Full Paper

β_2 -Adrenergic Receptor-Mediated Histamine H₁ Receptor Down-Regulation: Another Possible Advantage of β_2 Agonists in Asthmatic Therapy

Nozomi Kawakami¹, Katsuhiro Miyoshi¹, Shuhei Horio¹, and Hiroyuki Fukui^{1,*}

¹Department of Pharmacology, Faculty of Pharmaceutical Sciences, The University of Tokushima, 1-78-1 Sho-machi, Tokushima, Tokushima 770-8505, Japan

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Abstract. To clarify heterologous regulation of a receptor is important in considering medication. Histamine constricts the airway smooth muscle through the action to the H₁ receptor (H₁R), which contributes to asthma. β_2 -Adrenergic receptor (β_2 R) agonists are widely used in asthmatic therapy for their bronchodilating effects. In this study, we investigated the effect of β_2 R activation on the H₁R function using Chinese hamster ovary cells stably co-expressing human histamine H₁R and β_2 R (CHO-H₁/ β_2 cell). The stimulation of β_2 R resulted in the decrease of H₁R in the membrane. Heterologous H₁R down-regulation was significantly reversed in the presence of the cyclic AMP-dependent protein kinase (PKA) inhibitor KT5720. Since phosphorylation of G protein-coupled receptor (GPCR) by second messenger-dependent kinases, is proposed to be a key step initiating heterologous receptor desensitization, we examined whether heterologous H₁R down-regulation was accompanied by H₁R phosphorylation. H₁R was phosphorylated by β_2 R stimulation; however, a PKA inhibitor did not inhibit heterologous H₁R phosphorylation. Our results suggest that H₁R was heterologously regulated by β_2 R. Not only a direct action of β_2 R agonist to β_2 R causing bronchodilation but also indirect action that reduces the number of H₁R responsible for bronchoconstriction might contribute to a decrease in the bronchial resistance, which proposes another possible advantage of β_2 R agonists for asthmatic medication.

Keywords: heterologous down-regulation, histamine H₁ receptor, β_2 -adrenergic receptor, asthma, β_2 -receptor agonist

Introduction

Heterologous regulation of G protein-coupled receptor (GPCR) has been recognized as the form of regulation in which activation of one receptor by its agonist will modify the function of other receptors (1). In many GPCRs, loss of their biological responses (desensitization) is induced by continuous and repetitive stimulation with the receptors in order to protect cells from excess stimulation. Two types of desensitization have been observed in studies on GPCRs. Homologous desensitization is agonist specific, whereas heterologous desensitization is not. Heterologous desensitization is

one example of regulation caused by heterologous receptor stimulation. Homologous and heterologous desensitization can be initiated by receptor phosphorylation. Heterologous receptor stimulation-induced other receptor phosphorylation followed by desensitization has been observed in combinations of various receptors, that is, chemokine receptor and opioid receptor (2) and m₃-muscarinic receptor and β_2 -adrenergic receptor (3). Both G protein-coupled receptor kinases (GRKs) and second messenger-dependent kinases, such as protein kinase C (PKC) or cyclic AMP-dependent protein kinase (PKA) have been implicated in the desensitization of GPCRs. According to the conventional idea, agonist-dependent, GRK-mediated phosphorylation of receptors and second messenger-dependent kinase-mediated phosphorylation were thought to be the

*Corresponding author. FAX: +81-88-633-9513
E-mail: hfukui@ph.tokushima-u.ac.jp

property of homologous and heterologous desensitization, respectively (4). However, it is now recognized that GPCRs spontaneously isomerize to an activated conformation in the absence of agonist (5, 6), which suggests that GRKs may play a role not only in the homologous desensitization but also in the heterologous desensitization. Moreover, PKC-mediated receptor phosphorylation has been implicated in the homologous desensitization of prostacyclin receptors (7). Therefore, the contributions and mechanisms by which GRKs and second messenger-dependent kinases regulate the homologous and heterologous desensitization are not fully understood.

The β_2 -adrenergic receptor (β_2R) in airway smooth muscle cells that is responsible for bronchodilation plays important roles in the pathophysiology and the management of asthma. Since the β_2R agonists are widely used for asthmatic therapy, β_2R of the patients who have taken β_2R agonists medication is supposed to be usually in the activated state. In this circumstance where β_2R is excessively activated, the β_2R number of the airway smooth muscle cell surface is decreased by endocytosis (internalization) or degradation (down-regulation) (8). Moreover, there is a possibility that β_2R stimulation can lead to heterologous desensitization of other receptors co-expressed in airway smooth muscle cells. There have been some reports that demonstrated the use of high doses of β_2R agonists has been linked to the development of bronchial hyper-responsiveness (9, 10) and an increase in asthma exacerbations and mortality (11, 12). Nevertheless, β_2R agonists are widely used for asthmatics and are indeed effective for the relief of the asthmatic symptoms, which contradict the previous reports about the β_2R agonists possessing some effects that worsen asthma. Why are β_2R agonists so frequently prescribed for asthmatic patients in spite of those adverse effects on asthma? This discrepancy led us to speculate that not all patients who have taken β_2R agonists medication would suffer from those adverse effects described previously and that most people who could be cured effectively with this medication have some mechanisms that offset the β_2R agonists-induced harmful effects. According to this idea, we hypothesized that β_2R stimulation that occurred in the patients with β_2R agonists medication might induce heterologous desensitization of other receptors, such as histamine H_1 receptor (H_1R) (13), cysteinyl leukotriene receptors (14), or adenosine receptor (15), that are responsible for bronchoconstriction, although β_2R stimulation-induced such heterologous regulation might be lacking in the patients who are troubled with undesired effects of β_2R agonists.

It is well established that allergic bronchospasm in

asthmatics involves mast cell degranulation, which results in the release of histamine among other mediators (16). H_1R , a Gq-coupled receptor, associates with phospholipase C; and stimulation of the receptor leads to the formation of two second messengers, inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (17, 18). IP_3 induces the release of Ca^{2+} from intracellular stores, and Ca^{2+} and 1,2-diacylglycerol activate PKC. In airway smooth muscle cells, these H_1R -mediated responses result in bronchoconstriction, which contribute to the exacerbation of asthma. If we can demonstrate that H_1R is desensitized by the heterologous stimulation of β_2R , we can find out another valuable property of β_2R agonists and propose that one cause of asthma would be regarded as the dysfunction of heterologous receptor interaction.

In this study, we investigated the effect of β_2R activation on the H_1R function using Chinese hamster ovary (CHO) cells stably co-expressing human histamine H_1R and β_2R . Activation of β_2R by isoproterenol induced heterologous phosphorylation and loss of H_1R (heterologous down-regulation). Our results suggest that H_1R was heterologously regulated by β_2R . Not only a direct action of β_2R agonist to β_2R causing bronchodilation but also indirect action that reduces the number of H_1R responsible for bronchoconstriction might contribute to decrease the bronchial resistance, which proposes another possible advantage of β_2R agonist use for asthmatics.

Materials and Methods

Materials

[^{32}P]Orthophosphoric acid (314–377 TBq/mmol), [pyridimyl 5- 3H]mepyramine ([3H]mepyramine) (1.07 TBq/mmol) and *myo*-[1,2- 3H]inositol (3.40 TBq/mmol) were purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). [3H]Dihydroalprenolol was from Amersham Pharmacia Biotech. (Little Chalfont, Buckinghamshire, UK). Isoproterenol, propranolol, and 8-bromo-cyclic AMP were from Sigma (St. Louis, MO, USA). KT5720 (PKA inhibitor) and KT5823 (PKG inhibitor) were purchased from Alexis Biochemicals (San Diego, CA, USA). Ro 31-8220 (PKC inhibitor) was from Calbiochem (La Jolla, CA, USA). Anti-human β_2 receptor (rabbit, polyclonal), anti-c-myc (9E10; mouse, monoclonal), and anti-c-myc conjugated protein A/G plus agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and generation of CHO- H_1/β_2 cells

The plasmid vector pCMV5 containing human β_2R gene (β_2 -pCMV5) was a kind gift from Dr. H. Kurose

(Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka). The EcoRI/SalI fragment of β -pCMV5 was subcloned into the EcoRI/XhoI site of the pcDNA3.1 plasmid, which contains the constitutively expressed zeocin resistance gene. The resultant vector was transfected into CHO cells stably expressing the c-myc-epitope-tagged human H1R (CHO-H1 cells) using the Polyfect reagent (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. β 2R-expressing clones were selected using 300 μ g/ml zeocin and expression of β 2R was screened using [3 H]dihydroalprenolol binding assay. Cells co-expressing H1R and β 2R (CHO-H1/ β 2 cells) were cultured in α -minimum essential medium without ribonucleosides and deoxyribonucleosides (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Sigma), Antibiotics-Antimycotics (Gibco-BRL) and 150 μ g/ml zeocin.

Down-regulation of H1R and membrane preparation

Following exposure to different concentrations of isoproterenol for varying lengths of time, CHO cells grown in 100-mm dishes (80–90% confluence) were washed twice with cold Ca^{2+} , Mg^{2+} -free phosphate-buffered saline (PBS(-)) and scraped in 2 ml of 50 mM Na-phosphate buffer (PB). The cell suspension was centrifuged at $45,000 \times g$ for 30 min. The pellets (crude membrane) were resuspended in PB at a protein concentration of 0.5–1 mg/ml and sonicated for 20 s by using a handy sonic UR-20P (Tomy Seiko Co., Ltd., Tokyo) at a setting of 5. Remaining H1Rs were estimated by radioligand binding assays with [3 H]mepyramine. To study the effects of some drugs on the H1R down-regulation, cells were pretreated with drugs for 10 min before adding isoproterenol, and treatment was continued through the stimulation with isoproterenol.

Binding assays

Membrane suspension (50–100 μ g of protein) was incubated with [3 H]mepyramine or [3 H]dihydroalprenolol in the absence (total binding) or presence (non-specific binding) of 10 μ M antagonist (triprolidine for H1R, propranolol for β 2R, respectively) at 25°C for 60 min in a final volume of 500 μ l. The membrane-bound radioligands were separated from free radioligands by rapid filtration through a Whatman GF/B glass fiber filter (Whatman, Maidstone, UK). The filter was placed in 10 ml of Aquasol II (PerkinElmer Life Sciences, Inc.), and then the radioactivity on the filter was counted in a liquid scintillation counter (Aloka, Tokyo).

Measurement of inositol phosphates

Measurement of inositol phosphates (IP) was carried out as described previously (19). Briefly, cells in 12-well culture plates were labeled with *myo*-[3 H]inositol (37 kBq/well) in inositol-free Dulbecco's modified Eagle's medium (Nikken Seibutsu-Igaku Kenkyusho, Kyoto), supplemented with 10% dialyzed fetal calf serum for 24 h. Cells were washed twice and incubated with HEPES-buffered saline solution (HBS; 125 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl_2 , 1.2 mM KH_2PO_4 , 15 mM NaHCO_3 , 11 mM glucose, and 15 mM HEPES, pH 7.4) containing 10 mM LiCl. Cells were stimulated with 100 μ M histamine for varying lengths of time in 1 ml of HBS at 37°C. Reactions were terminated by rapid aspiration of the medium and addition of 0.5% trichloroacetic acid. Total [3 H]IP was isolated by anion exchange chromatography using AG1-X8, 100–200 mesh (Bio-Rad, Hercules, CA, USA); and radioactivities were determined in a liquid scintillation counter using Sintisol EX-H (Dojin, Tokyo).

In measurement of IP in long-term isoproterenol-treated cells, cells were exposed with ('down-regulated') or without ('control') the indicated concentration of isoproterenol in the labeling medium at 37°C for 24 h. The medium was then removed from all wells. Next, during the 'signaling period', cells were exposed for the indicated times at 37°C to HBS with 10 mM LiCl containing vehicle or 100 μ M histamine. Reactions were then terminated and total [3 H]IP was measured, as mentioned above. To analyze the basal lipid labeling, we referred to the method described by Budd et al. (3).

CHO cell membrane preparation and Western blot analysis

After various treatment, CHO cells grown in 100-mm dishes (80–90% confluence) were washed twice with ice-cold PBS(-) and scraped in 1 ml of TE (10 mM Tris-HCl, 2.5 mM EDTA, pH 7.4) containing 1 mM sodium orthovanadate, 100 μ g/ml benzamidine, and 1 mM phenylmethylsulfonyl fluoride. The cells were left in ice-cold TE for 10 min and subsequently homogenized for 20 s by using a handy sonic UR-20P at a setting of 5. Cell lysate was centrifugated at $500 \times g$ for 5 min and unbroken-cell debris was discarded. The supernatant was further centrifugated at $15,000 \times g$ for 15 min at 4°C. The resulting crude membrane pellet was resuspended in sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β -mercaptoethanol, and 0.005% bromophenol blue at a protein concentration of 3–5 mg/ml and sonicated for 1 min in a bath sonicator. Fifty micrograms of crude membrane sample/lane was resolved on an 8% SDS-PAGE gel, and the resolved proteins were transferred onto nitro-

cellulose filters (Schleicher & Schuell, Dassel, Germany). The filters were then blocked with 5% skim milk powder in TS buffer (20 mM NaH_2PO_4 , pH 7.4, containing 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature, and probed with the mouse monoclonal anti-c-myc (9E10) antibody (Santa Cruz Biotechnology) at a 1:150 dilution or with the rabbit polyclonal anti-human- $\beta 2\text{R}$ antibody (Santa Cruz Biotechnology) at a 1:200 dilution for 1 h at room temperature. Detection of immunoreactivity was achieved by use of a 1:2000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Santa Cruz Biotechnology) and enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Phosphorylation of H1R in intact CHO-H1/ $\beta 2$ cells

Cells grown to 80–90% confluence in 100-mm dishes were equilibrated in phosphate-free Dulbecco's modified Eagle's medium (Gibco-BRL) for 2 h and then incubated in the same medium containing [^{32}P]orthophosphate (50 $\mu\text{Ci}/\text{ml}$) for 2 h at 37°C. Metabolically labeled cells were incubated with different concentrations of isoproterenol for varying lengths of time. To study the effects of some drugs on H1R phosphorylation, cells were pretreated with drugs for 10 min before adding isoproterenol, and treatment was continued through the incubation with isoproterenol. After treatment, cells were washed three times with ice-cold PBS (–) and scraped in 1 ml of TE containing protease inhibitors (see above). The cells were left in ice-cold TE for 10 min and subsequently homogenized for 20 s by using a handy sonic UR-20P at a setting of 5. Cell lysate was centrifugated at $500 \times g$ for 5 min and unbroken-cell debris was discarded. The supernatant was further centrifugated at $15,000 \times g$ for 15 min at 4°C. The resulting crude membrane pellets were 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 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, 10 $\mu\text{g}/\text{ml}$ benzamidine), drawn through a 26-gauge needle 10 times and rotated at 4°C for 30 min. After centrifugation at $20,000 \times g$ 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 ($20,000 \times g$, 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).

Cyclic AMP assay

One day prior to the analysis, CHO cells were seeded in 6-well plates at the density of 100,000 cells/well. Cells were incubated for 10 min in culture medium supplemented with 1 mM isobutylmethylxanthine (IBMX) and exposed to 1 μM of isoproterenol for 5, 15, and 30 min. Reactions were terminated by rapid aspiration of the medium and addition of 500 μl of 0.1 M HCl. Agonist-induced cyclic AMP production was determined using a direct cyclic AMP enzyme immunoassay kit according to the manufacturer's instructions (Sigma).

Results

Functional expression of $\beta 2\text{R}$ in CHO-H1 cells

Saturation binding analysis of $\beta 2\text{R}$ -cDNA transfected CHO-H1 cell membranes demonstrated that the expressed receptor bound the $\beta 2\text{R}$ antagonist radioligand [^3H]dihydroalprenolol with K_d and B_{max} values of 0.065 ± 0.01 nM and 0.91 ± 0.08 pmol/mg protein, respectively (Fig. 1A). Stimulation of intact CHO-H1/ $\beta 2$ cells with the β -adrenergic agonist isoproterenol resulted in the elevation of cyclic AMP levels (Fig. 1B). As shown in Fig. 1C, using antibodies directed against human $\beta 2\text{R}$, $\beta 2\text{R}$ was found to be expressed only in the membranes from CHO-H1/ $\beta 2$ cells. $\beta 2\text{R}$ migrated as a relatively broad band centered at approximately 65 kD.

$\beta 2\text{R}$ stimulation induced H1R down-regulation in CHO-H1/ $\beta 2$ cells

Isoproterenol treatment (3–24 h) induced decrease in H1R population in a time- and concentration-dependent manner (Fig. 2: A–C). Maximal effect induced by isoproterenol led to about 50% H1R down-regulation of untreated control cells, which was comparable to those of histamine-induced homologous down-regulation (Fig. 2A). We could observe H1R down-regulation induced by isoproterenol at concentrations ranging from 0.1 nM up to 100 μM (Fig. 2B), and H1R down-regulation was reversed by the β -adrenergic antagonist 3 μM propranolol (Fig. 3). Following treatment of isoproterenol (1 μM) for 24 h, Scatchard analysis was performed. The K_d value and B_{max} were determined to be 0.67 ± 0.13 nM and 3.85 ± 0.66 pmol/mg protein in untreated control cells, and 0.46 ± 0.07 nM and 2.20 ± 0.47 pmol/mg protein in isoproterenol-treated cells, respectively. H1R down-regulation induced by isoproterenol was not accompanied by a change in the K_d of [^3H]mepyramine for the remaining H1R.

Fig. 1. Functional expression of β 2R in CHO-H1 cells. A: Representative saturation curves of [3 H]dihydroalprenolol binding to membranes from β 2R-cDNA transfected CHO-H1 cells. Inset: Scatchard analysis of the specific binding data from the same experiment. Non-specific binding was defined by the use of 10 μ M propranolol. B: Isoproterenol-induced cyclic AMP accumulation in CHO-H1/ β 2 cells. The cells were challenged with 1 μ M of isoproterenol for 0, 5, 15, or 30 min in the presence of 1 mM IBMX, and intracellular cyclic AMP level was determined. The data shown are mean values $\pm$ S.E.M. from three independent experiments. C: Immunodetection of β 2R expressed in CHO-H1/ β 2 cells. Western blot of membranes prepared from non-transfected CHO cells (lane 1) or cells expressing H1R alone (lane 2) or coexpressing H1/ β 2R (lane 3). Fifty micrograms of membrane protein was loaded per lane and the blot was probed with anti- β 2R antibody at a 1:200 dilution. Arrow indicates the position of β 2R. The mobility of protein molecular mass standards (Bio-Rad) is indicated on the left. Shown is a blot representative of three independent experiments.

Inhibition of histamine-stimulated IP accumulation by prolonged β 2R activation

In CHO-H1/ β 2 cells exposed to 1 μ M isoproterenol for 24 h, the subsequent histamine (100 μ M)-induced IP accumulation was reduced by approximately 40% of untreated control cells (Fig. 4). Thus, long-term isoproterenol treatment induced H1R down-regulation, and H1R loss was accompanied by a reduction of H1R-mediated signaling. Basal lipid labeling was determined to be 37806 ± 686 dpm/well in untreated control cells and 38387 ± 412 dpm/well in isoproterenol-treated cells, respectively ($n = 3$). The treatment with isoproterenol for 24 h did not affect basal lipid labeling of CHO-H1/ β 2 cells.

Effects of protein kinase inhibitors on isoproterenol-induced H1R down-regulation

As shown in Fig. 5, PKA inhibitor KT5720 (5 μ M) (20) partially reversed H1R down-regulation induced by 1 nM and 1 μ M isoproterenol (24 h) to 85% and 75%, respectively, of untreated control cells. However, the PKC inhibitor Ro 31-8220 (1 μ M) and PKG inhibitor KT5823 (5 μ M) could not reverse H1R down-regulation induced by isoproterenol at both concentrations of 1 nM and 1 μ M. PKC or PKG inhibitor did not affect isoproterenol-induced down-regulation of H1R (Fig. 5). Treatment with these protein kinase inhibitors alone had no effect on the K_d and B_{max} values of [3 H]mepyramine binding to H1R (data not shown).

Phosphorylation of H1R in intact CHO-H1/ β 2 cells

PKA inhibitor significantly impaired isoproterenol-induced H1R down-regulation, suggesting the possibility that PKA-mediated direct H1R phosphorylation was involved in H1R down-regulation induced by heterologous β 2R stimulation. To investigate this possibility, we performed an *in vivo* phosphorylation study in intact CHO-H1/ β 2 cells. After exposure of isoproterenol to metabolically [32 P]orthophosphate-labeled cells, H1R in the membrane was phosphorylated by the stimulation with histamine (Fig. 6A) or with isoproterenol (Fig. 6B). These homologous and heterologous H1R phosphorylations were impaired in the presence of the respective receptor antagonist (diphenhydramine for H1R, propranolol for β 2R). Isoproterenol-induced heterologous H1R phosphorylation occurred rapidly (within 1 min) and in a concentration-dependent manner (Fig. 7: A and B). Phosphorylation returned to the basal level when stimulation was prolonged for longer than 30 min. H1R antagonist diphenhydramine (10 μ M) had no effect on isoproterenol-induced heterologous H1R phosphorylation (data not shown). PKA inhibitor KT5720 (10 μ M) had no effect on isoproterenol

Fig. 2. Down-regulation of H1R. A: Time course for histamine- or isoproterenol-induced down-regulation of H1R. CHO-H1/ β 2 cells were incubated with 100 μ M histamine (open circle) or 1 μ M isoproterenol (closed circle) for the indicated time. Total H1R remaining was assessed by [3 H]mepyramine binding assays using a saturating concentration of 8 nM. The data shown are mean values $\pm$ S.E.M. from three to five independent experiments performed in triplicate and expressed as a percent of H1R number in untreated control cells studied in parallel. B: Concentration dependence of isoproterenol-induced down-regulation of H1R. CHO-H1/ β 2 cells were incubated with indicated concentrations of isoproterenol (Iso) for 24 h, and the H1R population was determined, as described above. The data shown are mean values $\pm$ S.E.M. from three to five independent experiments performed in triplicate and expressed as a percent of H1R number in untreated control cells studied in parallel. Dotted lines in A and B indicate the 100% H1R number (3.85 ± 0.66 pmol/mg protein) in untreated control cells. C: Immunodetection of H1R in isoproterenol-treated CHO-H1/ β 2 cell membranes. Following exposure to 1 μ M of isoproterenol (Iso) for 0 (lane 1), 6 (lane 2), 24 (lane 3) h, membranes were prepared. Fifty micrograms of membrane protein was loaded per lane, and the blot was probed with anti-c-myc antibody at a 1:150 dilution. Arrow indicates the position of H1R. The mobility of protein molecular mass standards is indicated on the right. Shown is a blot representative of three independent experiments with essentially identical results.

Fig. 3. Effect of propranolol on isoproterenol-induced H1R down-regulation. CHO-H1/ β 2 cells were pretreated with 3 μ M of propranolol (Prop) (+) or vehicle (-) for 10 min, and then incubated with the indicated concentrations of isoproterenol (Iso) for 24 h. Number of H1R was determined by [3 H]mepyramine binding assays using a saturating concentration of 8 nM. The data shown are the mean values $\pm$ S.E.M. from three to five separate experiments performed in triplicate and are expressed as a percent of H1R number in untreated control cells studied in parallel.

Fig. 4. Histamine-induced IP accumulation in H1R down-regulated cells. CHO-H1/ β 2 cells were exposed with (Iso) or without (control) 1 μ M isoproterenol in the labeling medium containing *myo*-[3 H]inositol at 37°C for 24 h, and then they were treated with 100 μ M histamine in the presence of LiCl for the indicated periods of time. Results are presented as increase in IP accumulation above the basal levels in the absence of histamine. The data shown are the mean $\pm$ S.E.M. of triplicate values from three independent experiments.

Fig. 5. Reversal of isoproterenol-induced HIR down-regulation by the PKA inhibitor KT5720. CHO-H1/ β 2 cells were incubated with (hatched column) or without (open column) the indicated concentrations of isoproterenol (Iso) in the presence of 5 μ M KT5720 (PKA inhibitor), 1 μ M Ro 31-8220 (PKC inhibitor), or 5 μ M KT5823 (PKG inhibitor) for 24 h; and the HIR population was determined by [3 H]mepyramine binding assays using a saturating concentration of 8 nM. The data shown are the mean values $\pm$ S.E.M. from three to five separate experiments performed in triplicate and expressed as a percent of untreated control cells studied in parallel. Dotted line indicates the HIR level (%) of isoproterenol (1 nM, 24 h) treated cells. * P <0.025: significance of difference from HIR levels of isoproterenol treated cells with Student's t -test.

Fig. 7. Time-course (A) and concentration-dependency (B) of isoproterenol-induced heterologous HIR phosphorylation in the intact CHO-H1/ β 2 cells. CHO-H1/ β 2 cells were labeled with [32 P]orthophosphate for 2 h and then exposed to different concentrations of isoproterenol (Iso) for 0 to 360 min. In the study for concentration-dependency of heterologous HIR phosphorylation, isoproterenol treatment was prolonged for 3 min. The data shown are the representative of at least three independent experiments that produced identical results.

Fig. 6. Homologous and heterologous HIR phosphorylation in the intact CHO-H1/ β 2 cells. CHO-H1/ β 2 cells were labeled with [32 P]orthophosphate for 2 h and then exposed to 100 μ M histamine (HA) (panel A) or 1 μ M isoproterenol (Iso) (panel B) in the presence (+) or absence (-) of 10 μ M diphenhydramine (DPH) or 3 μ M propranolol (Prop) for 5 min. The cell membranes were solubilized and the c-myc epitope-tagged HIR was immunoprecipitated with anti-c-myc antibody. The phosphorylated HIR in membrane was resolved by SDS-PAGE and transferred onto a nitrocellulose filter. Phosphorylated HIR was visualized by autoradiography. Arrows indicate the position of HIR. The mobility of protein molecular mass standards is indicated on the left. The data shown are the representative of three independent experiments that produced identical results.

(1 μ M)-induced HIR phosphorylation (Fig 8: A and C) in spite of its inhibitory effect on isoproterenol-induced HIR down-regulation (Fig. 5). When PKA was directly activated by the treatment of a nonhydrolyzable cyclic AMP analog, 8-bromo-cyclic AMP (3 mM), HIR phosphorylation was also observed; and PKA activator-induced HIR phosphorylation was inhibited by KT5720 (10 μ M) (Fig. 8: A and C). PKC inhibitor Ro 31-8220 (1 μ M) and PKG inhibitor KT5823 (5 μ M) could not inhibit isoproterenol (1 μ M)-induced HIR phosphorylation (Fig. 8: B and C).

Discussion

Evaluation of the β 2R agonists-induced heterologous receptor regulation at the protein level is also of clinical importance because of their general use for asthmatic medication. Therefore, in this present study, we investigated whether β 2R activation could induce HIR desensitization, especially focusing on HIR down-regulation, by using CHO cells co-expressing human HIR and β 2R. The co-expressing cells would be the simplest model for observing the heterologous receptor down-regulation, and the events that could occur in the body of asthmatic subjects who have taken β 2R agonists medication would be also mimicked in this system. The phenomena observed in our experiments using CHO-

Fig. 8. Effects of second messenger-dependent protein kinase inhibitors on H1R phosphorylation. A: Effects of PKA inhibitor on isoproterenol (Iso)-induced heterologous H1R phosphorylation and cyclic AMP analog (8-Br-cAMP)-induced direct phosphorylation of H1R by PKA. In the presence (+) or absence (-) of KT5720 (10 μ M), both types of H1R phosphorylation were also investigated. B: Effects of PKC and PKG inhibitors on isoproterenol (Iso)-induced heterologous H1R phosphorylation. In the presence (+) or absence (-) of Ro 31-8220 (1 μ M) or KT5823 (5 μ M), heterologous H1R phosphorylation was also investigated. Phosphorylated H1R was visualized by autoradiography as described above. The data shown are representative of at least three independent experiments that produced identical results. C: Three replicates of the experiments using protein kinase inhibitors are summarized. Intensity of the phosphorylated H1R band was determined by densitometric analysis and the extent of H1R phosphorylation was calculated in relation to that under basal conditions. The data in histograms represent means $\pm$ S.E.M. * P <0.025; significance of difference from 8-Br-cAMP-induced H1R phosphorylation levels of KT5720-untreated cells with Student's t -test.

H1/ β 2 cells where the receptor expression level was higher than that of native cells might be amplified from

the real ones in the patients; however, we believe that our present experiments would provide information enabling easy understanding of the β 2R activation-mediated heterologous regulation of H1R.

In CHO-H1/ β 2 cells, the β -adrenergic receptor agonist isoproterenol induced the loss of H1R number (down-regulation) in a time- and concentration-dependent manner; furthermore, maximal level of isoproterenol-induced heterologous H1R down-regulation was comparable to that of histamine-induced homologous down-regulation. We could demonstrate that β 2R stimulation had strong effect to induce H1R down-regulation, although it was heterologous stimulation.

A concept that has been the most generally accepted today is that phosphorylation of GPCR plays a key role in the GPCR desensitization. The β 2R, Gs-adenylyl cyclase-coupled receptor, is known to elevate the intracellular cyclic AMP level in response to its activation. The cellular effects mediated by cyclic AMP are subserved by PKA. Some reports have demonstrated that in heterologous desensitization, a second messenger-dependent kinase, such as PKC and PKA, could phosphorylate GPCR and their phosphorylation of GPCR was essential for heterologous desensitization (2, 3). Thus, we supposed the involvement of direct H1R phosphorylation by PKA in isoproterenol-induced heterologous H1R down-regulation. In the presence of the PKA inhibitor KT5720, isoproterenol-induced H1R down-regulation was significantly reversed. The PKC inhibitor Ro 31-8220 could not attenuate the isoproterenol-induced H1R down-regulation. PKG inhibitor KT5823, a structurally related compound to KT5720, also had no effect on isoproterenol-induced H1R down-regulation. Stimulation of β 2R also induced H1R phosphorylation, although it returned to the basal level within 30 min. Isoproterenol-induced heterologous H1R phosphorylation was not affected by the inhibition of PKA. On the other hand, treatment with the PKA activator 8-bromo-cyclic AMP induced phosphorylation of H1R, and PKA activator-induced H1R phosphorylation was attenuated by PKA inhibitor. These results suggest that H1R can become the substrate for PKA in intact cells, although isoproterenol-induced heterologous phosphorylation of H1R does not seem to be mediated by PKA. Moreover, PKC inhibitor and PKG inhibitor had no effects on isoproterenol-induced H1R phosphorylation, suggesting that neither PKC nor PKG is involved in heterologous H1R phosphorylation induced by β 2R activation. Involvement of GRKs in heterologous receptor phosphorylation has been demonstrated (21). This report demonstrated that μ -opioid receptor stimulation with its agonist led to translocate the GRKs to the membrane, and these kinases are involved in the other

receptor desensitization. In our case, the same possibility that GRKs are involved in isoproterenol-induced phosphorylation of H1R can be considered. However, due to the lack of specific inhibitors to the GRKs, it is not possible to assign the PKA inhibitor-insensitive heterologous H1R phosphorylation identified in this study to the GRKs. Further investigation is required to clarify which kinases are responsible for heterologous H1R phosphorylation, and to understand the detailed mechanism of β 2R-mediated heterologous down-regulation of H1R. However, as shown in Fig. 5, it is certain that β 2R-mediated H1R down-regulation would be controlled by PKA-dependent signaling pathways.

Our present data that β 2R stimulation reduced the number of H1R suggest another possible advantage of β 2R agonists. Mak et al. (22) has demonstrated that β 2R stimulation induced up-regulation of H1R mRNA, protein, and function, and that was completely contradictory to our present results. We speculated the differences in the results obtained by us and them were dependent on whether the H1R gene was native one or the recombinant one. To generate the CHO cells expressing H1R, we used recombinant human H1R gene lacking its intrinsic promoter subcloned into plasmid vector pDKCR. In our system, the expression of H1R is controlled by SV40 promoter on the vector plasmid, and we verified that isoproterenol had no effect on H1R mRNA levels in CHO-H1/ β 2 cells (data not shown). On the other hand, Mak et al. (22) used native bovine tracheal smooth muscle cells to demonstrate the up-regulation of H1R by β 2R stimulation. In their cases, regulation of H1R expression was in the native state that was different from our system where the gene transcription was kept constant. Certainly, the expression systems we employed were suitable to observe the H1R down-regulation. Therefore, we speculate that both up- and down-regulations have taken place in H1R regulation. When the increased amounts of receptor were more than the decreased ones, final amounts of receptor would increase. Not only in the experimental conditions but also in asthmatic subjects, there is a possibility that stimulation of one receptor can induce both up-regulation and down-regulation of the other receptor, and the stimulation of one receptor may determine the other receptor level depending on whether up- or down-regulation is preferred in a certain condition. Up- and down-regulations may be laid to an individual difference of receptor regulation. Mak et al. (22) concluded in their report that β 2R stimulation-induced up-regulation of H1R was the cause of β 2R agonists-induced aggravation of asthma. Nevertheless, β 2R agonists are widely used and are effective for most asthmatic subjects, suggesting that up-regulation of H1R

by β 2R agonists does not occur in all subjects. If the H1R levels regulated by β 2R stimulation may be inclined to increase in some patients, undesired effects of β 2R agonists that can induce the aggravation of asthma might easily appear. On the contrary, in the patients in whom β 2R agonists can effectively reduce the asthmatic symptoms, the H1R levels regulated by β 2R stimulation may be inclined to decrease like in our present experiment. Such individual difference of receptor regulation may contribute to the effectiveness and the risk of β 2R agonists used for asthmatic medication.

This study demonstrated that β 2R stimulation induced the heterologous H1R down-regulation. Not only a direct action of β 2R agonist to β 2R causing bronchodilation but also indirect action that reduces the number of H1R responsible for bronchoconstriction are assumed, which proposes another possible advantage of β 2 agonists use for asthma. Moreover, our data raise the idea that impairment of heterologous down-regulation of H1R that prefers bronchoconstriction may become the cause of asthma.

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