Inverse agonism of histamine H₂ antagonists accounts for upregulation of spontaneously active histamine H₂ receptors

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ABSTRACT Histamine H₂ receptors transfected in Chinese hamster ovary (CHO) cells are time- and dose-dependently upregulated upon exposure to the H₂ antagonists cimetidine and ranitidine. This effect appears to be H₂ receptor-mediated as no change in receptor density was observed after H₁ or H₃ antagonist treatment or after incubation with the structural analogue of cimetidine, VUF 8299, which has no H₂ antagonistic effects. By using transfected CHO cells expressing different densities of wild-type H₂ receptors or an uncoupled H₂Leu⁴⁸Ala receptor, the histamine H₂ receptor was found to display considerable agonist-independent H₂ receptor activity. Cimetidine and ranitidine, which both induce H₂ receptor upregulation, actually functioned as inverse agonists in those cell lines displaying spontaneous agonist-independent H₂ receptor activity. Burimamide, on the other hand, was shown to act as a neutral antagonist and did as expected not induce H₂ receptor upregulation after long-term exposure. The displayed inverse agonism of H₂ antagonists appears to be a mechanistic basis for the observed H₂ antagonist-induced H₂ receptor upregulation in transfected CHO cells. These observations shed new light on the pharmacological classification of the H₂ antagonists and may offer a plausible explanation for the observed development of tolerance after prolonged clinical use.

Following the discovery of burimamide as a selective histamine H₂ receptor antagonist (1) various related drugs (cimetidine, ranitidine, famotidine, nizatidine) have proven to be of great importance in the regulation of gastric acid secretion (2). The actual target of these drugs have recently been questioned (3, 4), but is generally considered to be the H₂ receptor on the gastric parietal cell (4). As such, the H₂ antagonists constitute currently one of the prominent therapies for duodenal and gastric ulcers (5). These drugs have been widely prescribed and are currently also clinically evaluated as immunosuppressants (6) and for the treatment of central nervous system disorders (7–10).

The histamine H₂ receptor is a member of the large multigene family of G-protein coupled receptors (GPCR) (11). Functionality and expression of members of the GPCR family are generally dynamically regulated after agonist or antagonist exposure (12, 13). Recently, histamine H₂ receptors were shown to be rapidly desensitized (14–16), internalized (17), and downregulated upon histamine or H₂ agonist exposure (18). Yet, despite their therapeutic importance, so far no detailed insights on the modulation of H₂ receptor function by H₂ antagonists have been published. In vivo modulation of H₂ receptor function after treatment of H₂ antagonists has been reported (2, 19–22). Prolonged H₂ receptor blockade was found to result in increased parietal cell sensitivity to H₂ agonists (22), increased intragastric hyperacidity after abrupt withdrawal (21), and the development of tolerance (2, 5, 19, 23, 24). Coruzzi and Bertaccini (22) and Nwokolo et al. (21) hypothesized that these observations could be explained by an upregulation of H₂ receptors. So far no data on the regulation of H₂ receptor expression by H₂ receptor antagonists have been presented, however.

Studies examining the molecular mechanism underlying H₂ receptor function have been greatly facilitated by the cloning of the genes encoding the histamine H₂ receptor (25–28). Model systems, expressing a homogeneous population of (mutant) H₂ receptors, are currently available for studies regarding H₂ receptor function and regulation (18, 29, 30). In the present study we describe upregulation of H₂ receptors after prolonged treatment of transfected Chinese hamster ovary (CHO) cells with some H₂ antagonists. In our study, we observed that the histamine H₂ receptor shows a spontaneous, histamine-independent activity. For some GPCRs, increased basal agonist-independent receptor activity can be inhibited by certain antagonists referred to as inverse agonists (for review, see refs. 31–34). Although this new pharmacological phenomenon is still a matter of debate (33–35), Milligan et al. (34) recently extended the concept of inverse agonism to GPCR regulation. While GPCR expression is reduced upon long-term agonist exposure, GPCR upregulation was hypothesized for inverse agonists (34). We found that the H₂ antagonists cimetidine and ranitidine act as inverse agonists at the H₂ receptor. The displayed inverse agonism of these compounds indeed appears to be the mechanistic basis for the observed H₂ antagonist-induced H₂ receptor upregulation in transfected CHO cells (34). These observations shed new light on the pharmacological classification of the H₂ antagonists and may offer a plausible explanation for the observed tolerance development after prolonged clinical use.

MATERIALS AND METHODS

Materials. Histamine dihydrochloride, isotubutymethylxanthine (IBMX), bovine serum albumine, gelatine, polylethyleneimine [50% (wt/wt) solution], cAMP, triprolidine hydrochloride, and forskolin were obtained from Sigma. [2,8-³H]cAMP (40 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham. VUF 8299 [N-[2-(benzylthio)ethyl]-N'-cyano-N''-methylguanidine] and thioperamide were taken from laboratory stock. Gifts of cimetidine and burimamide (SmithKline Beecham), ranitidine dihydrochloride (Glaxo), tiotidine (Imperial Chemical Industries), and the pSVratH₂ expression

Abbreviations: GPCR, G-protein coupled receptor, IBMX, isotubutymethylxanthine; 1²⁵I-AP, [1²⁵I]iodoaminopotentidine; WT, wild type; VUF 8299, N-[2-(benzylthio)ethyl]-N'-cyano-N''-methylguanidine.

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**Cell Culture and Transfection.** CHO cells, deficient in dihydrofolate reductase, were transfected with 15 μg pSVrH2 (29) by using Transfectam (Promega). Following ring cloning, cell lines expressing 96 ± 26, 286 ± 52, or 975 ± 12 fmol wild-type (WT) H2 receptors/mg protein, referred to as CHOrH2WT6, CHOrH2WT9, and CHOrH2WT, respectively, were selected and grown at 37°C in a humidified atmosphere with 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% (vol/vol) dialyzed fetal calf serum supplemented with 2 mM l-glutamine, minimal essential medium amino acids, 50 units of penicillin per ml, and 50 μg of streptomycin per ml. The same medium was used for CHO cells expressing 980 ± 7 fmol/mg protein of the rat histamine H2Receptor H2Leu124Ala receptor mutant (CHOrH2Leu124Ala cells) (18) and 459 ± 44 fmol/mg protein of the guinea-pig histamine H2 receptor (36).

**H2 Receptor Binding Studies.** The radiolabeled H2 receptor antagonist [125I]iodoaminopotentidine ([125I]-APT) (37) was synthesized as described (30). Triplicate assays were performed in polyethylene tubes in 50 mM Na2/potassium phosphate buffer containing gelatine (0.1%) to prevent adsorption of the radioligand. [125I]-APT was incubated with 5-10 μg of membrane proteins in the absence or presence of 1 μM tiotidine to define the nonspecific binding in a total volume of 400 μl. After 90 min at 30°C the incubations were stopped by rapid dilution with 3 ml ice-cold 20 mM Na2/potassium phosphate buffer (pH 7.4) supplemented with 0.1% bovine serum albumin. The bound radioactivity was separated by filtration with a Brandel cell harvester (Semat) through Whatman GF/B glass fiber filters that had been treated with 0.3% polyethyleneimine. Filters were washed twice with 3 ml buffer, and the radioactivity retained on the filters was counted with a LKB gamma counter at an efficiency of 63%. Changes in H2 receptor density were denoted as a percentage up- or downregulation compared to nontreated control cells. During the 24 hr of incubation of cells with various histamine H2 receptor ligands, cells were maintained in medium without fetal calf serum.

**cAMP Measurements.** CHOrH2WT, CHOrH2WT6, CHOrH2WT9, and CHOrH2Leu cells were grown to confluence overnight in 12-well plates, washed twice with DMEM supplemented with 50 mM Hepes (pH 7.4), preincubated for 30 min at 37°C in the same medium, and incubated with the appropriate drugs for 10 min at 37°C in the presence of 300 μM of the phosphodiesterase inhibitor IBMX. The determination of cAMP levels was performed in triplicate as described by Leurs et al. (30). Protein levels were determined according to Bradford (38) by using bovine serum albumin as standard.

**Statistical Analysis.** All data shown are expressed as mean ± SEM of at least three independent experiments. Statistical analysis was carried out by Student’s t test. P values <0.05 were considered to indicate a significant difference.

**RESULTS**

**Effect of Long-Term Incubation of the H2 Antagonist Cimetidine on [125I]-APT Binding in CHOrH2WT Cells.** As shown in previous studies (18), exposure of CHOrH2WT cells to 100 μM of histamine for prolonged periods of time resulted in a time-dependent decrease of [125I]-APT binding sites (Fig. 1). Remarkably, exposure of CHOrH2WT cells to 100 μM of the H2 antagonist cimetidine for prolonged periods of time resulted in a time- (Fig. 1) and concentration-dependent (EC50 value, 6.0 ± 1.1 μM, mean ± SEM; n = 4) increase of [125I]-APT binding. This antagonist-induced increase (100 μM cimetidine for 24 hr) could be ascribed to an increase in H2 receptor binding sites (control, Bmax = 975 ± 12 fmol/mg protein;
Table 1. Basal cAMP levels and forskolin-induced formation of cAMP in untransfected CHO cells and CHO cell lines expressing different levels of H₂ receptors and effect of cimetidine on ¹²⁵I-APT binding after long-term treatment of the different cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>H₂ receptor density, fmol/mg protein</th>
<th>cAMP, pmol/mg protein</th>
<th>% upregulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>0</td>
<td>37 ± 4</td>
<td>2148 ± 169</td>
</tr>
<tr>
<td>CHOrH₂WT6</td>
<td>96 ± 26</td>
<td>42 ± 7</td>
<td>2054 ± 393</td>
</tr>
<tr>
<td>CHOrH₂WT9</td>
<td>286 ± 52</td>
<td>62 ± 5*</td>
<td>2472 ± 130</td>
</tr>
<tr>
<td>CHOrH₂WT</td>
<td>975 ± 12</td>
<td>198 ± 17*</td>
<td>2945 ± 132*</td>
</tr>
<tr>
<td>CHOrH₂Leu¹²⁴Ala</td>
<td>980 ± 7</td>
<td>44 ± 3</td>
<td>2123 ± 71</td>
</tr>
</tbody>
</table>

The basal cAMP levels and forskolin (10 μM)-induced formation of cAMP in different CHO cell lines was determined for 10 min at 37°C in DMEM in the presence of 300 μM IBMX and 25 mM Hepes (pH 7.4). Data represent the mean ± SEM of at least three independent experiments. The asterisks indicate a significant difference (P < 0.05) from untransfected CHO cells. For the % upregulation the different CHO cell lines were incubated for 24 hrs with 100 μM cimetidine and ¹²⁵I-APT binding in membranes was measured. The ¹²⁵I-APT binding is expressed as a percentage upregulation compared to nontreated cells. Data were calculated as means ± SEM from at least three independent experiments. The asterisks indicate a significant difference (P < 0.05) from nontreated cells.

(Fig. 2A). Basal levels of cAMP (62 ± 5 pmol cAMP/mg protein, mean ± SEM; n = 5) in CHOrH₂WT9 cells were also found to be significantly reduced by 100 μM cimetidine (48 ± 5 pmol/mg protein, 23% reduction, mean ± SEM; n = 3), while no effect was observed when CHOrH₂WT6 cells were exposed to 100 μM cimetidine (control, 42 ± 7 pmol cAMP/mg protein; cimetidine-treated cells, 46 ± 9 pmol cAMP/mg protein, mean ± SEM; n = 4). In CHOrH₂Leu¹²⁴Ala cells in which basal cAMP levels were comparable to untransfected CHO cells (Table 1), cimetidine did not exhibit any negative intrinsic activity either (Fig. 2B). Moreover, no effect on basal cAMP levels was observed when untransfected CHO cells were exposed to 100 μM cimetidine (control, 37 ± 4 pmol cAMP/mg protein; cimetidine-treated cells, 39 ± 2 pmol cAMP/mg protein, mean ± SEM; n = 5).

Besides cimetidine, another H₂ receptor ranitidine was shown to display negative intrinsic activity as well (Fig. 3A). As can be seen in Fig. 3A, a dose-dependent decrease of basal cAMP levels was observed when CHOrH₂WT cells were incubated with increasing concentrations of ranitidine. Ranitidine displayed a higher negative intrinsic activity than cimetidine (Fig. 3A). As found for cimetidine, the IC₅₀ value of ranitidine for the inhibition of basal levels of cAMP correlated with its H₂ receptor affinity (Table 2). Remarkably, burimamide, the first identified H₂ antagonist showing reasonable affinity for the H₂ receptor (Kᵢ value, 5.5 μM), did not affect the basal levels of cAMP in CHOrH₂WT cells (Fig. 3A). Although burimamide did not display negative intrinsic activity, 100 μM burimamide was able to inhibit the histamine (30 nM)-induced cAMP production in CHOrH₂WT cells significantly (Fig. 3B) and may thus be classified as a neutral antagonist. According to the characteristics of a neutral antagonist, 100 μM burimamide inhibited the agonist response as well as the cimetidine (1 μM)-induced reduction of basal levels of cAMP significantly (Fig. 3B).

Inverse Agonism of H₂ Antagonists and H₂ Receptor Upregulation. Long-term (24-hr) incubation of CHOrH₂WT9 cells, displaying increased basal activity, with 100 μM of the inverse agonist cimetidine also led to a significant increase in ¹²⁵I-APT binding sites (83 ± 9% upregulation, mean ± SEM; n = 3) (Table 1). Yet, in cells that did not show increased basal receptor activity, CHOrH₂WT6 and CHOrH₂Leu¹²⁴Ala cells, no significant increase in ¹²⁵I-APT binding sites was observed after long-term incubation with 100 μM cimetidine (Table 1). Moreover, long-term incubation (24 hr) of CHOrH₂WT cells with 10 μM of the inverse agonist ranitidine was shown to induce a significant increase in ¹²⁵I-APT binding sites, whereas 1 mM of the neutral antagonist burimamide did not induce a significant change in H₂ receptor density (Table 2).

![Fig. 2](image)

(A) Effect of cimetidine and its phenyl analogue VUF 8299 on basal levels of cAMP in CHOrH₂WT cells. CHOrH₂WT cells were exposed to increasing concentrations of cimetidine (●) or VUF 8299 (○) for 10 min at 37°C in DMEM in the presence of 300 μM IBMX and 25 mM Hepes (pH 7.4). (B) Effect of cimetidine on basal levels of cAMP in CHOrH₂WT cells and CHOrH₂Leu¹²⁴Ala cells. CHOrH₂WT (●) and CHOrH₂Leu¹²⁴Ala cells (○) were exposed to increasing concentrations of cimetidine for 10 min at 37°C in DMEM in the presence of 300 μM IBMX and 25 mM Hepes (pH 7.4). Experiments were performed in parallel, and data represent the mean ± SEM of four independent experiments. The basal level of cAMP in CHOrH₂WT cells for cimetidine and VUF 8299 were 207 ± 33 pmol cAMP/mg protein and 193 ± 31 pmol cAMP/mg protein, respectively. The basal level of cAMP in CHOrH₂Leu¹²⁴Ala cells was 36 ± 2 pmol cAMP/mg protein.
FIG. 3. Effect of H2 antagonists on basal levels of cAMP in CHOReH2WT cells. (A) CHORh2WT cells were exposed to increasing concentrations of ranitidine (○), cimetidine (□), or burimamide (●) for 10 min at 37°C in DMEM in the presence of 300 μM IBMX and 25 mM Hepes (pH 7.4). Data represent the mean ± SEM of at least three independent experiments. The basal level of cAMP in CHOReH2WT cells for the experiments with ranitidine and burimamide were 171 ± 11 pmol cAMP/mg protein and 196 ± 15 pmol cAMP/mg protein, respectively. The curve of the dose-dependent inhibition of basal cAMP levels by cimetidine was inserted for comparison (basal cAMP levels, 207 ± 33 pmol cAMP/mg protein). (B) CHORh2WT cells were exposed to either 100 μM burimamide (Bur), 30 nM histamine (HA), or 1 μM cimetidine (Cim) or a combination of histamine and burimamide (HA + Bur) or cimetidine and burimamide (Cim + Bur) for 10 min at 37°C in DMEM in the presence of 300 μM IBMX and 25 mM Hepes (pH 7.4). Data represent the mean ± SEM of three independent experiments. NS, not statistically significant.

DISCUSSION

In CHOReH2WT cells histamine H2 receptors are upregulated by long-term exposure to the H2 antagonist cimetidine as shown by a significant elevation of 125I-APT binding sites. The cimetidine-induced H2 receptor upregulation appears to be H2 receptor-mediated as VUF 8299, a cimetidine-analogue devoid of H2 receptor activity (39) or H1 or H3 antagonists do not affect H2 receptor expression. According to classical models for drug–GPCR interaction, GPCR antagonists are believed to prevent the binding of agonists to the receptor (40). Consequently, their physiological effect, including receptor upregulation, is ascribed to their ability to prevent activation/downregulation of receptors by endogenous hormones or neurotransmitters. However, there is now substantial evidence that contradicts this concept. For some GPCRs, certain antagonists were shown to induce effects opposite to those observed by agonists, thereby displaying negative intrinsic activity, also referred to as inverse agonism (see refs. 31–34). Evidence of inverse agonism by GPCR antagonists came from experiments with constitutively active GPCR mutants (41–44), certain overexpressed WT GPCRs (45–51), and transgenic mice with myocardial overexpression of the β2-adrenergic receptor (52). In the various models, the respective GPCRs exhibit a spontaneous, agonist-independent receptor activity. This basal receptor activity was shown to be inhibited by some antagonists (inverse agonists), but not by all antagonists (neutral antagonists). Currently, an intensive debate on the new pharmacological phenomenon of inverse agonism or negative antagonism of GPCR antagonists is going on (33–35). Major criticism is often directed against the fact that spontaneous, agonist-independent GPCR activity was observed in transfected cell lines with receptor densities exceeding expression levels of 1 pmol/mg protein (45–51) or with constitutively active GPCR mutants (41–44). Yet, inverse agonism has recently also been observed in myometrial cells (53), erythrocytes (54), and cardiomyocytes (55, 56), suggesting that inverse agonism might be of physiological relevance.

In the present study we have shown that the histamine H2 receptor displays agonist-independent basal activity, which increases upon elevation of receptor density. Already, at a H2 receptor density of 286 fmol/mg protein (CHORh2WT9 cells), a significant increase of basal and forskolin-induced cAMP formation is observed. Evidence of spontaneous, agonist-independent activity of H2 receptors was supported by experiments using the H2Leu124Ala receptor mutant. Previously, we observed that this mutation resulted in a loss of agonist high-affinity binding sites and concomittant loss of histamine potency to induce the production of cAMP (18). CHO cells expressing this uncoupled H2 receptor mutant (CHORh2Leu124Ala cells) did not display increased basal or forskolin-induced formation of cAMP, even though expression levels were comparable to those in CHOReH2WT cells.

The agonist-independent H2 receptor activity in CHORh2WT and CHORh2WT9 cells was accompanied by the occurrence of inverse agonism exerted by cimetidine. Its structural analogue VUF 8299, which does not show H2 antagonism (39), did not display negative intrinsic activity in CHOReh2WT cells. Moreover, in CHOReH2Leu124Ala and CHORh2WT6 cells, two cell lines that both do not display elevated levels of basal or forskolin-induced cAMP production, cimetidine did not affect the cAMP levels. In CHOReh2WT cells ranitidine also exhibited negative intrinsic properties, whereas burimamide acted as a neutral antagonist. The different levels of intrinsic activity exerted by these compounds excludes the possibility that they act as competitive antagonists inhibiting H2 receptor activity induced by contaminating histamine.

As reported earlier (18), H2 receptor density is readily downregulated upon receptor stimulation. In view of the observed spontaneous agonist-independent receptor activity, we assumed a basal turnover of H2 receptors. We therefore
hypothesized that the observed inverse agonism displayed by
cimetidine could be a mechanistic basis for the observed H2
receptor upregulation by H2 antagonists. This hypothesis was
supported by the absence of cimetidine-induced H2 receptor
upregulation in CHO_H2WT6 and CHO_H2Leu124Ala cells.
Moreover, in CHO_H2WT cells burimamide, which was
shown to act as a neutral antagonist, did not induce H2
receptor upregulation. The lack of upregulation by burimi-
amide furthermore excludes the possibility that the H2
receptor upregulation is caused by a competitive antagonism
of histamine-induced H2 receptor downregulation. Thus, as
previously hypothesized by Milligan et al. (34) our findings
show that inverse agonists cause GPCR upregulation, when
spontaneous, agonist-independent receptor activity is dis-
played.

As already stated, although very effective in humans for
treating peptic ulcers, long-term H2 antagonist treatment is
complicated in some patients by the development of toler-
ance and recurrence (2, 5, 19, 23, 24, 57). It is tempting to
speculate that the inverse agonism displayed by the tested H2
antagonists, consequently resulting in an upregulation of H2
receptors, contributes to the development of tolerance after
chronic treatment (2, 5, 19, 23, 24). In view of this hypothesis,
the use of a neutral H2 antagonist might offer advantages,
as inverse agonists are more likely to cause H2 receptor up-
regulation and thus development of tolerance. Unfortu-
nately, the neutral antagonist burimamide is clinically not
applicable (58) and is therefore at present not available for
investigation in humans.

Taken together, our study shows that already at moderate
receptor densities histamine H2 receptors are spontaneously
active and the H2 antagonists cimetidine and ranitidine, pre-
viously thought to act as competitive antagonists, actually
function as inverse agonists. The inverse agonism displayed
appears to be a mechanistic basis for the observed H2 antag-
onist-induced H2 receptor upregulation in CHO cells. It is
clear that these findings should alter the view regarding the
action of H2 antagonists and may have implications for effec-
tive drug therapy. Moreover, our study shows that an inverse
agonist and a neutral antagonist differentially regulate H2
receptor expression. These observations may be of general
importance for the understanding of GPCR regulation.

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