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Histamine H_3-receptor-mediated [^{35}S]GTPγ[S] binding: evidence for constitutive activity of the recombinant and native rat and human H_3 receptors

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1 Constitutive activity of the recombinant and native rat and human H_3 receptors (H_3Rs) was studied using H_3R-mediated [^{35}S]GTPγ[S] binding and [^{3}H]-arachidonic acid release.

2 Ciproxifan, an inverse agonist at the rat H_3R (rH_3R), decreased [^{3}H]arachidonic acid release from CHO cells expressing moderate densities ($\sim$200–300 fmol mg^{-1} protein) of the human H_3R (hH_3R). This effect occurred with the same magnitude than at the rH_3R.

3 The expression of the hH_3R was associated with an increase in [^{35}S]GTPγ[S] binding to membranes of CHO cells. Ciproxifan decreased [^{35}S]GTPγ[S] binding to membranes of CHO (hH_3R) cells. Both effects were correlated to receptor density and revealed that constitutive activity of the hH_3R, although lower than that of the rH_3R in this assay, was again observed at physiological densities ($<$500 fmol mg^{-1} protein). Ciproxifan was less potent at the human than the rat receptor, not only as an antagonist ($K_i = 45$ nM), but also as an inverse agonist ($EC_{50} = 15$ nM).

4 Constitutive activity of the hH_3R was also evidenced using inhibition of [^{35}S]GTPγ[S] binding by unlabelled GTPγS. The expression of the hH_3R generated a high affinity binding for GTPγS which was increased by imetit, but partially decreased by ciproxifan, therefore acting as a partial inverse agonist.

5 [^{35}S]GTPγ[S] binding to rat brain membranes was decreased in several regions by thioperamide, ciproxifan and FUB 465, three inverse agonists at the H_3R, whose effects were blocked by proxyfan, a neutral antagonist. [^{35}S]GTPγ[S] binding was also decreased by an A_1-adenosine receptor inverse agonist, but remained unchanged in the presence of inverse agonists at D_2/D_3 dopamine, H_1 and H_2 histamine, α_2-adrenergic and δ opioid receptors.

6 In conclusion, the present study shows that the recombinant rat and human H_3 receptors expressed at physiological densities display constitutive activity and suggests that constitutive activity of native H_3Rs is one of the highest among G-protein-coupled receptors present in rat brain.

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Keywords: Histamine; H_3 receptor; G-protein-coupled receptors; recombinant receptors; native receptors; rat brain; [^{35}S]GTPγ[S] binding; constitutive activity; ciproxifan

Abbreviations: BSA, bovine serum albumin; CPDPX, 8-cyclopentyl-1,3-dipropylxanthine; GPCR, G-protein-coupled receptor; H_3R, histamine H_3 receptor; rH_3R, rat histamine H_3 receptor; hH_3R, human histamine H_3 receptor

Introduction

The histamine H_3 receptor (H_3R) was initially characterized as an autoreceptor regulating histamine release in brain (Arrang *et al*., 1987; 1988). Subsequently, it has been shown to modulate the release of other monoamines, glutamate, GABA and tachykinins in brain or peripheral tissues (Schlicker *et al*., 1994; Hill *et al*., 1997; Brown *et al*., 2001). The sensitivity of agonist binding and various H_3R-mediated responses to guanylnucleotides and pertussis toxin suggested that it was a $G_{i/o}$ protein-coupled heptahelical receptor (Clark & Hill, 1996; Takeshita *et al*., 1998). This proposal was confirmed with the recent cloning of H_3R cDNAs from human (Lovenberg *et al*., 1999), guinea-pig (Tardivel-Lacombe *et al*., 2000) and rat (Morisset *et al*., 2000; Lovenberg *et al*., 2000; Drutel *et al*., 2001).

Data accumulated over the last years strongly suggested that G-protein-coupled receptors (GPCRs) could be spontaneously active even in the absence of an agonist. This constitutive activity was mainly evidenced for recombinant receptors overexpressed and/or mutated (Lefkowitz *et al*., 1993; Milligan *et al*., 1995). However, indirect indications suggested that it could occur for native receptors endogenously expressed in cells or tissues (De Ligt *et al*., 2000). Consistent with the physiological relevance of the phenomenon, we recently demonstrated high constitutive activity of native rat H_3Rs (rH_3Rs) (Morisset *et al*., 2000). Using functional assays, we showed that several prototypic antagonists such as thioperamide or ciproxifan (Arrang *et al*., 1987; Ligneau *et al*., 1998) were, in fact, acting as inverse agonists not only at recombinant rH_3Rs but also at H_3Rs present in rodent brain. Moreover we showed that constitutive activity of H_3 autoreceptors regulated histamine neurons

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(Morisset *et al*., 2000). In the present study, we have assessed whether the recombinant human H_3R (hH_3R) also displays the constitutive activity that we previously detected in rodents. We have also analysed constitutive activity of the native H_3R in various rat brain regions using three inverse agonists.

Methods

Cloning of the rH_3R and hH_3R cDNAs

The rH_3R and hH_3R were cloned as described (Ligneau *et al*., 2000). Briefly, a rat striatal cDNA library was screened with a cDNA fragment (third transmembrane domain/third intracellular loop) amplified from rat cerebral cortex using primers based on the sequence of the hH_3R (Lovenberg *et al*., 1999). Several clones exhibited a full-length open reading frame encoding a 445-amino acid protein corresponding to the rH_3R (Morisset *et al*., 2000). A human striatum cDNA library was screened with the same probe. One clone exhibited a full-length cDNA sequence corresponding to the hH_3R (Lovenberg *et al*., 1999).

Stable transfection of CHO-K1 cells

cDNA inserts corresponding to the full-length coding sequences of the rH_3R and hH_3R, were ligated into the mammalian expression vector pCIneo (Promega, Charbonnières, France). CHO-K1 cells were transfected using SuperFect (Qiagen, Courtaboeuf, France). Stable transfectants were selected with 2 mg ml^{-1} of G418 and tested for [^{125}I]-iodoproxyfan binding (Ligneau *et al*., 1994). Several clones, named CHO(rH_3R) and CHO(hH_3R), expressing various receptor densities, were selected for further characterization and maintained in presence of 1 mg ml^{-1} of G418. Histamine levels present in the cell culture media were determined using an enzymoimmunoassay (Beckman Coulter, Roissy, France).

[^{125}I]-iodoproxyfan binding assay

CHO(rH_3R or hH_3R) cells were washed and homogenized with a Polytron in ice-cold binding buffer (Na_2HPO_4/KH_2PO_4 50 mM, pH 6.8) and assays performed as described (Ligneau *et al*., 1994).

[^{35}S]GTPγ[S] binding assay

[^{35}S]GTPγ[S] binding assays were performed according to Clark & Hill (1996) with slight modifications. Brain tissues from male Wistar rats (160–200 g, Iffa-Credo, L'Arbresle, France) and CHO(rH_3R or hH_3R) cells were homogenized in ice-cold buffer (Tris HCl 50 mM, pH 7.4). Homogenates were centrifuged twice at 20,000 × *g* for 10 min and the final pellet was resuspended in 50 volumes of buffer. Membranes (20–50 μg) were pretreated with adenosine deaminase (1 U ml^{-1} Roche, Meylan, France) and incubated for 60 min at 25°C with 0.1 nM [^{35}S]-GTPγ[S] and, when required, the various drugs tested, in 1 ml of assay buffer (50 mM Tris HCl, 50 mM NaCl, 5 mM $MgCl_2$, 10 μM GDP, 0.02% bovine serum albumin (BSA) pH 7.4). The nonspecific binding was determined using GTPγS (10 μM). Incubations were stopped by rapid filtration under vacuum through Whatman GF/B filters. Filters were washed twice with 4 ml ice-cold water and the radioactivity retained on the filters was measured by liquid scintillation spectrometry.

[^{3}H]-arachidonic acid release

CHO (rH_3R or hH_3R) cells were incubated for 2 h at 37°C with 0.5 μCi of [^{3}H]-arachidonic acid in DMEM-Nut mix F12 (Life Technologies, Cergy-Pontoise, France) containing 0.2% BSA. After washing, cells were incubated for 30 min with 2 μM A23187 (Roche) and, when required, the H_3-receptor ligands and [^{3}H]-arachidonic acid release was determined by liquid scintillation counting.

Analysis of data

For determination of EC_{50} and IC_{50} values of imetit and ciproxifan on [^{35}S]GTPγ[S] binding, the total curves were analysed with an iterative least-squares method derived from that of Parker & Waud (1971). The K_i value of ciproxifan acting as an antagonist was calculated from its IC_{50} value, assuming a competitive antagonism and by using the relationship (Cheng & Prussoff, 1973):

$$K_i = IC_{50}/(1 + (S/EC_{50}))$$

where S represents the concentration of imetit and EC_{50} the imetit concentration required for a half-maximal stimulation of [^{35}S]GTPγ[S] binding. Protein contents were determined according to the method of Lowry *et al*. (1951), using BSA as the standard. Statistical evaluation of the results was performed by ANOVA followed by Newman–Keuls test.

Radiochemicals and drugs

[^{125}I]-Iodoproxyfan (2000 Ci mmol^{-1}) was prepared as described (Krause *et al*., 1997). [^{35}S]GTPγ[S] (1250 Ci mmol^{-1}) was from NEN Life Science (Zaventem, Belgium). Ciproxifan and thioperamide were from Bioprojet (Paris, France). FUB 465 (ethyl-3-(1*H*-imidazol-4-yl)propyl ether) and proxyfan (3-(1*H*-imidazol-4-yl)propylphenylmethyl ether) were provided by Pr Schunack (Freie Universität Berlin, Germany). Imetit was provided by Pr Ganellin (University College, London, U.K.). Mepyramine was from Specia (Paris, France) and cimetidine from Smith Kline Beecham (London, U.K.). ICI-174,864 was obtained from Fisher Bioblock Scientific (Illkirch, France). Yohimbine, haloperidol and CPDPX (8-cyclopentyl-1,3-dipropylxanthine) were from Sigma (St-Quentin-Fallavier, France). All other chemicals were obtained from commercial sources and were of the highest purity available.

Results

Effects of H_3-receptor ligands on two responses mediated by the recombinant rat or human H_3 receptor expressed in CHO cells

The basal specific [^{35}S]GTPγ[S] binding to membranes of wild type CHO cells incubated with 0.1 nM [^{35}S]GTPγ[S] represented 22.2 ± 0.3 fmol mg^{-1} protein. It was significantly

($P<0.001$) increased to membranes of CHO cells expressing ~200–300 fmol mg^{-1} protein of rat or human H_3R (39.5 ± 1.1 and 26.7 ± 0.1 fmol mg^{-1} protein, respectively). Imetit, a selective H_3-receptor agonist (Garbarg *et al.*, 1992) used at a maximal concentration (1 μM), induced a similar increase (by ~100%) of $[^{35}S]GTP\gamma[S]$ binding to membranes of CHO(rH_3R) and CHO(hH_3R) cells which represented 71.2 ± 1.3 and 54.4 ± 0.5 fmol mg^{-1} protein, respectively (Figure 1A). In contrast, ciproxifan, a H_3-receptor inverse agonist (Morisset *et al.*, 2000), significantly decreased $[^{35}S]GTP\gamma[S]$ binding to membranes of CHO(rH_3R) and CHO(hH_3R) cells (by 44 and 15%, respectively). Both compounds did not modify specific $[^{35}S]GTP\gamma[S]$ binding to membranes of wild type CHO cells (Figure 1A).

Ciproxifan (1 μM) decreased significantly, and with a similar amplitude (by 44 and 49%, respectively), $[^3H]$-arachidonic acid release evoked by the Ca^{2+}-ionophore A23187 from CHO(rH_3R) and CHO(hH_3R) cells without affecting $[^3H]$-arachidonic acid release alone (Figure 1B). All these effects were observed in total absence of histamine in the culture medium.

Effects of H_3-receptor ligands on specific $[^{35}S]GTP\gamma[S]$ binding to membranes of CHO(hH_3R) cells

Changes in the effect of imetit and ciproxifan associated with receptor expression were assessed on specific $[^{35}S]GTP\gamma[S]$ binding to membranes of CHO(hH_3R) cells expressing increasing receptor densities. Following incubation with 0.1 nM $[^{35}S]GTP\gamma[S]$, the basal specific $[^{35}S]GTP\gamma[S]$ binding itself remained unchanged to membranes of cells expressing 100 fmol mg^{-1} protein (20.4 ± 1.2 fmol mg^{-1} protein) and then progressively increased (from a receptor density of 170 fmol mg^{-1} protein) to reach 41.3 ± 1.4 and 44.0 ± 2.0 fmol mg^{-1} protein at a receptor density of ~700 fmol mg^{-1} protein and 1 pmol mg^{-1} protein, respectively.

In the presence of imetit (1 μM), the basal $[^{35}S]GTP\gamma[S]$ binding significantly increased from a receptor density of 170 fmol mg^{-1} protein to reach 92.1 ± 4.5 fmol mg^{-1} protein ($+120\pm4\%$) and 124.4 ± 3.9 fmol mg^{-1} protein ($+183\pm6\%$) in membranes of cells expressing ~700 fmol mg^{-1} protein and 1 pmol mg^{-1} protein, respectively (Figure 2). The decrease in basal binding induced by the inverse agonist ciproxifan (1 μM) was observed in membranes of cells expressing 250–400 fmol mg^{-1} protein of the hH_3R and reached ~20% at a density of 1 pmol mg^{-1} protein (Figure 2).

The effects of imetit and ciproxifan on specific $[^{35}S]GTP\gamma[S]$ binding to membranes of CHO(hH_3R) cells expressing 400 fmol mg^{-1} protein were concentration dependent and occurred with EC_{50} values of 2.2 ± 0.4 nM and 15.2 ± 2.0 nM, respectively (Figure 3). In addition, ciproxifan progressively inhibited the effect of imetit (30 nM) with an IC_{50} value of 654 ± 74 nM leading (Cheng & Prussoff, 1973) to a K_i value of 45 ± 14 nM for the drug tested as an antagonist. At the highest concentrations tested against imetit, ciproxifan tended to decrease $[^{35}S]GTP\gamma[S]$ binding and the amplitude of this decrease was similar to that observed when the drug was added alone (Figure 3).

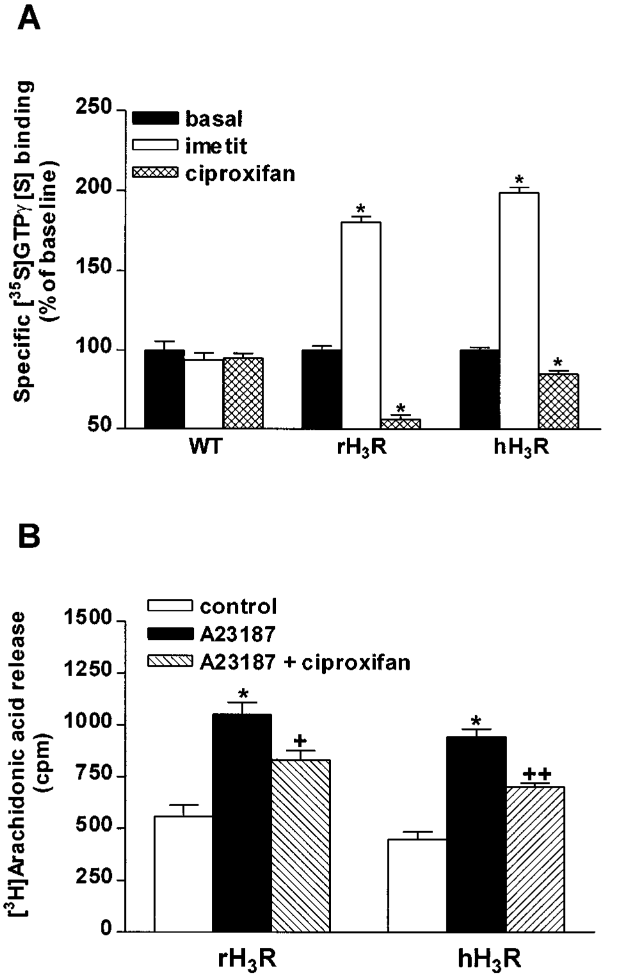

Figure 1 Effects of H_3-receptor ligands on two responses mediated by the recombinant rat and human H_3 receptors. (A) Effects of H_3-receptor ligands on specific $[^{35}S]GTP\gamma[S]$ binding. Membranes of wild-type CHO cells (WT) or cells expressing ~200–300 fmol mg^{-1} protein of rat (rH_3R) or human (hH_3R) receptors were incubated with 0.1 nM $[^{35}S]GTP\gamma[S]$ and, when required, 1 μM imetit or ciproxifan. Data represent means±s.e.mean of 11–16 determinations from two separate experiments. $^*P<0.001$ vs the corresponding basal. (B) Effect of ciproxifan on A23187-evoked $[^3H]$-arachidonic acid release. After incubation with 0.5 μCi of $[^3H]$-arachidonic acid, CHO(rH_3R) and CHO(hH_3R) cells were incubated with 2 μM A23187 and, when required, 1 μM ciproxifan. Data are means±s.e.m. of 11–32 determinations from three to four experiments. $^*P<0.001$ vs the corresponding control; $^+P<0.01$, $^{++}P<0.001$ vs A23187.

The inhibitition curve of $GTP\gamma S$ on $[^{35}S]GTP\gamma[S]$ binding to membranes of CHO(WT) cells was found to be shallow, its pseudo-Hill coefficient calculated in a one-site model being $n_H=0.54\pm0.03$. Nonlinear regression revealed that a two-site model analysis best accounted than a one-site model for the inhibition curve ($c=0.9982$ and 0.9684; $\chi^2=2.60$ and 41.95, respectively; $P<0.0001$ in *F*-test). The latter could be resolved in a medium-affinity population of sites, termed sites 2 (40% of maximal specific binding) and a low-affinity population of sites, termed sites 1, with pIC_{50} values of 7.3 ± 0.1 and 5.5 ± 0.1, respectively (Figure 4 and Table 1). Nonlinear regression analysis revealed that expression of the recombinant hH_3R generated a third population of sites, termed sites 3, ($c=0.9983$) which was not observed in membranes of CHO(WT) cells, sites 1 and 2 being unchanged. This additional binding site displayed a higher affinity for GTPγS ($pIC_{50} = 8.2 \pm 0.2$) and represented 17% of specific binding in membranes of CHO(hH_3R) cells (Figure 4). A three-site model analysis best accounted than a two-site model for the inhibition curve ($\chi^2 = 0.67$ and 2.37, respectively) and fitted the data significantly better in the presence of imetit ($P < 0.01$ in F-test). Imetit (1 μM) significantly increased (by ~300%) the capacity of the site 3 which, then, represented ~50% of specific binding, but did not modify its affinity or the parameters of sites 1 and 2. In contrast, the inverse agonist ciproxifan significantly reduced (by 33%) the capacity of the high-affinity site 3 without changing its affinity, the low and medium affinity sites being also unaffected (Table 1 and Figure 4).

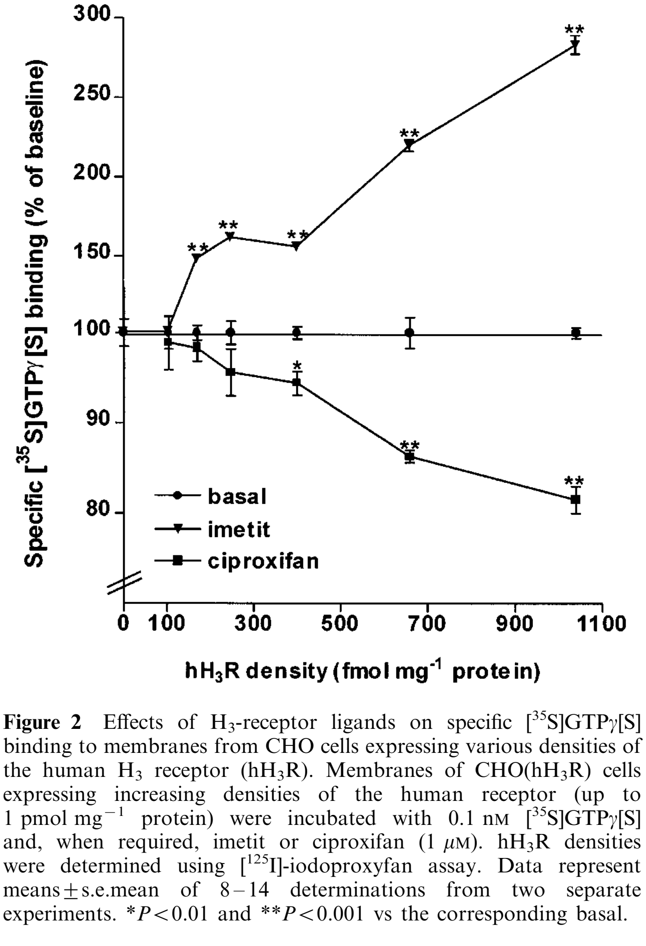

Figure 2 Effects of H_3-receptor ligands on specific [^{35}S]GTPγ[S] binding to membranes from CHO cells expressing various densities of the human H_3 receptor (hH_3R). Membranes of CHO(hH_3R) cells expressing increasing densities of the human receptor (up to 1 pmol mg^{-1} protein) were incubated with 0.1 nM [^{35}S]GTPγ[S] and, when required, imetit or ciproxifan (1 μM). hH_3R densities were determined using [^{125}I]-iodoproxyfan assay. Data represent means ± s.e.mean of 8–14 determinations from two separate experiments. *$P < 0.01$ and **$P < 0.001$ vs the corresponding basal.

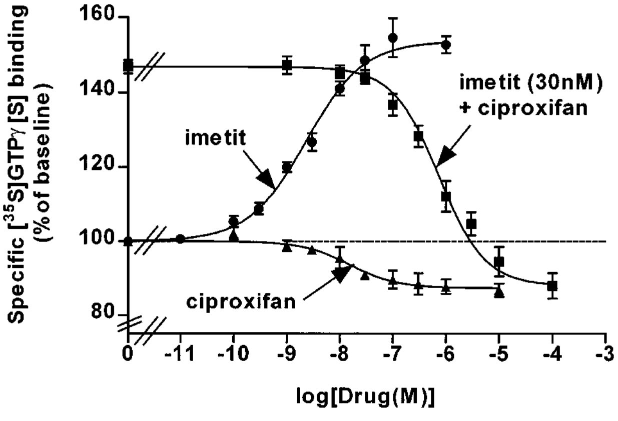

Figure 3 Effects of H_3-receptor ligands on specific [^{35}S]GTPγ[S] binding to membranes of CHO (hH_3R) cells expressing 400 fmol mg^{-1} protein. Membranes were incubated with 0.1 nM [^{35}S]GTPγ[S] in the presence, when required, of increasing concentrations of imetit, and ciproxifan alone or in the presence of 30 nM imetit. Means ± s.e.mean of 7–16 determinations from two separate experiments.

Effects of H_3-receptor ligands on specific [^{35}S]GTPγ[S] binding to membranes from various rat brain regions

Following incubation with 0.1 nM [^{35}S]GTPγ[S], specific [^{35}S]GTPγ[S] binding to rat brain membranes represented ~5,000–10,000 d.p.m., i.e., 169 ± 25 fmol mg^{-1} protein (hippocampus) to 261 ± 41 fmol mg^{-1} protein (hypothalamus). It was increased significantly (by 10–20%) by imetit (10 nM) in all the regions studied, the effect of the agonist being stronger in the cerebral cortex and striatum, however (Figure 5). In all regions, the increase in binding elicited by imetit was blocked by the antagonist proxyfan (1 μM) (Morisset *et al.*, 2000). In contrast, FUB 465, ciproxifan and thioperamide (10 nM), three compounds acting as inverse agonists (Morisset *et al.*, 2000), reduced significantly [^{35}S]GTPγ[S] binding, and their effect was also blocked by 1 μM proxyfan. Proxyfan did not itself significantly affect binding in all brain regions studied (Figure 5).

Effects of inverse agonists at various G protein coupled-receptors on specific [^{35}S]GTPγ[S] binding to membranes from rat cerebral cortex and striatum

The effects of various ligands described previously as inverse agonists in responses mediated by recombinant GPCRs ([^{35}S]GTPγ[S] binding, GTPase assay, cyclic AMP formation, prolactin release, [^{3}H]-thymidine incorporation, [^{3}H]-inositol-phosphates accumulation), were assessed on specific [^{35}S]GTPγ[S] binding to membranes of rat cerebral cortex and striatum. Mepyramine, cimetidine, ICI-174,864, yohimbine and haloperidol that were reported in cell culture systems to act as inverse agonists at recombinant or endogenously expressed histamine H_1- (Bakker *et al.*, 2000), histamine H_2- (Smit *et al.*, 1996; Alewijnse *et al.*, 1998), δ opioid (Costa & Herz, 1989; Milligan *et al.*, 1997), α_2-adrenergic (Tian *et al.*, 1994; Pauwels *et al.*, 2000) and dopamine D_2/D_3- (Nilsson & Eriksson, 1993; Griffon *et al.*, 1996) receptors, respectively, did not affect specific [^{35}S]GTPγ[S] binding to membranes of rat cerebral cortex (Figure 6) and striatum (not shown). In contrast, ciproxifan and CPDPX, an inverse agonist at recombinant adenosine A_1-receptors (Shryock *et al.*, 1998), reduced significantly specific [^{35}S]GTPγ[S] binding to membranes from cerebral cortical (Figure 6) and striatal (not shown) membranes.

Discussion

The present findings based upon two H_3-receptor-mediated responses, i.e., phospholipase A_2 activation (Morisset *et al.*, 2000) and specific [^{35}S]GTPγ[S] binding (Clark & Hill, 1996), provide the first direct evidence that the human histamine H_3R displays constitutive activity. Ciproxifan, behaving as a

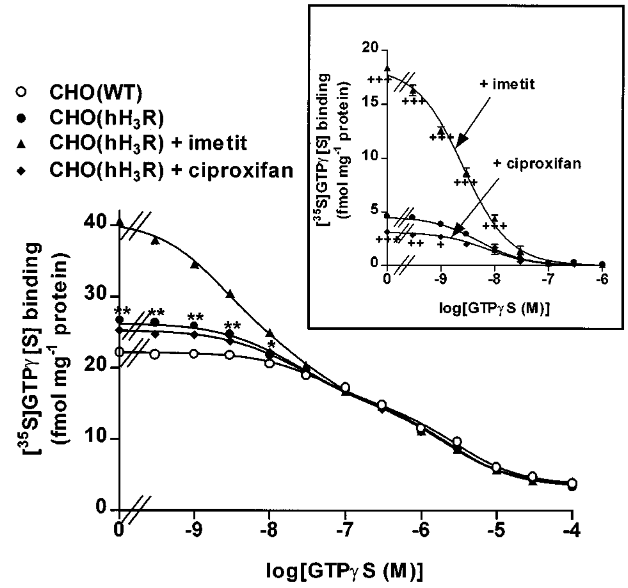

Figure 4 Inhibition of [^{35}S]GTPγ[S] binding to membranes of CHO(WT) and CHO(hH$_3$R) cells by GTPγS. Membranes of CHO(WT) or CHO(hH$_3$R) cells expressing ~300 fmol mg^{-1} protein, were incubated with 0.1 nM [^{35}S]GTPγ[S] and increasing concentrations of GTPγS, in the presence, when required, of 1 μM imetit or ciproxifan. The inset shows the same data after subtraction of [^{35}S]GTPγ[S] binding to membranes of CHO(WT) cells. Means ± s.e.mean of 23–25 determinations from two independent experiments. $^{*}P<0.01$; $^{**}P<0.001$ vs CHO(WT) cells; $^{+}P<0.05$; $^{++}P<0.01$; $^{+++}P<0.001$ vs CHO(hH$_3$R) cells in the absence of ligand.

Table 1 Effect of H$_3$-receptor ligands on the inhibition by GTPγS of [^{35}S]GTPγ[S] binding to membranes of CHO (WT) and CHO(hH$_3$R) cells

	CHO(WT)	*CHO(hH$_3$R)*		
	Basal	*Basal*	*Imetit (1μM)*	*Ciproxifan (1μM)*
Site 1:				
(fmol mg^{-1})	13.1±0.2	13.1±0.1	12.2±0.1	12.9±0.1
pIC$_{50}$	5.5±0.1	5.6±0.1	5.7±0.1	5.7±0.1
Site 2:				
(fmol mg^{-1})	9.1±0.1	9.1±0.1	9.3±0.1	9.4±0.1
pIC$_{50}$	7.3±0.1	7.4±0.2	7.4±0.3	7.4±0.2
Site 3:				
(fmol mg^{-1})	–	4.6±0.1	19.1±0.1 (+315%)	3.1±0.1 (−33%)
pIC$_{50}$	–	8.1±0.2	8.6±0.1	8.2±0.2

Untransformed data shown in Figure 4 were analysed by nonlinear regression using a least square curve fitting procedure. Analysis of isotherms provides estimates and standard errors of these estimates of pIC$_{50}$ values and maximal capacities (expressed in fmol mg^{-1} protein) of three different sites of low (site 1), medium (site 2) and high (site3) affinity for GTPγS. Per cent change of the capacity of site 3 induced by 1 μM imetit or ciproxifan is indicated between brackets.

potent inverse agonist (Morisset *et al.*, 2000), decreased [^{3}H]-arachidonic acid release from CHO cells expressing moderate densities of the human receptor. Moreover, its effect occurred with a magnitude similar to that observed with the rat receptor, thereby suggesting that the H$_3$R displays the same high level of constitutive activity for this signalling pathway in both species. The carboxy-terminal portion of the third intracellular loop is critical for constitutive activity of GPCRs (Kjelsberg *et al.*, 1992; Parma *et al.*, 1993; Ren *et al.*, 1993; Samama *et al.*, 1993). The rH$_3$R contains in this region a motif (SRDKKVAK) that is maintained in the sequence of the hH$_3$R, with seven identical and one conserved amino acids (SRDRKVAK) (Lovenberg *et al.*, 1999). As we recently noticed (Morisset *et al.*, 2000), this motif is highly similar to the corresponding sequence of a mutated human β_2-adrenergic receptor in which the mutations confer constitutive activity (Samama *et al.*, 1993). This conserved motif may therefore account for constitutive activity of rat and human H$_3$Rs.

The expression of the human receptor in CHO cells was also associated with an increase in basal [^{35}S]GTPγ[S] binding. This increment of agonist-independent [^{35}S]GTPγ[S] binding above the basal level of the parent untransfected CHO cells, revealed again the constitutive activity of the transfected receptor. In agreement, ciproxifan alone significantly decreased the basal [^{35}S]GTPγ[S] binding to membranes of cells expressing the human receptor. However, both the effect of the inverse agonist and the increase in [^{35}S]GTPγ[S] binding were lower than those observed with the rat receptor. The increment of [^{35}S]GTPγ[S] binding measured at ~200–300 fmol mg^{-1} protein of hH$_3$R represented ~5 fmol mg^{-1} protein whereas that found at the same density of the rat receptor represented ~20 fmol mg^{-1} protein, suggesting that constitutive activity of the human receptor was about four times lower than that of the rat receptor in this test system. This apparent lower constitutive activity may result from a lower coupling efficiency of the human receptor to some G-protein subtypes. In agreement, the propensity of a given GPCR to produce constitutive activity is known to be an inherent property of the receptor and has been shown to be dependent on the G-proteins and signalling pathways activated by the receptor (Perez *et al.*, 1996; Pauwels *et al.*, 2000; Chen *et al.*, 2000). The H$_3$-receptor-mediated stimulation of [^{35}S]GTPγ[S] binding being sensitive to pertussis toxin (Clark & Hill, 1996), may involve distinct G$\alpha_{i/o}$ subunits inasmuch as the recombinant receptor couples to distinct signalling pathways involving G$_i$/G$_o$ proteins, i.e., adenylyl cyclase inhibition, phospholipase A$_2$ and MAP kinase activation (Lovenberg *et al.*, 1999; Morisset *et al.*, 2000; Drutel *et al.*, 2001; Héron *et al.*, 2001).

As previously shown for other GPCRs (Chidiac *et al.*, 1994; Pozvek *et al.*, 1997; Claeysen *et al.*, 1999; Newman-Tancredi *et al.*, 2000), we reported that constitutive activity of the recombinant rH$_3$R was positively correlated to receptor density and was favoured upon overexpression (Morisset *et al.*, 2000). This observation, attributable to an increased receptor/G-protein stoichiometry (Kenakin, 1997), could be made with the hH$_3$R. Indeed, the increment of [^{35}S]GTPγ[S] binding due to the transfected receptor was 4 fold greater (~20 and 5 fmol mg^{-1} protein, respectively) at ~700 fmol mg^{-1} protein versus ~300 fmol mg^{-1} protein of hH$_3$R. The extended ternary complex model which describes two receptor states whereby the active state interacts with G proteins (Samama *et al.*, 1993), predicts that the same maximal response that is produced by an agonist should be observed constitutively with high receptor expression levels. However, the increase in [^{35}S]GTPγ[S] binding (i.e. constitutive activity) observed at the highest densities of hH$_3$R was

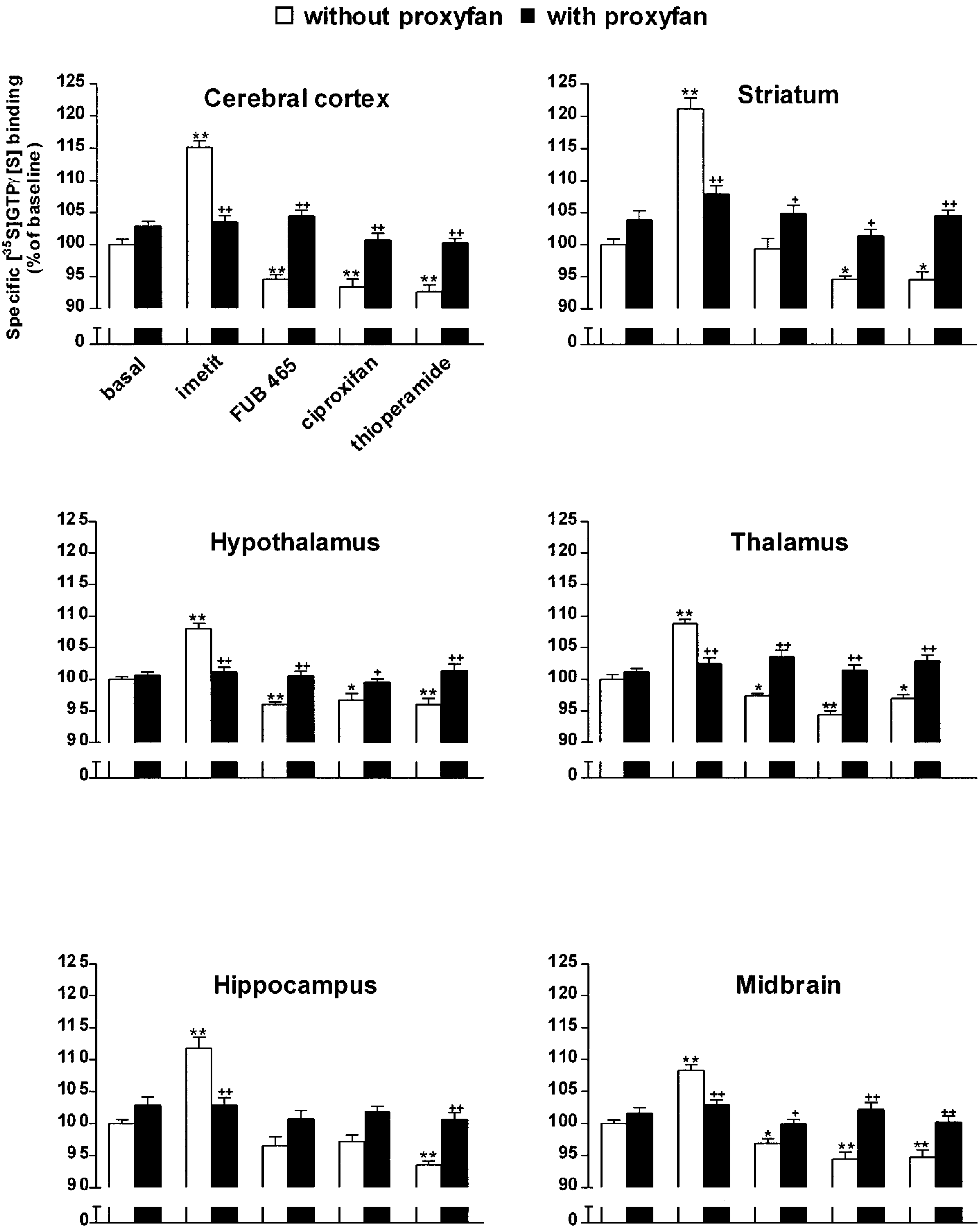

Figure 5 Effects of H_3-receptor ligands on specific [^{35}S]GTPγ[S] binding to membranes from various rat brain regions. The effects of imetit, a selective agonist, and FUB 465, ciproxifan and thioperamide (10 nM), three inverse agonists, were studied in the absence (open bars) or presence (solid bars) of 1 μM proxyfan. The H_3-receptor density was measured using [^{125}I]-iodoproxyfan binding assay (Ligneau *et al*., 1994) and represented 130±1, 136±6, 108±2, 82±3, 103±4 and 99±6 fmol mg^{-1} protein in the cerebral cortex, striatum, hypothalamus, thalamus, hippocampus and midbrain respectively. Data are means±s.e.mean of 10–38 determinations from four to six separate experiments. $^{*}P<0.05$, $^{**}P<0.001$ vs basal; $^{+}P<0.05$, $^{++}P<0.001$ vs without proxyfan.

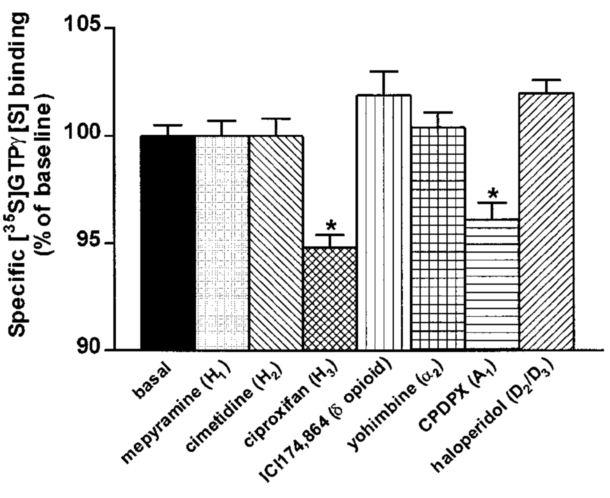

Figure 6 Effects of inverse agonists at various G protein coupled-receptors on specific [^{35}S]GTPγ[S] binding to rat cerebral cortical membranes. Rat cerebral cortical membranes were incubated with 0.1 nM [^{35}S]GTPγ[S] in the presence of the drugs at a 1 μM (mepyramine, cimetidine, ciproxifan, ICI-174,864 and CPDPX) or 0.1 μM (yohimbine and haloperidol) final concentration. Data represent means ± s.e.mean of 8–39 determinations from two to four separate experiments. *$P<0.001$ vs basal.

still substantially lower than the increase in binding induced by imetit (i.e. the agonist-induced maximal response), suggesting that the amount of receptor may be limiting for constitutive activity of the hH$_3$R. Consistent with this proposal, the increment in [^{35}S]GTPγ[S] binding observed at a receptor density of 1 pmol mg^{-1} protein was not significantly higher than that observed at ~700 fmol mg^{-1} protein. However, it was already significant at moderate densities (<500 fmol mg^{-1} protein), i.e., consistent with natural cellular levels of receptors, indicating that the threshold expression level for constitutive activity, which varies with the different types of receptors (Chen *et al*., 2000), is rather small for the hH$_3$R. This suggests that constitutive activity of the native H$_3$R may be present not only in rodent brain (Morisset *et al*., 2000) but also in human brain.

We also analysed the inhibition of [^{35}S]GTPγ[S] binding by unlabelled GTPγS to further investigate constitutive G-protein activation by the hH$_3$R expressed at a moderate density (~300 fmol mg^{-1} protein). This new approach was recently used successfully to examine directly constitutive activity of 5-HT receptors in CHO cells (Audinot *et al*., 2001). It shows that the increment in [^{35}S]GTPγ[S] binding generated upon overexpression of the H$_3$R corresponded to a high-affinity site able to bind the low concentration (0.1 nM) of [^{35}S]GTPγ[S] used in the present study. In agreement with the low affinity binding previously reported (pIC$_{50}$ = 6.2 to 6.6) (Newman-Tancredi *et al*., 2000; Audinot *et al*., 2001), GTPγS bound to low and medium affinity components (pIC$_{50}$ = 5.5 and 7.3, respectively) in membranes of wild-type CHO cells. hH$_3$R expression generated an additional high affinity binding site for GTPγS, similar to that associated with the expression of recombinant human serotonin 5-HT$_{1B}$ or 5-HT$_{1D}$ receptors in the same cells (pIC$_{50}$ = 8.1 and 8.7, respectively) (Newman-Tancredi *et al*., 2000; Audinot *et al*., 2001). This high affinity site reflected the constitutive activity of the hH$_3$R since it was increased by imetit, but decreased by ciproxifan, acting again as an inverse agonist abrogating the constitutive activation of G proteins. In contrast, the two ligands did not alter the density and affinity of the two lower affinity binding sites confirming that the latter are not related to H$_3$ receptor/G-protein coupling events. The capacity of the high affinity site in the absence of agonist (~5 fmol mg^{-1} protein at a receptor density of ~300 fmol mg^{-1} protein) was similar to the total increment of binding generated by the transfected receptor. Moreover, both the K_D value of [^{35}S]GTPγ[S] to membranes from transfected CHO cells (Newman-Tancredi *et al*., 2000) and the pIC$_{50}$ value of GTPγS for the high-affinity site (Table 1) were two orders of magnitude higher than the concentration of [^{35}S]GTPγ[S] that we used (0.1 nM), indicating that the absolute increases in [^{35}S]GTPγ[S] binding observed in the present study represented only a small fraction of G proteins activated per receptor. As discussed above, the capacity of the high affinity site was further increased by imetit. Since it represented ~20 fmol mg^{-1} protein versus ~5 fmol mg^{-1} protein in the presence and absence of imetit, respectively (at a receptor density of ~300 fmol mg^{-1} protein), it can be concluded that approximately 25% of hH$_3$Rs exist in a precoupled state. Similar values (29 and 20%, respectively) could be calculated from the constitutive increase in binding and from the binding induced by imetit at a receptor density of ~700 fmol mg^{-1} protein (~20 and ~70 fmol mg^{-1} protein, respectively) or ~1 pmol mg^{-1} protein (~20 and ~100 fmol mg^{-1} protein, respectively). These levels of precoupling are in the same range as those previously reported for other G$_i$-protein-coupled receptors (Neubig *et al*., 1988; Chen *et al*., 2000). Although it may be dependent on the receptor density and G-protein subtypes, the high affinity binding of GTPγS may allow to quantify the degree of constitutive activity and, therefore, the intrinsic activity of inverse agonists (Audinot *et al*., 2001). According to this model, ciproxifan, which did not abolish totally the high affinity component, would be acting as a partial inverse agonist at the hH$_3$R.

Previous functional or binding studies revealed distinct pharmacological profiles of the rat and human H$_3$Rs (Arrang *et al*., 1988; West *et al*., 1999; Lovenberg *et al*., 2000). Several agonists were found to be equipotent at both receptors but thioperamide and ciproxifan, tested as antagonists, displayed significantly higher potencies at the rat receptor when compared to the human receptor. These differences, ascribed to two amino acids in the third transmembrane domain (Ligneau *et al*., 2000), were confirmed here using another test. In agreement, whereas imetit increased [^{35}S]GTPγ[S] binding to membranes expressing the human receptor, with a potency very similar to that displayed at the rat autoreceptor (Garbarg *et al*., 1992), this effect was blocked by ciproxifan with an antagonist potency (K_i = 45 nM) consistent with that obtained using binding assays (K_i = 46 nM) (Ligneau *et al*., 2000), i.e., lower than that displayed at the rat autoreceptor (K_i = 0.5 nM) (Ligneau *et al*., 1998).

As expected from the extended ternary complex model (Samama *et al*., 1993; Lefkowitz *et al*., 1993; Weiss *et al*., 1996) and the higher affinity of inverse agonists for the inactive conformation of the receptor (Samama *et al*., 1994), ciproxifan was slightly more potent as an inverse agonist (EC$_{50}$ = 15 nM) than as an antagonist (K_i = 45 nM) at the human receptor. We recently reported a similar ratio between the inverse agonist and antagonist potencies of the drug at the rat receptor (EC_{50} and K_i values of 0.1 and 0.5 nM, respectively). It can be concluded that ciproxifan is less potent at the human than the rat receptor, not only as an antagonist, but also as an inverse agonist.

We recently established inverse agonism at native H_3Rs expressed at a normal level in mouse brain (Morisset *et al*., 2000). This observation brought direct evidence for the physiological relevance of constitutive activity of GPCRs (De Ligt *et al*., 2000). In agreement, [^{35}S]GTPγ[S] binding to mouse cerebral cortical membranes was decreased by FUB 465, thioperamide and ciproxifan, three inverse agonists whose effects were blocked by proxyfan, a neutral antagonist. Moreover, the constitutive activity of H_3Rs, as determined by the [^{35}S]GTPγ[S] binding assay, was further established for H_3 autoreceptors regulating histamine release *in vitro* and *in vivo* (Morisset *et al*., 2000). A very similar pattern of [^{35}S]GTPγ[S] binding was obtained in the present study using rat cerebral cortex and the same ligands. In addition, the magnitude of the decrease evoked by the three inverse agonists was similar in all rat brain regions studied, suggesting that constitutive activity of native H_3Rs occurs at the same level in all cerebral areas. As expected, the overall regional distribution of the imetit-induced binding response paralleled the known distribution of H_3Rs (Pollard *et al*., 1993). Consistent with autoradiographic studies (Laitinen & Jokinen, 1998), it was the highest in the striatum and cerebral cortex. All these findings show that [^{35}S]GTPγ[S] binding is a useful assay to analyse the interactions of native H_3 receptors with G proteins, inasmuch as the signaling pathways to which they couple in brain and peripheral tissues remain unclear (Hill *et al*., 1997).

Among authors suggesting an inverse agonism at native GPCRs (De Ligt *et al*., 2000), Costa & Herz (1989) were pioneers by demonstrating for the first time that ICI-174864, an antagonist at the δ opioid receptor, behaved in fact as an inverse agonist at the δ opioid receptor endogenously expressed in NG 108-15 cells. However, the same authors reported that the inverse agonism induced by the drug occurred only in isolated membranes but not in intact cells (Costa *et al*., 1992). They suggested that constraints imposed by cytosolic factors such as GTP prevent constitutive activity, making inevitable its observation in membranes upon separation from cytosol leading to removal of these factors. However, the present findings show that, in contrast to H_3 receptors, not all native receptors display constitutive activity in rat brain membranes. Indeed, in contrast to its inverse agonist effect on GTPase activity (Costa & Herz, 1989) and [^{35}S]GTPγ[S] binding (Szekeres & Traynor, 1997) in membranes of NG108-15 cells, ICI-174864 did not decrease [^{35}S]GTPγ[S] binding to membranes of rat cerebral cortex or striatum. These findings suggest that the threshold expression level for constitutive activity inherent to δ opioid receptors is not reached in the brain. The same interpretation may account for the apparent lack of effect of inverse agonists at D_2/D_3 dopamine, H_1 and H_2 histamine and α_2-adrenergic receptors, that we report here in brain membranes, in spite of the inverse agonism that they induce in cells (Nilsson & Eriksson, 1993; Griffon *et al*., 1996; Bakker *et al*., 2000; Alewijnse *et al*., 1998; Pauwels *et al*., 2000). It should be pointed out, however, that an apparent lack of inverse agonism does not furnish definitive evidence for the absence of constitutive activity since the intrinsic activity of a given inverse agonist is dependent on receptor systems and experimental conditions (Newman-Tancredi *et al*., 1997; Szekeres & Traynor, 1997; Pauwels *et al*., 1997; 2000; Audinot *et al*., 2001). Besides H_3Rs, A_1 adenosine receptors display a high constitutive activity in rat brain. Constitutive inhibition of adenylyl cyclase by A_1 adenosine receptors was previously shown using CPDPX. This drug known as a potent and highly selective antagonist, was acting as an inverse agonist not only at overexpressed receptors in CHO cells (Shryock *et al*., 1998) but also at endogenously expressed receptors of embryonic chick ventricular myocytes (Ma & Green, 1992). Although endogenous adenosine is present in high enough concentrations to stimulate [^{35}S]GTPγ[S] binding in rat brain sections (Laitinen & Jokinen, 1998), the decrease evoked by CPDPX in membranes is more likely to reflect high constitutive activity of cerebral A_1 receptors rather than antagonism of endogenous adenosine since it was observed after pretreatment of the membranes with adenosine deaminase.

In conclusion, the present study suggests that constitutive activity of native H_3Rs is one of the highest among GPCRs present in rat brain. Recombinant hH_3Rs also display high constitutive activity. The latter is easily detected at moderate concentrations, suggesting that it is present in human brain. Since we recently showed that constitutive activity of H_3Rs regulates histamine neurons in brain, the present findings further suggest that inverse agonists should find therapeutic applications. Ciproxifan, which is known to display distinct affinities at the rat and human receptors, is less potent not only as an antagonist, but also as an inverse agonist at the human receptor when compared to its rat counterpart. The evaluation of inverse agonist potency at human receptors should facilitate the rational design of novel compounds to be used in therapeutics.

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