JPP Journal of Pharmacy and Pharmacology

JPP 2008, 60: 747–752

Received November 11, 2007
Accepted March 5, 2008
DOI 10.1211/jpp.60.6.0010
ISSN 0022-3573

Down-regulation of histamine H_1 receptors by β_2-adrenoceptor-mediated inhibition of H_1 receptor gene transcription

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Abstract

Histamine H_1 receptor (H1R) levels vary under various pathological conditions, and these changes may be responsible for some pathogenesis such as in allergic rhinitis. Several stimulants, including histamine, muscarinic agonists and platelet-activating factor, have now been shown to regulate H1R levels and may have roles in regulating the H1R level in physiological and pathological conditions. Results for β_2-adrenoceptor (β2AR) stimulation are conflicting, however. β2AR up-regulated H1R in bovine tracheal smooth muscle, but down-regulated human H1R expressed in Chinese hamster ovary (CHO) cells. It is possible that this discrepancy comes from the differences in the preparations used for each study: the former cell expressed bovine H1R and the latter cell expressed human H1R. Moreover, CHO cells have been shown to be inadequate for studying the effects on H1R gene expression, because the cells express non-endogenous stably transfected H1R under the control of the SV40 promoter. Therefore, in this study, we have investigated the role of β2AR stimulation in H1R gene regulation using human U373 astrocytoma cells that express endogenous H1R and transfected β2AR. Stimulation of β2AR significantly reduced H1R promoter activity and H1R mRNA levels. H1R mRNA stability was slightly reduced by β2AR stimulation, although this was not significant. The decrease of H1R mRNA by β2AR stimulation was blocked by the protein kinase A (PKA) inhibitor KT5720, suggesting the involvement of PKA. These results indicate that the β2AR is involved in the down-regulation of human H1R by inhibiting H1R gene transcription through a PKA-dependent process.

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Acknowledgements: This work was supported in part by Grant-in-aid for Scientific Research from Japan Society for the Promotion of Science and by a fund from the Osaka Medical Research Foundation for Incurable Diseases.

Introduction

Histamine plays important pathophysiological roles in central and peripheral tissues; its actions are mediated through four histamine receptor subtypes: H_1, H_2, H_3 and H_4 (Hill et al 1997; Nguyen et al 2001). Histamine H_1 receptors (H1R) are distributed in a wide variety of tissues, including mammalian brain and several peripheral tissues (Hill et al 1997). The level of H1R changes under various conditions. Up-regulation of H1R or H1R mRNA is observed in the electrical foci in the temporal cortex of patients with epilepsy (Iinuma et al 1993) and in the nasal mucosa of patients with allergic rhinitis (Iriyoshi et al 1996; Hamano et al 1998). Down-regulation of H1R is seen in the frontal cortex of patients with chronic schizophrenia (Nakai et al 1991) and in the frontal and temporal areas of patients with Alzheimer's disease (Higuchi et al 2000). These changes in the H1R level may be related to such pathological conditions, and thus it will be important to delineate the regulatory mechanisms of H1R expression.

Several stimulants, including histamine, muscarinic agonists and platelet-activating factor (PAF), have been shown to regulate H1R levels (Nakasaki et al 1999; Horio et al 2004; Miyoshi et al 2004; Das et al 2007; Miyoshi et al 2007), indicating that H1R expression is regulated by various factors. Stimulation of the β_2-adrenoceptor (β2AR) also regulates H1R levels. In this case, however, some conflicting results have been reported: β2AR stimulation up-regulated H1R in bovine tracheal smooth muscle (Mak et al 2000), while it down-regulated human H1R expressed in Chinese hamster ovary (CHO) cells (Kawakami et al 2004). This discrepancy may come from the differences in the preparations used for each

study: the former cells expressed bovine H1R whereas the latter cells expressed human H1R. Moreover, the CHO cells have been shown to be inadequate for studying the effects on H1R gene regulation, because the cells express exogenously introduced H1R under the control of SV40 promoter, in which the level of H1R is usually maintained constant and is not influenced by the stimulation of the cell. Therefore, to clarify the effect of β2AR stimulation on the regulation of human H1R gene expression, cells that express endogenous H1R should be examined. In this study we used human U373 astrocytoma cells that express endogenous H1R and stably transfected β2AR (U373-β2 cells) to investigate the effect of β2AR stimulation on H1R gene regulation.

Materials and Methods

Materials

The plasmid vector pCMV5 containing the human β2AR gene was kindly provided by Dr H. Kurose (Kyusyu University, Fukuoka, Japan). [Pyridinyl-5-^{3}H] mepyramine ([^{3}H]mepyramine, 20 Ci mmol^{-1}) was obtained from Perkin Elmer (Boston, MA, USA). L-[4,6-propyl-^{3}H] Dihydroalprenolol ([^{3}H]DHA, 90 Ci mmol^{-1}) was from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). PolyFect transfection reagent was from Qiagen K.K. (Tokyo, Japan). The dual-luciferase reporter assay system, pRL-TK vector and pcDNA3.1/Zeo (+) vector were from Promega (Madison, WI, USA). Predeveloped TaqMan assay reagents for human and rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Applied Biosystems (Foster City, CA, USA). All other reagents, unless otherwise stated, were of analytical grade and were from Wako Pure Chemicals (Osaka, Japan) or Sigma (St Louis, MO, USA).

Preparation of U373 astrocytoma cells stably expressing β2AR

U373-β2 cells (human U373 astrocytoma cells stably expressing β2AR) were prepared according to the method described previously, with minor modifications (Miyoshi et al 2004). In brief, pcDNA3.1/Zeo (+) vector encoding β2AR was transfected into U373 astrocytoma cells using the PolyFect transfection reagent. Then, U373 astrocytoma cells stably expressing β2AR (U373-β2 cells) were cloned from individual colonies cultured in Dulbecco's modified Eagle's medium (DMEM) plus 8% fetal bovine serum containing 100 μg mL^{-1} zeocin. Cloned cells expressing β2AR were cultured in the same medium without zeocin, and the expression of β2R was measured by binding assay using [^{3}H]DHA.

Radioligand binding assay

Cultured cells grown to 70% confluency in 150 mm dishes were harvested with a cell scraper into 1.5 mL ice-cold 50 mM Na/K phosphate buffer (37.8 mM Na_2HPO_4, 12.2 mM KH_2PO_4, pH 7.4). Then the cell suspension was homogenized with an ultrasonic disrupter, and the homogenate was centrifuged at 45000 *g* for 30 min. The pelleted membranes were resuspended in 50 mM Na/K phosphate buffer and used for the radioligand binding assay. The [^{3}H]mepyramine binding assay was carried out by incubation of the membranes with [^{3}H]mepyramine (0.1–4 nM) in the presence and absence of 10 μM triprolidine at 25°C for 60 min in a final volume of 500 μL. The [^{3}H]DHA binding assay was performed by incubation of the membranes with [^{3}H]DHA (0.02–0.4 nM) in the presence and absence of 10 μM propranolol at 30°C for 60 min in a final volume of 500 μL. Assays were terminated by rapid vacuum filtration through Whatman GF/B filters (Whatman, Maidstone, UK). In the [^{3}H]mepyramine binding assay, filters were presoaked with 1% polyethyleneimine. Filters were soaked in 10 mL Aquasol II (Packard Instrument, Inc., Meriden, CT, USA), and the radioactivity on the filter was counted in a liquid scintillation counter.

Real-time RT-PCR for the measurement of human H1R mRNA

Cells cultured to 70% confluency in 35 mm dishes were cultured without serum for the next 24 h, and then treated with various drugs for appropriate times. After the treatment, the cells were scraped with 700 μL trizol reagent (Gibco-BRL, Carlsbad, CA, USA), and 200 μL chloroform was added to the cell suspension. After centrifugation of the cell suspension at 15000 *g* for 15 min at 4°C, the aqueous phase was collected and the RNA was precipitated by the addition of isopropanol. Five micrograms of each RNA sample was reverse-transcripted to cDNA in 25 μL reaction buffer (375 mM KCl, 250 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 0.8 mM concentrations of each deoxyribonucleoside triphosphate (dNTPs), 40 μM oligo (dT) primers, 0.1 units of RNase inhibitor and 200 units of reverse transcriptase). The transcripts were amplified using a GeneAmp 5700 Sequence Detection System (Applied Biosystems) in 28 μL reaction mixture containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 200 μM dNTPs, 660 nM forward and reverse primer, 330 nM probe and 14 μL platinum quantitative PCR super-mix UDG (Invitrogen, Carlsbad, CA, USA). The sequences of the primers and the probe were as follows: forward primer for H1R: 5′-CAG AGG ATC AGA TGT TAG GTG ATA GC-3′; reverse primer for H1R: 5′-AGC GGA GCC TCT TCC AAG TAA-3'; probe: FAM-CTT CTC TCG AAC GGA CTC AGA TAC CAC C-TAMRA. The human GAPDH gene was used to standardize starting material. Data are expressed as the ratio of H1R mRNA level to GAPDH mRNA level.

Luciferase reporter assay

Human H1R reporter plasmid (pH1R) was constructed as follows. A 2.1-kbp 5′-upstream fragment (–2029 to +64, +1 indicates the putative transcription initiation site of human H1R gene (De Backer et al 1998)) was amplified by PCR using a forward primer (5′-GCTAGCCGAATGT-GGGAAGATCAGTAGTAG-3′) and a reverse primer (5′-AGATCTGAAGGTCTTCTCCATGATGGGCTTC-3′), and ligated to pGEM-T Easy vector (Promega). After confirming the sequence, the fragment was then subcloned into the NheI-BglII site of the promoter-less luciferase reporter plasmid pGL3-Basic vector (Promega). Cells cultured in

12-well plates were co-transfected with pH1R and pRL-TK vector (Promega) at a ratio of 200:1. One microgram of the plasmid mixture was transfected into the cells using PolyFect transfection reagent or Lipofectamine plus reagent according to the manufacturer's instructions. After 5 h of incubation, the medium was replaced with 1 mL DMEM without serum. After another 24 h, the cells were stimulated with various drugs for appropriate times in the same medium. Pretreatment with several drugs was performed 30 min before stimulation. After the treatment, the cells were washed twice with 500 μL cold phosphate-buffered saline and lysed with 100 μL passive lysis buffer (Promega). The lysates were frozen at −85°C for at least 3 h, thawed at room temperature and then analysed using a dual-luciferase assay System (Promega). Luminescence was measured using a BLR 302 photoluminescence reader (ALOKA, Tokyo, Japan). The measurement was integrated over 20 s with no delay.

Assay of H1R level

Membrane preparations from U373-β2 cells treated with vehicle (control) or drugs for appropriate times were subjected to a radioligand binding assay using 4 nM [^{3}H]mepyramine. Changes in H1R level were expressed as the percentage of [^{3}H]mepyramine binding of the treated cells compared with that of vehicle-treated cells.

Statistical analysis

Data are expressed as mean ± s.e.m. Statistical analysis was performed by one-way analysis of variance, followed by Dunnett's test, or using the Mann–Whitney *U*-test. A value of $P < 0.05$ was considered significant.

Results

Expression of H1R and β2AR in U373 astrocytoma cells

Human U373 astrocytoma cells were transfected with pcDNA3.1 vector encoding the human β2AR gene. The stable expression of β2AR in the cells (U373-β2 cells) was examined by radioligand binding assay using the β-adrenoceptor antagonist [^{3}H]DHA. The K_d (dissociation constant) and B_{max} (binding capacity) were 0.07 ± 0.01 nM and 3.5 ± 0.5 pmol mg^{-1} protein, respectively (n = 4). The K_d was comparable to reported values (Jockers et al 1996; Kawakami et al 2004). U373 astrocytoma cells express endogenous H1R; expression in U373-β2 cells was measured using the specific H1R antagonist [^{3}H]mepyramine. K_d and B_{max} were 8.6 ± 2.1 nM and 85.1 ± 12 fmol mg^{-1} protein, respectively (n = 4), which were comparable to reported values (Arias-Montaño et al 1994).

Effects of β2AR activation on H1R mRNA level

First, we examined the effect of β2AR activation on H1R mRNA levels in U373-β2 cells using real-time RT-PCR. Treatment with 0.1 μM or 1 μM fenoterol (β2AR agonist) for 4 h decreased H1R mRNA levels significantly (Figure 1A). Treatment with the PKA activator 8-Br-cAMP (3 mM) for 4 h also decreased H1R mRNA levels (Figure 1A). The decrease in H1R mRNA induced by fenoterol was transient, reaching a minimum by 4 h, which was maintained for the next 4 h (Figure 1B). The fenoterol-induced decrease in H1R mRNA level was blocked by propranolol (a non-selective β-adrenoceptor antagonist) and ICI 118551 (a selective β_2AR antagonist) but not by CGP 20712A (a selective β_1-adrenoroceptor antagonist), indicating the involvement of β2AR in this process (Figure 1C). The PKA inhibitor KT5720 inhibited the fenoterol-induced decrease in H1R mRNA levels (Figure 1D), suggesting that PKA activation is involved in this process.

To examine the effect of fenoterol on H1R mRNA stability, U373-β2 cells were treated with and without 0.1 μM fenoterol in the presence of actinomycin D (10 μg mL^{-1}). The treatment with fenoterol slightly accelerated the degradation of H1R mRNA, but this effect was not significant (Figure 2A).

Effects of β2AR activation on H1R gene promoter activity

For the H1R gene promoter assay, U373-β2 cells were co-transfected with pGL3 basic vector encoding the promoter region of H1R and pRL-TK vector (Promega) for the luciferase reporter assay. H1R promoter activity was decreased about 20% by treatment with 1 μM fenoterol for 8 h. Treatment with 3 mM 8-Br-cAMP decreased H1R promoter activity by about 30% (Figure 2B).

Effects of β2AR activation on the expression of H1R

We examined the effect of β2AR activation on the expression level of H1R in U373-β2 cells. Treatment with fenoterol significantly decreased the H1R level after 48 h exposure (Figure 2C).

Discussion

Stimulation of β2AR in U373-β2 cells caused a time-dependent decrease in the H1R mRNA level, reaching a minimum by 4 h, which was then maintained for up to 8 h of incubation. This down-regulation of H1R mRNA was blocked by the specific β_2AR antagonist ICI 118551, but not by the specific β_1-receptor antagonist CGP 20712A, confirming a β_2AR-mediated process. Activation of β2AR decreased H1R promoter activity, but had no significant effect on H1R mRNA stability, indicating that down-regulation of H1R mRNA by β2AR was mainly due to a decrease in the synthesis of H1R mRNA. Treatment with 8-Br-cAMP (a non-hydrolysable analogue of cAMP) reduced both the level of H1R mRNA and H1R gene promoter activity in U373-β2 cells, suggesting that a PKA-dependent process is involved in the down-regulation of H1R mRNA. This was confirmed by the finding that β2AR-mediated down-regulation of H1R mRNA was inhibited by the PKA inhibitor KT5720. We suggest two possible mechanisms to explain the role of PKA in H1R regulation.

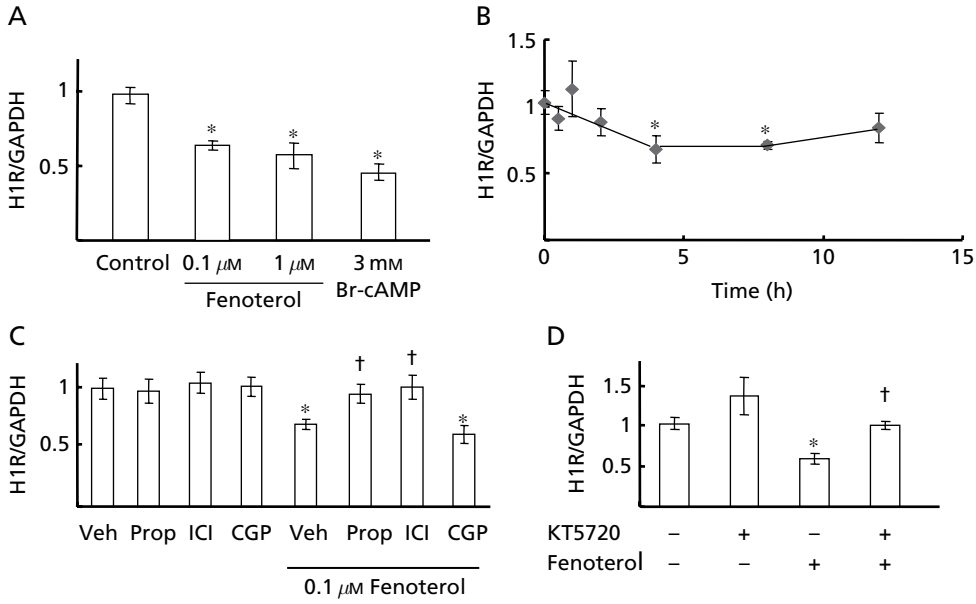

Figure 1 β_2-adrenoceptor (β2AR)-mediated down-regulation of histamine H_1 receptor (H1R) mRNA in U373-β2 cells. The levels of H1R mRNA were determined by real-time RT-PCR. (A) Decrease in H1R mRNA level by the β2AR agonist fenoterol or 8-Br-cAMP. U373-β2 cells were treated with 0.1–1 μM fenoterol, or 3 mM 8-Br-cAMP for 4 h. *$P < 0.05$ vs control (n = 6). (B) Time course of decrease in H1R mRNA level in U373-β2 cells induced by 0.1 μM fenoterol. *$P < 0.05$ vs control (n = 6). (C) Effects of β2AR antagonists on fenoterol-induced decrease in H1R mRNA level. U373-β2 cells were pretreated with 0.1 μM propranolol (Prop), ICI 118551 (ICI) or CGP 20712A (CGP) for 30 min, and then treated with 0.1 μM fenoterol for 4 h. * $P < 0.05$ vs control (vehicle): $^{\dagger}P < 0.05$ vs fenoterol (n = 6). (D) Effect of the protein kinase A inhibitor KT5720 on the fenoterol-induced decrease in H1R mRNA level. U373-β2 cells were pretreated with 3 μM KT5720 for 30 min and then treated with 0.1 μM fenoterol for 4 h. *$P < 0.05$ vs control; $^{\dagger}P < 0.05$ vs fenoterol (n = 6).

Several potential transcription factor binding sites in the human H1R promoter region have been reported, including Sp1 transcription factor (SP1), activating protein-1 (AP1) and nuclear factor (NF)-κB (De Backer et al 1998). H1R stimulation activates NF-κB (Bakker et al 2001), and NF-κB may promote H1R mRNA synthesis (Das et al 2007). PKA has been reported to inhibit the NF-κB-mediated transcription process and may therefore inhibit H1R mRNA synthesis (Ollivier et al 1996; Parry & Mackman 1997; Takahashi et al 2002). Secondly, cAMP-responsive element binding protein (CREB), which is activated by PKA, is reported to bind to AP-1 sites and to inhibit the AP-1-mediated transcription process (Masquilier & Sassone-Corsi 1992). Thus, PKA may inhibit AP-1-mediated up-regulation of H1R (Das et al 2007) by this mechanism.

Previously we have shown that activation of β2AR caused down-regulation of H1R through degradation of the H1R protein (Kawakami et al 2004). In those studies, we used CHO cells that expressed exogenously introduced H1R that was under the regulation of the SV40 promoter. Such a cell system has been widely used to express exogenous genes, and the expression of the introduced gene was usually maintained constant and was not influenced by stimulation of the cell. Our previous study therefore concluded that β2AR stimulation in CHO cells down-regulated H1R by accelerating H1R protein degradation. The U373 cells used in the current study express H1R that is under the regulation of an endogenous promoter. In this case, it is possible for stimulants to modulate H1R expression by influencing H1R mRNA synthesis. This cell was therefore suitable for studying the effect of stimulants on H1R gene regulation. Our results in U373 cells indicate that β2AR stimulation suppressed H1R mRNA synthesis. Taken together, these two works indicate that β2AR stimulation regulates levels of human H1R via two main mechanisms: one is to accelerate the degradation of H1R protein and the other is to inhibit H1R mRNA synthesis. Both mechanisms will contribute to a decrease in the level of H1R.

It is interesting to note that, in contrast to our results, β2AR stimulation has been shown to up-regulate H1R and H1R mRNA in bovine trachea smooth muscle (Mak et al 2000). This discrepancy could be attributed to differences in the structure of the promoter regions between human and bovine H1Rs. It may also be attributed to differences in the cell types. Differences between these two cell types has also been found regarding protein kinase C (PKC)-mediated regulation of H1R mRNA level. Muscarinic M3 receptor stimulation up-regulated H1R via a PKC-mediated process in human U373 astrocytoma cells (Miyoshi et al 2007), whereas PKC activating phorbol ester down-regulated H1R in bovine trachea smooth muscle (Pype et al 1998). Analysis of the promoter regions of human and bovine H1R may help to clarify these discrepancies. At present, however, we have no data on the structure of the promoter region of bovine H1R and we must therefore await future work to solve this problem.

We have previously reported that various stimulants regulate H1R levels. Stimulation of H1R up-regulated H1R by accelerating H1R mRNA synthesis (Das et al 2007) and down-regulated H1R by accelerating H1R degradation (Horio et al 2004). M3 receptor stimulation also up-regulated H1R by

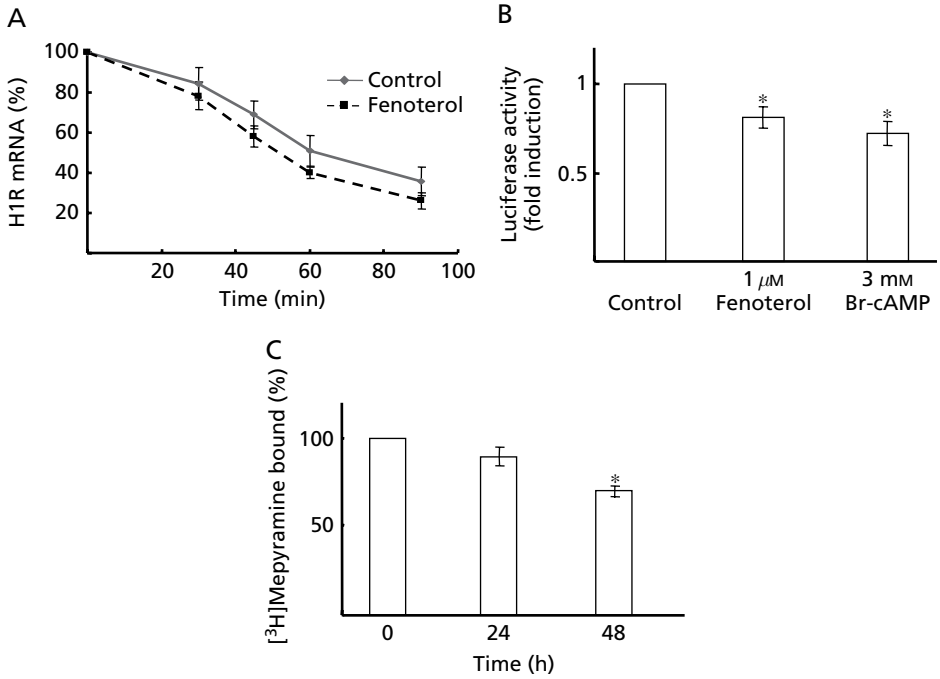

Figure 2 (A) Stability assay of histamine H_1 receptor (H1R) mRNA. U373-β2 cells were incubated with 0.1 μM fenoterol or vehicle in the presence of actinomycin D 10 μg mL^{-1} (n = 6). (B) Effects of β_2-adrenoceptor (β2AR) activation on H1R promoter activity in U373-β2 cells (using the luciferase reporter assay). U373-β2 cells were treated with 1 μM fenoterol or 3 mM 8-Br-cAMP for 8 h. *$P < 0.05$ vs control. (C) β2AR-mediated down-regulation of H1R in U373-β2 cells (measured using the [^{3}H]mepyramine binding assay). U373-β2 cells were treated with 0.1 μM fenoterol for 24 or 48 h. *$P < 0.05$ vs control (n = 4).

increasing H1R mRNA synthesis (Miyoshi et al 2007), and down-regulated H1R by increasing H1R degradation (Miyoshi et al 2004). PAF has been reported to increase H1R mRNA level in rat trigeminal nerve ganglion cells (Nakasaki et al 1999). To add to these data, we provide evidence that β2AR stimulation regulates H1R levels by decreasing H1R mRNA synthesis. The present study suggests that most of the agonists that increase cAMP have similar effects on the H1R level as with β2AR stimulation. For example, histamine H_2 receptor (H2R) stimulation will probably decrease H1R levels because this stimulation will increase cAMP. It is interesting that histamine will increase H1R levels through H1R stimulation but will decrease it through H2R activation. Further studies are required to clarify these points. Thus, many types of receptor, including β2AR, M3, H1R and H2R, and possibly others, are involved in the regulation of H1R level.

Levels of H1R are reported to become abnormal in some pathological conditions, such as in the temporal cortex of patients with epilepsy (Iinuma et al 1993), in the nasal mucosa of patients with allergic rhinitis (Iriyoshi et al 1996; Hamano et al 1998), in the frontal cortex of patients with chronic schizophrenia (Nakai et al 1991) and in the frontal and temporal areas of patients with Alzheimer's disease (Higuchi et al 2000). Thus, regulation of H1R level is probably important in both central and peripheral tissues to maintain their functions. As described above, many stimulants are involved in this regulation and the β2AR also plays a role in these processes. Further studies are required to clarify the physiological and pathophysiological role of this agonist-mediated regulation of H1R level.

Conclusion

We have investigated the possible role of β2R stimulation in H1R gene regulation by using human U373 astrocytoma cells that express endogenous H1R and transfected β2R. Stimulation of β2R significantly decreased H1R promoter activity, H1R mRNA level and the H1R level. Treatment with 8-Br-cAMP (a non-hydrolysable analogue of cAMP) reduced H1R mRNA levels in the U373-β2 cells, and the β2AR-mediated decrease of H1R mRNA was blocked by the PKA inhibitor. These results suggest that the β2AR is involved in the down-regulation of human H1R by inhibiting H1R gene transcription through a PKA-dependent process. Such heterologous regulation of H1R may play an important role in regulating the H1R level in both the central nervous system and in the peripheral tissues.

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