Modulation of forskolin-mediated adenylyl cyclase activation by constitutively active G_s-coupled receptors

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Abstract In transfected CHO cells constitutively active histamine H_2 receptors not only increase the basal cAMP level, but also enhance forskolin-induced cAMP production. The increased forskolin response was inhibited by inverse H_2 agonists with potencies similar to those determined at basal levels. The modulation of the forskolin response was also observed after H_2 receptor expression in HEK-293 and Sf9 cells or TSH receptor expression in COS-7 cells. The enhancement of forskolin-induced cAMP production seems to be a general characteristic of constitutively active G_s-coupled receptors and can be very useful to study inverse agonism at wild-type receptors.

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Key words: G-protein-coupled receptor; Constitutive activity; Forskolin; Histamine H_2 receptor; Inverse agonism; TSH receptor

1. Introduction

Classical models of G-protein-coupled receptors (GPCRs) require agonist occupation of the receptor to activate signal transduction pathways. Nowadays, it is well documented that GPCRs can be spontaneously active and this agonist-independent receptor activity is often referred to as constitutive receptor activity [1–5]. Inverse agonists reduce the constitutive GPCR activity, whereas neutral antagonists do not affect the basal GPCR activity. Constitutive activity was first shown for mutant GPCRs and has proved to be the mechanistic basis for several genetic diseases [6]. Moreover, a detailed understanding of constitutive GPCR activity will give better insights of agonist-induced GPCR activation [5]. Because of these important implications the phenomenon of constitutive GPCR activity and inverse agonism currently receives considerable attention [1–5].

To study inverse agonism GPCRs should possess a detectable level of constitutive activity. Constitutive activity of wild-type receptors is generally low and inverse agonism is therefore often studied using constitutively active mutant (CAM) GPCRs. However, as a result of introduced mutations, the pharmacological behaviour of ligands can be seriously affected [7,8]. For example, as a result of a single point mutation in TM4 of the δ-opioid and μ-opioid receptors antagonists became surprisingly full agonists [8]. Moreover, the β-blocker propranolol was reported to change from an inverse agonist at the wild-type β_2-adrenergic receptor [9] to a neutral antagonist at a constitutively active β_2-adrenergic receptor mutant [10]. In view of these findings the use of wild-type receptors in the study of inverse agonism is preferred.

Recently, the use of highly sensitive reporter gene technology has been introduced to observe constitutive activity of wild-type G_q-coupled receptors [11]. Similarly, modifications of the extracellular sodium concentration have been shown to increase the constitutive activity of bradykinin B_2 [12], TSH [13] and LH receptor [13]. Moreover, overexpression of $G\alpha_q$ in NIH-3T3 cells results in a detectable constitutive activity of the coexpressed, wild-type muscarinic m_3 receptor [14]. However, this latter approach is not always feasible. Overexpression of $G\alpha_s$ in NG108-15 cells did not affect the constitutive activity of the β_2-adrenergic receptor [15], probably because $G\alpha_s$ is not the limiting factor in the signal transduction.

Activation of G_s-coupled receptors and reconstitution of adenylyl cyclase isoenzymes with purified $G\alpha_s$ subunits are known to have a synergistic effect on the forskolin-induced adenylyl cyclase activation [16,17]. Consequently, constitutively active G_s-coupled receptors can be anticipated to potentiate forskolin responses by increasing the levels of free $G\alpha_s$ subunits. We previously noticed that stable expression of the rat H_2 receptor in CHO cells not only enhanced the basal cAMP level but also potentiated the forskolin-induced cAMP production [18]. In this study, we provide evidence that the enhancement of the forskolin response is indeed the consequence of the constitutive activity of the wild-type H_2 receptor. We show that modulation of the forskolin response offers a useful method to investigate constitutive GPCR activity in several cell lines and inverse agonism at wild-type G_s-coupled receptors under normal experimental conditions.

2. Materials and methods

2.1. Chemicals

Histamine dihydrochloride, isobutylmethylxanthine (IBMX), aminopotentidine, cyclic AMP (cAMP), DEAE-dextran, chicken egg albumin and forskolin were obtained from Sigma Chemical Company (USA). [2,8-^{3}H]cAMP (40 Ci/mmol) was obtained from Amersham. VUF 8299 was taken from laboratory stock [19]. Gifts of cimetidine (SmithKline Beecham, United Kingdom), ranitidine dihydrochloride (Glaxo, United Kingdom) and famotidine (Merck Sharp and Dohme, The Netherlands) are greatly acknowledged.

2.2. Cell culture and transfection

Chinese Hamster ovary (CHO) cells deficient in dihydrofolate reductase ($CHOdhfr^-$), H_2 receptor expressing CHO ($CHOH_2$) and human embryonic kidney (HEK-293H_2) cells, African green monkey kidney (COS-7) cells and Sf9 insect cells were grown as described previously [20–22].

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COS-7 cells were transiently transfected with the expression vectors pSVL, pSVL-TSHI[486]F, pSVL-TSHF[631]L and pSVL-TSH (5 μg DNA/10^6 cells) using DEAE-dextran [23]. Sf9 cells were infected with a recombinant baculovirus containing the epitope-tagged H_2 receptor as described by Beukers et al. [22].

2.3. Biochemical measurements

The determination of H_2 receptor binding with the H_2 antagonist [^{125}I]iodoaminopotentidine ([^{125}I]APT) was performed as described by Leurs et al. [24]. The cAMP production in CHO, HEK-293, COS-7 (48 h after transfection) and Sf9 cells was determined as described previously [18]. Protein concentrations were determined according to Bradford using bovine serum albumin as a standard [25].

2.4. Statistical analysis

All data shown are expressed as mean ± standard error (mean ± S.E.M.) of at least three independent experiments. Statistical analysis was carried out by Student's *t*-test. *P*-values < 0.05 were considered to indicate a significant difference.

3. Results

In CHOdhfr$^-$ cells, stably expressing the H_2 receptor [24] at a density of 1079 ± 313 fmol/mg protein (mean ± S.E.M., $n = 6$) ($CHOH_2$ cells), the basal cAMP level was about 2-fold higher (170 ± 37 pmol cAMP/mg protein, mean ± S.E.M., $n = 6$) than in CHOdhfr$^-$ cells (78 ± 13 pmol cAMP/mg protein, mean ± S.E.M., $n = 6$, $P < 0.05$). Forskolin dose-dependently increased the cAMP formation in both CHOdhfr$^-$ and $CHOH_2$ cells (Fig. 1). At each tested concentration the forskolin-induced cAMP production in $CHOH_2$ cells was significantly higher than in CHOdhfr$^-$ cells. The absolute difference in cAMP production was maximal (1600 pmol cAMP/mg protein) at a concentration of 1 μM of forskolin. A potentiation of the forskolin response was also seen at higher concentrations of forskolin, but due to the increased response in the CHOdhfr$^-$ cells the relative increase was lower at these concentrations (Fig. 1).

The inverse agonist cimetidine dose-dependently reduced the elevated basal cAMP level of $CHOH_2$ cells to 52 ± 3% (mean ± S.E.M., $n = 12$) (Table 1). Cimetidine also inhibited the forskolin-induced cAMP production in $CHOH_2$ cells, whereas in CHOdhfr$^-$ cells cimetidine did not exhibit any negative intrinsic activity (Fig. 2A). VUF8299, a cimetidine analogue with strongly reduced H_2 receptor affinity [19], showed up to 10 μM no negative intrinsic activity in $CHOH_2$ cells (Fig. 2B). The pIC_{50} values for cimetidine, as determined by inhibition of either the basal cAMP level or forskolin-induced (1 or 10 μM) cAMP formation, were not significantly different (Table 1). Moreover, these values correspond with the observed pK_i value of 6.2 ± 0.2 (mean ± S.E.M., $n = 3$), as determined from [^{125}I]APT displacement studies. The percentage maximal inhibition by the inverse agonist decreased at higher concentrations of forskolin (Table 1), as expected from the data in Fig. 1. Besides cimetidine, ranitidine and famotidine were also tested for their effect on the forskolin response in $CHOH_2$ cells. The pIC_{50} values for these inverse agonists, determined by inhibition of the forskolin response, also correspond well with the values determined by inhibition of the basal cAMP level (Table 2). The negative intrinsic activity (α_{inv}) of cimetidine determined by inhibition of the forskolin response in $CHOH_2$ cells was significantly lower than that of famotidine and ranitidine (Table 2). At the basal cAMP level no significant differences in intrinsic activity of the inverse agonists were found (Table 2).

The modulation of the forskolin response by constitutively active H_2 receptors was not restricted to CHO cells. Stable expression of the H_2 receptor in HEK-293 cells at a density of 1594 ± 17 fmol/mg protein [21] resulted in an approximately 10-fold increase in the cAMP production induced by 10 μM forskolin (HEK-293 cells: 805 ± 245 pmol/mg protein (mean ± S.E.M., $n = 6$), HEK-293H$_2$ cells: 7471 ± 998 pmol/

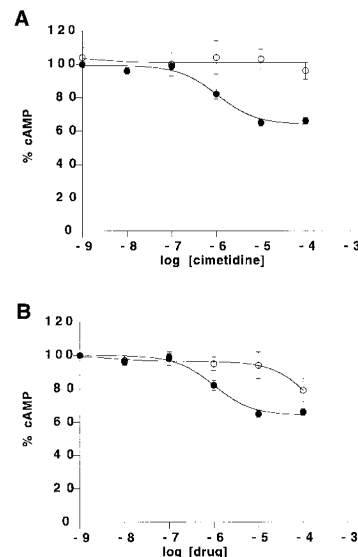

Fig. 2. A: Effect of cimetidine on forskolin-induced (10 μM) cAMP production in CHOdhfr$^-$ (open circles) and $CHOH_2$ cells (filled circles). The data shown represent the mean ± S.E.M. of respectively four or eight experiments. B: Effect of cimetidine (filled circles) and the inactive analogue, VUF 8299 (open circles), on forskolin-induced (10 μM) cAMP production in $CHOH_2$ cells. The data shown represent the mean ± S.E.M. of respectively eight or six experiments.

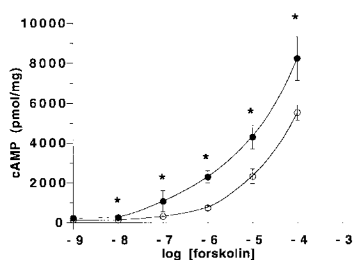

Fig. 1. Forskolin-induced increase in cAMP production in untransfected (open circles) and $CHOH_2$ cells (filled circles). The data shown represent the mean ± S.E.M. of six experiments. The asterisk indicates a significant difference ($P < 0.05$) compared to CHOdhfr$^-$ cells.

Table 1
The pIC_{50} and the maximal inhibitory response of cimetidine in $CHOH_2$ cells determined on the basal cAMP level and forskolin-induced cAMP formation

Conditions	pIC_{50}	% inhibition	*n*
Basal	6.3 ± 0.2	48 ± 3	12
1 μM forskolin	6.3 ± 0.1	41 ± 2*	4
10 μM forskolin	6.0 ± 0.2	36 ± 2*	8

The pIC_{50} and the maximal inhibition were determined from the inhibition curve of cimetidine.
* indicates a significant difference ($P < 0.05$) from the % inhibition of the basal cAMP level.
n, number of experiments.

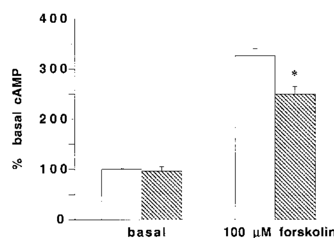

Fig. 3. Effect of cimetidine (hatched bars) on basal and forskolin-induced cAMP production in Sf9 cells expressing the H_2 receptor. Sf9 cells infected with recombinant baculovirus containing an epitope-tagged H_2 receptor were exposed to 100 μM cimetidine, in the presence or absence of 100 μM forskolin. Basal cAMP level in Sf9 cells expressing the H_2 receptor: 10.5±3.9 pmol/10^6 cells. The data shown represent the mean±S.E.M. of four experiments. The single asterisk indicates a significant difference ($P<0.05$).

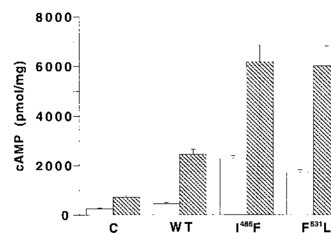

Fig. 4. Basal (white bars) and forskolin-induced (10 μM) cAMP production (hatched bars) in COS-7 cells transiently transfected with pSVL (C), pSVL-TSH (WT), pSVL-TSHI^{486}F (I^{486}F) and pSVL-TSHF^{631}L (F^{631}L). The data shown represent the data of a typical experiment, from three independent experiments, each performed in triplicate.

mg protein (mean±S.E.M., $n=6$)). The increase in the basal cAMP level was approximately 15-fold (HEK-293 cells: 33±5 pmol/mg protein (mean±S.E.M., $n=6$), HEK-293H_2 cells: 572±83 pmol/mg protein (mean±S.E.M., $n=6$)). The increase in the forskolin response by the constitutive activity of the H_2 receptor in HEK-293H_2 cells was inhibited by cimetidine ($pIC_{50}=6.0\pm0.1$, mean±S.E.M., $n=3$). Furthermore, in previous studies we showed that the wild-type H_2 receptor can also be functionally expressed in insect Sf9 cells [22]. Infection of Sf9 cells with recombinant baculovirus resulted in high expression levels of H_2 receptor proteins (2.7 pmol/mg protein). Expression of the H_2 receptor did not significantly elevate the basal cAMP level in Sf9 cells [22]. Yet, the response to 100 μM forskolin was enhanced after H_2 receptor expression (53±14 pmol cAMP/10^6 cells, mean±S.E.M., $n=3$) compared to mock-infected Sf9 cells (15±4 pmol cAMP/10^6 cells, mean±S.E.M., $n=3$). Cimetidine showed no significant effect on the basal cAMP production in these cells (Fig. 3). However, a small, but significant, inhibition of the forskolin-induced (100 μM) cAMP production by cimetidine was observed (Fig. 3).

Finally, we transiently expressed the thyrotropin (TSH) receptor and the constitutively activated Ile^{486}Phe and Phe^{631}Leu TSH receptor mutants [26] in COS-7 cells. Expression of the wild-type receptor resulted in a small, but significant elevation of the basal cAMP level compared to mock-(pSVL) transfected COS-7 cells (Fig. 4). As reported previously [26], the two clinically important CAM TSH receptors both caused a marked elevation of the basal cAMP level. In the presence of forskolin the constitutive activity of the wild-type TSH receptor became quite prominent and easy to detect in comparison to the minor increase in the basal cAMP level (Fig. 4). The constitutive activity of the two CAM receptors was further magnified (Fig. 4).

4. Discussion

In this study we show that the modulation of forskolin response by constitutively active G_s-coupled receptors offers a simple way to investigate inverse agonism at wild-type G_s-coupled receptors. Stable expression of the H_2 receptor in CHO cells both enhanced basal and forskolin-induced cAMP production, probably by increasing the level of free $G\alpha_s$ subunits. The increase in forskolin-induced cAMP production was maximal at a concentration of 1 μM. At higher forskolin concentrations the relative increase in forskolin-induced cAMP production decreased due to the increased response in CHOdhfr$^-$ cells. If the increase in forskolin-induced cAMP production is a direct effect of the constitutive activity of the H_2 receptor, inverse agonists should, as shown at the basal cAMP level [18], be able to inhibit the H_2 receptor-mediated increase of the forskolin-induced cAMP production. Indeed, a dose-dependent inhibition of the forskolin-induced cAMP production by cimetidine was observed. As expected, the maximal inhibition by cimetidine in CHOH_2 cells was slightly higher at a forskolin concentration of 1 μM than at a concentration of 10 μM. The pIC_{50} values of cimetidine determined from the inhibition of the forskolin responses were not significantly different from the values determined from the inhibition of the basal cAMP level (Table 1). In addition, investigation of the inverse agonistic properties of famotidine and ranitidine in CHOH_2 cells revealed no significant differences in inverse agonistic behaviour determined in either the absence or presence of forskolin (Table 2). Modulation of the forskolin response by constitutively active H_2 receptors thus represents a useful tool to study inverse agonism at the H_2 receptor. The method seems quite sensitive as in CHOH_2 cells cimetidine could be classified as a partial inverse agonist at forskolin-stimulated cAMP levels. At the

Table 2
pIC_{50} and α_{inv} of different inverse agonists in CHOH_2 cells determined on the basal cAMP level and forskolin-induced cAMP formation

Drug	Basal		Forskolin (1 μM)	
	pIC_{50}	α_{inv}	pIC_{50}	α_{inv}
Cimetidine	6.3±0.2	0.88±0.04	6.3±0.1	0.83±0.02*
Famotidine	6.7±0.2	0.88±0.05	6.7±0.3	0.93±0.04
Ranitidine	6.8±0.2	1.00±0.03	6.7±0.3	1.00±0.02

pIC_{50} and the α_{inv} were determined from the inhibition curve of the indicated drug on either the basal cAMP level or the forskolin-induced cAMP production. The data shown represent the mean±S.E.M. of three independent experiments performed in duplicate.
* indicates a significant difference ($P<0.05$) from α_{inv} of ranitidine which caused a maximal inhibition in this cell line.

basal cAMP level no differences in negative intrinsic activity (α_{inv}) between cimetidine, famotidine and ranitidine could be detected. This is probably due to the fact that the forskolin-induced cAMP levels are about 20-fold higher than basal cAMP levels and small differences can consequently be detected easier.

The increase of the forskolin response by H_2 receptor expression is not limited to a particular cell type. Compared to $CHOH_2$ cells the constitutive activity of the H_2 receptor was more pronounced in HEK-293 cells. Stable expression of the H_2 receptor in this cell line resulted in a strongly enhanced basal and forskolin-induced cAMP production, that was inhibited by the inverse agonist cimetidine with comparable potency as observed in $CHOH_2$ cells. In insect Sf9 cells the H_2 receptor is not very efficiently coupled to adenylyl cyclase activation [22]. Consequently, in these cells no inverse agonism by cimetidine could be detected at the basal cAMP level. Nevertheless, this inverse agonist was able to decrease the forskolin-induced cAMP production in Sf9 cells expressing the H_2 receptor. These data further support the high sensitivity of the described method to measure inverse agonism.

Importantly, transient expression of the G_s-coupled TSH receptor and constitutively active TSH mutant receptors [26] in COS-7 cells also modulated the forskolin response. A clear constitutive activity of the wild-type TSH receptor was observed after evaluation of the forskolin responses. Our method to study inverse agonism at the H_2 receptor may thus be of general use in the study of inverse agonism at G_s-coupled receptors. Further evidence supporting this suggestion is available in literature [27–29]. Takuma et al. [29] showed e.g. that propranolol reduces the forskolin response in rat parotid acinar cells endogenously expressing the β_1 receptor. This inhibition was not attributed to inverse agonism as this phenomenon was relatively new at that moment. From experimental data obtained with heterologous expression systems and/or CAM receptors, the modulation of the forskolin response by constitutively active G_s-coupled receptors can often be deduced [30,31]. In line with our findings, constitutively active G_i-coupled receptors also affect forskolin response [32,33]. As expected, the forskolin response is modulated in a way opposite to that of G_s-coupled receptors and consequently the elevation of the forskolin response by inverse agonists can be regarded as a related assay for inverse agonism at G_i-coupled receptors [32,33].

In conclusion, we observed that constitutively active G_s-coupled receptors (H_2 and TSH receptor) not only enhance basal cAMP production but also increase the forskolin response in a variety of cell lines (CHO, HEK-293, COS-7 and Sf9 cells). Inverse agonists are able to reduce the increased forskolin response. Thorough investigation of the inverse agonistic properties of cimetidine, famotidine and ranitidine in $CHOH_2$ cells revealed no significant differences in inverse agonistic potency determined in either the absence or presence of forskolin. Inhibition of forskolin responses may thus be very useful to investigate inverse agonism at wild-type receptors and eliminate the need of using CAM receptors.

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