Role of the C-terminus in histamine H2 receptor signaling, desensitization and agonist-induced internalization

ヒスタミンH２受容体を介するシグナル伝達、脱感作、インタナリゼーションにおけるC末端の役割

福嶋康之

Role of the C-terminus in histamine H2 receptor signaling, desensitization and agonist-induced internalization

ヒスタミンＨ２受容体を介するシグナル伝達、脱感作、インタナリゼーションにおけるＣ末端の役割

福　嶋　康　之

目次

SUMMARY

To evaluate the role of the histamine H2 receptor C-terminus in signaling, desensitization and agonist-induced internalization, canine H2 receptors with truncated C-termini were generated. Wild-type (WT) and truncated receptors were tagged at their N-termini with a hemagglutinin (HA) epitope and expressed in COS7 cells. Most of the C-terminal intracellular tail could be truncated (51 of 70 residues, termed T^{308} mutant) without loss of functions: cAMP production, tiotidine binding and plasma membrane targeting. In fact, the T^{308} mutant produced more cAMP than the WT when cell-surface expression per cell was equivalent. Pretreatment of cells with 10^{-5} M histamine desensitized cAMP productions via WT and T^{308} receptors to similar extents. Incubation of cells expressing WT receptors with 10^{-5} M histamine reduced cell-surface anti-HA antibody binding by approximately 30% (by 30 min, $t_{1/2}$ ~ 15 min), but did not affect the B_{max} of tiotidine in membrane fractions, which represents total receptor amounts, suggesting that WT receptors were internalized from the cell-surface. In contrast, no internalization was observed with T^{308} receptors following histamine treatment. A mutant with a deletion of the 30 C-terminal amino acids, termed T^{329}, was functional but was as potent as the WT in terms of cAMP production. Besides being desensitized by histamine, the internalization of the receptor was indistinguishable from that of the WT. Internalization was observed in the T^{320} but not in T^{313} mutant, narrowing the region involved in internalization to that between Glu^{314} and Asn^{320} (ETSLRSN). Of these seven

residues, either Thr^{315}, Ser^{316}, or both, were replaced with Ala. Thr^{315} and Ser^{316} are conserved among species. The mutation at Thr^{315}, but not that at Ser^{316} abolished internalization. Taken together, these results demonstrate that Thr^{315} is involved in agonist-induced internalization. Furthermore, the finding that T^{308} receptors were desensitized in the absence of internalization suggests that internalization and desensitization are meditated by independent mechanisms.

INTRODUCTION

Histamine is involved in acid production in gastric parietal cells through histamine H2 receptor (6). Molecular cloning of the genes for canine, human and rat H2 receptor was recently accomplished (13, 14, 22). Analysis of the deduced amino acid sequences of the receptors revealed structural homology with guanine nucleotide binding protein-coupled receptors (GPCRs) with putative seven-transmembrane motifs. An important feature of GPCRs is desensitization, a phenomenon in which exposure of cells to an agonist results in attenuation of the subsequent cellular response to the agonist. It is difficult to investigate desensitization of the H2 receptor in gastric parietal cells prepared from stomachs of animals, since preparation procedures may damage the parietal cells. Therefore, these studies were mainly performed in fibroblast cell lines overexpressing H2 receptors.

As observed in a number of guanine nucleotide binding protein-coupled receptors (GPCRs), cAMP responses occurring via the histamine H2 receptor are rapidly desensitized after agonist stimulation (2, 8, 9, 21, 24). The H2 receptor also exhibits an agonist-induced internalization from the cell surface (9, 25). However, the mechanisms underlying these phenomena have yet to be identified. Recently, a number of studies focusing on GPCRs have examined the role of the C-terminus in agonist-induced desensitization and internalization, with varying results. For example, C-terminal truncation of the angiotensin II receptor inhibited agonist-induced internalization of this receptor, but not

desensitization of the calcium response mediated via the receptor (29). On the other hand, C-terminal truncation of ß2-adrenergic, α_{1B}-adrenergic, lutropin/choriogonadotropin, PAF and neurokinin-2 receptors resulted in impairment of homologous desensitization (1, 7, 20, 23, 27). Thus, the C-termini of GPCRs have functional importances, which may differ among receptors. The present study was designed to analyze the role of this important portion of the histamine H2 receptor in signaling, desensitization and agonist-induced internalization. To this end, we constructed H2 receptor cDNAs, devoid of either the 71 or the 51 amino acids at the C-terminus and expressed these cDNAs in COS7 cells. Herein, we present evidence that the 51 C-terminal amino acids of the histamine H2 receptor are important for agonist-induced internalization, but not for either signaling or homologous desensitization of cAMP response which occurs via this receptor. In addition, these amino acids exert inhibitory effects on cAMP production via the H2 receptor.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]-tiotidine was purchased from Du Pont NEN (Boston, MA). Goat anti-mouse [^{125}I]-IgG was purchased from ICN (Costa Mesa, CA). Cimetidine was obtained from Sigma. Anti-hemagglutinin (anti-HA) monoclonal antibody 12CA5 was purchased from Boehringer Mannheim (Germany). DEAE-dextran was obtained from Pharmacia Biotech Inc (Uppsala, Sweden).

Cell culture

COS7cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS), 100 units/ml penicillin G, and 0.1 mg/ml streptomycin sulfate.

Construction of cDNAs for the H2 receptors with C-terminal truncation

cDNAs for mutant canine H2 receptors with mutations involving C-terminal truncation of 70, 51, 46, 39, 30 and 15 amino acids, respectively, were constructed by polymerase chain reactions. They were termed T^{289}, T^{308}, T^{313}, T^{320}, T^{329} and T^{344}, respectively. cDNAs for mutant receptors, in which Thr^{315}, Ser^{316}, or both, were replaced with Ala (A^{315}, A^{316} and $A^{315, 316}$), were also constructed. The hemagglutinin (HA) epitope (YPYDVPDYA) was inserted into the extracellular N-termini of the wild-type (WT) and mutant receptors. After confirming the sequences, the cDNAs encoding the WT histamine H2 receptor and its truncated mutants with or without the HA-tag were subcloned into pCAGGS. COS7 cells were transfected with varying amounts of plasmid DNAs by the DEAE-dextran

method.

Immunocytochemical localization of the H2 receptor in COS7 cells

For immunofluorescence microscopy, COS7 cells were fixed in 3% formaldehyde/ phosphate-buffered saline (PBS), scraped off the dish with a rubber blade, and embedded in 10% gelatin/PBS. Semithin frozen sections (1 μm thick) were cut and incubated with anti-HA antibody or antibody against the C-terminus of the canine H2 receptor (anti-$H2R^{CT}$ antibody) (2). These sections were then incubated with rhodamine-labeled affinity purified goat anti-mouse or anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) (13).

Quantification of cell-surface H2 receptors

At 24 h post-transfection, COS7 cells expressing the WT and truncated H2 receptors with the HA epitope or parental COS7 cells were plated onto 24-well plates at a density of 10^5 cells/well. At 48 h post-transfection, cells were incubated in Hepes-tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.37 mM NaH_2PO_4, 5.6 mM glucose, 25 mM Hepes, pH 7.4) containing 0.1% bovine serum albumin (BSA) at 37°C for 30 min. After incubation in the presence or absence of 10^{-5} M histamine at 37°C for the indicated times, the cells were fixed with PBS containing 3% paraformaldehyde, washed again with PBS and incubated with 5% skim milk/PBS for one hour at room temperature. They were incubated with 200 μl of 12CA5 (5 μg/ml) in PBS for 2 h at room temperature, washed again with PBS, and incubated for one additional hour with 200 μl of [^{125}I]-labeled goat anti-mouse IgG (1:200

dilution) at room temperature. The wells were then washed twice with 5% skim milk/PBS, twice with PBS, and three times with 0.05% Tween 20 / PBS. Bound [^{125}I]-labeled goat anti-mouse IgG was solubilized in 1% SDS, and radioactivities were determined in a gamma-counter. Specific bindings were determined by subtraction of the non-specific bindings observed in parental COS7 cells.

Tiotidine binding assay

Tiotidine binding assays involving intact cells were performed as described previously (14). COS7 cells, grown in 24-well plates, were assayed at a density of 1 x 10^5 cells / well. The cells were incubated for 2 h at 37°C in 200 µl of Hepes-tyrode's buffer containing 1 nM [^{3}H]-tiotidine and various concentrations of unlabeled tiotidine (15). All samples were analyzed in triplicate. After incubation, cells were washed three times with ice-cold PBS, then removed from the wells in 0.1% SDS, and radioactivities were determined by liquid scintillation counting. Tiotidine bindings in membranes were assessed as described previously, with some modifications (2). Membrane fractions (200 µg) from COS7 cells were incubated with 1 nM [^{3}H]-tiotidine and varying concentrations of unlabeled tiotidine in 25 mM Hepes, 0.1% BSA, pH 7.4, in a final volume of 200 µl at 37 °C for 2 h. The binding reaction was terminated by filtration over Whatman GF/C glass fiber filters, followed by 10-ml washes with ice-cold incubation buffer. Radioactivity on the filters was determined by liquid scintillation counting. In both experiments, specific bindings were calculated by subtraction of the nonspecific bindings determined in the presence

of 10^{-4} M cimetidine. No specific binding was observed in either parental COS7 cells or COS7 cells transfected with the expression vector alone.

Measurement of cAMP productions

COS7 cells, plated onto 24-well plates, were assayed at a density of 1×10^5 cells / well, as described previously (14). The cells were incubated for 30 min at 37 °C in 450 μl of Hepes-tyrode's buffer containing 0.1% BSA and 0.1 mM 3-isobutyl-1-methylxanthine, then 50 μl of histamine solution was added to initiate the reaction. After 10 min of incubation at 37°C, the reaction was terminated by the addition of 500 μl of 12% trichloroacetic acid. The samples were centrifuged for 5 min at 3000 x g at 4°C. Following extraction of the supernatants three times with diethylether, cAMP contents in the samples were measured using a radioimmunoassay (16). Histamine-dependent and forskolin-dependent cAMP productions were determined by subtracting basal cAMP productions.

RESULTS

Expression of wild-type and C-terminus truncated histamine H2 receptors

To investigate the role of the cytoplasmic tail of the histamine H2 receptor in signaling and desensitization, we generated C-terminus-truncated receptor cDNAs. Fig. 1 presents a schematic representation of the 359-amino acid canine histamine H2 receptor. Consensus sites for post-translational modifications, N-glycosylation and palmitoylation are also indicated (17). We chose to delete 51 and 70 amino acids from the C-terminal tail, yielding two distinct mutant receptors, termed T^{308} and T^{289}, lacking 11 and 13 serine/threonine residues, respectively. The T^{289} receptor was truncated at the beginning of the cytoplasmic C-terminus and the T^{308} receptor just distal to the putative palmitoylation site (Cys^{305}). All receptor constructs were tagged at their N-termini with a nine-amino acid HA epitope, as shown in Fig. 1. As the HA-epitope is located in the extracellular region, the cell-surface receptor amount can be estimated utilizing the antibody against the HA-epitope, 12CA5. The cDNA constructs were subcloned into an expression vector, pCAGGS and transfected into COS7 cells. COS7 cells do not have detectable endogenous histamine H2 receptors as demonstrated by the absence of histamine-dependent cAMP production and the absence of specific [^{3}H]-tiotidine binding (not shown) and are thus a suitable model system for these studies. At 48 h after transfection, the cells were subjected to immunocytochemistry using the anti-HA antibody or the anti-$H2R^{CT}$ antibody. Expression of the receptors is shown in Fig. 2. The

WT, the HA-wild-type (HA-WT), and the HA-T^{308} receptors were distributed in the plasma membranes, while the HA-T^{289} receptor, the 70 C-terminal deleted amino acids of which include Cys 305, a consensus site for palmitoylation, was distributed intracellularly as well as in the plasma membranes. Whether the different distribution of the HA-T^{289} receptor was due to the loss of palmitoylation or to lack of the proximal amino acid residues from 290 to 307 was not determined. In either case, it can be concluded that the 51 C-terminal amino acid residues of the histamine H2 receptor, a large part of the C-terminus, are not involved in trafficking of the H2 receptor to the plasma membrane. Similar results were obtained when various amounts of plasmid DNAs were used for transfection of COS7 cells (not shown), indicating that the different distribution of the receptors is not a function of receptor numbers.

Tiotidine binding of the WT and truncated receptors

To examine the effect of C-terminal truncation on ligand binding, intact COS7 cells on 24-well plates were subjected to binding of tiotidine (15), an H2 receptor antagonist, at 48 h post-transfection. The affinities of the WT and truncated receptors for tiotidine were determined by Scatchard plot analysis. K_ds for tiotidine of the WT and T^{308} receptors were comparable (Table 1). In addition, HA-tagging did not affect the affinity of these receptors for tiotidine (Table 1). However, irrespective of the HA-tagging, the T^{289} receptor showed no tiotidine binding. The third and fifth transmembrane regions are reportedly important for ligand binding of the H2 receptor (18). This finding might be

accounted for by a conformational change in the T^{289} receptor induced by the deletion of amino acid residues from 290 to 307. Since tiotidine is membrane-permeable and is capable of binding to receptors distributed intracellularly, the inability of the T^{289} mutant to bind to tiotidine is not due to the difference in the intracellular distribution of the receptor shown in Fig. 2.

cAMP productions via the WT and truncated receptors

To examine the role of the C terminus in H2 receptor signaling, we measured histamine-dependent cAMP productions via the WT and truncated receptors in COS7 cells. cAMP productions mediated by these receptors can be compared only when the amounts of cell-surface receptors are equivalent. Iida-Klein et al. reported that in a COS7 transfection system using the DEAE-dextran method the amount of plasmid DNA used for transfection determined the number of cell-surface PTH/PTHrp receptors expressed per cell but not the percentage of transfected cells (19). We have obtained similar results, in that the amounts of plasmid DNA affected the number of H2 receptors expressed per cell and the percentage of transfected cells was constant even when the amount of plasmid DNA used was not equivalent (Table 2). We transfected various amounts of plasmid DNA for HA-WT, HA-T^{308} and HA-T^{289} receptors and measured the number of receptors expressed per cell. As shown in Fig. 3A, the amounts of cell-surface receptors were dependent on the amounts of plasmid DNA used for transfection and, in addition, differed markedly among the WT and mutant receptors even when equivalent amounts (molar) of plasmid DNAs were used

for transfection. The number of cell-surface HA-WT receptors expressed was larger than that of HA-T^{308} receptors when the equivalent amounts of plasmid DNAs were used for transfection. It is essential to study the functions of these receptors at similar expression level. Therefore, we measured histamine-dependent cAMP productions via the WT and mutant receptors at each transfection levels. This allowed comparison of the functions of these receptors. As shown in Fig. 3B, histamine-dependent cAMP productions were observed in HA-WT and HA-T^{308} cells but not in HA-T^{289} cells. Histamine-dependent productions of cAMP observed in HA-WT and HA-T^{308} were inhibited by cimetidine but not by diphenhydramine (not shown). Interestingly, as compared with HA-WT receptors, HA-T^{308} receptors produced more cAMP than the WT receptor (Fig. 3B) if the numbers of cell-surface receptors per cell were equivalent. Therefore, the 51 C-terminal amino acids are not only essential for, but rather may actually inhibit, cAMP production mediated by the H2 receptor. It is also possible that inhibitory effects on the H2 receptor are exerted via the C-terminal region. Although less marked than those in HA-WT and HA-T^{308} cells, specific cell-surface anti-HA antibody bindings were also observed in HA-T^{289} cells (Fig. 3A). Thus, the absence of histamine-dependent cAMP production via T^{289} receptors was not due to a lack of cell-surface T^{289} receptors, but rather to the inability of the receptor itself to couple to G_s. It is not clear whether this is due to the absence of the amino acid residues from 289 to 307, or to a conformational change induced by the deletion. Furthermore, the possible contributions of

these amino acid residues to cAMP production could not be examined.

Desensitization of WT and T^{308} receptor-mediated cAMP responses

The histamine H2 receptor reportedly shows homologous desensitization of the cAMP response following preincubation with histamine. We examined whether the C-terminal tail of the H2 receptor is involved in desensitization of the cAMP response which occurs via this receptor. Since COS7 cells expressing the HA-T^{289} mutant did not mediate cAMP production in response to histamine (Fig. 3B), this mutant was omitted from the experiment. COS7 cells were transfected with plasmid DNAs containing HA-WT (0.4 μg / 10 cm plate) or HA-T^{308} (1.0 μg / 10 cm plate) receptors. Transfection with these DNA amounts resulted in similar expression levels of the HA-WT and HA-T^{308} receptors (Fig. 3A). Preincubation of transfected COS7 cells with 10^{-5} M histamine for 30 min led to reduced cAMP production in both the HA-WT and HA-T^{308} cells as compared with untreated cells (Fig. 4A), indicating desensitization, while forskolin-dependent cAMP production was slightly increased (Fig. 4B). It is noteworthy that the transfection efficiency of the COS7 cell system is approximately 20% at best (Table 2). Furthermore, the results shown in Fig. 4B do not necessarily mean that forskolin-dependent cAMP production in COS7 cells expressing the receptors was not altered. Therefore, we performed similar experiments in CHO cells stably expressing HA-WT or HA-T^{308} receptors and found that histamine-dependent cAMP production via these receptors was reduced by preincubation with 10^{-5} M histamine, while forskolin-dependent cAMP

production was not (not shown). Similar results were obtained following 15 min preincubation with 10^{-5} M histamine (not shown). Taken together, these results suggest that the 51 amino acids of the C-terminus of the histamine H2 receptor are not essential for desensitization of the receptor-mediated cAMP response.

Effect of C-terminal truncation on agonist-induced internalization of the H2 receptor

A number of GPCRs have been reported to be internalized into compartments inaccessible to agonists acting at the plasma membrane. Agonist-induced internalizations of several other GPCRs have been detected by measuring the acid-resistant forms of radiolabeled agonists (7, 20-23). However, this experiment can not be performed for the H2 receptor because of the high levels of non-specific uptake of histamine by cells. To circumvent this difficulty, we utilized the HA-tag, which is located at the N-terminal extracellular region, to quantify the cell-surface H2 receptor amount. Specific cell-surface bindings of anti-HA antibody were observed in COS7 cells expressing HA-WT and HA-T^{308} receptors (Fig. 3A), but not in parental or mock transfected COS7 cells (not shown). We examined the effect of incubation with 10^{-5} M histamine on the cell-surface H2 receptor amount. As shown in Fig. 5A, in COS7 cells expressing HA-WT receptors the amount of cell-surface anti-HA antibody binding decreased by approximately 30% with a 30 min incubation with 10^{-5} M histamine and the $t_{1/2}$ was approximately 15 min. However, neither the binding maximum (B_{max}) of

tiotidine in membrane fractions (Fig. 5B), which represents the total receptor amount, nor the K_d for tiotidine (not shown) was decreased. These findings indicate that HA-WT receptors were internalized from the cell-surface. Immunocytochemical examination after a 30 min incubation with histamine revealed the HA-WT receptors to be on the plasma membrane, possibly indicating that the receptors were in coated pits or caveola. In contrast, incubation of COS7 cells expressing HA-T^{308} receptors with histamine affected neither cell-surface anti-HA antibody binding (Fig. 5A) nor the B_{max} of tiotidine (Fig. 5B), indicating that the 51 C-terminal amino acids of the H2 receptor play a role in agonist-induced internalization of the receptor.

Effects of truncation of 15 and 30 C-terminal amino acids on H2 receptor signaling, desensitization and internalization

To define more clearly the region involved in internalization, two mutant receptors, T^{329} and T^{344}, with deletion of 30 and 15 C-terminal amino acids, respectively, were generated. These mutants were also tagged with the HA-epitope at their N-termini and were expressed in COS7 cells. Both receptors were capable of binding tiotidine with affinities similar to that of the WT receptor (not shown). In contrast to the finding in the HA-T^{308} mutant receptor, cAMP productions via both receptors were comparable to those mediated by the HA-WT receptor provided that the amounts of cell-surface receptors per cell were equivalent (Fig. 6A). These observations indicate that these 30 amino acids of the C-terminus are not involved in inhibition of cAMP production. In addition,

cAMP productions via these receptors were desensitized by 10^{-5} M histamine preincubation (not shown). Finally, upon histamine exposure, HA-T^{329} and HA-T^{344} receptors showed internalization indistinguishable from that of the HA-wild-type receptor (Fig. 6B).

Identification of the amino acid residues involved in internalization of the H2 receptor

The results obtained indicate that the region responsible for agonist-induced internalization and inhibition of cAMP production resides between amino acid residues 308 and 328. To identify the amino acid residues involved in agonist-induced internalization, we generated additional mutants, HA-T^{313} and HA-T^{320}, with deletions of 39 and 46 C-terminal amino acids, respectively. Interestingly, agonist-induced internalization was observed in the HA-T^{320} , but not in the HA-T^{313} receptor (not shown). Thus, the amino acid residues involved in agonist-induced internalization of the histamine H2 receptor are likely to reside between Glu^{314} and Asn^{320} (ETSLRSN). Of these seven amino acid residues, assuming that the hypothesis that serine and/or threonine phosphorylation is involved in agonist-induced internalization of the H2 receptor is correct, the likely candidates are Thr^{315} and Ser^{316}. This is because these two residues are conserved among species (Table 3). To determine whether these amino acid residues are involved in agonist-induced internalization, we generated three mutant receptors, HA-A^{315}, HA-A^{316} and HA-$A^{315,316}$, in which either Thr^{315}, Ser^{316}, or both were replaced with Ala. As shown in Fig. 7, the HA-A^{316} receptor internalization was

indistinguishable from that of the WT, whereas those of the HA-$A^{315, 316}$ and HA-A^{315} were not. This finding clearly demonstrates that Thr^{315} is involved in agonist-induced internalization of the histamine H2 receptor and that the inabilities of the HA-T^{308} and HA-T^{313} mutants to internalize were not due to nonspecific effects induced by the truncations.

DISCUSSION

The histamine H2 receptor plays a central role in acid production in gastric parietal cells via production of cAMP (24). Inappropriate gastric acid production can lead to the generation or exacerbation of peptic ulcer disorders. Tight control of signaling via the H2 receptor is thus vital for normal gastric function. It has been accepted that the histamine H2 receptor undergoes rapid desensitization of the cAMP response upon exposure to an agonist (1-5). Although homologous desensitization of agonist-induced signaling via GPCRs is an essentially ubiquitous phenomenon, the functional roles of the C-termini vary among receptors. In a number of GPCRs, agonist-induced serine/threonine phosphorylation of the C-terminus has been implicated in receptor desensitization (8-10), while in others the C-terminus has been suggested to be involved in agonist-induced internalization, but not in desensitization (20, 25, 26). To understand the as yet unidentified roles of the C-terminus in the histamine H2 receptor, we generated two mutant receptors, T^{289} and T^{308}, which were truncated at the beginning of the cytoplasmic C-terminal portion and just distal to putative palmitoylation site Cys^{305}, respectively. The WT and mutant receptors were tagged at their N-termini with the HA epitope and expressed in COS7 cells.

One of the primary observations made in our present study was that the T^{308} receptor with a truncation of 51 amino acids located distal to the putative palmitoylation site was desensitized upon histamine exposure. It is hypothesized that serine/threonine phosphorylation is involved in agonist-induced

desensitization of GPCRs. The T^{308} mutant, which is missing 11 serine/threonine residues present in the C-terminus of the histamine H2 receptor, did not show altered agonist-induced uncoupling of the H2 receptor from adenylyl cyclase. Therefore, it is likely that the serine/threonine residues, which may be phosphorylated as part of the desensitization process, reside in other intracellular regions or the proximal C-terminal residues, namely Thr^{290} and Thr^{297}. Possible contributions of this proximal carboxyl region could not be examined because T^{289} receptors, which are devoid of all C-terminal amino acid residues, were nonfunctional (Table 1, Fig. 3B). It is noteworthy that the lack of T^{289} receptor activity is not due to the absence of cell-surface expression but rather to the receptor itself being devoid of functional activity (Fig. 3).

Secondly, the WT receptor was internalized upon histamine exposure while the T^{308} mutant was not. In addition, the T^{329} mutant with a deletion of 30 amino acids was internalized. Thus, the region involved in internalization of the histamine H2 receptor is present within the area including amino acid residues from 308 to 328. The observation that the T^{320}, but not the T^{313} receptor, was internalized further confined the amino acid residues involved in agonist-induced internalization. Finally, the finding that internalization occurred in the HA-A^{316} receptor, but in neither the HA-A^{315} nor the HA-$A^{315, 316}$ receptor, identified the amino acid residue involved in agonist-induce internalization of the H2 receptor as Thr^{315}. Our preliminary observations indicated that both the HA-WT and the HA-T^{308} receptors were phosphorylated after histamine stimulation (not shown).

Thus, although we can speculate that phosphorylation does actually play a part in desensitization, we cannot determine whether it is involved in agonist-induced internalization of the H2 receptor. Recently, Barak et. al. reported that a tyrosine residue highly conserved in G-protein coupled receptors and in the NPXXY motif is involved in sequestration of the $ß_2$-adrenergic receptor (27). This tyrosine residue is conserved in the histamine H2 receptor (Tyr 288) (17, 28, 29). The T^{308} receptor contains this tyrosine, such that the tyrosine residue Tyr 288 appears to be necessary for, but is not by itself sufficient to produce, internalization of the histamine H2 receptor. Furthermore, desensitization of the cAMP response occurring via the T^{308} receptor, which was not internalized, indicates that internalization per se is not essential for agonist-induced desensitization of the cAMP response and that desensitization and internalization are mediated by independent mechanisms.

It is noteworthy that desensitization of the WT receptor was slightly more extensive than that of the T^{308} receptor (Fig. 4). A possible explanation for this observation is that receptor internalization, which occurred in WT but not in T^{308} receptors, might have affected cAMP productions via the WT receptors. However, at the expression levels used for these studies, cAMP productions via these receptors correlated with the cell-surface receptor amounts (Fig. 3). Therefore, internalization of the WT receptor should have had an additive effect on desensitization of cAMP productions and the cAMP productions via WT receptors in treated cells should have been less than those observed in this

study. At present, we can not provide an appropriate explanation for this observation.

Thirdly, we have shown the T^{308} receptor to be more active than the WT receptor in terms of adenylyl cyclase stimulation. In contrast, the T^{329} and the T^{344} receptors showed essentially the same levels of activity as the WT receptor. Thus, the 51 C-terminal amino acids of the histamine H2 receptor contain sequences that suppress the interaction of this receptor with G-proteins and these sequences apparently lie within the region from amino acids 308 to 329. When these sequences are removed a more productive interaction takes place, and hormonal responsiveness upon truncation is enhanced. As desensitization still took place in the T^{308} receptor, this inhibitory effect is unlikely to be related to desensitization.

In conclusion, we have shown in COS7 cells that the C-terminus of the histamine H2 receptor is involved in agonist-induced internalization, but not in desensitization. Furthermore, the C-terminus is likely to exert an inhibitory effect on cAMP production via the receptor. Since these experiments were performed using fibroblast cell lines transfected with histamine H2 receptor cDNAs, the results presented show that the processes involved in histamine-induced uncoupling of the histamine H2 receptor are not specific to gastric parietal cells. However, the extent of internalization observed in this system is minimal compared with those of other receptors. We can not rule out the possibility that some other cofactor, which is not present in COS7, might function

in histamine H2 receptor internalization. Future studies must be designed to identify the specific amino acid residues involved in agonist-induced desensitization and internalization.

REFERENCES

1. **Alblas, J., I. van Etten, A. Khanum, and W. H. Moolenaar.** 1995. C-terminal truncation of the neurokinin-2 receptor causes enhanced and sustained agonist-induced signaling. Role of receptor phosphorylation in signal attenuation. J. Biol. Chem. **270:**8944-8951.

2. **Arima, N., Y. Kinoshita, A. Nakamura, Y. Yamashita, and T. Chiba.** 1993. Homologous desensitization of histamie H2 receptors in the human gasric carcinoma cell line MKN-45. Am. J. Physiol. **265:**G987-G992.

3. **Asano, T., K. Takata, H. Katagiri, K. Tsukuda, J.-L. Lin, H. Ishihara, K. Inukai, H. Hirano, Y. Yazaki, and Y. Oka.** 1992. Domains responsible for the differential targeting of glucose transporter isoforms. J. Biol. Chem. **267:**19636-19641.

4. **Barak, L. S., M. Tiberi, N. J. Freedman, M. M. Kwatra, R. J. Lefkowitz, and M. G. Caron.** 1994. A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated beta 2-adrenergic receptor sequestration. J. Biol. Chem. **269:** 2790-2795.

5. **Benya, R. V., Z. Fathi, J. F. Battey, and R. T. Jensen.** 1993. Serines and threonines in the gastrin-releasing peptide receptor carboxyl terminus mediate internalization. J. Biol. Chem. **268:** 20285-20290.

6. **Black, J. W., W. A. Duncan, C. J. Durant, C. R. Ganellin, and E. M. Parsons.** 1972. Definition and antagonism of histamine H 2 -receptors. Nature **236:**385-390.

7. **Bouvier, M., W. P. Hausdorff, A. De Blasi, B. F. O'Dowd, B. K. Kobilka, M. G. Caron, and R. J. Lefkowitz.** 1988. Removal of phosphorylation sites from the beta 2-adrenergic receptor delays onset of agonist-promoted desensitization. Nature **333:**370-373.

8. **Fukushima, Y., T. Asano, H. Katagiri, M. Aihara, T. Saitoh, M. Anai, M. Funaki, T. Ogihara, K. Inukai, N. Matsuhashi, Y. Oka, Y. Yazaki, and K. Sugano.** 1996. Interaction between the two signal transduction systems of the histamine H2 receptor; desensitizing and sensitizing effects of histamine stimulation on histamine-dependent cAMP production in Chinese hamster ovary cells. Biochem. J. **320:**27-32.

9. **Fukushima, Y., Y. Oka, H. Katagiri, T. Saitoh, T. Asano, H. Ishihara, N. Matsuhashi, T. Kodama, Y. Yazaki, and K. Sugano.** 1993. Desensitization of canine histamine H2 receptor expressed in Chinese hamster ovary cells. Biochem. Biophys. Res. Commun. **190:**1149-1155.

10. **Fukushima, Y., Y. Oka, T. Saitoh, H. Katagiri, T. Asano, N. Matsuhashi, K. Takata, E. van Breda, Y. Yazaki, and K. Sugano.** 1995. Structural and functinal analysis of the canine histamine H2 receptor by site-directed mutagenesis: N-glycosylation is not vital for its action. Biochem. J. **310:**553-558.

11. **Gajtkowski, G. A., D. B. Norris, T. J. Rising, and T. P. Wood.** 1983. Specific binding of 3H-tiotidine to histamine H2 receptors in guinea pig cerebral cortex. Nature **304:**65-67.

12. **Gantz, I., J. DelValle, L. D. Wang, T. Tashiro, G. Munzert, Y. J. Guo, Y. Konda, and T. Yamada.** 1992. Molecular basis of the interaction of histamine with the histamine H2 receptor. J. Biol. Chem. **267:**20840-20843.

13. **Gantz, I., G. Munzert, T. Tashiro, M. Schäffer, L. Wang, J. DelValle, and T. Yamada.** 1991. Molecular cloning of the human histamine H2 receptor. Biochem. Biophys. Res. Commun. **178:**1386-1392.

14. **Gantz, I., M. Schäffer, J. DelValle, C. Logsdon, V. Campbell, M. Uhler, and T. Yamada.** 1991. Molecular cloning of a gene encoding the histamine H2 receptor. Proc. Natl. Acad. Sci. U. S. A. **88:**429-433.

15. **Grady, E. F., L. W. Slice, W. O. Brant, J. H. Walsh, D. G. Payan, and N. W. Bunnett.** 1995. Direct observation of endocytosis of gastrin releasing peptide and its receptor. J. Biol. Chem. **270:**4603-4611.

16. **Hermans, E., J. N. Octave, and J. M. Maloteaux.** 1996. Interaction of the COOH-terminal domain of the neurotensin receptor with a G protein does not control the phospholipase C activation but is involved in the agonist-induced internalization. Mol. Pharmacol. **49:**365-372.

17. **Homma, M., T. Satoh, J. Takezawa, and M. Ui.** 1977. An ultrasensitive method for the simultaneous determination of cyclic AMP and cyclic GMP in small-volume samples from blood and tissues. Biochem. Med. **18:**257-273.

18. **Huang, Z., Y. Chen, and R. A. Nissenson.** 1995. The cytoplasmic tail of the G-protein-coupled receptor for parathyroid hormone and parathyroid hormone-related protein contains positive and negative signals for endocytosis.

J. Biol. Chem. **270:**151-156.

19. **Iida-Klein, A., J. Guo, L. Y. Xie, H. Juppner, J. Potts, Jr., H. M. Kronenberg, F. R. Bringhurst, A. B. Abou-Samra, and G. V. Segre.** 1995. Truncation of the carboxyl-terminal region of the rat parathyroid hormone (PTH)/PTH-related peptide receptor enhances PTH stimulation of adenylyl cyclase but not phospholipase C. J. Biol. Chem. **270:**8458-8465.

20. **Lattion, A. L., D. Diviani, and S. Cotecchia.** 1994. Truncation of the receptor carboxyl terminus impairs agonist-dependent phosphorylation and desensitization of the alpha 1B-adrenergic receptor. J. Biol. Chem. **269:**22887-22893.

21. **Prost, A., S. Emani, and C. Gespach.** 1984. Desensitization by histamine H2 receptor-mediated adenylate cyclase activation in the human gastric cancer cell line HGT-1. FEBS Lett. **177:**227-230.

22. **Ruat, M., E. Traiffort, J. M. Arrang, R. Luers, and J. C. Schwartz.** 1991. Cloning and tissue expression of a rat histamine H2-receptor gene. Biochem. Biophys. Res. Commun. **179:**1470-1478.

23. **Sanchez-Yague, J., M. C. Rodriguez, D. L. Segaloff, and M. Ascoli.** 1992. Truncation of the cytoplasmic tail of the lutropin/choriogonadotropin receptor prevents agonist-induced uncoupling. J. Biol. Chem. **267:**7217-7220.

24. **Smit, M. J., R. Leurs, S. R. Shukrula, A. Bast, and H. Timmerman.** 1994. Eur. J. Pharmacol. **288:**17-25.

25. **Smit, M. J., H. Timmerman, A. E. Alewijnse, M. Punin, I. van den**

Nieuwenhof, J. Blauw, J. van Minnen, and R. Leurs. 1995. Visualization of agonist-induced internalization of histamine H2 receptors. Biochem. Biophys. Res. Commun. **214:**1138-1145.

26. **Soll, A. H., and A. Wollin.** 1979. Histamine and cyclic AMP in isolated canine parietal cells. Am. J. Physiol. **237:**E444-E450.

27. **Takano, T., Z. Honda, C. Sakanaka, T. Izumi, K. Kameyama, K. Haga, T. Haga, K. Kurokawa, and T. Shimizu.** 1994. Role of cytoplasmic tail phosphorylation sites of platelet-activating factor receptor in agonist-induced desensitization. J. Biol. Chem. **269:**22453-22458.

28. **Thomas, W. G., K. M. Baker, T. J. Motel, and T. J. Thekkumkara.** 1995. Angiotensin II receptor endocytosis involves two distinct regions of the cytoplasmic tail. J. Biol. Chem. **270:**22153-22159.

29. **Thomas, W. G., T. J. Thekkumkara, T. J. Motel, and K. M. Baker.** 1995. Stable expression of a truncated AT1A receptor in CHO-K1 cells. The carboxyl-terminal region directs agonist-induced internalization but not receptor signaling or desensitization. J. Biol. Chem. **270:**207-213.

30. **Wang, L. H., S. A. Mantey, J. T. Lin, H. Frucht, and R. T. Jensen.** 1993. Ligand binding, internalization, degradation and regulation by guanine nucleotides of bombesin receptor subtypes: a comparative study. Biochim. Biophys. Acta **1175:**232-242.

TABLES

Table 1

Dissociation constants (K_d) for tiotidine of wild-type and truncated H2 receptors.

	Kd (nM)		Kd (nM)
WT	23.2 ± 1.1	HA-WT	25.6 ± 0.8
T^{308}	25.2 ± 2.7	HA-T^{308}	22.1 ± 1.5
T^{289}	no binding	HA-T^{289}	no binding

Table 2

Transfection efficiency as assessed by immunostaining with the anti-HA antibody

	[cDNA]	HA-WT %	HA-T^{308} %	HA-T^{289} %
Exp. 1	5 µg	17.3 ± 2.3	16.1 ± 2.7	17.6 ± 2.3
	0.1 µg	16.1 ± 1.2	17.2 ± 3.2	19.2 ± 3.2
Exp. 2	5 µg	19.4 ± 2.1	18.8 ± 4.7	16.1 ± 2.5
	0.1 µg	21.1 ± 3.5	17.7 ± 5.8	17.9 ± 4.1
Exp .3	5 µg	18.5 ± 3.3	19.1 ± 3.6	16.9 ± 3.3
	0.1 µg	19.3 ± 1.2	17.7 ± 2.4	18.1 ± 0.9

Table 3

Comparison of amino acid sequences of histamine H2 receptor C-termini

Truncation at		**-46** (T^{313})	**-39** (T^{320})	**-30** (T^{329})
Canine	CRPASHNAQ	ETSLRSN	SSQLARNQS	REPMRQEEKPLKLQVWSGTEVTAPRGATDR
Rat	CKFASHNSH	KTSLRLN	NSLLPRSQS	REGRWQEEKPLKLQVWSGTELTHPQGNPIR
Human	CRLANRNSH	KTSLRSN	ASQLSRTQS	REPRQQEEKPLKLQVWSGTEVTAPQGATDR
Guinea pig	CRLASHNSH	ETSLRLN	NSQLNRSQC	QEPRWQEDKPLNLQVWSGTEVTAPQGATNR

FIGURE LEGENDS

Figure 1

Schematic representation of the canine histamine H2 receptor

The sites of truncation for the deletion mutants, T^{308} and T^{289}, are indicated by arrows. Serine and threonine residues in the C-terminus are shown in bold characters. The palmitoylation and membrane anchorage of Cys 305 are presumed. Consensus sequences of N-glycosylation sites, Asn 4, Asn 162 and Asn 168, are also presented.

Figure 2

Immunocytochemistry of the WT and truncated receptors expressed in COS7 cells

COS7 cells were transfected with each plasmid DNA. At 48 h post-transfection COS7 cells were fixed in PBS with 3% paraformaldehyde. Thin sections were prepared and immunostained with anti-HA antibody (**a**, **b**, **c**) or anti-$H2R^{CT}$ antibody (**d**). **a**. HA-T^{289}; **b**. HA-T^{308}; **c**. HA-WT; **d**. WT. Scale bar, 10 μm.

Figure 3

A. Relationship between the amount of plasmid used to transfect COS7 cells and the number of cell-surface receptors expressed

COS7 cells were transfected with varying amounts of plasmid DNA containing

cDNAs for the WT and truncated receptors. The cell were fixed in 3% paraformaldehyde/PBS and cell-surface anti-HA antibody bindings were determined as described in Experimental Procedures. HA-WT (●), HA-T^{308} (■), HA-T^{289} (△). Data shown are means ± S.E. from three separate experiments, each performed in triplicate. When not shown, S.E. values were so small that they fell within the symbols.

B. Relationship between the numbers of cell-surface receptors expressed and cAMP productions via WT and truncated receptors.

At 48 h post-transfection, COS7 cells on 24-well plates at a density of 10^5 cells / well were preincubated in Hepes-Tyrode's buffer containing 0.1% BSA and 0.1 mM 3-isobutyl-1-methylxanthine for 30 min, then incubated for an additional 10 min in the absence or presence of 10^{-7} M (left panel) or 10^{-5} M (right panel) histamine. Incubation was terminated by the addition of 500 μl of 12% trichloroacetic acid. cAMP contents were measured by a radioimmunoassay. HA-WT (●), HA-T^{308} (■), HA-T^{289} (△). Data (mean ± S.E.) are from one of three representative experiments performed in triplicate. When not shown, S.E. values were so small that they fell within the symbols.

Figure 4

Effect of histamine treatment on subsequent cAMP accumulation

At 48 h post-transfection cells were preincubated in the presence or absence

of 10^{-5} M histamine for 30 min at 37°C. Subsequently, the cells were washed and either varying concentrations of histamine (**A**) or 10^{-4} M forskolin (**B**) were added to the cells which had been preincubated with histamine. Incubations continued for an additional 10 min at 37°C and reactions were terminated by the addition of trichloroacetic acid (6% w/v final concentration). ●, preincubation with vehicle; ■, with histamine. Shown are the means ± S.E. of three individual experiments, each performed in triplicate. When not shown, S.E. values were so small that they fell within the symbols.

Figure 5

Effects of histamine treatment on the amounts of cell-surface receptors or of total receptors.

A. COS7 cells expressing HA-WT or HA-T^{308} receptors on 24-well plates were incubated with 10^{-5} M histamine or vehicle for the indicated times, and the cells were then fixed in 3% paraformaldehyde/PBS. Cell-surface anti-HA antibody bindings were determined as described in the Experimental Procedures. HA-WT (●), HA-T^{308} (■). Shown are the means ± S.E. of three individual experiments, each performed in triplicate. When not shown, S.E. values were so small that they fell within the symbols.

B. After incubation in the presence or absence of 10^{-5} M histamine at 37°C for 60 min, COS7 cells were scraped from the plates, and membrane fractions thus prepared were incubated with 1 nM [^{3}H]-tiotidine and increasing concentrations

of unlabeled tiotidine at 37°C for 2 h. Bound [^{3}H]-tiotidine was trapped by filtration through GF/C filters. Radioactivies remaining on filters after washing were counted by liquid scintillation. Open bars, HA-WT; filled bars, HA-T^{308}. Shown are the means ± S.E. of three individual experiments, each performed in triplicate. When not shown, S.E. values were so small that they fell within the symbols.

Figure 6

Effects of truncation of 15 and 30 C-terminal amino acids

A. Relationship between the numbers of cell-surface receptors expressed and cAMP productions COS7 cells transfected with varying amounts of plasmid DNA containing cDNAs for the HA-WT, HA-T^{329} and HA-T^{344} receptors were plated onto 24-well plates. At 48 h post-transfection, histamine (10^{-7} M, left panel; 10^{-5} M, right panel)-dependent cAMP productions and cell-surface anti-HA antibody bindings were measured. HA-WT (●), HA-T^{329} (□), HA-T^{344} (▲). Data (mean ± S.E.) are from one of three representative experiments performed in triplicate. When not shown, S.E. values were so small that they fell within the symbols. B. **Effects of histamine treatment on the amounts of cell-surface receptors or of total receptors.** (left panel) COS7 cells expressing HA-WT, HA-T^{329} or HA-T^{344} receptors on 24-well plates were incubated with 10^{-5} M histamine or vehicle for the indicated times and cell-surface anti-HA antibody bindings were determined. HA-WT (●), HA-T^{329} (□), HA-T^{344} (▲).

Shown are the means ± S.E. of three individual experiments, each performed in triplicate. When not shown, S.E. values were so small that they fell within the symbols. (right panel) After incubation in the presence or absence of 10^{-5} M histamine at 37°C for 60 min, COS7 cells were scraped from the plates, and tiotidine bindings in membrane fractions thus prepared were measured. Open bars, HA-WT; filled bars, HA-T^{329}; hatched bars, HA-T^{344}. Shown are the means ± S.E. of three individual experiments, each performed in triplicate. When not shown, S.E. values were so small that they fell within the symbols.

Figure 7

Effects of histamine treatment on the amounts of cell-surface HA-A^{315}, HA-A^{316} and HA-$A^{315,316}$ receptors

COS7 cells expressing HA-A^{315}, HA-A^{316} or HA-$A^{315,316}$ receptors on 24-well plates were incubated with 10^{-5} M histamine or vehicle for the indicated times and cell-surface anti-HA antibody bindings were determined. HA-A^{315} (●), HA-A^{316} (■), HA-$A^{315,316}$ (▲). Shown are the means ± S.E. of two individual experiments, each performed in triplicate. When not shown, S.E. values were so small that they fell within the symbols.

Figure 1

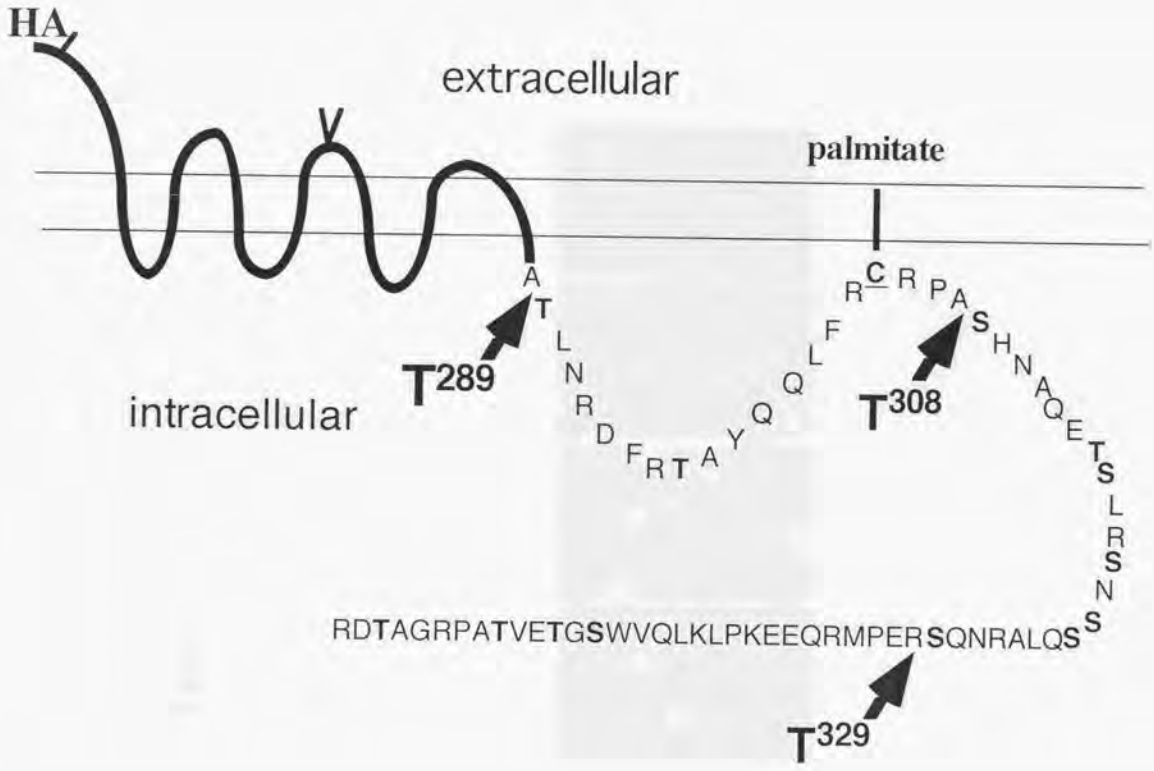

HA
extracellular
palmitate
intracellular
T289
T308
T329
RDTAGRPATVETGSWVQLKLPKEEQRMPERSQNRALQS

Figure 2

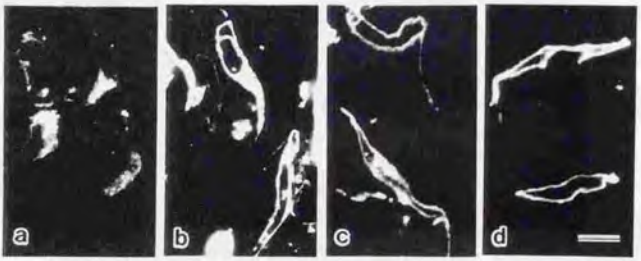


Figure 3

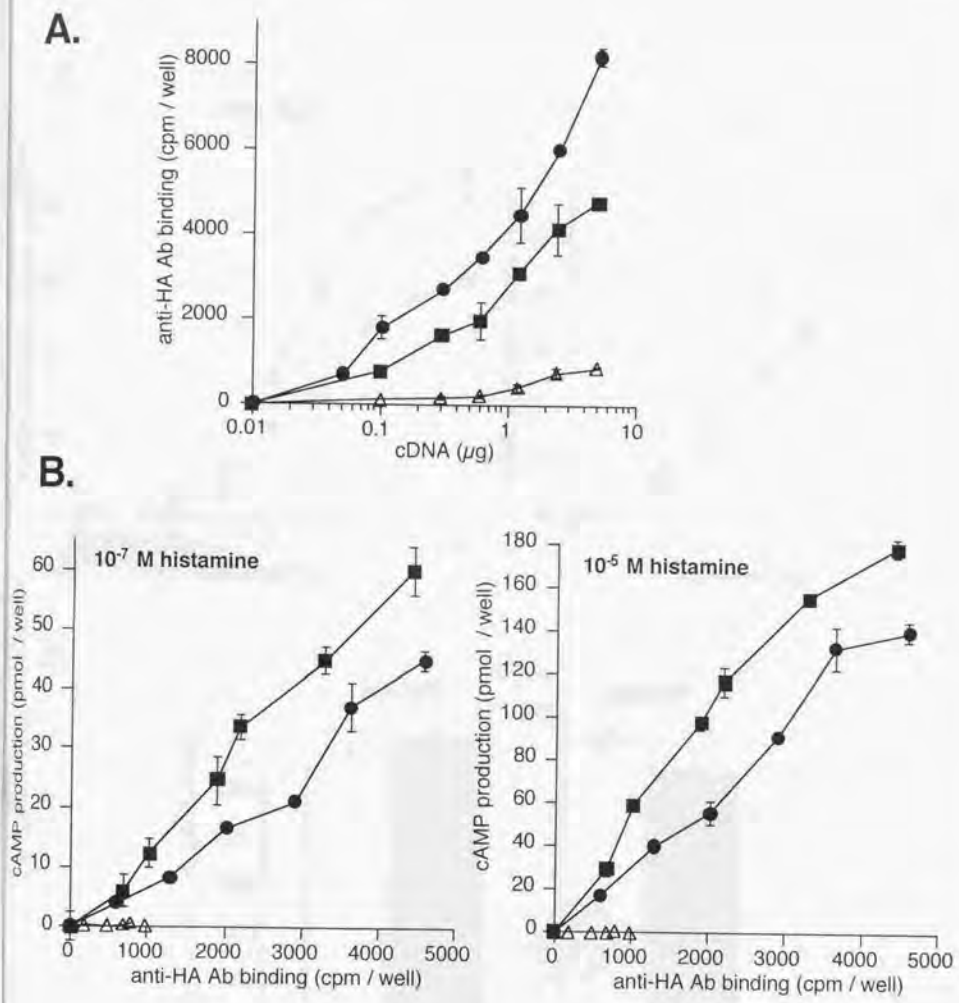

A.
anti-HA Ab binding (cpm / well)
8000
6000
4000
2000
0
0.01
0.1
1
10
cDNA (μg)
B.
10-7 M histamine
cAMP production (pmol / well)
60
50
40
30
20
10
0
0
1000
2000
3000
4000
5000
anti-HA Ab binding (cpm / well)
10-5 M histamine
cAMP production (pmol / well)
180
160
140
120
100
80
60
40
20
0
0
1000
2000
3000
4000
5000
anti-HA Ab binding (cpm / well)

Figure 4

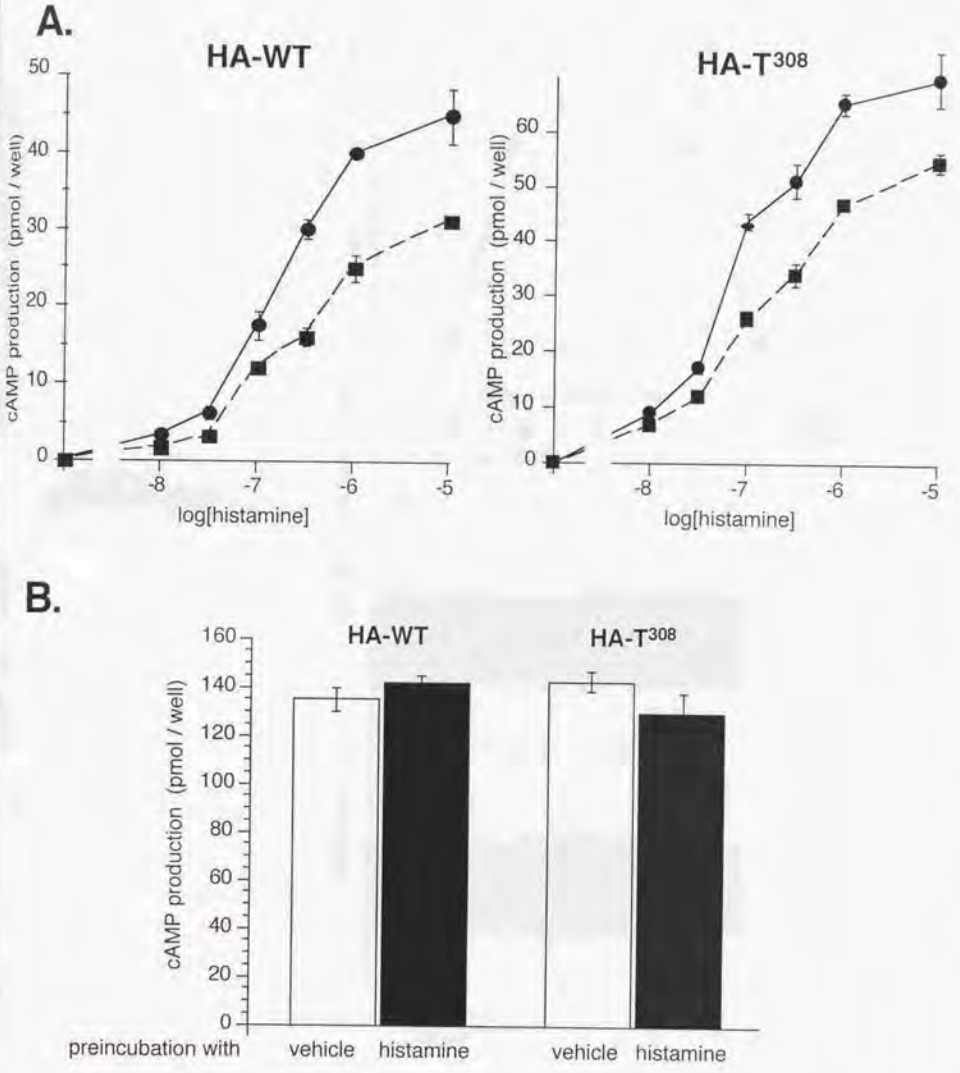

A.
HA-WT
HA-T308
cAMP production (pmol / well)
log[histamine]
B.
HA-WT
HA-T308
cAMP production (pmol / well)
preincubation with
vehicle
histamine
vehicle
histamine

Figure 5

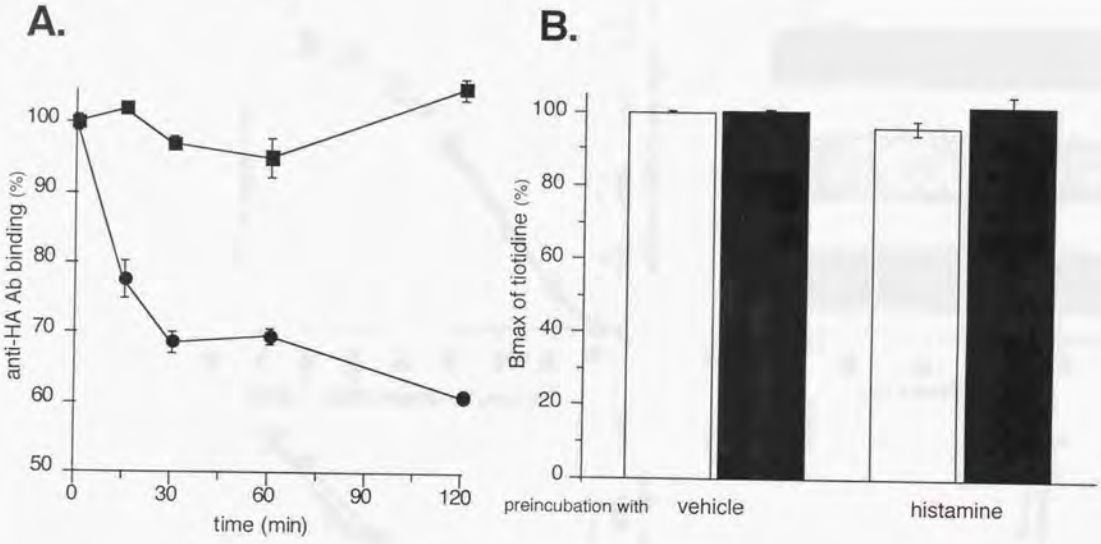

A.
B.
anti-HA Ab binding (%)
100
90
80
70
60
50
0
30
60
90
120
time (min)
Bmax of tiotidine (%)
100
80
60
40
20
0
preincubation with
vehicle
histamine

Figure 6

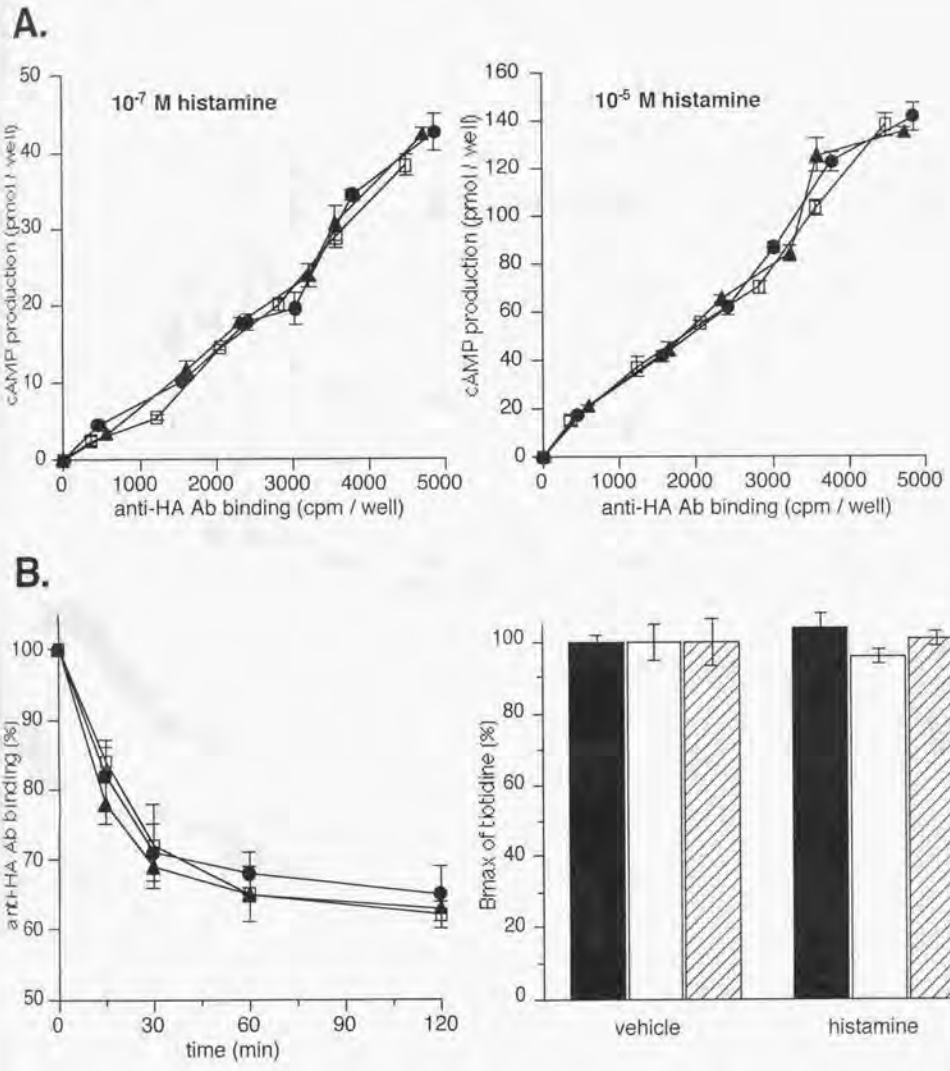
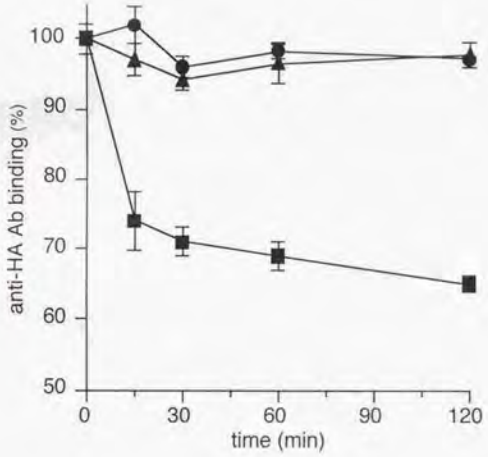
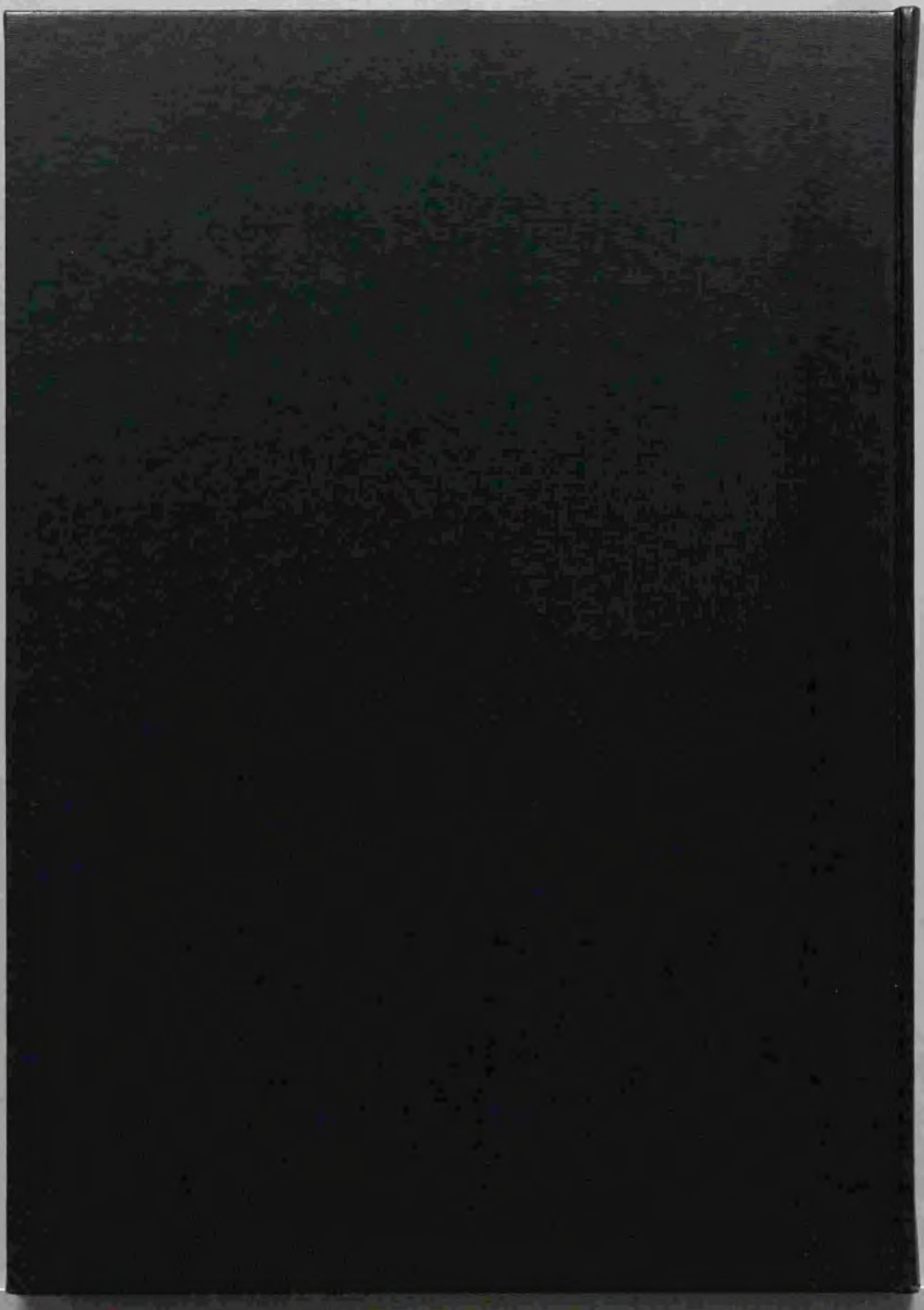
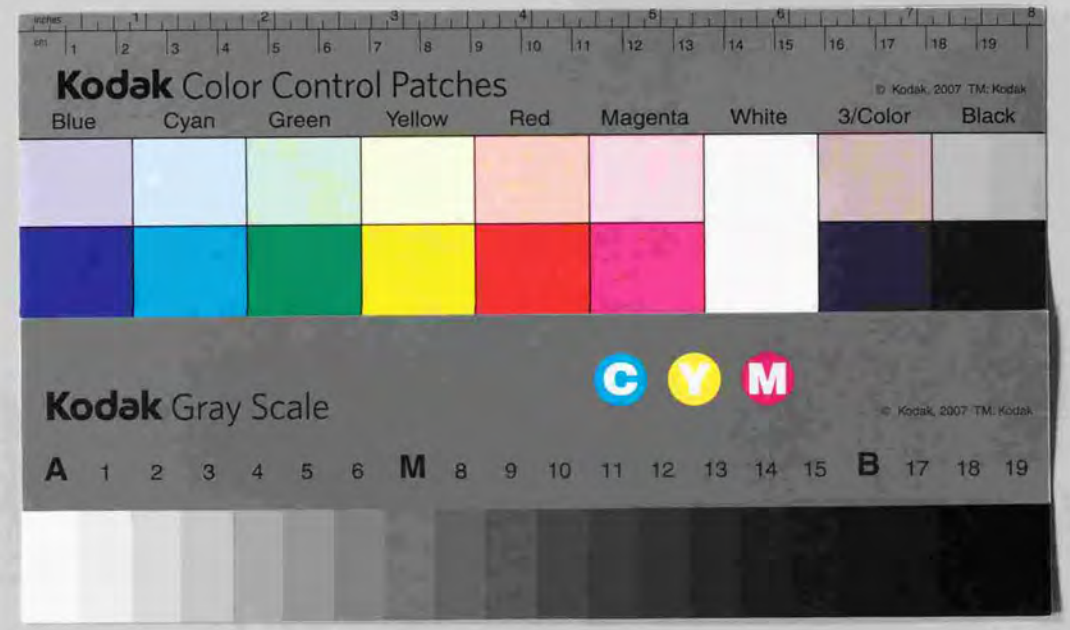

A.
10-7 M histamine
cAMP production (pmol / well)
anti-HA Ab binding (cpm / well)
10-5 M histamine
cAMP production (pmol / well)
anti-HA Ab binding (cpm / well)
B.
anti-HA Ab binding [%]
time (min)
Bmax of tiotidine [%]
vehicle
histamine

Figure 7






Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19