Two Distinct Pathways for Histamine H2 Receptor Down-regulation

H2 LEU124 → ALA RECEPTOR MUTANT PROVIDES EVIDENCE FOR A cAMP-INDEPENDENT ACTION OF H2 AGONISTS*

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The introduction of molecular biology in the field of histamine receptor research has greatly improved the possibilities to study molecular aspects of histamine receptor proteins. In 1991, Gantz et al. (1) cloned the cDNA encoding the canine histamine H2 receptor, which was followed by the cloning of both the rat and human homologues (2, 3). The deduced amino acid sequence of the H2 receptor proteins reveals the existence of seven putative transmembrane domains, indicating that this receptor is a member of the large family of G-protein-coupled receptors (GPCRs).1 This family of receptors is known to readily subjected to regulatory processes in order to control receptor signaling and thus cellular communication (4). Short-term exposure of receptors to high concentrations of agonists is often followed by a decrease in cellular responsiveness, called desensitization (5). Long-term exposure, on the other hand, results in a reduction of receptor number (6) and is referred to as receptor down-regulation. Since the histamine H2 receptor is a member of this family of GPCRs, it is not surprising that this receptor is also susceptible to such regulatory mechanisms.

Recently, we have shown that in human U937 cells the endogenously expressed histamine H2 receptors are indeed rapidly desensitized when exposed to histamine (7). Similar observations have been reported in other cellular systems (8, 9). Yet, so far, no detailed information is available on long-term desensitization of the histamine H2 receptor such as receptor down-regulation. Such processes may become apparent under several pathophysiological conditions (e.g. asthmatic attack or allergic reactions in general), during which histamine is released in large quantities, but might also occur under normal physiological conditions. Recently, Diaz et al. (10) suggested for example that in vivo receptor down-regulation might explain the inverse relationship between H2 receptor expression and the localization of histamine-synthesizing cells in the rodent gastric wall. The regulation of H2 receptor expression has gained further interest due to the potential therapeutic application of H2 receptor agonists in patients suffering from congestive heart failure (11).

Investigation of the regulation of H2 receptor expression has so far been hampered by the availability of suitable model systems. Cellular systems (7–9, 12) have been used to investigate second messenger responses coupled to the histamine H2 receptor stimulation, but the used systems such as U937 cells for example do not express a sufficiently high density of H2 receptors to permit radioligand binding studies, which are essential for the investigation of long-term regulatory mechanisms (7). Following the recent cloning of cDNAs or genes encoding histamine H2 receptors, cell lines expressing considerable amounts of histamine H2 receptors can be obtained (13, 14). Additionally, the availability of the H2 receptor gene allows the construction of receptor mutants, which can provide mechanistic insights in phenomena like receptor down-regulation.

In the present study we have examined the effects of long-term exposure of the rat histamine H2 receptor stably expressed in Chinese hamster ovary (CHO) cells (referred to as CHOrH2 cells) (13) to H2 agonists and cAMP-mobilizing agents with regard to H2 receptor protein expression and H2 receptor mRNA levels. In order to get more insight into the mechanisms underlying H2 receptor regulation, we constructed a H2 receptor mutant, in which leucine 124 in the second intracellular

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1 The abbreviations used are: GPCR, G-protein-coupled receptor; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; [35S]JAPT, [35S]iodoaminopentidetidine; IBMX, isobutylymethanxthine; CTX, chola toxin; PCR, polymerase chain reaction; GTPγS, guanosine 5'-O-(thiodiphosphate).
loop was substituted by an alanine. This H₂ Leu¹²⁴ → Ala receptor mutant was partially uncoupled from its G-protein and proved to be a suitable tool for investigating the existence of possible cAMP-dependent and independent pathways in the process of agonist-induced H₂ receptor down-regulation.

MATERIALS AND METHODS

Cell Culture—CHO cells expressing the rat histamine H₂ receptor (CHOH₂) (13) and the mutated H₂ Leu¹²⁴ → Ala receptor (CHOH₂Leu¹²⁴Ala) were grown at 37°C in a humidified atmosphere with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum. Appropriate drugs in DMEM/HEPES (55 mM HEPES) were added, and the cells were incubated for 10 min at 37°C. The reaction was stopped by the rapid aspiration of the culture medium and the addition of 200 μl of 1 M cold HCl. The cells were kept on ice and disrupted by sonification (5 s, 50 watts, Labsonic 1510, Braun-Melsungen). The resulting homogenate was frozen at −20°C or directly neutralized with 1 N NaOH and assayed for the presence of cAMP. In order to determine the long-term effects of histamine treatment on H₂ receptor signaling, CHOH₂ cells were preincubated with 100 μM histamine for 24 h in DMEM without fetal calf serum. Thereafter, the cells were thoroughly washed and preincubated in DMEM/HEPES at 37°C for 30 min before actual incubation with the indicated drugs.

Cyclic AMP Assay—The amount of cAMP in the CHOH₂ and CHOH₂Leu¹²⁴Ala cells was determined according to Nordstedt and Fredholm (17), with some minor modifications. Briefly, a protein kinase A-mediated fraction was isolated from bovine adrenal glands. Adrenal cortex was homogenized in 10 volumes of 100 mM Tris-HCl, 250 mM NaCl, 10 mM EDTA, 0.25 mM sucrose, and 0.1% 2-mercaptoethanol (pH 7.4 at 4°C, buffer A) using an Omni-Sorval mixer (30 s, maximal speed) and a Polytron homogenizer (10 s, maximal speed). The homogenate was centrifuged for 60 min at 30,000 g at 4°C. The supernatant, containing protein kinase A, was carefully recovered and frozen in 1 ml aliquots at −80°C. Before use, the binding protein was diluted 5-fold in ice-cold buffer A without sucrose and 2-mercaptoethanol and kept on ice. Subsequently, 200 μl of the binding protein was mixed with 50–100 μM of the CHO homogenate or cAMP standards and 30,000 dpm of [3H]cAMP. After incubation for 150 min at 37°C, the mixture was rapidly diluted with 3 ml of cold 50 mM Tris-HCl (pH 7.4 at 4°C) and filtered through Whatman GF/B filters, with a Brandel cell harvester (Semat). The radioactivity retained on the filters was measured by liquid scintillation counting.

RNA Slot Blot Analysis—RNA was analyzed by means of mRNA slot blot assay as described by Zhang et al. (18), with minor modifications. Briefly, RNA was isolated according to the method of Chomczynski and Sacchi (19), using Trizol reagent (Life Technologies, Inc.), and RNA was slot-blotted onto a nitrocellulose filter (GeneScreen Plus, DuPont NEN) and prehybridized for 2 h at 65°C in 7% SDS, 0.5 M NaPO₄ (pH 7.0). The RNA was hybridized overnight with a radioactively labeled 18-mer antisense rat H₂ receptor oligonucleotide (5'-GATGTCGGCTCTCACTACGAGCTGTGTGCTTGTG-3', corresponding to nucleotides 2062 to 2107) at 65°C in 7% SDS, 0.5 M NaPO₄, and 1 M EDTA (pH 7.2). The rat H₂ receptor oligonucleotide (1.5 pmol) was 32P-labeled by 3'-end tailing using 16 pmol of [α-32P]dATP (3000 Ci/mmol; Amersham) and 1 unit of terminal deoxynucleotidyl transferase (Boehringer) for 30 min at 37°C. The 32P-labeled oligonucleotide (5 pmol) was labeled using 10 pmol of [γ-32P]dATP (3000 Ci/mmol; Amersham) and 4 units of polyadenylate kinase (Boehringer) for 30 min at 37°C. The blasts were washed twice for 5 min at room temperature in 2× SSC (0.3 M NaCl, 0.03 M Na₂HPO₄/NaH₂PO₄) supplemented with 0.1% SDS, which was followed by two 45-min washes at 65°C with 2× SSC supplemented with 0.1% SDS. The blot with a Phosphor imager was scanned and analyzed. Densitometric and signals were quantified with a Phosphorimager 425 (Molecular Dynamics) using the computer program ImageQuant (Molecular Dynamics). H₂ receptor mRNA levels were expressed as the ratio of the values of the H₂ receptor mRNA signals and the corresponding β-actin signals.

Analysis of H₂ Receptor mRNA Stability—H₂ receptor mRNA levels were determined after incubation of the CHOH₂ cells with actinomycin D to block transcription as described previously (20). Cells were preincubated with or without 100 μM histamine or with 10 μM forskolin for 1 h in DMEM. Thereafter, actinomycin D (10 μg/ml) was added. Cells were harvested from 0 to 90 min after addition of actinomycin D. Total RNA was isolated at each point, and RNA was stored at −80°C. The RNA was analyzed by means of the mRNA slot blot assay as described above.

Chemicals—Histamine dihydrochloride, isobutylmethylxanthine (IBMX), cyclic AMP (cAMP), forskolin, 1,9-dideoxyforskolin, cholera toxin, and GTP-γ-S were obtained from Sigma. Actinomycin D was purchased from Boehringer Mannheim. [2,8-3H]cAMP (40 Ci/mmol) was obtained from Amersham. H₂ agonists or antagonists were purchased from Calbiochem. Dimaprit dihydrocholrine, homol- and nor-dimaprit dihydrocholrine, amantidine dihydrocholrine, amesine dihydrocholrine, and amantadine were taken from laboratory stock.

Statistical Analysis—All data shown are expressed as mean ± S.E. of at least three independent experiments. Statistical analysis was carried out using analysis of variance with Student's t-test.
Histamine-induced H\textsubscript{2} Receptor Down-regulation—Exposure of CHOrH\textsubscript{2} cells (13) to 100 \mu M histamine for prolonged periods of time resulted in a time-dependent decrease of \[^{125}\text{I}\text{APT}\] binding (Fig. 1A). Maximum reduction of \[^{125}\text{I}\text{APT}\] binding was observed after a 16-h incubation of cells with 100 \mu M histamine. Under this condition, histamine induced 44 \pm 10% (p < 0.05) reduction of \[^{125}\text{I}\text{APT}\] binding. Half-maximum reduction of the \[^{125}\text{I}\text{APT}\] binding was recorded at an incubation period of approximately 7 h. A 24-h incubation of CHOrH\textsubscript{2} cells with increasing concentrations of histamine led to a dose-dependent reduction of \[^{125}\text{I}\text{APT}\] binding (EC\textsubscript{50} value = 18 \pm 6 nM, mean \pm S.E., n = 7) (Fig. 1B). The observed reduction of \[^{125}\text{I}\text{APT}\] binding was not reflected by a change in affinity of \[^{125}\text{I}\text{APT}\] for the H\textsubscript{2} receptor, as its dissociation constant (K\textsubscript{d}), determined by means of saturation studies, remained unaffected in CHOrH\textsubscript{2} cells incubated with 100 \mu M histamine for 24 h (Table I).

Exposure of CHOrH\textsubscript{2} cells to histamine resulted only in a marked decrease of the total number of \[^{125}\text{I}\text{APT}\] binding sites (B\textsubscript{max}) (Table I). The recently described selective H\textsubscript{2} receptor agonists amsel-amine and amthamine (21, 22) induced cAMP production in CHOrH\textsubscript{2} cells, with EC\textsubscript{50} values lower and maximum responses comparable to histamine (Table II). Long-term exposure (24 h) of CHOrH\textsubscript{2} cells with 100 \mu M dimaprit induced a maximal decrease of \[^{125}\text{I}\text{APT}\] binding with EC\textsubscript{50} values, which were correlated with their respective EC\textsubscript{50} values for the cAMP response. Amselamine and amthamine induced a maximal decrease of \[^{125}\text{I}\text{APT}\] binding sites of 50 \pm 3% and 43 \pm 4%, respectively. As shown in Table II, 24-h incubation of CHOrH\textsubscript{2} cells with 100 \mu M dimaprit, which exhibits a lower potency as compared to histamine, induced a 40 \pm 3% decrease of \[^{125}\text{I}\text{APT}\] binding sites. Dimaprit’s structural analogues, homodimaprit and nordimaprit, showed strongly reduced capacities to generate cAMP with EC\textsubscript{50} values of 1.4 \pm 0.9 \mu M and higher than 10 \mu M, respectively (Table I). The reduced ability of these dimaprit analogues to induce a CAMP response was paralleled by a lack of H\textsubscript{2} receptor down-regulation after 24 h of incubation of CHOrH\textsubscript{2} cells with 100 \mu M concentrations of the analogues (Table I).

Effect of Long-term Histamine Treatment on Histamine- and Forskolin-induced Signaling in CHOrH\textsubscript{2} Cells—Long-term exposure (24 h) of CHOrH\textsubscript{2} cells with 100 \mu M histamine resulted in a rightward shift of the dose-response curve of the histamine-induced cAMP production (EC\textsubscript{50} of histamine-induced cAMP response in nontreated cells: 36 \pm 3 nm, mean \pm S.E., n = 7, and histamine-treated cells: 1.2 \pm 0.05 \mu M, mean \pm S.E., n = 4) (Fig. 2A). The forskolin-induced rise in cAMP was not found to be affected as no change in dose dependence or im-
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Cholera toxin (CTX), which irreversibly activates the Gs-protein, thereby generating cAMP, also induced a dose-dependent decrease of [125I]APT binding sites when incubated for 24 h (EC50 = 32 ± 1 ng/ml, mean ± S.E., n = 4, Fig. 4). CTX pretreatment of CHOrH2 cells resulted in a maximum down-regulation of H2 receptors of 46 ± 3%. Finally, exposure of CHOrH2 cells for 24 h to 300 μM IBMX, a cAMP phosphodiesterase inhibitor, also resulted in an attenuation of [125I]APT binding (26 ± 7% H2 receptor down-regulation, n = 4, mean ± S.E., p < 0.05).

H2 Receptor mRNA Levels and Stability in Control, Histamine-treated, and Forskolin-treated CHOrH2 Cells—Exposure of CHOrH2 cells to 100 μM histamine for increasing periods of time resulted in a rapid transient decrease of H2 receptor mRNA (maximum reduction of 71 ± 4%, mean ± S.E., n = 4) (Fig. 5). This effect was at its peak after 4 h of incubation of cells with histamine (100 μM), while the amount of H2 receptor mRNA returned to approximately 50% of control after 12 h of histamine treatment. Long-term incubation of CHOrH2 cells with 10 μM forskolin also induced a time-dependent transient decrease (maximum reduction: 75 ± 7%, mean ± S.E., n = 4) of H2 receptor mRNA to levels similar to those observed after histamine treatment (Fig. 5).

To study the role of mRNA stability, CHOrH2 cells were incubated for 1 h in the absence or presence of histamine (100 μM) or forskolin (10 μM), whereafter actinomycin D (10 μg/ml) was added to block mRNA transcription. Cells were collected at different time intervals ranging from 0 to 90 min after addition of actinomycin D and were analyzed for H2 receptor mRNA content. The H2 receptor mRNA in nontreated cells was hardly affected during the 90 min of incubation with actinomycin D (inset, Fig. 5). Incubation of cells with 100 μM histamine, however, resulted in a significant breakdown of H2 receptor mRNA levels (inset, Fig. 5). Similar results were obtained after forskolin treatment (inset, Fig. 5).

Fig. 2. Effect of long-term histamine treatment on histamine- and forskolin-induced signaling in CHOrH2 cells. CHOrH2 cells were treated with (open circles) or without (filled circles) 100 μM histamine for 24 h in DMEM without fetal calf serum. Thereafter, cells were washed several times and incubated for 1 h with DMEM supplemented with 25 mM HEPES, pH 7.4. CHOrH2 cells were subsequently incubated with increasing concentrations of histamine (A) or forskolin (B) for 10 min at 37°C in DMEM in the presence of 300 μM IBMX and 25 mM HEPES, pH 7.4. The data represent the mean ± S.E. of 4 independent experiments.

Role of cAMP in the Process of H2 Receptor Down-regulation—Forskolin, which directly activates adenyllylcyclase, dose-dependently induced the formation of cAMP in CHOrH2 cells (Fig. 3A). Prolonged exposure (incubation periods ranging from 4 to 32 h) of CHOrH2 cells with 10 μM forskolin led to a marked reduction of 58 ± 2% [125I]APT binding (Fig. 3B). Again, no major change in affinity of [125I]APT for the H2 receptor was apparent, only a decrease in Bmax was observed when CHOrH2 cells were incubated for 24 h with 10 μM forskolin (Table I). Maximum and half-maximum down-regulation was recorded after 16 h and approximately 7 h of incubation of CHOrH2 cells with 10 μM forskolin, respectively (Fig. 3B). The H2 receptor binding sites appeared to be dose-dependently down-regulated by increasing concentrations of forskolin, with an EC50 value of 0.3 ± 0.06 μM (mean ± S.E., n = 4) (Fig. 3C). Concentrations up to 10 μM of the inactive analogue 1,9-dideoxyforskolin, which does not generate cAMP in CHOrH2 cells (Fig. 3A), did not attenuate the H2 receptor density after 24 h of pretreatment (Fig. 3D).

M. J. Smit, unpublished observations.
induces down-regulation, suggesting that a cAMP-independent pathway is responsible for the histamine-induced down-regulation.

Functional Analysis of the Leu124 → Ala Mutation of the Rat Histamine H2 Receptor—Using the polymerase chain reaction, leucine 124 in the second intracellular loop of the rat histamine H2 receptor (Fig. 7) was mutated into an alanine residue. Transfection of the H2 Leu124 → Ala receptor cDNA into CHO cells, resulted in the formation of several clonal cell lines expressing [125I]APT binding sites. A clonal cell line expressing amounts of [125I]APT binding comparable to those of the CHOrH2 cells was chosen for further analysis and referred to as CHOrH2Leu124Ala (CHOrH2 cells: 975 ± 6 12 fmol/mg of protein, CHOrH2Leu124Ala cells: 980 ± 7 fmol/mg of protein, mean ± S.E., n = 3). There were no major differences in the binding of the H2 antagonists to the wild-type receptor or the mutated receptor. The affinity of [125I]APT for the mutated receptor was hardly affected (Kd of [125I]APT in CHOrH2 cells: 0.43 ± 0.06 nM, in CHOrH2Leu124Ala cells: 0.61 ± 0.03 nM, mean ± S.E., n = 3). Moreover, cimetidine and ranitidine had similar Kd values for both receptors (Table III). In contrast, the introduced Leu124 → Ala mutation significantly affected the agonist binding characteristics. In CHOrH2 cells, histamine displacement curves were shallow and could be analyzed best by a two-site model (Fig. 8, Table III). The addition of 10 μM GTP-Y-S resulted in a steepening and a rightward shift of the histamine displacement curve, which could be analyzed best by a single site model (Fig. 8, Table III). The asterisks indicate a significant difference (p < 0.05) from control, represented by the [125I]APT binding measured in untreated cells. Data from B, C, and D were calculated as mean ± S.E. from 4 independent experiments.
histamine-induced response was also found to be affected in CHOrH2Leu124Ala cells. The CHOrH2 cells were treated with increasing concentrations of CTX for 24 h. [125I]APT binding is expressed as a percentage of [125I]APT binding measured in nontreated cells. Data shown are mean ± S.E. of 4 independent experiments.

FIG. 4. Effect of long-term treatment with CTX on [125I]APT binding in CHOrH2 cells. The CHOrH2 cells were treated with increasing concentrations of CTX for 24 h. [125I]APT binding is expressed as a percentage of [125I]APT binding measured in nontreated cells. Data shown are mean ± S.E. of 4 independent experiments.

Histamine- and forskolin-induced modulation of H2 receptor mRNA levels. CHOrH2 cells were incubated for the indicated times with 100 μM histamine (filled circles) or 10 μM forskolin (open circles). Cells were harvested, and total RNA was extracted and quantified by means of a RNA slot blot assay as described under "Materials and Methods." The results displayed are the mean ± S.E. of two separate experiments, performed in duplicate. Histamine treatment and forskolin treatment on H2 receptor mRNA stability. CHOrH2 cells were incubated with (open circles) or without (open squares) 100 μM histamine or with 10 μM forskolin (filled squares) for 1 h, before actinomycin D (10 μg/ml) was added. Cells were harvested at 0, 15, 30, 60, and 90 min after the addition of actinomycin D. The data are the mean ± S.E. of three separate experiments, each performed in duplicate. The asterisks indicate a significant difference (p < 0.05) from control, represented by untreated cells.

FIG. 5. Histamine- and forskolin-induced modulation of H2 receptor mRNA levels. CHOrH2 cells were incubated for the indicated times with 100 μM histamine (filled circles) or 10 μM forskolin (open circles). Cells were harvested, and total RNA was extracted and quantified by means of a RNA slot blot assay as described under "Materials and Methods." The results displayed are the mean ± S.E. of two separate experiments, performed in duplicate. Histamine treatment and forskolin treatment on H2 receptor mRNA stability. CHOrH2 cells were incubated with (open circles) or without (open squares) 100 μM histamine or with 10 μM forskolin (filled squares) for 1 h, before actinomycin D (10 μg/ml) was added. Cells were harvested at 0, 15, 30, 60, and 90 min after the addition of actinomycin D. The data are the mean ± S.E. of three separate experiments, each performed in duplicate. The asterisks indicate a significant difference (p < 0.05) from control, represented by untreated cells.

Histamine-induced Down-regulation of Rat H2 Leu124 → Ala Receptors—Long-term exposure (24 h) of CHOrH2Leu124Ala cells to increasing concentrations of histamine resulted in a dose-dependent reduction of [125I]APT binding sites (Fig. 9B). Whereas in CHOrH2 cells an EC50 of 18 ± 6 nM (mean ± S.E., n = 7) was observed for histamine, in CHOrH2Leu124Ala cells histamine induced down-regulation with an EC50 value of 288 ± 89 nM (mean ± S.E., n = 4). Comparing the histamine-induced cAMP production and H2 Leu124 → Ala receptor down-regulation (Fig. 9B), a discrepancy in dose relationships is observed. Almost 40-fold higher concentrations of histamine are required to induce cAMP production, compared to receptor down-regulation. Pretreatment of CHOrH2Leu124Ala cells for 24 h with 1 μM histamine resulted in a significant degree of H2 receptor down-regulation (51 ± 2%, mean ± S.E., n = 4), whereas no significant cAMP production was observed after 10 min of incubation (Fig. 9B). Even after 24 h of incubation of CHOrH2Leu124Ala cells with 1 μM histamine, no significant increase in cAMP was observed (data not shown). Moreover, even at 0.1 μM histamine, significant H2 receptor down-regulation was observed.

In the CHOrH2Leu124Ala cells, the maximal histamine-induced down-regulation was more pronounced (68 ± 4%, mean ± S.E., n = 4) than was observed for the CHOrH2 cells (43 ± 4%, mean ± S.E., n = 7). The forskolin (10 μM)-induced H2 receptor down-regulation was also found to be more pronounced in the CHOrH2Leu124Ala cells (67 ± 3%, mean ± S.E., n = 3) than in CHOrH2 cells (58 ± 2%, mean ± S.E., n = 4).
Down-regulation of the Histamine H₂ Receptor

In the present study we have demonstrated that the rat histamine H₂ receptor density in CHO cells is reduced about 50% by long-term exposure to histamine or selective H₂ agonists. Long-term treatment of CHO H₂ cells with histamine resulted in a time-dependent (t₁/₂ ~ 7 h at a concentration of 100 μM) and dose-dependent (EC₅₀ = 18 nM at 24 h of incubation) decrease in the number of H₂ receptor binding sites. Yet, incubation of CHO H₂ cells with homo- or nondiarylput, two side chain homologues of the H₂ agonist dimaprit with weak H₂ agonistic activity (23, present study), did not significantly reduce the number of H₂ receptors. These findings show that the observed H₂ agonist-induced down-regulation is a H₂ receptor-mediated process. Long-term exposure of CHO H₂ cells to histamine resulting in a reduction of H₂ receptor binding sites is paralleled by a decrease of H₂ receptor responsiveness, characterized by a 34-fold shift of the histamine dose-response curve. The observed shift cannot be ascribed to decreased adenylyl cyclase activity as forskolin dose-response curves remained unaffected after long-term histamine exposure.

As was found for the β₂-adrenergic receptor (24), a cAMP-dependent pathway can also regulate the H₂ receptor density. Forskolin, generating cAMP upon addition, time dependently (t₁/₂ ~ 7 h at a concentration of 10 μM) and dose dependently (EC₅₀ = 0.3 μM at 24 h of incubation) induced H₂ receptor down-regulation. CTX and IBMX, agents that also elevate intracellular levels of cAMP in CHO H₂ cells, induced down-regulation of the H₂ receptor as well. Thus, the H₂ receptor does not need to be stimulated by an agonist in order to be down-regulated. This mechanism might be involved in heterologous H₂ receptor down-regulation as previously shown for other GPCRs (see Refs. 4 and 25). The time course of the forskolin-induced decrease of H₂ receptor number in CHO H₂ cells parallels the time-dependent decrease of H₂ receptors induced by histamine. For both histamine and forskolin, half-maximal H₂ receptor down-regulation is reached after approximately 7 h of incubation. Moreover, the maximum decrease of H₂ receptor numbers induced by forskolin is comparable to the maximum agonist-mediated H₂ receptor down-regulation.

DISCUSSION

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Agonist-induced receptor down-regulation is a commonly occurring regulatory process of the large family of GPCRs (see for reference reviews, Refs. 4 and 25). Enhanced degradation and/or decreased synthesis of the receptor protein are thought to contribute to receptor down-regulation (4, 25). Agonist-induced down-regulation of GPCRs is often accompanied by a decline of receptor mRNA levels, presumably contributing to the overall reduction in receptor number and responsiveness (26). Indeed, incubation of CHO H₂ cells with histamine or forskolin resulted in a transient decrease of H₂ receptor mRNA levels (70% reduction) within 4 h, which was followed by a gradual increase of H₂ receptor mRNA to 50% of control mRNA levels in the following hours. The reduced H₂ receptor mRNA levels, 50% of the control levels, at later time points are considered to represent a new steady-state level of receptor mRNA to maintain the down-regulated state of H₂ receptors. The reduction of H₂ receptor mRNA is most likely explained by post-transcriptional events, such as receptor mRNA destabilization. For example, the β₂-adrenergic receptor and thrombin receptor in DDT, MF-2 smooth muscle cells, the endothelin ET₉ receptor in ROS17/2 rat osteosarcoma cells, and also for the β₂-adrenergic receptor and muscarine m1 receptor expressed in CHO and CHO cells, respectively, the decline in receptor mRNA has been ascribed to destabilization of the mRNA (24, 27–30). In the presence of actinomycin D, breakdown of the H₂ receptor mRNA in CHO H₂ cells was stimulated significantly upon histamine-treated and forskolin-treated compared to non-treated cells. Recently, it was shown that a so-called M₉ = 35,000 β-adrenergic receptor mRNA-binding protein, involved in the destabilization of β₂-adrenergic receptor mRNA, also recognizes other GPCR transcripts (29). As such, our observations of H₂ receptor mRNA destabilization fit well in an appar-
Down-regulation of the Histamine H2 Receptor

For the β2-adrenergic receptor, the most extensively studied GPCR, receptor down-regulation is ascribed to two pathways: an agonist-dependent, protein kinase A-independent, and a protein kinase A-dependent process (4, 25). Evidence for a protein kinase A-independent pathway was obtained by studies which showed unaffected profiles of β2-adrenergic receptor down-regulation in mutant S49 mouse lymphoma cells defective in signal transduction components (32–35). Receptor-Gs coupling seems to be important for the process of β2-adrenergic receptor down-regulation, since defects in this coupling induced by mutations of the receptor or Gs-protein have lead to impaired β2-adrenergic receptor down-regulation (33–37). Agents responsible for the elevation of intracellular levels of cAMP, such as forskolin and IBMX, or cAMP analogues, e.g., dibutyryl cAMP, were shown to induce β2-adrenergic receptor down-regulation as well, providing evidence for the existence of cAMP-dependent receptor down-regulation (24, 25, 36). In CHW cells, the time course of the cAMP-promoted down-regulation of the β2-adrenergic receptor was much slower than the β-agonists-induced down-regulation, suggesting that distinct pathways can lead to down-regulation of the β2-adrenergic receptor (24). Yet, protein kinase A-independent phosphorylation of the β2-adrenergic receptor appears to enhance down-regulation, since receptor mutants lacking protein kinase A phosphorylation sites showed impaired agonist-induced down-regulation (24). Taken together, β2-adrenergic receptor down-regulation seems to require receptor-Gs coupling for the initial loss of receptor binding sites, while the cAMP-dependent decrease of receptor mRNA levels serves to maintain the down-regulated state by establishing a new steady-state of receptor expression (25). The underlying biochemical mechanisms responsible for each of these events is, however, unclear so far.

In our study on CHORe2 cells, comparable time courses and a maximum extent of histamine-induced and forskolin-induced H2 receptor down-regulation as well as H2 mRNA down-regulation suggest the involvement of cAMP in the process of agonist-induced H2 receptor down-regulation. The initial reduction of H2 receptor mRNA upon histamine or forskolin exposure can, however, not explain the 50% reduction of the H2 receptor numbers, since relatively short (<4 h) treatments of CHORe2 cells with histamine or forskolin followed by a wash-out up to 24 h led to a more pronounced H2 receptor down-regulation upon histamine than forskolin exposure. Thus, apparently there is no direct link between H2 receptor mRNA and H2 receptor expression. Moreover, these data are a first indication that histamine and forskolin induce H2 receptor down-regulation by different mechanisms. The existence of a cAMP-dependent and cAMP-independent pathway was further corroborated by the fact that the protein kinase A inhibitor H-89 (38) inhibited the forskolin-induced, but not the histamine-induced, H2 receptor down-regulation. Moreover, recently we have shown that down-regulation of H2 receptors stably expressed into human embryonal kidney cells (HEK-293 cells) is mediated via cAMP-dependent and cAMP-independent processes as the histamine-induced down-regulation was found to be more pronounced than the forskolin-induced H2 receptor down-regulation (39).

In order to assess the role of cAMP in the process of agonist-induced H2 receptor down-regulation in CHORe2 cells directly, we constructed a mutant H2 receptor which showed impaired G-protein coupling. Recently, Moro et al. (40) have shown that hydrophobic amino acids within a highly conserved GPCR motif DRYXXV(I)XXPL (X is any amino acid and L is leucine or other lipophilic amino acid) in the second intracellular loop are involved in receptor-G-protein coupling (40). In the H2 receptor protein, a DRYCAVTDPL sequence is found at an equivalent position of the highly conserved motif (2). Substitution of the Leu124 residue by an alanine residue had no effect on H2 receptor expression nor on H2 antagonist binding properties. However, the mutation induced a marked impairment of the ability of the receptor to physically couple to its G-protein as assessed by alterations in its agonist-binding parameters (disappearance high affinity binding site, no detectable GTPγS shift). The physical uncoupling of the H2, Leu124 → Ala mutant was paralleled by a functional uncoupling, characterized by an impairment of the histamine-induced cAMP production (160-fold reduction of the EC50 value and 55% decrease of the maximal cAMP response). These findings are in agreement with the functional uncoupling reported by Moro et al. (40) after mutation of a hydrophobic amino acid at similar position in the muscarinic m1, m3, and β2-adrenergic receptor.

Interestingly, long-term exposure of CHORe2Leu124Ala cells to 0.1 μM and 1 μM histamine, concentrations that do not elicit
cAMP production, resulting in a significant reduction of [125I]APT binding sites, indicating that a cAMP-independent pathway is involved in the observed agonist-induced H2 receptor down-regulation in CHOrH2Leu124Ala cells. Previous findings in mutant S49 mouse lymphoma cells defective in signal transduction components also showed the existence of cAMP-independent pathways in the agonist-induced β2-adrenergic receptor down-regulation (32–35). However, it should be noted that the EC50 value of histamine-induced H2 receptor down-regulation was shifted 16-fold to the right for the H2 Leu124 → Ala receptor compared to the wild-type receptor. These data suggest that agonist-induced H2 receptor down-regulation depends on intact receptor-G-protein coupling. As already stated earlier, previous findings for the β2-adrenergic receptor have shown that defective receptor-Gi coupling leads to impaired receptor down-regulation (33–37). Remarkably, both the maximum histamine-induced and forskolin-induced down-regulation of H2 Leu124 → Ala receptor were found to be more pronounced than for the wild-type H2 receptor, suggesting that the mutated receptor has become more susceptible to receptor down-regulation. Although we do not have an explanation for this finding, we hypothesize that the Leu124 → Ala mutation induces a conformational change in the second intracellular loop of the H2 receptor protein, causing an uncoupling from the Gα-protein but also an increase of the accessibility of molecular entities involved in receptor degradation. Recent studies with the parathyroid hormone receptor (41) and β2-adrenergic receptor (42) support this hypothesis. Small changes in the conformation of intracellular receptor domains have been shown to augment receptor internalization (41, 42). Unfortunately, no data on receptor down-regulation are available for these mutant receptors (41, 42).

In conclusion, for the first time we have demonstrated that the histamine H2 receptor is down-regulated by prolonged treatment with H2 agonists. Elevation of cAMP by long-term incubation of CHOrH2 cells with forskolin, CTX, and IBMX, is also shown to induce H2 receptor down-regulation. These data suggest the involvement of protein kinase A in the process of H2 receptor down-regulation and provides a mechanism for heterologous H2 receptor regulation. Also, H2 receptor mRNA levels were rapidly down-regulated upon both histamine treatment and forskolin treatment. However, the agonist-induced and forskolin-induced H2 receptor down-regulation do appear to be differentially regulated, by a cAMP-dependent and cAMP-independent pathway. Substitution of the hydrophobic amino acid leucine 124, located within the highly conserved G-protein coupling motif DRYXVXLXXPL in the second intracellular loop of the H2 receptor, by an alanine generated a mutant receptor with impaired ability to couple to its G-protein. Interestingly, the H2 Leu124 → Ala mutant receptor was still down-regulated by histamine, at concentrations which showed no increase of cAMP, thereby providing additional evidence for a cAMP-independent pathway in the process of agonist-induced H2 receptor down-regulation. Thus, H2 receptor down-regulation appears to be induced by two distinct pathways, a cAMP-dependent and cAMP-independent pathway.

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