Constitutive Activity of Histamine H₃ Receptors Stably Expressed in SK-N-MC Cells: Display of Agonism and Inverse Agonism by H₃ Antagonists

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ABSTRACT

Agonist-independent activity of G-protein-coupled receptor, also referred to as constitutive activity, is a well-documented phenomenon and has been reported recently for both the histamine H₁ and H₂ receptors. Using SK-N-MC cell lines stably expressing the human and rat H₃ receptors at physiological receptor densities (500–600 fmol/mg of protein), we show that both the rat and human H₃ receptors show a high degree of constitutive activity. The forskolin-mediated cAMP production in SK-N-MC cells is inhibited strongly upon expression of the constitutive activity. The forskolin-mediated cAMP production can be further inhibited upon agonist stimulation of the H₃ receptor and can also refered to as constitutive activity, is a well-documented phenomenon and has been reported recently for both the histamine H₁ and H₂ receptors. Using SK-N-MC cell lines stably expressing the human and rat H₃ receptors at physiological receptor densities (500–600 fmol/mg of protein), we show that both the rat and human H₃ receptors show a high degree of constitutive activity. The forskolin-mediated cAMP production in SK-N-MC cells is inhibited strongly upon expression of the constitutive activity. The forskolin-mediated cAMP production can be further inhibited upon agonist stimulation of the H₃ receptor and can also be enhanced by a variety of H₃ antagonists acting as inverse agonists at the H₃ receptor. Thioperamide, clobenpropit, and iodophenpropit raise the cAMP levels in SK-N-MC cells with potencies that match their receptor binding affinities. Surprisingly, impentamine and burimamide act as effective H₃ agonists. Modification of the amine group of impentamine dramatically affected the pharmacological activity of the ligand. Receptor affinity was reduced slightly for most impentamine analogs, but the functional activity of the ligands varied from agonist to neutral antagonist and inverse agonist, indicating that subtle changes in the chemical structures of impentamine analogs have major impact on the (de)activation steps of the H₃ receptor. In conclusion, upon stable expression of the rat and human H₃ receptor in SK-N-MC cells constitutive receptor activity is detected. In this experimental system, H₃ receptors ligands, previously identified as H₃ antagonists, cover the whole spectrum of pharmacological activities, ranging from full inverse agonists to agonists.

ABBREVIATION: GPCR, G-protein-coupled receptor.
al., 2000), and we reported that the therapeutically important $H_3$ and $H_2$ antagonists, in fact, act as inverse agonists. In the present study, we describe that the human and rat histamine $H_3$ receptors stably expressed in SK-N-MC cells (Lovenberg et al., 1999, 2000) show a high level of constitutive activity, resulting in the identification of several standard $H_3$ antagonists (thioperamide and clobenpropit) as inverse agonists in this cell system. Moreover, burimamide and impentamine, previously identified as $H_3$ antagonists (Arrang et al., 1983; Vollinga et al., 1995a, 1995b), behave as $H_3$ agonists at the recombinant $H_3$ receptors. The agonistic effects of impentamine could also be demonstrated on the hypothalamic histamine release in the rat brain using in vivo microdialysis. Moreover, in a series of impentamine analogs we were able to manipulate the intrinsic activity. VUF4904, an impentamine analog with an isopropyl group at the amino group of the side chain, bound with a relatively high affinity (12 nM) and acted as a neutral antagonist in the transfected SK-N-MC cells. These data indicate that ligands, previously identified as $H_3$ antagonists, can cover the whole spectrum of pharmacological activities, ranging from full inverse agonism to agonism, at the recombinant $H_3$ receptors heterologously expressed in SK-N-MC cells.

**Experimental Procedures**

**Materials.** ($R$)-$\alpha$-Methylhistamine dihydrobromide was obtained from Sigma Research Biochemicals Inc. (Zwijndrecht, The Netherlands). Burimamide was a kind gift of GlaxoSmithKline (Welwyn Garden City, Hertfordshire, UK). All other $H_3$ ligands were taken from laboratory stock or (re-)synthesized at the Vrije Universiteit Amsterdam (details will be published elsewhere). Forskolin, 3-isobutyl-1-methylxanthine, cyclic 3',5'-adenosine monophosphate (cAMP), pertussis toxin, and bovine serum albumin were obtained from Sigma. Dulbecco's modified Eagle's medium, trypsin-EDTA, penicillin, nonessential amino acids, $\alpha$-glutamine, streptomycin, and so-}

dium-pyruvate were from Invitrogen (Breda, The Netherlands). Eagle's minimal essential medium was from BioWhittaker (Verviers, Belgium), and fetal calf serum was from Integral (Zaanstad, The Netherlands). Culture dishes and 24-well plates were from Costar (Haarlemmermeer, The Netherlands). G418 was obtained from Calbiochem (Amsterdam, The Netherlands). $[^{3}H]$(cyclic 3',5'-adenosine monophosphate ($[^{3}H]$cAMP), 40 Ci/mmol, was from Amersham (s'Hertogenbosch, The Netherlands); $[^{3}H]$N$^{-}$methylhistamine, 85 Ci/mmol, was from PerkinElmer Life Sciences (Zaventem, Belgium).

**Cell Culture.** SK-N-MC cells, a human neuroblastoma cell line stably expressing the human histamine $H_3$ receptor (the 445-amino acid isoform) or the rat histamine $H_3$ receptor (Lovenberg et al., 1999, 2000), were grown in 10-cm$^2$ dishes at $37\,\text{°C}$ in a humidified atmosphere with 5% CO$_2$ in Eagle's minimal essential medium, supplemented with 10% v/v fetal calf serum, 50 IU/ml penicillin, nonessential amino acids, 2 mM $\alpha$-glutamine, 50 $\mu$g/ml streptomycin, and 50 $\mu$g/ml sodium-pyruvate in presence of 600 $\mu$g/ml G418. Cells were diluted from the dishes with 0.05% trypsin-EDTA.

**$[^{3}H]$N$^{-}$Methylhistamine Binding.** Confluent 10-cm dishes of SK-N-MC cells stably expressing the rat or human histamine $H_3$ receptor were harvested using a cell scraper and centrifuged (3 min, 500g), and the pellets were stored at $-20\,\text{°C}$ until the day of the experiment. Before use the pellets were dissolved in distilled water and homogenized for 2 s by sonication (40 Watt, Labsonic 1510). The cell homogenates (30-100 $\mu$g) were incubated for 40 min at $25\,\text{°C}$ with 1 nM $[^{3}H]$N$^{-}$methylhistamine (85.0 Ci/mmol) in 50 mM sodium phosphate buffer, pH 7.4, with or without competing ligands. The reaction was terminated by rapid dilution with 3 ml of ice-cold buffer, pH 7.4, and filtration over 0.2% polyethyleneimine-pretreated What-
The forskolin-induced cAMP levels of the parental SK-N-MC (99% an overnight pretreatment with 100 ng/ml pertussis toxin forskolin-induced cAMP levels) were abolished completely by incubation the cAMP levels were determined by a competitive binding assay. Of clobenpropit on nontransfected SK-N-MC cells is shown. Cells were incubated for 10 min with the indicated ligands. After termination of the incubation the cAMP levels were determined by a competitive binding assay.

Results

The generation of the SK-N-MC cells stably expressing either the rat or human histamine H3 receptor was described previously (Lovenberg et al., 1999, 2000). In the present study, we used SK-N-MC cell lines, expressing 516 fmol/mg of protein (n = 3) of the human histamine H3 receptor or 627 ± 87 fmol/mg of protein (n = 3) of the rat histamine H2 receptor, as assessed by [3H]-Nα-methylhistamine binding.

As described previously, the H3 agonists (R)-α-methylhistamine (pEC50 = 9.26 ± 0.08) and imetit (pEC50 = 9.28 ± 0.04) potently inhibited the 10 μM forskolin-stimulated production of cAMP in human H3 receptor expressing cells (Fig. 1A; Table 1). In contrast, (R)-α-methylhistamine had no effect in the parental SK-N-MC cell line (Fig. 1A). As expected for a G1-coupled receptor, the 1 μM (R)-α-methylhistamine effects at the human H3 receptor (reduction to 11 ± 3% of the forskolin-induced cAMP levels) were abolished completely by an overnight pretreatment with 100 ng/ml pertussis toxin (99 ± 10% of forskolin-induced cAMP level, not shown).

Interestingly, we noticed an important difference between the forskolin-induced cAMP levels of the parental SK-N-MC cell line and the H3 receptor expressing cells (Fig. 1B). As this could be an indication of constitutive H3 receptor activation, we tested a variety of previously identified H3 antagonists on the SK-N-MC cell line expressing the human H3 receptor. Standard H3 antagonists as thioperamide, clobenpropit, and idopropentin concentration-dependently increased the forskolin-induced cAMP levels in the transfected SK-N-MC cells (Fig. 1B; Table 1), whereas clobenpropit had no effect on the forskolin response in the parental cell line (Fig. 1B). In our experiments, idopropentin and clobenpropit acted as full inverse agonists (α = −1.0−0.9), whereas thioperamide acted as a partial inverse agonist (Table 1). The obtained pEC50 values for the various inverse agonists correspond well with the respective affinities, as obtained in [3H]-Nα-methylhistamine competition experiments (Table 1).

In search of neutral antagonists, we tested a variety of other H3 antagonists. Surprisingly, the presumed H3 antagonists burimamide, impentamine, and the imetit homolog VUF 8328 (Van der Goot et al., 1992) all acted as potent H3 agonists at the human H3 receptor with intrinsic activities between 0.8 and 0.9 (Fig. 2; Table 1). Comparing the pKi values of various agonists with their pEC50 values revealed that in general the potencies of the agonists nicely parallel their affinities (Table 1). Only for the full agonists (R)-α-methylhistamine and perhaps imetit may some sort of receptor reserve be noticed (Table 1).

Based on the identification of agonism of impentamine, we studied several impentamine analogs to identify a neutral H3 receptor antagonist. In this series of H3 ligands, the amine function of impentamine was substituted or incorporated in a

### Table 1

Affinities and functional activities of several H3 receptor ligands at the human H3 receptor

<table>
<thead>
<tr>
<th>Ligand</th>
<th>pKi</th>
<th>pEC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R)-α-Methylhistamine</td>
<td>8.36 ± 0.07</td>
<td>9.26 ± 0.08</td>
</tr>
<tr>
<td>Imetit</td>
<td>8.79 ± 0.08</td>
<td>9.28 ± 0.04</td>
</tr>
<tr>
<td>VUF 8328</td>
<td>8.48 ± 0.01</td>
<td>8.86 ± 0.06</td>
</tr>
<tr>
<td>Burimamide</td>
<td>8.29 ± 0.14</td>
<td>8.63 ± 0.23</td>
</tr>
<tr>
<td>Idopropentin</td>
<td>7.11 ± 0.11</td>
<td>6.67 ± 0.14</td>
</tr>
<tr>
<td>Thiopepamide</td>
<td>7.18 ± 0.03</td>
<td>6.73 ± 0.20</td>
</tr>
<tr>
<td>Clobenpropit</td>
<td>8.42 ± 0.02</td>
<td>8.44 ± 0.15</td>
</tr>
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</table>

*indicates a significant difference compared with (R)-α-methylhistamine. ** indicates a significant difference compared with idopropentin.

Fig. 1. Modulation of 10 μM forskolin induced cAMP production in SK-N-MC cells, expressing the human H3 receptor. A, effects of the H3 agonist (R)-α-methylhistamine on the forskolin response in SK-N-MC cells or SK-N-MC cells expressing the human H3 receptor. B, effects of the H3 inverse agonists clobenpropit and idopropentin on the forskolin response in SK-N-MC cells expressing the human H3 receptor. For comparison, the effects of clobenpropit on nontransfected SK-N-MC cells is shown. Cells were incubated for 10 min with the indicated ligands. After termination of the incubation the cAMP levels were determined by a competitive binding assay.
piperidine ring. Modification of the amine group results in a series of compounds with a wide spectrum of pharmacological activity, including the neutral antagonist VUF4904 (Fig. 3; Table 2).

Constitutive activity is not restricted to the human $H_3$ receptor. Whereas the rat and human $H_3$ receptor were expressed at similar levels (627 fmol/mg of protein versus 516 fmol/mg of protein), the forskolin-induced cAMP levels were always lower in the SK-N-MC cells expressing the human $H_3$ receptor (Fig. 4). These data indicate that in our experimental model the level of constitutive activity of the human $H_3$ receptor is more pronounced than that of the rat $H_3$ receptor. At the recombinant rat receptor, the $H_3$ ligands burimamide and impentamine also behave as $H_3$ agonists. In contrast to the human $H_3$ receptor, both ligands behave as full agonists at the rat $H_3$ receptor (Table 3). The constitutive activity, displayed by the rat $H_3$ receptor, can also be inhibited by compounds such as clobenpropit (Fig. 4; Table 3). Especially for the inverse agonists thioperamide and iodophenpropit, we have confirmed the reported species differences (Lovénberg et al., 1999, 2000) with respect to their potencies in both receptor binding and functional assays (Table 3).

To investigate the predictive value of the data obtained with the recombinant receptors, impentamine and clobenpropit were tested in vivo. Previously, we showed by microdialysis the $H_3$ receptor-mediated effect on in vivo histamine release in the rat hypothalamus (Jansen et al., 1998). Using the same experimental set-up, we first evaluated the effects of impentamine. The mean values ± S.E.M. of the basal histamine release in the experiments in Fig. 5 were 0.078 ± 0.008 (n = 4) pmol/20 min. This value remained constant throughout the experimental period of 5 h under anesthesia (data not shown). After infusion of impentamine the histamine levels in the hypothalamus rapidly decreased to approximately 40% of the basal levels. Concomitant infusion of clobenpropit reversed the effect of impentamine and even caused an increase (±40%) in the histamine release above basal levels (Fig. 5).

**Discussion**

The recent cloning of the rat and human $H_3$ receptor cDNAs by Lovénberg et al. (1999, 2000) has had a great impact in the field of histamine research. The new information has been instrumental in identifying $H_3$ receptor isoforms (Drutel et al., 2001) and the $H_4$ receptor (Nakamura et al., 2000; Oda et al., 2000; Liu et al., 2001; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001) and has also been essential in deriving important information about the signaling properties of the $H_3$ receptor. Whereas for many years the actual signaling pathways for the $H_3$ receptor had been unknown, the use of cell lines expressing the $H_3$ receptor has led to the identification of at least three signal transduction pathways for the $H_3$ receptor: a G i-mediated inhibition of adenylyl cyclase (Lovénberg et al., 1999, 2000; Drutel et al., 2001), the activation of the MAP kinase pathway (Drutel et al., 2001), and the stimulation of Na + /H + exchange (Silver et al., 2001).

Using SK-N-MC cell lines, stably expressing either the human and rat $H_3$ receptors at physiological receptor densi-
TABLE 2
Affinities and functional activities of several impentamine analogs at the human H₃ receptor
H₃ receptor affinity (pKi) was determined by [³H]-N-methylhistamine binding to membranes of SK-N-MC cells expressing the human H₃ receptor. For agonists the pEC₅₀ values were determined by the inhibition of the forskolin-stimulated (10 μM) cAMP production, whereas the inverse agonistic activity was determined by the increase of the forskolin response in SK-N-MC cells, expressing the human H₃ receptor. All data shown are the mean ± S.E. of at least three experiments.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>pKi</th>
<th>pEC₅₀</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impentamine</td>
<td>8.29 ± 0.14</td>
<td>8.63 ± 0.23</td>
<td>0.9 ± 0.08</td>
</tr>
<tr>
<td>VUF5300</td>
<td>8.05 ± 0.09</td>
<td>8.67 ± 0.18</td>
<td>1.0 ± 0.02</td>
</tr>
<tr>
<td>VUF5207</td>
<td>7.81 ± 0.18</td>
<td>7.89 ± 0.1</td>
<td>0.7 ± 0.05</td>
</tr>
<tr>
<td>VUF4904</td>
<td>7.89 ± 0.05</td>
<td>-0.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>VUF4903</td>
<td>7.97 ± 0.03</td>
<td>8.10 ± 0.17</td>
<td>-0.6 ± 0.1</td>
</tr>
<tr>
<td>VUF5202</td>
<td>8.63 ± 0.04</td>
<td>8.66 ± 0.11</td>
<td>-0.9 ± 0.1</td>
</tr>
</tbody>
</table>

a Indicates a significant difference compared with (R)-α-methylhistamine.
b Indicates a significant difference compared with iodophenpropit.

ties (500–600 fmol/mg of protein) (Yanai et al., 1994; Brown et al., 1996), we now show that both the rat and human H₃ receptor display a high degree of constitutive activity. The forskolin-mediated cAMP production in SK-N-MC cells is inhibited strongly upon expression of the Gi-coupled H₃ receptor. The cAMP production can be further inhibited upon agonist stimulation of the H₃ receptor and can be enhanced by a variety of H₃ antagonists acting as inverse agonists at the H₃ receptor. Thiopropamide, clofenpropit, and iodophenpropit raise the cAMP levels in SK-N-MC cells expressing either the human or rat H₃ receptor with potencies that match their receptor binding affinities. As reported previously (Lovenberg et al., 1999, 2000), an important species difference is noticed for thiopropamide in both the binding and cAMP assay.

Burimamide was one of the key compounds used by Arrang et al. (1983) to demonstrate pharmacologically the existence of the H₃ receptor in rat cerebral cortex slices. Remarkably, at recombinant H₃ receptors, the presumed H₃ antagonist burimamide acts as a H₃ agonist. Whereas at the human H₃ receptor burimamide acts as a partial agonist (α = 0.8), full agonism is observed at the rat receptor. It is interesting to note that via the use of heterologous expression systems, burimamide is reclassified for the second time. Previously, we showed that at the human H₃ receptor burimamide acts as a weak partial agonist (Arrang et al., 1998). Because burimamide was developed originally as an H₂ antagonist using histamine as a starting point (Black et al., 1972), the discovery of residual agonistic activity at histamine H₂ and H₃ receptors is perhaps not too surprising.

The use of transfected cell lines also suggests a reclassification for impentamine, the histamine homolog previously suggested to differentiate between H₃ receptors in the guinea pig intestine and rat or guinea pig brain (Leurs et al., 1996; Harper et al., 1999). Impentamine is a potent H₃ antagonist in the guinea pig intestine (pA₂ = 8.4), but a partial agonist in the rat brain (pD₅₀ = 8.2, α = 0.6) (Leurs et al., 1996). Moreover, radioligand binding studies at the H₃ receptor in the guinea pig brain and intestine indicated that impentamine can discriminate between the receptors in the two prep-

<table>
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<tr>
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<th>Rat H₃</th>
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<tr>
<td>(R)-α-Methylhistamine</td>
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a Indicates a significant difference compared with (R)-α-methylhistamine.
b Indicates a significant difference compared with iodophenpropit.
Constitutive Activity of the Human H₃ Receptor

Fig. 5. Effect of impentamine and clobenpropit on the in vivo histamine release in the rat hypothalamus as measured by microdialysis. Drugs (10 µM) were infused in the hypothalamus via the microdialysis probe. Fractions were collected every 20 min and the amount of histamine was determined by HPLC, as described under Experimental Procedures. *, a significant (P < 0.05) difference compared with basal levels of histamine release.

activity when expressed at physiological expression levels in SK-N-MC cells. This observation has important consequences for the classification of H₃ receptor ligands, which can now be classified as inverse agonists, neutral antagonists, and agonists.

Constitutive activity of the rat H₃ receptor was also reported very recently by Morisset et al. (2000). Interestingly, the constitutive activity of the rat H₃ receptor was suggested to regulate brain histamine release in both rat and mouse (Morisset et al., 2000). The H₃ receptor is, therefore, one of the few GPCRs for which it is known that they modulate important physiological processes by means of its constitutive activity. In light of the foregoing therapeutic application of H₃ antagonists (Leurs et al., 1998), it remains to be established whether inverse agonists or neutral antagonists will be favored for clinical application.

References


Chen Y and Prusoff W (1973) Relationship between the inhibition constant (Kᵢ) and the concentration of inhibitor which causes 50% inhibition (IC₅₀) of the enzymatic reaction. Biochem Pharmacol 22:3099–3108.


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References


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