Histamine H\textsubscript{1} Receptor Down-Regulation Mediated by M\textsubscript{3} Muscarinic Acetylcholine Receptor Subtype

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Abstract. Heterologous down-regulation of histamine H\textsubscript{1} receptor (H1R) mediated by muscarinic acetylcholine receptor subtype was investigated using five kinds of Chinese hamster ovary (CHO) cells stably co-expressing the human H1R and one of the five (M\textsubscript{1} – M\textsubscript{5}) muscarinic acetylcholine receptors, CHO-H1/M1, CHO-H1/M2, CHO-H1/M3, CHO-H1/M4, and CHO-H1/M5 cells. Among the CHO-H1/M1, CHO-H1/M3, and CHO-H1/M5 cells, carbachol treatment of the CHO-H1/M3 cells time-dependently led to remarkable down-regulation of the H1R to 60% of the control level. In contrast, stimulation of CHO-H1/M1 cells by carbachol induced negligible effect on the down-regulation. Stimulation of CHO-H1/M5 cells by carbachol induced significant but only small H1R down-regulation. M\textsubscript{2} and M\textsubscript{4} muscarinic receptors showed negligible effect on the down-regulation. H1R-mediated accumulation of inositol phosphates in CHO-H1/M3 cells with long-term exposure to carbachol was decreased to 60% compared with non-treated cells. Heterologous phosphorylation of H1R was induced by the stimulation of each muscarinic receptor. H1R was phosphorylated by about twofold from the basal level through five subtypes of muscarinic receptor. The M\textsubscript{3} muscarinic receptor-mediated phosphorylation of H1R was reversed by the inhibition of protein kinase C. In the present study we demonstrated that the M\textsubscript{3} muscarinic acetylcholine receptor mediated remarkable down-regulation of the H1R with decreased receptor signaling.

Keywords: histamine H\textsubscript{1} receptor, M\textsubscript{3} muscarinic receptor, down-regulation, desensitization, phosphorylation

Introduction

The histamine H\textsubscript{1} receptor (H1R), one of the G protein-coupled receptors (GPCRs), predominantly couples with Gq/11 protein and phospholipase C-\textbeta and mediates type I allergy in peripheral tissues and histaminergic neurotransmission in the central nervous system (1, 2). Desensitization of various GPCRs including H1R has been documented and is thought to be a protection mechanism of receptor-bearing cells against excess stimulation. Two types of desensitization, homologous and heterologous desensitizations, have been extensively investigated by pharmacological methods. There are many reports about heterologous desensitization between different GPCRs. It is generally accepted that receptor phosphorylation is involved in receptor desensitization (3, 4). Phosphorylation of GPCRs by second messenger-dependent protein kinases was postulated to trigger receptor desensitization. G-protein coupled receptor kinases (GRKs) were found to function in homologous desensitization.

Receptor desensitization has been considered to advance in three steps, namely, uncoupling with effectors, internalization, and down-regulation, as determined by studies at the molecular level. Down-regulation of GPCRs, which is thought to lead to long-term suppression of the receptor signaling, has been focused on with great interest (5 – 7). Long-term homologous stimulation of H1R by histamine also induced H1R down-regulation (8), and heterologous up-regulation of H1R...
by β2-adrenergic receptor activation was reported (9). Down- or up-regulation of GPCRs alters the receptor function by regulation of receptor signaling. Previous study in human of type I allergy revealed that the number of H1R was changed (10), which is of importance in its treatment.

Muscarinic receptors are distributed in various tissues, such as smooth muscle, cardiac muscle, and brain (11, 12), and regulate numerous biological actions. The M1, M2, and M3 muscarinic receptors couple predominantly to Gq-protein and activate phospholipase C, while M4 and M5 muscarinic receptors couple to Gi-protein and inhibit adenyl cyclase. H1R and each subtype of muscarinic receptors are co-expressed in various tissues. Especially, in regulation of airway smooth muscle contraction, these receptors are postulated to have physiological importance via the parasympathetic nervous system. H1R and each muscarinic receptor-induced down-regulation of H1R signaling by muscarinic receptors were co-localized on airway smooth muscle. Heterologous regulation of H1R signaling by muscarinic receptors via the parasympathetic nervous system is postulated to have physiological importance in the airway.

In the present study, we report M3 muscarinic receptor-induced down-regulation of H1R, leading to a long-term regulation of H1R signaling mediated by some mechanism other than H1R phosphorylation.

Materials and Methods

Materials

Plasmid DNAs for human M1, M2, M3, M4, and M5 muscarinic receptors were kindly provided by Tom I. Bonner (National Institute of Mental Health, Bethesda, MD, USA). [Pyridinyl-5-3H]mepyramine ([3H]mepyramine, 1 mCi/ml, 20 Ci/mmol), quinuclidinyl [phenyl-4-3H]benzilate ([3H]QNB, 1 mCi/ml, 30 Ci /mmol), [32P]orthophosphoric acid (314–377 TBq /mmol), and myo-[3H]inositol (1 mCi/ml, 20 Ci/mmol) were obtained from Perkin Elmer (Boston, MA, USA). KT5823, a protein kinase G (PKG) inhibitor, was purchased from Alexis (Lausen, Switzerland). PolyFect Transfection Reagent was from Qiagen K.K. (Tokyo). Ro 31-8220, a protein kinase C (PKC) inhibitor, and other reagents were purchased from Wako (Osaka).

Preparation of CHO cells stably co-expressing the human H1R and each muscarinic acetylcholine receptor subtype

CHO cells stably expressing H1Rs (CHO-H1R cells) were prepared according to the previously described method (13). Plasmids encoding muscarinic receptor subtype cDNAs were transfected into CHO-H1R cells. In brief, the mammalian expression vector pdkKR-dhfr, containing the wild-type human H1R gene was introduced into CHO cells with the calcium phosphate precipitation method. Transfected CHO cells were cultured in α-minimum essential medium without ribonucleosides and deoxyribonucleosides, and the CHO cell stably expressing the recombinant H1R was cloned. Then, the pcDNA3.1/Zeo (+) vector (Promega K.K., Tokyo) encoding one of the M1–M5 muscarinic receptor subtypes was transfected into CHO-H1R cells using PolyFect Transfection Reagent. CHO-H1R cells stably expressing one of the M1–M5 muscarinic receptor subtypes (CHO-H1/M1, CHO-H1/M2, CHO-H1/M3, CHO-H1/M4, CHO-H1/M5 cells) were cloned from individual colonies cultured in α-minimum essential medium without ribonucleosides and deoxyribonucleosides, containing 350 µg/ml zeocin. Cloned cells were cultured in α-minimum essential medium containing 150 µg/ml zeocin. CHO cells stably co-expressing human H1R and one of the muscarinic acetylcholine receptors were cultured at 37°C in a humidified incubator with 95% air and 5% CO2. CHO cells stably co-expressing c-myc-tagged human H1R and one of the muscarinic acetylcholine receptors were prepared by the same methods except using the mammalian expression vector pdkKR-dhfr, containing c-myc-tagged wild-type human H1R gene.

Assessment for H1R down-regulation

Receptor-bearing CHO cells incubated with 1 mM carbachol for 24 h and appropriate times were subjected to the [3H]mepyramine binding assay. The cells were pretreated with atropine or protein kinase inhibitors 30 min prior to the stimulation with carbachol.

Membrane preparation and radioligand binding assay

Cultured cells grown to 80% confluency in 100-mm dishes or 150-mm dishes were harvested with a cell scraper into 1.5 ml of ice-cold 50 mM Na2/K-phosphate buffer (378 mM Na2HPO4, 122 mM KH2PO4, pH 7.4). Then the cell suspension was homogenized with an ultrasonic disrupter, and the homogenate was centrifuged at 18,000 rpm for 30 min. The pellet was resuspended in 50 mM Na2/K-phosphate buffer using the ultrasonic disrupter and served for the radioligand binding assay as the membrane sample. The [3H]QNB and [3H]mepyramine binding assays were performed according to the described methods (5, 13). In brief, [3H]mepyramine binding assay was carried out by the incubation of membranes with [3H]mepyramine (0.1–
8 nM) in the absence or presence of 10 μM triprolidine at 25°C for 60 min in a final volume of 500 μl. [3H]QNB binding assay was performed by the incubation of membranes with [3H]QNB (0.01 to 2 nM) in the absence or presence of 10 μM atropine at 37°C for 60 min in a final volume of 500 μl. Assays were terminated by rapid vacuum filtration through Whatman GF/B filters. Filters were soaked in 10 ml of Aquasol-2, and then the radioactivities trapped on the filters were counted in a liquid scintillation counter.

**Western blotting**

CHO cells stably co-expressing c-myc-tagged human H1Rs and m3 muscarinic receptors (CHO-c-myc-H1/M3 cells) were harvested in ice-cold TE (10 mM Tris-HCl /2.5 mM EDTA) containing 1 mM sodium orthovanadate, 100 μg/ml benzamidine, and 1 mM phenylmethylsulfonyl fluoride at scheduled times and then centrifuged at 500 x g for 5 min after ultrasonic cell disruption. The supernatant was then recentrifuged at 15,000 x g for 15 min. The resultant crude membrane pellet was resuspended in SDS sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.005% bromophenol blue] by using the ultrasonic disruptor. Protein concentrations were determined by using a Bio-Rad assay kit with bovine serum albumin as the standard. Cell extracts containing 50 μg of protein in each sample were ultrasonically disrupted and served for electrophoresis using an 8% SDS polyacrylamide gel. After electro-blotting onto nitrocellulose filters, the filters were stained with Ponceau S to ensure quantitative transfer of proteins. The membranes were first blocked for 2 h at room temperature with 5% skim milk. Then, the blots were incubated for 2 h at room temperature with primary antibody (mouse monoclonal anti-c-myc), which was followed by incubation for 2 h with secondary antibody (horseradish peroxidase-conjugated anti mouse IgG). Immunoreactive bands were visualized by chemiluminescence using the ECL Western Blotting Detection System (Amersham, UK).

**Phosphorylation of H1R in CHO cells**

Cells grown to 60% confluency in 100-mm dishes were washed with phosphate-free Dulbecco’s modified Eagle’s medium (DMEM) and then incubated in the same medium containing [32P]orthophosphate (50 μCi /ml) for 2 h at 37°C. Metabolically labeled cells were incubated with 100 μM histamine for 5 min or with different concentrations of carbachol for varying lengths of time. To study the effects of some drugs on H1R phosphorylation, cells were pretreated with drugs for 30 min before adding carbachol, and treatment was continued through the incubation with carbachol. After treatment, cells were washed three times with ice-cold Ca2+, Mg2+-free phosphate-buffered saline (PBS(−)); lysed with 1 ml of solubilizing buffer (20 mM Tris-HCl, pH 7.4, containing 137 mM NaCl, 2 mM EDTA, 1% NP-40, 20 mM NaF, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamidine); drawn through a 26-gauge needle 10 times; and rotated at 4°C for 30 min. After centrifugation at 15,000 rpm for 30 min, the supernatants were collected. The solubilized receptors were immunoprecipitated with 5 μl of anti-c-myc (9E10) conjugated to protein A/G plus agarose during a 2-h rotation at 4°C. The beads were collected by centrifugation (20,000 × g, 1 min) and washed three times by solubilizing buffer. Phosphorylated receptors were eluted with SDS sample buffer and resolved by 8% SDS-PAGE. Phosphorylated proteins were electrophoretically transferred onto a nitrocellulose filter and visualized by using a BAS-1500 Fuji Bioimage analyzer (Fuji Film Co., Tokyo). To confirm the position of H1R in the autoradiogram, Western blot analysis using anti-c-myc antibody was also performed as described above.

**Measurement of inositol phosphates (IPs) and phosphatidylinositol 4,5-bisphosphate (PIP2)**

CHO-H1/M3 cells were cultured in a 12-well culture plate and labeled with myo-[3H]inositol at 37°C for 24 h with or without 1 mM carbachol. Then the labeled cells with myo-[3H]inositol were washed three times in HEPES buffered saline (HBS: 125 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl2, 1.2 mM KH2PO4, 15 mM NaHCO3, 11 mM glucose, 10 mM LiCl, and 15 mM HEPES, pH 7.4) and then incubated for several times in HBS containing 100 μM histamine at 37°C. The reaction was terminated by addition of 5% trichloroacetic acid. Total [3H]IPs was isolated by anion exchanger chromatography using AG1-X8, 100 – 200 mesh, and radioactivities were determined. PIP2 was determined as described previously (14).

**Statistical analysis**

All data except for those in Fig. 4 are expressed as means ± S.E.M. of at least three independent experiments. Statistical analysis was carried out by Student’s t-test. P values <0.05 were considered statistically significant.

**Results**

**Down-regulation of H1Rs mediated by muscarinic acetylcholine receptor subtypes**

To investigate the H1R down-regulation mediated by...
each muscarinic acetylcholine receptor subtype, five kinds of CHO cells stably co-expressing H1R and one of the muscarinic acetylcholine receptor subtypes (CHO-H1/M1, CHO-H1/M2, CHO-H1/M3, CHO-H1/M4, CHO-H1/M5 cells) were prepared. H1Rs and muscarinic receptor subtypes expressed in these CHO cells were characterized by radioligand binding assays using \(^{3}H\)mepyramine and \(^{3}H\)QNB, respectively; and CHO cells expressing H1Rs with \(B_{\text{max}}\) values ranging from 2.2 to 2.8 pmol/mg protein and muscarinic receptor subtypes with \(B_{\text{max}}\) values ranging 1.0 to 2.8 pmol/mg protein were selected for further experiments (Table 1). Heterologous down-regulation of H1Rs was induced in CHO-H1/M3 and CHO-H1/M5 cells among five kinds of CHO cells 24 h after the stimulation by 1 mM carbachol (Fig. 1). H1Rs were down-regulated to 60% of the initial level in CHO-H1/M3 cells. Down-regulation of H1Rs in CHO-H1/M5 cells was 20% of the control during the same time course. No obvious down-regulation was induced in CHO-H1/M1, CHO-H1/M2, and CHO-H1/M4 cells. The down-regulation of H1R in CHO-H1/M3 cells was completely blocked by the pretreatment with 10 \(\mu\)M atropine (Fig. 2). All drugs we used for determining H1R density did not alter the \(K_d\) values of \(^{3}H\)mepyramine binding (Table 2).

Time- and dose-dependent decrease in \(^{3}H\)mepyramine binding to CHO-H1/M3 cell membranes was induced by carbachol (Fig. 3: A and B). Decrease in \(^{3}H\)mepyramine binding was induced at least by 100 nM carbachol. Treatment of either 1 mM carbachol for 18 h or 100 \(\mu\)M carbachol for 24 h induced maximum decrease in H1Rs at 60% of the initial level. Stimulation by the combination of 100 \(\mu\)M histamine and 1 mM carbachol for 24 h induced a decrease in \(^{3}H\)mepyramine binding to 70% of the control (Fig. 3A). The decrease induced by histamine plus carbachol was larger than that by either carbachol or histamine alone.

In order to confirm the heterologous H1R down-regulation mediated by the \(M_3\) muscarinic receptor, immunoblot analysis was performed. The band of approx. 60 kd corresponding to c-myc-tagged H1Rs was successfully immuno-detected in CHO-c-myc-H1/M3 cells using an anti-c-myc antibody (Fig. 4). Remarkable down-regulation of c-myc-tagged H1Rs was induced by the treatment of 1 mM carbachol for 24 h. The down-regulation was completely reversed by the pretreatment of atropine (Fig. 4).

### Table 1. Binding properties for their radioligands of recombinant histamine H1 receptors and muscarinic \(M_1\) – \(M_5\) receptor subtypes stably co-expressed in Chinese hamster ovary (CHO) cells

<table>
<thead>
<tr>
<th></th>
<th>Histamine H1 receptor</th>
<th>Muscarinic receptors</th>
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<tr>
<td></td>
<td>(K_d) (nM)</td>
<td>(B_{\text{max}}) (pmol/mg protein)</td>
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<tr>
<td>CHO-H1/M1</td>
<td>1.01 ± 0.2</td>
<td>2.48 ± 0.5</td>
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<tr>
<td>CHO-H1/M2</td>
<td>1.16 ± 0.4</td>
<td>2.16 ± 0.3</td>
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<tr>
<td>CHO-H1/M3</td>
<td>0.98 ± 0.2</td>
<td>2.24 ± 0.4</td>
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<tr>
<td>CHO-H1/M4</td>
<td>1.20 ± 0.1</td>
<td>2.46 ± 0.6</td>
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<tr>
<td>CHO-H1/M5</td>
<td>0.94 ± 0.3</td>
<td>2.80 ± 0.4</td>
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Membrane preparations of CHO-H1/M1, H1/M2, H1/M3, H1/M4, and H1/M5 cells were used for \(^{3}H\)QNB and \(^{3}H\)mepyramine binding assays. Scatchard plot analysis was performed. Data represent means ± S.E.M. from 3 – 6 independent assays.
Fig. 2. Effect of atropine on M₁ muscarinic receptor-mediated histamine H₁ receptor (H₁R) down-regulation. CHO-H₁/M₃ cells pretreated with or without 10 μM atropine were incubated with 1 mM carbachol for 24 h, and Bₘₐₓ values of expressed H₁R were determined by the [³H]mepyramine binding assay. Data are expressed as percentages of the control Bₘₐₓ value from the untreated cells with the means ± S.E.M. from 6 independent assays in duplicate. *P<0.01, significant difference from the control value.

Table 2. Kᵦ values for [³H]mepyramine of recombinant histamine H₁ receptors in CHO-H₁/M₃ cells treated with some drugs

<table>
<thead>
<tr>
<th>Kᵦ value for [³H]mepyramine (nM)</th>
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<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Atropine</td>
</tr>
<tr>
<td>Carbachol</td>
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<tr>
<td>Atropine + Carbachol</td>
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<tr>
<td>Ro 31-8220</td>
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<td>Ro 31-8220 + Carbachol</td>
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<tr>
<td>KT5823</td>
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<td>KT5823 + Carbachol</td>
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CHO-H₁/M₃ cells incubated with some drugs (10 μM atropine, 10 μM Ro 31-8220, 3 μM KT5823) for 30 min were treated with or without 1 mM carbachol for 24 h. Membrane preparations of CHO-H₁/M₃ cells were used for the [³H]mepyramine binding assay. Scatchard plot analysis was performed. Data represent means ± S.E.M. from 4 independent assays.

Fig. 3. Time course and dose response of M₁ muscarinic receptor-mediated histamine H₁ receptor (H₁R) down-regulation. A: Chinese hamster ovary cells co-expressing H₁Rs and M₁ muscarinic receptors (CHO-H₁/M₃ cells) were incubated with 1 mM carbachol (open square) or 100 μM histamine (closed square) or a combination of carbachol and histamine (triangle) for the indicated hours, and H₁R expression levels were determined by the [³H]mepyramine binding assay. Data are expressed as percentages of the control [³H]mepyramine binding in untreated cell membranes with means ± S.E.M. from 4 independent assays in triplicate. *P<0.01, significant difference from the control level. **P<0.025, significant difference from histamine-treated cells. B: CHO-H₁/M₃ cells were incubated with various concentrations of carbachol for 24 h, and H₁R expression levels were determined by the [³H]mepyramine binding assay. Data are expressed as percentages of [³H]mepyramine binding in untreated cell membranes with means ± S.E.M. from 3 independent assays in triplicate.

Phosphorylation of H₁R mediated by muscarinic receptors

Treatment with 100 μM histamine to CHO-H₁/M₃ cells caused homologous phosphorylation of H₁R; the phosphorylated H₁R was increased about eightfold from the basal level (Fig. 6A). Carbachol-induced heterologous phosphorylation of H₁R was twofold higher than the basal level. Stimulation of each subtype
**Histamine H₁ Receptor Down-Regulation**

Fig. 4. Immunodetection of M₃ muscarinic receptor-mediated histamine H₁ receptor (H₁R) down-regulation. CHO cells co-expressing c-myc-tagged human H₁Rs and M₃ muscarinic receptors pretreated with 10 μM atropine for 30 min were incubated with 1 mM carbachol for 24 h, and c-myc-tagged H₁R was detected by Western blotting using mouse monoclonal anti-c-myc. Similar results were obtained from three independent experiments. A representative result is shown.

**Fig. 5.** Decrease in histamine-induced IPs accumulation through down-regulated histamine H₁ receptors (H₁Rs) by M₃ muscarinic receptors. CHO-H₁/M₃ cells labeled with myo-[³²H]inositol at 37°C for 24 h with or without 1 mM carbachol were stimulated by 100 μM histamine or 1 mM carbachol for 5 min, solubilization of the receptors was performed. Solubilized receptors immuno-precipitated with anti-c-myc antibody were subjected to SDS polyacrylamide gel electrophoresis. Phosphorylation of the receptor was visualized by autoradiography. The lower panel shows the representative results of autoradiography from 3 independent experiments. The upper panel shows the quantification of the autoradiography. *P<0.01, significant difference from the control cells.

**Fig. 6.** Phosphorylation of histamine H₁ receptors (H₁R) induced by the stimulation of muscarinic receptor subtypes. A: Metabolic labeling of CHO-H₁/M₃ cells were performed with [³²P]orthophosphate for 2 h at 37°C. After incubation of labeled cells with 100 μM histamine or 1 mM carbachol for 5 min, solubilization of the receptors was performed. Solubilized receptors immuno-precipitated with anti-c-myc antibody were subjected to SDS polyacrylamide gel electrophoresis. Phosphorylation of the receptor was visualized by autoradiography. The lower panel shows the representative results of autoradiography from 3 independent experiments. The upper panel shows the quantification of the autoradiography. *P<0.025, significant difference from the control cells.
(M1 – M5) of muscarinic receptors induced phosphorylation of H1Rs (Fig. 6B). Levels of phosphorylation were similar (about twofold of the control) by the stimulation of M1, M2, and M3 muscarinic receptor subtypes and lower (about 1.6-fold of the control) by the stimulation of M4 and M5 muscarinic receptor subtypes. Phosphorylation levels of the two groups were significantly different.

Effect of PKC inhibitor on H1R phosphorylation and H1R down-regulation mediated by M3 muscarinic receptors

Stimulation by 1 mM carbachol induced remarkable phosphorylation of H1Rs in CHO-H1/M3 cells (twofold increase from the basal level, Fig. 7). The phosphorylation of H1R was completely reversed by the pretreatment of 10 μM atropine, but not pretreatment of 10 μM diphenhydramine. This heterologous phosphorylation of H1Rs was completely inhibited by 10 μM Ro 31-8220, an inhibitor of PKC. In contrast, carbachol-induced H1R down-regulation in CHO-H1/M3 cells was not significantly affected by the pretreatment of 5 μM Ro 31-8220 (Fig. 8). The treatment of Ro 31-8220 alone was without effect. In spite of pretreatment with Ro 31-8220, H1R was down-regulated by carbachol at the concentrations of 10, 100, and 1,000 μM for 24 h to 70%, 68%, and 63% of the control, respectively.

Fig. 7. Effect of M3 receptor antagonist, PKC inhibitor, and histamine H1 receptor antagonist on M3 muscarinic receptor-mediated H1R phosphorylation. Metabolic labeling of CHO-H1/M3 cells was performed with [32P]orthophosphate. Labeled cells pretreated with 10 μM atropine, 5 μM Ro 31-8220, or 10 μM diphenhydramine for 30 min were incubated with 1 mM carbachol for 5 min. Solubilization of the cells was performed, and solubilized receptors were immuno-precipitated with anti-c-myc antibody. The precipitated receptors were subjected to SDS polyacrylamide gel electrophoresis, and detection of phosphorylated receptors was performed by radioautography. Graph shows the cumulative data and the gels shown are representative of 3 experiments. *P<0.01, significant difference from the control cells. **P<0.01, significant difference from carbachol-treated cells.

Fig. 8. Effect of PKC inhibitor on M3 muscarinic receptor-induced histamine H1 receptor (H1R) down-regulation. CHO-H1/M3 cells were pretreated with 5 μM Ro 31-8220 30 min prior to the stimulation of carbachol at various concentrations for 24 h. The amount of H1R was determined by the [3H]mepyramine binding assay. Data are expressed as percentages of the amount of H1R from untreated cells and represent means ± S.E.M. from 3 independent assays.

Effect of PKG inhibitor on H1R phosphorylation and H1R down-regulation mediated by M3 muscarinic receptors

We previously reported that histamine-induced homologous down-regulation of H1R was inhibited by...
the pretreatment with KT5823, a PKG inhibitor (15). Thus we examined the effect of KT5823 on M₃ muscarinic receptor-mediated H1R down-regulation. CHO-H1/M3 cells pretreated with 3 μM KT5823 for 30 min were stimulated by carbachol at concentrations of 10, 100, and 1,000 μM. KT5823 showed no inhibitory effect on the M₃ muscarinic receptor-mediated H1R down-regulation. M₃ muscarinic receptor-mediated H1R down-regulations in CHO-H1/M3 cells pretreated with KT5823 were similar to those without any pretreatment (Fig. 9). Stimulation of carbachol at concentrations of 10, 100, and 1,000 μM for 24 h induced decreases in H1R levels by 30%, 34%, and 32% of the initial levels, respectively, in CHO-H1/M3 cells pretreated with KT5823.

Discussion

In this study, we have clearly demonstrated H1R down-regulation mediated by M2 and M3 muscarinic receptors (Fig. 1). H1R down-regulation mediated by M3 muscarinic receptors was also successfully confirmed by Western blot analysis (Fig. 4). Antagonism by atropine of the H1R down-regulation either by [³H]mepyramine binding assay (Fig. 2) or by Western blot analysis (Fig. 4) clearly demonstrates the heterologous down-regulation mediated by the muscarinic receptor. Although M1, M2, and M3 muscarinic receptors are all GPCRs predominantly coupling with Gq/11 and phospholipase C-β, the M1 receptor was the strongest among them for the induction of H1R down-regulation. The H1R down-regulation was weakly induced by the stimulation of the M1 receptor and little observed by the stimulation of the M2 receptor. The molecular mechanisms of heterologous H1R down-regulation induced by M1 and M2 muscarinic receptors remain to be elucidated. H1Rs and M1 muscarinic receptors are co-localized in various smooth muscles in peripheral tissues and in the central nervous system, for example, in the airway smooth muscle and brain cortex (2, 11). The down-regulation may play an important role in H1R-mediated histamine functions in these tissues.

The H1R down-regulation by M3 receptors was induced in dose- and time-dependent manner (Fig. 3). It took 18 h to reach the maximum with 40% reduction of the initial level. In contrast, the homologous H1R down-regulation mediated by H1R proceeded over 24 h with 60% reduction of the initial level. Combination of histamine and carbachol showed a larger effect than each alone on H1R down-regulation. The results suggest that the mechanism of the heterologous H1R down-regulation is different from that of the homologous H1R down-regulation. It is also supported by the result that KT5823, a PKG inhibitor, reduced the homologous down-regulation (15), but not the heterologous one at all (Fig. 9).

H1R-mediated accumulation of IPs was decreased corresponding to the H1R down-regulation by M3 muscarinic receptors in CHO-H1/M3 cells (Fig. 5). Similar decrease in IPs accumulation due to β2-receptor-mediated H1R down-regulation was also documented (16). It was also observed that phorbol 12-myristate 13-acetate (PMA), a PKC-activating phorbol ester, induced H1R up-regulation, and H1R-mediated IPs accumulation was increased correspondingly. These results strongly suggest that H1R-mediated signaling is regulated by the expression level of H1R. The activation of M1 receptors may induce the reduction of H1R-mediated histamine signaling in vivo. Especially, H1R-mediated reaction in type I allergy may be strongly regulated by the autonomic parasympathetic system. Autonomic regulation of H1R-mediated reaction in type I allergy was reported in another report (9). Long-term use of β2-agonist in bronchial asthma is linked to the development of bronchial hyper-responsiveness due to the down-regulation of β2-adrenergic receptor (17) and also caused up-regulation of airway smooth muscle H1R mRNA and protein.

It has been generally accepted that phosphorylation of GPCRs is a key event in their desensitization. However, it has not been elucidated whether H1R phosphorylation is involved in the H1R down-regulation. The H1R was homologously phosphorylated in vivo (Fig. 6) and was phosphorylated in vitro by several protein kinases including PKC and protein kinase A (18). In this report, we showed that H1R was heterologously phosphorylated by the activation of each subtype of muscarinic receptor in five kinds of CHO cells (Fig. 6B). The phosphorylation of H1R by M1 muscarinic receptors was rapid but transient. Carbachol-induced H1R phosphorylation by M1 muscarinic receptor activation occurred after 30-s stimulation and returned to the basal level in 40 min (data not shown). The level of phosphorylation mediated by M1 muscarinic receptors was far weaker than that mediated by H1Rs. Histamine increased H1R phosphorylation by eightfold from the basal level, while carbachol induced H1R phosphorylation by about two-fold (Fig. 6A). H1R phosphorylation mediated by M2 muscarinic receptors was completely inhibited by Ro 31-8220 (Fig. 7), suggesting that this small amount of H1R phosphorylation was solely catalyzed by PKC.

A recent report has shown that H1R lacking putative phosphorylation sites was not down-regulated by histamine, suggesting that phosphorylation of H1R is the key step in homologous down-regulation of H1R (19). However, the heterologous down-regulation of H1R by
M3 muscarinic receptors seems not to be involved in HR phosphorylation because the down-regulation was still induced in the presence of Ro 31-8220 when H1R phosphorylation was completely inhibited. KT5823, a PKG inhibitor, was without effect on M3 muscarinic receptor-mediated heterologous H1R down-regulation, although the compound showed partial inhibition of homologous H1R down-regulation (15). Recently, there have been many reports about dimerization of GPCRs. Adrenergic $\alpha_2$- and $\beta_1$ receptors heterodimerize and stimulation of adrenergic $\alpha_2$- receptor causes internalization of adrenergic $\beta_1$ receptor (20). Heterodimerization of somatostatin and opioid receptors cross-modulate their pharmacological properties (21). Thus H1R and some muscarinic receptor subtypes (especially H1R and M3 muscarinic receptors) may heterodimerize, and cause heterologous down-regulation of H1R. The physiological significance of M3 muscarinic receptor-mediated heterologous H1R phosphorylation remains to be elucidated.

In conclusion, heterologous down-regulation of H1R was induced by the stimulation of M3 and M5 muscarinic receptors and resulted in a decrease in H1R-mediated signaling. The heterologous H1R phosphorylation catalyzed by PKC seemed not to be involved in heterologous H1R down-regulation. H1Rs and M3 muscarinic receptors are co-located in various peripheral tissues including smooth muscles, vascular endothelial cells, and the central nervous system. The heterologous down-regulation of H1Rs is hypothesized to play important physiological and pathophysiological roles in peripheral tissues under parasympathetic regulation and in the central nervous system.

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References

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