, Thr^{142}, Ser^{396}, Ser^{398} and Thr^{478} [17]. Substitution of all of these five residues with alanine did not affect histamine response or internalization of H_1R, but it completely impaired agonist-induced H_1R downregulation. In this study, we further delineated the residues involved in H_1R downregulation by using mutant receptors in which single or multiple residues were substituted with alanine. The data indicate that Thr^{140} and Ser^{398} are mainly responsible for receptor downregulation, and Thr^{142} and Ser^{396} have a modulatory role.

2. Materials and methods

2.1. Materials

[^{3}H]Mepyramine ([pyridinyl-5-^{3}H]pyrilamine, 0.74 TBq/mmol) was purchased from NEN Life Science Products (Boston, MA, USA). Cell culture reagents were from Life Technologies (Rockville, MD, USA). All other reagents, unless otherwise stated, were of analytical grade and were from Wako Pure Chemicals (Osaka, Japan) or Sigma (St. Louis, MO, USA).

2.2. Plasmid construction

The BclI fragment (1.8 kilobase pairs) of the human H_1R gene was subcloned into the M13 mp19 phage at the *Eco*RI site by using *Eco*RI–*Not*I–*Bam*HI adaptors (Takara Biochemicals, Kyoto, Japan). Then, the coding region of the H_1R gene was subcloned into a pBluescript SK(+) vector (Stratagene, La Jolla, CA, USA). Site-directed mutagenesis was

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Abbreviations: H_1R, histamine H_1 receptor; GPCR, G protein-coupled receptor; CHO, Chinese hamster ovary; PKG, cGMP-dependent protein kinase

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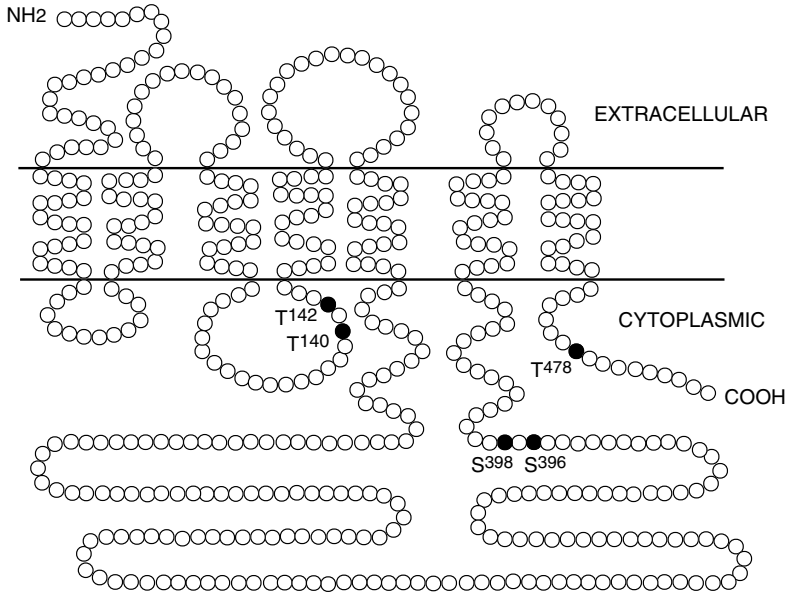
performed with an Oligonucleotide-directed In Vitro Mutagenesis System (Amersham, Buckinghamshire, UK) and long-range polymerase chain reaction system [18]. Three groups of mutant H_1Rs were constructed. First, each of the five possible phosphorylation residues, i.e., Thr^{140}, Thr^{142}, Ser^{396}, Ser^{398} and Thr^{478}, was substituted with alanine, forming mutant receptors designated T140A, T142A, S396A, S398A and T478A. Second, one of the above five residues was left intact, while the other four residues were all substituted with alanine, forming mutant H_1Rs designated 4A-140T, 4A-142T, 4A-396S, 4A-398S and 4A-478T. Third, the pair of residues Thr^{140} and Thr^{142} or Ser^{396} and Ser^{398} was left intact, while the other three residues were replaced with alanine (3A-140T142T and 3A-396S398S). The nucleotide sequences of the mutated H_1R genes were confirmed by the dideoxynucleotide method. Each coding region of wild-type and the mutant H_1R genes was subcloned into a mammalian expression vector, pdKCR-dhfr for stable expression in Chinese hamster ovary (CHO) cells. The constructs contained 5′ untranslated region (140 bp) and 3′ untranslated region (182 bp) of H_1R gene. The 5′ untranslated region does not contain any potential binding sites for transcription factors.

2.3. 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 [19]. The cells were cultured in α-minimum essential medium without ribonucleosides and deoxyribonucleosides supplemented with 10% dialyzed fetal calf serum. Then, individual colonies were screened for stable expression of the H_1R using a [^{3}H]mepyramine binding assay.

2.4. Radioligand binding assay

The [^{3}H]mepyramine binding assay was performed as described previously [20]. A suspension of cell membranes (150–300 μg of protein) was incubated with [^{3}H]mepyramine in the absence (total binding) or presence (non-specific binding) of 10 μM triprolidine in 50 mM sodium–potassium phosphate buffer (pH 7.4) at 25 °C for 60 min in a final volume of 600 μl. 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,



	[2nd IL]	[3rd IL]	[C terminus]
	* *	* *	*
WT	YLKYRTKTRAS 145	WKRLRSHSRQYV 403	ENFKKTFKRIL 483
5MT	-----A-A---	-----A-A----	-----A-----
T140A	-----A-----	----------	----------
T142A	-------A---	----------	----------
S396A	----------	-----A------	----------
S398A	----------	-------A----	----------
T478A	----------	----------	-----A-----
4A-140T	-------A---	-----A-A----	-----A-----
4A-142T	-----A-----	-----A-A----	-----A-----
4A-396S	-----A-A---	-------A----	-----A-----
4A-398S	-----A-A---	-----A------	-----A-----
4A-478T	-----A-A---	-----A-A----	----------
3A-140T142T	----------	-----A-A----	-----A-----
3A-396S398S	-----A-A---	----------	-----A-----

Fig. 1. Schematic representation illustrating the sites of mutation of human H_1R. Filled circles indicate serine or threonine residues substituted in various mutants by alanine. Shown underneath are the mutant H_1Rs used in this study. Partial amino acid sequences of the second and the third intracellular loops and the carboxyl terminus of human H_1R are shown, in which asterisked residues indicate residues that were replaced by alanine. The amino acid numbers in the sequence are shown to the right of the sequences. "A" indicates the residue that was replaced by alanine and hyphens indicate the residues without mutation. The naming for each mutant receptor is shown in Section 2.

USA) and the radioactivity on the filter was counted in a liquid scintillation counter.

2.5. Receptor downregulation assay

Nearly confluent cells were incubated at 37 °C for 24 h in the α-minimum essential medium described above supplemented with 10% fetal calf serum in the presence or absence of 100 μM histamine. 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 then homogenized with an ultrasonic disruptor (Tomy Seiko Co, Ltd., Tokyo, Japan) and 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. Downregulation was defined as the decrease in specific [^{3}H]mepyramine binding due to exposure of the cells to the desensitizing agent [21]. Changes in H_1R density were expressed as a percentage of [^{3}H]mepyramine binding of the histamine-treated cells to that of non-treated cells. Statistical evaluation of significant differences was performed with Student's *t* test.

3. Results

3.1. Expression of mutant H_1Rs in CHO cells

In a previous study, we constructed a mutant H_1R (5MT H_1R), in which all of the threonines and serines of the possible phosphorylation residues of human H_1R (Thr^{140}, Thr^{142}, Ser^{396}, Ser^{398} and Thr^{478}) were substituted with alanine [17]. Properties of CHO cells expressing 5MT H_1R were comparable with those of cells expressing wild-type H_1R (WT H_1R) in antagonist binding, agonist binding, histamine-induced inositol phosphate formation and histamine-induced receptor internalization. However, the mutant H_1R was completely resistant to histamine-induced receptor downregulation, suggesting that these five residues are responsible for H_1R downregulation [17]. In the present study, to delineate the role of each residue in H_1R downregulation, we studied three groups of mutant H_1Rs that were constructed as described in Fig. 1. First, each of the five residues was substituted with alanine (T140A, T142A, S396A, S398A and T478A). Second, each of the five residues was left intact while the other four residues were all replaced with alanine (4A-140T, 4A-142T, 4A-396S, 4A-398A and 4A-478T). Third, residues of Thr^{140} and Thr^{142} or residues of Ser^{396} and Ser^{398} were left intact, and the other three residues were replaced with alanine (3A-140T142T and 3A-396S398S). Then, the pdKCR-dhfr vectors containing wild-type or mutant H_1R gene were transfected by calcium phosphate precipitation method into dihydrofolate reductase-deficient CHO cells. Stable transfectants were selected in the medium devoid of nucleosides and clonal cell lines were isolated for each type of H_1R. The binding characteristics of wild-type and mutant H_1Rs expressed in CHO cells are shown in Table 1. Each cell had nearly the same binding properties.

3.2. Histamine-induced downregulation of mutant H_1Rs expressed in CHO cells

Downregulation or degradation of H_1R was assessed by measuring an agonist-induced decrease in binding sites of lipophilic tracer [^{3}H]mepyramine, which can penetrate the cell membrane and thus label total receptors in the cell [17,22]. CHO cells expressing wild-type or mutant H_1R were treated with 100 μM histamine at 37 °C for 24 h and then cells were 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 Section 2. Then, the membrane preparations were subjected to [^{3}H]mepyramine binding assays.

Table 1
Binding characteristics of wild-type (WT) and mutant H_1Rs expressed in CHO cells

Receptor	[^{3}H]Mepyramine binding	
	K_d (nM)	B_{max} (pmol/mg protein)
WT	1.17 ± 0.45	1.30 ± 0.15
5MT	1.19 ± 0.39	1.71 ± 0.13
T140A	0.93 ± 0.07	1.75 ± 0.06
T142A	1.30 ± 0.18	1.81 ± 0.13
S396A	1.09 ± 0.13	1.08 ± 0.06
S398A	1.14 ± 0.09	1.57 ± 0.08
T478A	1.78 ± 0.34	1.35 ± 0.10
4A-140T	0.59 ± 0.25	1.38 ± 0.13
4A-142T	0.84 ± 0.11	2.42 ± 0.14
4A-396S	1.37 ± 0.23	2.20 ± 0.26
4A-398S	0.74 ± 0.18	3.99 ± 0.19
4A-478T	1.67 ± 0.16	4.55 ± 0.13
3A-140T142T	0.64 ± 0.05	2.42 ± 0.20
3A-396S398S	0.40 ± 0.12	1.03 ± 0.16

Plasma membranes were prepared from CHO cells expressing wild-type or mutant H_1Rs and [^{3}H]mepyramine binding assays were performed as described in Section 2. K_d and B_{max} values for [^{3}H]mepyramine binding were obtained from Scatchard plots. Results represent means ± S.E.M. of three independent experiments.

As we showed previously [17], [^{3}H]mepyramine binding sites were decreased by approximately 60% upon treatment with histamine for 24 h in cells expressing wild-type H_1R, but were scarcely not in cells expressing 5MT H_1R. As the first step to identify the residue(s) responsible for H_1R downregulation, we studied mutant H_1Rs in which one residue was substituted with alanine. As shown in Fig. 2, H_1R downregulation was significantly inhibited in cells expressing T140A or S398A H_1R, but

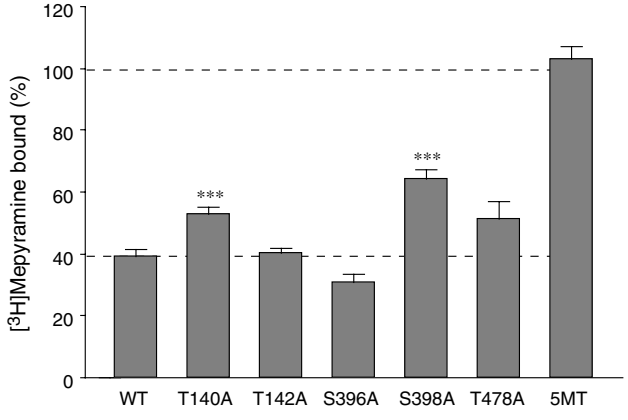


Fig. 2. Histamine-induced downregulation of wild-type (WT) and the mutant human H_1Rs expressed in CHO cells. In the mutant H_1Rs, each of the five residues Thr^{140}, Thr^{142}, Ser^{396}, Ser^{398} and Thr^{478} was substituted with alanine, forming mutant receptors designated T140A, T142A, T478A, S396A and S398A. Cells were treated with 100 μM histamine at 37 °C for 24 h and washed with ice-cold phosphate buffer. Then, membrane preparations were prepared and were subjected to [^{3}H]mepyramine binding assays. The [^{3}H]mepyramine binding for each point is expressed as a percentage of [^{3}H]mepyramine binding measured in non-treated cells. Each bar represent means ± S.E.M. of 4–8 independent experiments. The asterisks indicate a significant difference compared with wild-type H_1R (***$P < 0.001$).

was not in cells expressing T142A, S396A or T478A H_1R. This result indicates that both Thr^{140} and Ser^{398} are responsible for the downregulation process, since the mutation at either Thr^{140} or Ser^{398} affected H_1R downregulation. However, their contribution to downregulation remained unclear, since either mutation inhibited downregulation only partially.

Therefore, in the next step, we studied mutant H_1Rs in which one residue was left intact and the other four residues were all replaced with alanine. As shown in Fig. 3A, both mutants, 4A-140T and 4A-398S H_1Rs, were downregulated to the same extent as wild-type H_1R was. Other mutants, 4A-142T and 4A-396S, were downregulated significantly compared with 5MT H_1R ($P < 0.001$) but to a lesser extent compared with wild-type H_1R (significantly different). This result indicates that Thr^{140} alone or Ser^{398} alone could fully induce H_1R downregulation. On the other hand, Thr^{142} or Ser^{396} contributed to downregulation significantly but only partially. The 4A-478T mutant receptor was downregulated only slightly, indicating that Thr^{478} had marginal effect on downregulation.

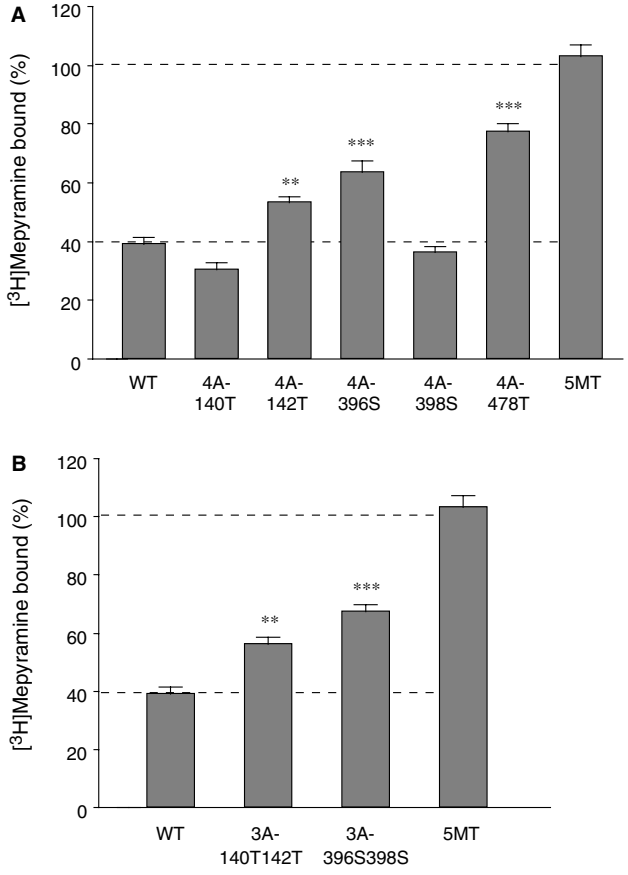


Fig. 3. Histamine-induced downregulation of wild-type (WT) and the mutant human H_1Rs expressed in CHO cells. (A) In the mutant H_1Rs, one of the five phosphorylation residues was left intact and the other four residues were substituted with alanine residues, forming mutant receptors designated 4A-140T, 4A-142T, 4A-396S, 4A-398S and 4A-478T. (B) Two of the five phosphorylation residues of H_1R were left intact and the other three residues were substituted with alanine, forming mutant receptors, 3A-140T142T and 3A-396S398S. Cells were treated with 100 μM histamine at 37 °C for 24 h and washed with ice-cold phosphate buffer. Then, membrane preparations were prepared and were subjected to [^{3}H]mepyramine binding assays. The [^{3}H]mepyramine binding for each point is expressed as a percentage of [^{3}H]mepyramine binding measured in non-treated cells. Each bar represent means ± S.E.M. of 4–8 independent experiments. The asterisks indicate a significant difference compared with wild-type H_1R (**$P < 0.01$, ***$P < 0.001$).

The above results suggest that there are two groups of residues in the intracellular domain of H_1R that are involved in H_1R downregulation; a pair consisting of Thr^{140} and Thr^{142} in the second intracellular loop and another pair consisting of Ser^{396} and Ser^{398} in the third intracellular loop. Thus, to examine the role of each pair of residues, we studied mutants in which residues Thr^{140} and Thr^{142} (3A-140T142T) or residues Ser^{396} and Ser^{398} (3A-396S398S) were intact and the other three residues were replaced with alanine. As shown in Fig. 3B, both mutants were downregulated significantly ($P < 0.001$, compared with 5MT H_1R), but the extent of downregulation was significantly less compared with wild-type H_1R. These results indicate that Thr^{142} and Ser^{396} may have inhibitory effect on Thr^{140}- and Ser^{398}-mediated downregulation, respectively. Thus, each pair of residues in the second and third intracellular loops contributed to downregulation only partially, and they probably took part in downregulation in an additive way for the process to proceed in full.

4. Discussion

The present study examined the role of residues Thr^{140}, Thr^{142}, Ser^{396}, Ser^{398} and Thr^{478} of human H_1R in agonist-induced receptor downregulation, and the data clearly indicate that residues Thr^{140} and Ser^{398} are responsible for this process. This conclusion is based on the results that mutation of either Thr^{140} or Ser^{398} to alanine affected H_1R downregulation (Fig. 2), and the results that the mutant H_1R in which either Thr^{140} or Ser^{398} was left intact and the other four residues were replaced with alanine underwent downregulation completely (Fig. 3A). The latter results indicate that Thr^{140} alone or Ser^{398} alone was sufficient for inducing full downregulation. Therefore, there seem to be two groups of residues that participate in H_1R downregulation, i.e., Thr^{140} in the second intracellular loop and Ser^{398} in the third loop. Point mutations at either Thr^{140} or Ser^{398} were not fully effective in inhibiting receptor downregulation, and this was probably because the downregulation process was mediated by the two group of residues and the mutation introduced into one group was insufficient to inhibit downregulation completely.

Downregulation mediated by these two residues, Thr^{140} and Ser^{398}, seems to be modulated by other residues that reside nearby, i.e., Thr^{142} and Ser^{396} may inhibit Thr^{140}- and Ser^{398}-mediated processes, respectively. This was elucidated from the results that mutant H_1Rs, 3A-140T142T or 3A-396S398S underwent only partial downregulation compared with full downregulation induced by mutant H_1Rs, 4A-140T or 4A-398S (Fig. 3). Interestingly, Thr^{142} alone (4A-142T) or Ser^{396} alone (4A-396S) effectively induced downregulation, although the extent of downregulation was significantly less compared to that of wild-type H_1R (Fig. 3A). This result may suggest that these residues contribute in part to the downregulation process. However, it is to be noted that this result was obtained in the condition that both Thr^{140} and Ser^{398} were replaced with alanine. Probably, in this condition Thr^{142} or Ser^{396} has mimicked the action of Thr^{140} or Ser^{398} that resided nearby and thus induced downregulation to some extent, respectively.

Moreover, as Fig. 2 shows, point mutations at Thr^{142} or Ser^{396} did not affect downregulation at all, indicating the minor role of these residues in inducing receptor downregulation. Therefore, although it is still speculative, it is probable that Thr^{142} and Ser^{396} have a modulatory role to inhibit Thr^{140}- and Ser^{398}-mediated process, respectively. Thus, it seems that two groups of residues participate in H_1R downregulation, one Thr^{140} and Thr^{142} in the second intracellular loop, and another Ser^{396} and Ser^{398} in the third loop. Each pair of residues contributes to downregulation partially, and they can induce full downregulation in conjunction with each other.

How Thr^{142} and Ser^{398} are involved in H_1R downregulation? In a previous study, we showed data that the mutant (5MT) H_1R, in which all of the five residues were replaced with alanine, was internalized properly but was completely resistant to downregulation, suggesting that the mutant receptor was impaired in the process of receptor trafficking from endosomes to lysosomes [17]. Therefore, one possible mechanism is that the residues Thr^{140} and Ser^{398} are responsible for the process of sorting the internalized receptor to lysosomes. Recent studies have identified cytoplasmic sequences present in certain GPCRs that promote sorting of internalized receptors to lysosomes [13], or those that promote or prevent recycling of receptors from endosomes to the plasma membrane [12,14,16]. The residues identified in this study can be a sorting signal that is responsible for receptor trafficking to lysosomes, but they have no similarity with any of the reported one.

In this study, H_1R downregulation was induced by stimulation with histamine. Since H_1R stimulation phosphorylates H_1R [23], it is possible that agonist-induced phosphorylation of the residues of Thr^{140} and Ser^{398} are involved in H_1R downregulation. In fact, our previous study suggested that these two residues are substrates for several kinases [17,24]. However, there have been no convincing data indicating that H_1R phosphorylation is involved in H_1R downregulation. For example, Smit et al. [22] showed that activation of protein kinase C by phorbol esters had no effect on H_1R downregulation. Miyoshi et al. [25] showed that a specific inhibitor of cGMP-dependent protein kinase (PKG) suppressed H_1R downregulation, but direct activation of PKG did not induce H_1R downregulation. Consequently, at present, whether phosphorylation of H_1R, especially that of residues of Thr^{140} and Ser^{398}, is involved in H_1R downregulation remains to be determined. It is interesting whether Thr^{140}-mediated and Ser^{398}-mediated downregulation occur by similar mechanisms or not. Although we have no data to clarify this point, a growing amount of data shows that multiple mechanisms contribute to downregulation of GPCRs [26], and thus, it is possible that downregulation mediated by Thr^{142} and that by Ser^{398} occur through quite different mechanisms.

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