Characterization of the binding of the first selective radiolabelled histamine H_3-receptor antagonist, [^{125}I]-iodophenpropit, to rat brain

[1]F.P. Jansen, T.S. Wu, H.-P. Voss, [2]H.W.M. Steinbusch, R.C. Vollinga, [3]B. Rademaker, A. Bast & H. Timmerman

Leiden/Amsterdam Center for Drug Research, Department of Pharmacochemistry, Faculty of Chemistry, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

1 The binding of the first selective radiolabelled histamine H_3-receptor antagonist [^{125}I]-iodophenpropit to rat cerebral cortex membranes was characterized.

2 [^{125}I]-iodophenpropit, radiolabelled to a high specific activity of 1900 Ci mmol^{-1}, saturably bound to a single class of sites with a K_D of 0.57 ± 0.16 nM ($n = 4$) and B_{max} of 268 ± 119 fmol mg^{-1} protein.

3 Specific binding at a concentration below 1 nM represented 50 to 60% of total binding.

4 Binding of [^{125}I]-iodophenpropit to rat cerebral cortex membranes was readily displaced by histamine H_3-agonists and antagonists. In contrast, the inhibitory potencies of selective histamine H_1- and H_2-receptor ligands were very low.

5 [^{125}]-iodophenpropit was biphasically displaced by the histamine H_3-receptor antagonists, burimamide and dimaprit, which may indicate the existence of histamine H_3-receptor subtypes. Other histamine H_3-receptor antagonists showed a monophasic displacement.

6 Competition binding curves of H_3-agonists were biphasic and showed a rightward shift upon the addition of the nonhydrolysable GTP analogue, guanosine $5'$-o-(3-thio) triphosphate (GTPγS; 100 μM) which implicates the interaction of histamine H_3-receptors with G-proteins. The affinities of the H_3-receptor antagonists iodophenpropit, thioperamide and burimamide were not altered by GTPγS.

7 Histamine competition binding curves were shifted to the right by different nucleotides (100 μM) with a rank order of potency GTPγS > Gpp(NH)p, GTP.

8 *In vitro* autoradiographic studies revealed a heterogeneous distribution of [^{125}I]-iodophenpropit binding sites in rat brain, with highest densities observed in specific cerebral cortical areas and layers, the caudate-putamen complex, the olfactory tubercles, the hippocampal formation, the amygdala complex, the hypothalamic area and the mammillary bodies.

9 It is concluded that the histamine H_3-receptor antagonist, [^{125}I]-iodophenpropit, meets the criteria for a suitable radioligand for histamine H_3-receptor binding studies in rat brain.

Keywords: Histamine H_3-receptor binding; radiolabelled H_3-receptor antagonist; [^{125}I]-iodophenpropit; rat cerebral cortex membranes; high affinity; guanine nucleotide shift; G-protein; autoradiography; distribution, histamine H_3-receptor subtypes

Introduction

The histamine H_3-receptor has been characterized as an autoreceptor inhibiting neuronal histamine release in the CNS, and as a heteroreceptor inhibiting the release of various neurotransmitters both in CNS and PNS (Leurs & Timmerman, 1992). Previously, in receptor binding experiments the agonists [^{3}H]-histamine, [^{3}H]-(R)α-methylhistamine and [^{3}H]-N$^\alpha$-methylhistamine were used to study the molecular pharmacology and distribution of histamine H_3-receptors. These studies suggested the interaction of histamine H_3-receptors with G-proteins. Moreover, from these experiments the presence of subtypes has been suggested (West *et al.*, 1990b). However, the binding profile of the radiolabelled agonists seems to be rather complex. Therefore, the development of radiolabelled histamine H_3-receptor antagonists is of utmost importance. Recently, a new class of highly potent histamine H_3-receptor antagonists has been described (Van der Goot *et al.*, 1992). Its most

potent member is clobenpropit (VUF9153) showing a pA_2-value of 9.9 as determined on the electrically contracted guinea-pig intestine. The series allowed the development of the first radiolabelled histamine H_3 receptor antagonist, [^{125}I]-iodophenpropit (Figure 1; Menge *et al.*, 1992). Preliminary results indicated that [^{125}I]-iodophenpropit is a promising tool for histamine H_3-receptor binding experiments (Jansen *et al.*, 1992). The present paper describes the full characterization of [^{125}I]-iodophenpropit as a radioligand for studying histamine H_3-receptors.

Methods

Preparation of rat cerebral cortex membranes

Male Wistar rats (200–250 g, Harlan C.P.B., Zeist, The Netherlands) were killed by decapitation and the brains were rapidly removed. The cerebral cortices were dissected and homogenized in 15 volumes (v/w) of ice-cold Tris-HCl buffer (50 mM Tris-HCl; 5 mM MgCl$_2$, 145 mM NaCl; pH 7.4 at 4°C) using an Ultra-Turrax homogenizer (8 s) and a glass-teflon homogenizer (four up and down strokes) subsequently. All subsequent steps preceding incubation were performed at a temperature of 0 to 4°C. The homogenate was centrifuged

[1] Author for correspondence.
[2] Research Institute Neurosciences, Department of Pharmacology, Faculty of Medicine, Vrije Universiteit, Amsterdam, The Netherlands.
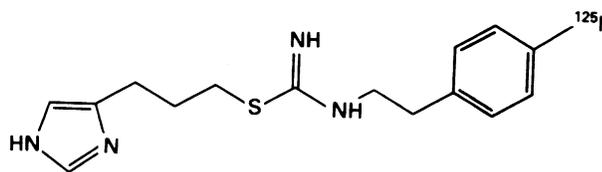[3] Rephartox bv., Fazantenkamp 83, 3607 CD Maarssen, The Netherlands

Figure 1 Structure of [^{125}I]-iodophenpropit.

at 800 g for 10 min. The pellet was discarded and the supernatant was centrifuged for 20 min at 40,000 g. The resulting pellet was resuspended and the last centrifugation step was repeated. The pellet was resuspended in 1.5 volumes (v/w) Tris-HCl buffer and stored at − 80°C. Before each receptor binding experiment the membranes were resuspended in Tris-HCl buffer and were centrifuged for 20 min at 40,000 g. Finally, the pellet was resuspended in 7 volumes (v/w) of incubation buffer (50 mM Tris-HCl containing 5 mM MgCl₂, 145 mM NaCl; pH 7.4 at 37°C. In some experiments NaCl was omitted).

Receptor binding experiments

Binding experiments were performed at 37°C in the Tris-HCl incubation buffer (pH 7.4) with a total incubation volume of 0.5 ml, using polyethylene tubes. Determinations were performed in triplicate. Drugs (except for thioperamide, see drugs and chemicals) were prepared in incubation buffer. Rat cerebral cortex membranes were incubated for 60 min to reach equilibrium (Jansen et al., 1992). In saturation experiments membranes were incubated with [^{125}I]-iodophenpropit in final concentrations ranging from 0.025 to 3 nM. In competition binding experiments a concentration of 0.25 nM [^{125}I]-iodophenpropit was used. Specific binding was defined as the difference between total binding and binding in the presence of 0.3 μM thioperamide and represented 50 to 60% of total binding. [^{125}I]-iodophenpropit was displaced to the same level by all of the histamine H₃-receptor ligands tested. Moreover, at this level [^{125}I]-iodophenpropit binding was not further displaced upon the addition of 0.1 μM thioperamide.

Incubations were started by the addition of 100 μl membranes (20–60 μg of protein per tube) and were terminated after 60 min by adding 2 ml of ice-cold Tris-HCl buffer (pH 7.4 at 4°C) immediately followed by filtration through Whatman GF/C filters using a Brandel filtration apparatus. Filters were pretreated (for 2 h) with 0.3% polyethyleneimine, reducing filter binding to less than 1% of the total radioactivity added. After filtration of the membranes the filters were washed twice with 2 ml of ice-cold Tris-HCl buffer. The amount of radioactivity bound to the membranes was not reduced by repetition of the washing procedure up to five times. The radioactivity bound to the filters was measured by an LKB gamma counter.

Data analysis

Saturation and competition binding experiments were evaluated using the non-linear curve fitting programme LIGAND (Munson & Rodbard, 1980) on a Macintosh computer. With the aid of this programme binding curves were fitted (unweighed) to a one and a two site model respectively, and statistically tested on the increasing goodness of the fit for a model with additional parameters, based on the 'extra sum of squares' principle (Draper & Smith, 1966) using a probability level of 5%.

Protein assays

Protein concentrations were determined with the Bio-Rad Protein Assay (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard.

Receptor autoradiography

Male Wistar rats (200–250 g, Harlan C.P.B., Zeist, The Netherlands) were killed by decapitation and the brains were removed. Brains were frozen in liquid CO₂. Cryostat sections (14 μm) were mounted on gelatine/chromalum-coated glass slides and stored at − 80°C before use. The brain sections were incubated with 0.3 nM [^{125}I]-iodophenpropit for 60 min at 37°C in 50 mM Tris-HCl buffer containing 145 mM NaCl, 5 mM MgCl₂ and 0.25% BSA (pH 7.4 at 37°C). Nonspecific binding was determined by incubation of adjacent sections in the presence of 0.3 μM thioperamide. To stop the incubation the slices were washed twice for 15 min in ice-cold Tris-HCl buffer (50 mM Tris-HCl; 5 mM MgCl₂, 145 mM NaCl; pH 7.4 at 4°C) and 15 s in ice-cold distilled water. Sections were dried by a stream of cold air and were exposed to Hyperfilm, (Amersham International, U.K.) for 20 h.

Drugs and chemicals

[^{125}I]-iodophenpropit was labelled to a specific activity of 1900 Ci mmol⁻¹ as described by Menge et al. (1992). The radiolabelled compound was stored at 4°C in a 1 mM H₂SO₄ solution in ethanol. H₂SO₄ did not affect the pH of the Tris-HCl buffer at the final concentrations of the radioligand used. High performance liquid chromatography (h.p.l.c.) analysis two months after preparation revealed that the radioactivity was almost quantitatively present in one peak with a retention time corresponding to the radioligand.

The following drugs and chemicals were used: thioperamide (Schering Corporation), iodophenpropit dihydrobromide (laboratory stock), clobenpropit dihydrobromide (VUF9153, laboratory stock), impromidine trihydrochloride (SK&F Laboratories), burimamide (SK&F Laboratories), dimaprit dihydrochloride (SK&F Laboratories), histamine dihydrochloride (Sigma), (R)α-methylhistamine maleate (gift from Prof. Dr J.-C. Schwartz, Paris), (S)α-methylhistamine dihydrobromide (Cookson Chemicals), imetit dihydrobromide (VUF8325, laboratory stock), immepip dihydrobromide (VUF4708; 4-(1H-imidazol-4-ylmethyl)piperidine dihydrobromide, laboratory stock), 5'-guanylylimidodiphosphate (Gpp(NH)p) (Sigma), guanosine 5'-o-(3-thio)triphosphate (GTPγS) (Sigma), guanosine 5'-triphosphate (GTP) (Sigma), betahistine dihydrochloride (Solvay Duphar), mepyramine hydrochloride (Sigma), triprolidine hydrochloride (Sigma), amthamine dihydrobromide (laboratory stock), tiotidine (ICI), imidazole (Merck) and polyethylenimine (Aldrich-Chemie). Drugs were dissolved in distilled water or in Tris-HCl buffer except for thioperamide which was diluted in buffer from 1 mM stock solutions prepared in dimethylsulphoxide (DMSO). At the final concentrations used, DMSO did not affect [^{125}I]-iodophenpropit receptor binding.

Statistical analysis

Dissociation constants for agonists in the absence and presence of guanine nucleotides were compared by Student's one tailed, unpaired t test. The effect of NaCl on dissociation constants for antagonist was analysed by Student's two tailed, unpaired t test. Differences were considered using a probability level of 5%.

Results

[^{125}I]-iodophenpropit saturation binding experiments
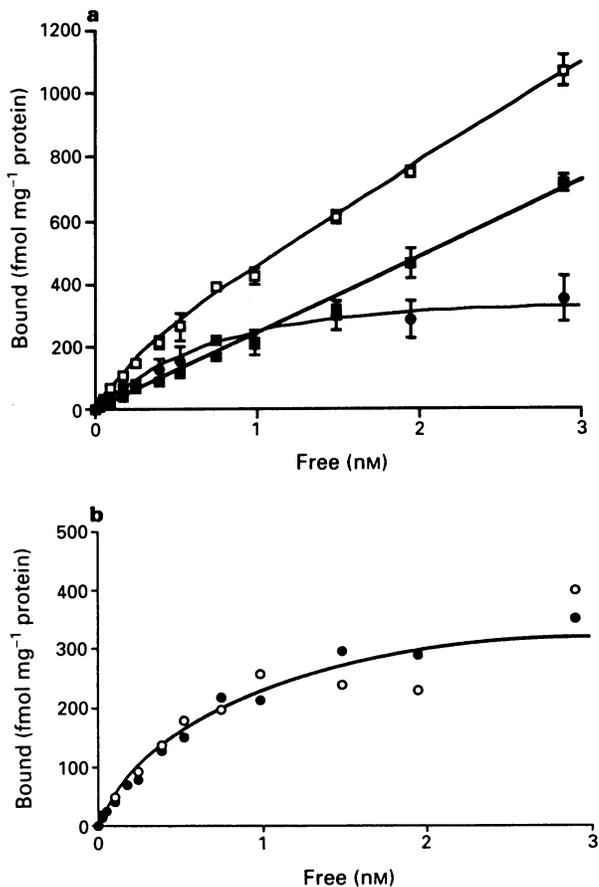
Specific binding of [^{125}I]-iodophenpropit (0.025–3 nM) to rat cerebral cortex membranes was saturable (Figure 2a) and yielded linear Scatchard plots. Computer analysis of the saturation binding curves revealed that [^{125}I]-iodophenpropit bound to a single class of sites with a K_D of 0.57 ± 0.16 nM and a B_{max} of 268 ± 119 fmol mg⁻¹ of protein (n = 4). Hill

coefficients were not significantly different from unity (1.00 ± 0.02). Non-specific binding determined in the presence of 0.3 μM thioperamide was linear with the [^{125}I]-iodophenpropit concentration up to 3 nM. Specific binding at concentrations below 1 nM accounted for 50 to 60% of total binding.

Both dissociation constant and receptor density of the [^{125}I]-iodophenpropit binding sites were unaffected by the non-hydrolysible GTP analogue 5'-guanylylimidodiphosphate (Gpp(NH)p) in a concentration of 100 μM ($K_D = 0.55 \pm 0.19$ nM; $B_{max} = 272 \pm 101$ fmol mg^{-1} protein; $n = 4$) (Figure 2b).

Competition binding curves of H₃-antagonists

[^{125}I]-iodophenpropit was displaced from rat cerebral cortex membranes by histamine H₃-receptor antagonists (Figure 3). The selective histamine H₃-receptor antagonists, clobenpropit (VUF9153) and iodophenpropit, showed the highest potency with K_D values of 0.9 ± 0.4 nM and 1.0 ± 0.1 nM respectively (Table 1). Omitting sodium chloride from the buffer significantly reduced the affinity of iodophenpropit and thioperamide for [^{125}I]-iodophenpropit binding sites (Table 1). Competition binding curves of clobenpropit, iodophenpropit, thioperamide ($K_D = 4.3 \pm 1.6$ nM) and impromidine ($K_D = 51 \pm 9$ nM) fitted best to a one site model ($P > 0.05$).
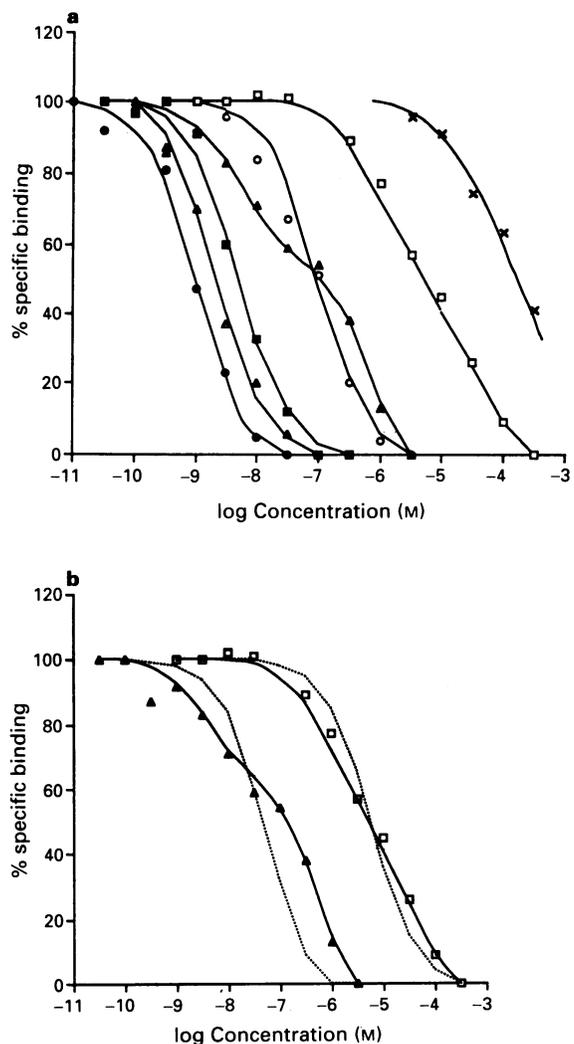
In contrast, competition binding curves of the histamine H₃-receptor antagonists burimamide and dimaprit were significantly better described by a two site model ($P < 0.05$; Figure 3b). The dissociation constants for the two different sites were 18 ± 9 nM and 725 ± 392 nM for burimamide and 0.42 ± 0.11 μM and 38 ± 37 μM for dimaprit (Table 2). The histamine H₃-receptor antagonist, betahistine, had a K_D value of 131 ± 37 μM (Figure 3a).

Displacement by other ligands

Selective ligands for histamine H₁- (mepyramine and triprolidine) and H₂-receptors (tiotidine and amthamine) and imidazole showed a very low affinity ($K_D > 10$ μM) towards the [^{125}I]-iodophenpropit binding sites (Table 1).

[^{125}I]-iodophenpropit displacement by H₃-agonists; the effect of guanine nucleotides

Competition binding curves for histamine H₃-receptor agonists were shallow and all fitted a two site model best

Figure 2 (a) Saturation binding of [^{125}I]-iodophenpropit (0.025–3 nM) to rat cerebral cortex membranes; (□) total binding; (■) non-specific binding; determined by the addition of 0.3 μM thioperamide (●) specific binding. (b) Specific binding of [^{125}I]-iodophenpropit to rat cerebral cortex membranes in the absence (●) and presence (O) of 100 μM Gpp(NH)p. The dissociation constant and receptor density in the absence of Gpp(NH)p were 0.57 ± 0.16 nM and 268 ± 119 fmol mg^{-1} protein, and in the presence of Gpp(NH)p 0.55 ± 0.19 nM and 272 ± 101 fmol mg^{-1} protein respectively ($n = 4$). Results shown are from one representative experiment with triplciate determinations.

Figure 3 (a) Inhibition of [^{125}I]-iodophenpropit binding (0.25 nM) by histamine H₃-receptor antagonists. (b) Competition binding curves for burimamide and dimaprit compared with the corresponding theoretical one site competition curves (dashed curves). Data are expressed as % specific binding. Specific binding was determined using 0.3 μM thioperamide and represented 50 to 60% of the total binding. Each curve represents a single representative experiment with triplicate determinations. Symbols used: (●) clobenpropit; (△) iodophenpropit; (■) thioperamide; (O) impromidine; (▲) burimamide; (□) dimaprit; (×) betahistine.

($P<0.05$), showing high and low affinity binding sites. The rank order of potency of the five agonists used for the high affinity sites was imetit ($K_H = 2.7 \pm 0.8$ nM), immepip ($K_H = 2.7 \pm 0.5$ nM), (R)α-methylhistamine ($K_H = 3.5 \pm 1.2$ nM) > histamine ($K_H = 38 \pm 10$ nM) > (S)α-methylhistamine ($K_H = 230 \pm 97$ nM) (Table 3).

Upon the addition of GTPγS (100 μM) the competition binding curves of the agonists showed a rightward shift. GTPγS completely abolished the high affinity sites for histamine, (R)α-methylhistamine and (S)α-methylhistamine (curves fitted best to a one site model, $P>0.05$; Table 3, Figure 4). For imetit and the new histamine H_3-receptor agonist, immepip (Vollinga *et al.*, 1994) a significant increase of the high affinity dissociation constant was observed upon addition of GTPγS (Table 3). With respect to competition binding curves for histamine, the nucleotides GTP and Gpp(NH)p at a concentration of 100 μM were less potent than GTPγS. Both GTP and Gpp(NH)p significantly increased the high affinity dissociation constants for histamine (Table 3). GTPγS (100 μM) did not affect the dissociation constants for the histamine H_3-receptor antagonists, iodophenpropit (K_D-value without GTPγS: 1.4 ± 0.4 nM, K_D value with GTPγS: 2.2 ± 0.8 mM, $n = 3$), thioperamide (K_D-value without GTPγS: 1.2 ± 0.4 nM, K_D value with GTPγS:

1.3 ± 0.7 nM, $n = 3$) and burimamide ($K_{D,1}$- and $K_{D,2}$- value without GTPγS: 18 ± 9 nM and 0.7 ± 0.4 μM respectively, $n = 8$; $K_{D,1}$ and $K_{D,2}$ value with GTPγS: 62 ± 44 nM and 3.1 ± 2.8 μM respectively, $n = 3$).

Distribution of [^{125}I]-iodophenpropit binding sites in rat brain studied by receptor autoradiography

Incubation of rat brain cryostat sections with [^{125}I]-iodophenpropit showed that the radioligand binding sites were heterogeneously distributed (Figure 5a–c). Non-specific binding, defined by incubation of adjacent sections in the presence of 0.3 μM thioperamide, was very low and homogeneously distributed (Figure 5d–f). The highest levels of specific binding were observed in the upper frontal layers and lower temporal layers of the cerebral cortex, the caudate-putamen complex, the olfactory tubercles, the hippocampal formation, the amygdala complex, the substantia nigra, the hypothalamic area and the mammillary bodies. Densities were low in the cerebellum.

Discussion

Recently, we described the preliminary pharmacological characterization of the first radiolabelled histamine H_3-receptor antagonists [^{125}I]-iodophenpropit showing it to be a potential tool for histamine H_3-receptor binding studies (Jansen *et al.*, 1992). In the present study, we describe the full characterization of the binding of [^{125}I]-iodophenpropit to rat brain. [^{125}I]-iodophenpropit meets the basic criteria for a suitable radioligand for receptor binding studies, i.e. high affinity,

Table 1 Affinities of different ligands towards [^{125}I]-iodophenpropit binding sites on rat cerebral cortex membranes and the effect of NaCl on histamine H_3-receptors antagonist affinity

Ligand	Tris-buffer + MgCl_2 (5 mM)		Tris-buffer + MgCl_2 (5 mM) + NaCl (145 mM)	
	(K_D nM)		(K_D, nM)	
H_3-antagonists:				
Clobenpropit	ND		0.93 ± 0.39	(3)
Iodophenpropit	1.7 ± 0.3	(3)	0.97 ± 0.06*	(3)
Thioperamide	11 ± 2.3	(7)	4.3 ± 1.6*	(7)
Impromidine	107 ± 68	(3)	51 ± 9	(3)
Other ligands:				
Mepyramine	> 10,000	(5)	ND	
Triprolidine	> 10,000	(3)	ND	
Tiotidine	> 10,000	(4)	ND	
Amthamine	> 10,000	(2)	ND	
Imidazole	> 10,000	(2)	ND	

All compounds listed were fitted best to a one-site model ($P>0.05$). Values are given as the mean ± s.d. (number of experiments). ND; not determined. *$P<0.05$ as compared with buffer without NaCl.

Table 2 Affinities of histamine H_3-receptor antagonists discriminating between two [^{125}I]-iodophenpropit binding sites

Antagonist				
Burimamide	$K_{D,1}$ (nM)	R_1 (%)	$K_{D,2}$ (nM)	R_2 (%)
	18 ± 9	54 ± 10	725 ± 392	46 ± 9
Dimaprit	$K_{D,1}$ (μM)	R_1 (%)	$K_{D,2}$ (μM)	R_2 (%)
	0.42 ± 0.11	52 ± 6	38 ± 37	47 ± 6

Values are given as the mean ± s.d. of eight (burimamide) and five (dimaprit) separate experiments with triplicate determinations. $K_{D,1}$ and $K_{D,2}$ are the dissociation constants for the two different binding sites, R_1 and R_2 the corresponding percentages of each site.

Table 3 The effect of guanine nucleotides on the high and low affinity binding sites of histamine H_3-receptor agonists

Agonist:	Nucleotide	High affinity		Low affinity	
		K_H (nM)	R_H (%)	K_L (μM)	R_L (%)
Histamine	–	38 ± 10	52 ± 4	2.5 ± 0.6	48 ± 4
	GTP	85 ± 35*	51 ± 8	2.5 ± 0.9	49 ± 8
	Gpp(NH)p	137 ± 100*	42 ± 13	3.2 ± 1.5	58 ± 13
	GTPγs	–	–	0.8 ± 0.1*	100**
(R)α-methylhistamine	–	3.5 ± 1.2	52 ± 4	1.2 ± 0.3	48 ± 3
	GTPγs	–	–	0.1 ± 0.1*	100**
(S)α-methylhistamine	–	230 ± 97	39 ± 11	9.5 ± 1.8	61 ± 5
	GTPγs	–	–	12 ± 3.9	100**
Imetit	–	2.7 ± 0.8	59 ± 5	40 ± 12	41 ± 2
	GTPγs	18 ± 10*	52 ± 14	35 ± 20	48 ± 13
Immepip	–	2.7 ± 0.5	63 ± 3	1.0 ± 0.2	37 ± 2
	GTPγs	20 ± 5*	67 ± 5	3.4 ± 3.4	33 ± 8

Values are given as the mean ± s.d. of six (histamine ± GPP(NH)p) or four (remaining) separate experiments with triplicate determinations. The concentrations of GTP, Gpp(NH)p and GTPγS were 100 μM. K_H and K_L correspond to the dissociation constants for the high and low affinity binding sites respectively. R_H and R_L are the percentages of high and low affinity sites. *$P<0.05$ as compared to value without the nucleotide. **curve best fitted to a one-site model ($P>0.05$).
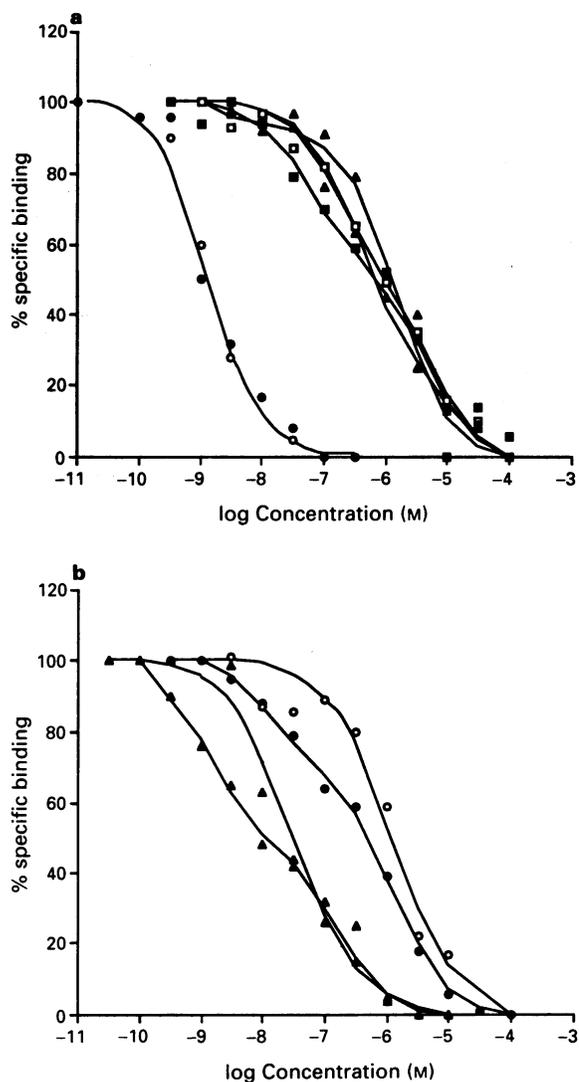
Figure 4 The effect of different nucleotides on displacement of [^{125}I]-iodophenpropit (0.25 nM). (a) The effect of nucleotides on histamine (HA) and thioperamide competition binding curves; (●) thioperamide; (○) thioperamide + GTPγS; (■) HA; (△) HA + GTP; (□) HA + Gpp(NH)p; (▲) HA + GTPγS: (b) The effect of GTPγS on (R) and (S)α-methylhistamine competition binding curves; (▲) (R)α-methylhistamine; (△) (R)α-methylhistamine + GTPγS; (●) (S)α-methylhistamine; (○) (S)α-methylhistamine + GTPγS. Curves displayed are from representative experiments, each point determined in triplicate. The concentration of the nucleotides used was 100 μM.

saturability, reversibility, selectivity and high specific activity. The pK_D value (9.3) observed in saturation binding experiments is close to the pA$_2$ value (9.6) determined on the guinea-pig intestine. Binding of the radioligand to histamine H$_3$-receptors was validated by competition binding studies using various selective histamine receptor ligands and by receptor autoradiography.

The binding results obtained with [^{125}I]-iodophenpropit largely correlate to data obtained from receptor binding studies using the radiolabelled agonists [^{3}H]-histamine, [^{3}H]-N^π-methylhistamine and [^{3}H]-(R)α-methylhistamine; however, some clear discrepancies also exist. The receptor density found using [^{125}I]-iodophenpropit (268 fmol mg^{-1} protein) was 3 to 8 fold higher than the densities reported using radiolabelled agonists (30 to 80 fmol mg^{-1} protein). The observation that the histamine H$_3$-receptor antagonists, clobenpropit, iodophenpropit, thioperamide and impromidine displaced [^{125}I]-iodophenpropit according to a one site model is a clear indication that all displaced binding represents

histamine H$_3$-receptors. Considering the variation in chemical structure of the four ligands mentioned, it seems unlikely that the high densities observed using [^{125}I]-iodophenpropit can be explained by binding of the radioligand to an additional high affinity binding component in the membrane preparation for which all these compounds have the same affinity as for the histamine H$_3$-receptor. Another indication that the specific [^{125}I]-iodophenpropit binding sites represent histamine H$_3$-receptors is the stereoselective displacement by (R)α-methylhistamine and (S)α-methylhistamine. Moreover, in our laboratory, receptor densities of 200 fmol mg^{-1} protein were found using [^{3}H]-N^π-methylhistamine (P. Kuyt, personal communication, 1991). Hence, the differences between B_{max}-values obtained with the currently used radiolabelled H$_3$-agonists compared with the B_{max}-values obtained with [^{125}I]-iodophenpropit might be the result of differences in receptor isolation and incubation procedures.

Displacement of [^{125}I]-iodophenpropit by the histamine H$_3$-receptor antagonists, burimamide and dimaprit, was shown to be biphasic. This phenomenon may be due to discrimination between subtypes of histamine H$_3$-receptors by these two compounds. A two site displacement of radiolabelled histamine H$_3$-agonists by burimamide in rat brain has previously been reported using [^{3}H]-(R)α-methylhistamine (rat cerebral cortex membranes; Arrang et al., 1990) and [^{3}H]-N^π-methylhistamine (whole rat brain membranes; West et al., 1990b). However, in another report in which [^{3}H]-(R)α-methylhistamine was used (whole rat brain membranes; West et al., 1990a) burimamide displacement was monophasic. A two site displacement has also been reported for thioperamide (West et al., 1990b), but was not observed in other studies. Variation in the experimental conditions (membrane preparation, composition of the buffer) might account for the discrepancies. Considering the different results, the existence of histamine H$_3$-receptor subtypes from receptor binding studies needs to be explored further. To validate the existence of possible subtypes, more ligands are needed which clearly discriminate between possible subtypes.

The present study provides evidence for the interaction of histamine H$_3$-receptors with G-proteins. Displacement of [^{125}I]-iodophenpropit by histamine H$_3$-receptor agonists was biphasic and was modified by guanine nucleotides. Hence, the two sites found for agonists is probably related to the formation of a ternary complex between the agonist, the receptor and a G-protein rather than discrimination between receptor subtypes by these agonists. The observation that biphasic competition binding curves for the histamine H$_3$-receptor antagonist, burimamide, was not affected by GTPγS is in accordance with this conclusion. The results obtained with [^{125}I]-iodophenpropit to characterize histamine H$_3$-receptors were similar to those from binding studies to other G-protein coupled receptors, such as α- (Weinshank et al., 1990) and β-adrenoceptors (Voss et al., 1992), histamine H$_1$-(Hattori et al., 1991) and histamine H$_2$-receptors (Ruat et al., 1990), using radiolabelled antagonists. Generally, guanine nucleotides bind to G-proteins, inducing the uncoupling of the G-protein-receptor complex. In a receptor binding study this shows up as a conversion of agonist high affinity binding sites into low affinity binding sites, since the high affinity binding sites represent the binding of an agonist to the receptor – G-protein complex and the low affinity binding sites represent the binding of an agonist to the receptor alone. In our experiments GTPγS completely abolished the high affinity sites for histamine, (R)α-methylhistamine and (S)α-methylhistamine. However, a significant increase of the high affinity dissociation constant of imetit and immepip was observed upon addition of GTPγS. Similar results were described for α$_2$-adrenoceptors (Weinshank et al., 1990). These observations are different from the generally observed reduction of the ratio between the amount of high and low affinity binding sites by guanine nucleotides. At present, the explanation for these differences remains to be established.

The interaction of histamine H$_3$-receptors with G-proteins
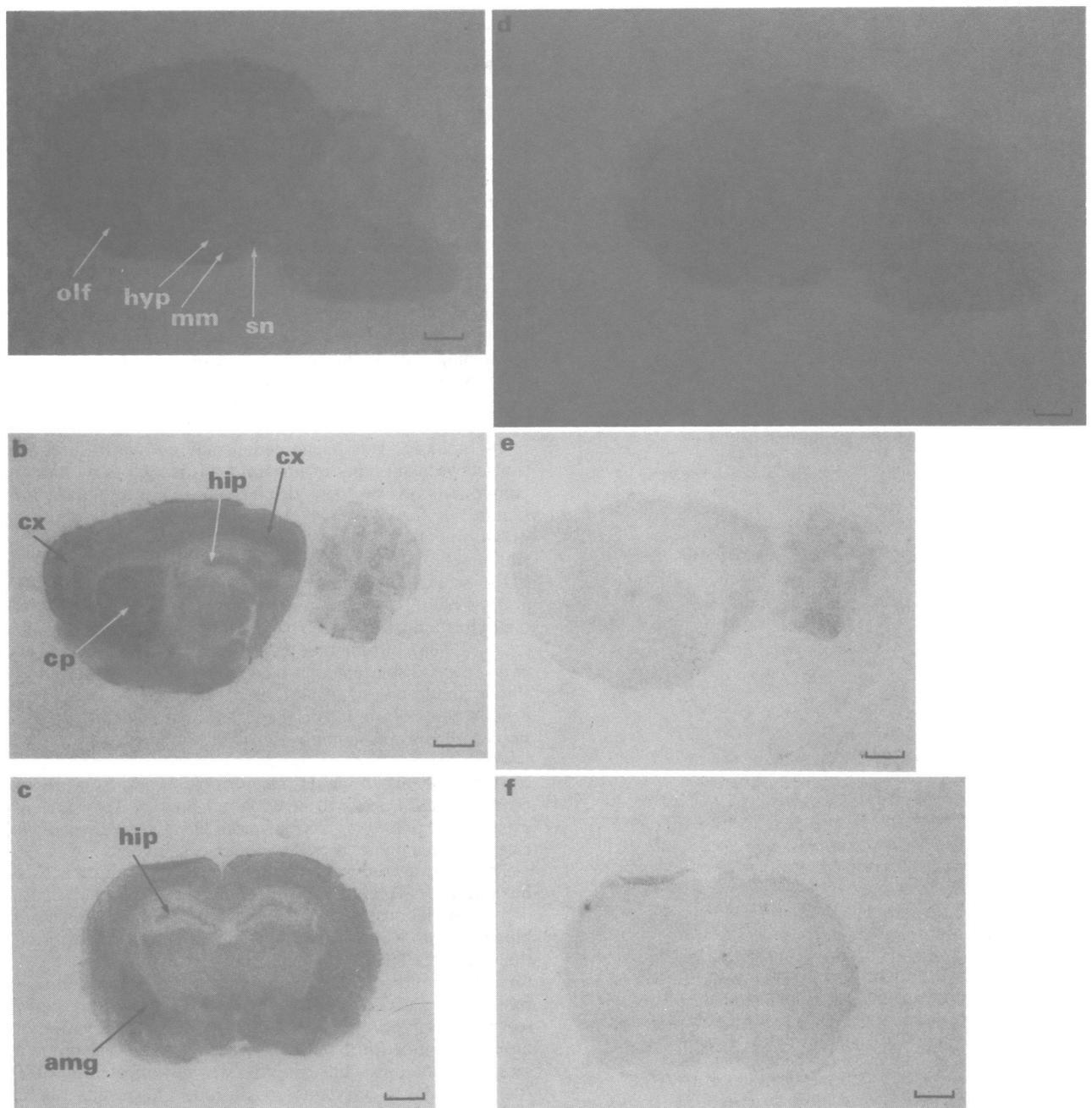
Figure 5 Autoradiographic localization of [^{125}I]-iodophenpropit binding sites in rat brain. On the left side (a to c) total binding is shown using 0.3 nM [^{125}I]-iodophenpropit (a and b: sagittal sections; c: transverse section). On right side (d to f), the corresponding non-specific binding is shown as defined by the inclusion of 0.3 μM thioperamide. Indicated areas: caudate-putamen (cp), hippocampal formation (hip), amygdala complex (amg), substantia nigra (sn), hypothalamic region (hyp), cortex (cx), olfactory tubercles (olf) and mammillary bodies (mm).

has also been indicated by binding studies using the tritium labelled agonists histamine, (R)α-methylhistamine and N^{α}-methylhistamine. In 1980, Barbin *et al.* described the high affinity binding of [^{3}H]-histamine to rat brain. Although at that time histamine H$_{3}$-receptors were not yet identified, the [^{3}H]-histamine binding sites presumably represented histamine H$_{3}$-receptors. In their study, the density of the [^{3}H]-histamine high affinity binding sites was reduced by Gpp(NH)p without altering the affinity. More recently, a reduction of [^{3}H]-N^{α}-methylhistamine binding by GTPγS was observed which may be due both to a reduced density and affinity of [^{3}H]-N^{α}-methylhistamine binding sites (West *et al.*, 1990b). In this last study biphasic displacement of the radiolabelled agonist by thioperamide and burimamide was observed. As the low affinity sites of both antagonists were

abolished by GTPγS it was concluded that [^{3}H]-N^{α}-methylhistamine bound to two subtypes of histamine H$_{3}$-receptors (H$_{3A}$ and H$_{3B}$) of which one was not detectable in the presence of GTPγS. Their conclusion is not in agreement with the results of our experiments using [^{125}I]-iodophenpropit, as GTPγS had no effect on displacement curves for burimamide. In another report an effect of guanine nucleotides on [^{3}H]-(R)α-methylhistamine binding was observed only when calcium was present in the incubation medium (Arrang *et al.*, 1990). When calcium was included in the buffer saturation curves showed a high and low affinity component, the latter being abolished by Gpp(NH)p. From these rather unexpected results the authors suggested that the low affinity site may be its functional receptor. Reduction of [^{3}H]-N^{α}-methylhistamine binding sites by Gpp(NH)p has also

Table 4 The affinity of histamine H₃-receptor agonists and antagonists for [¹²⁵I]-iodophenpropit binding sites on rat cerebral cortex compared with their functional potencies

H₃-antagonists:	(pK_D)	(pA_2)
Clobenpropit	9.0	9.9[a]
Iodophenpropit	9.0	9.6[b]
Thioperamide	8.4	8.5[a]
Impromidine	7.3	7.2[c]

	(p$K_{D,1}$)	(p$K_{D,2}$)	(pA_2)
Burimamide	7.7	6.1	7.2[c]
Dimaprit	6.4	4.4	5.5[c]

H₃-agonists:	(pK_D)	(pD_2)
Histamine	7.4	7.4[c]
(R)α-methylhistamine	8.5	8.4[c]
(S)α-methylhistamine	6.6	6.3[c]
Imetit	8.7	9.3[c]
Immepip	8.6	8.0[d]

The affinities of the various ligands for the [¹²⁵I]-iodophenpropit binding sites are expressed as $-\log K_D$ ($= pK_D$). The pK_D-value for agonists correspond to their high affinity binding site. The functional potencies of H₃-receptor agonists and antagonists are expressed as pD_2 and pA_2 values respectively.
[a]Determined on guinea-pig intestine (Van Der Goot et al., 1992); [b]Determined on guinea-pig intestine (Jansen et al., 1992); [c]Determined on rat cerebral cortex (Leurs et al., 1992); [d]Determined on guinea-pig jejunum (Vollinga et al., 1994).

been shown by in vitro receptor autoradiography (Cumming et al., 1991). Summarizing, various studies using radio-labelled histamine H₃-receptor agonists provided evidence for coupling of the histamine H₃-receptor to a G-protein. However, the binding characteristics of these agonists are complex and results obtained by several authors appear to be to a certain extent controversial.

The affinities of histamine H₃-receptor antagonists largely correlate to the antagonistic activities obtained from functional studies (Table 4). With respect to histamine H₃-receptor agonists, the agonistic activities are closely related to their high affinity binding sites (Table 4). In contrast, in binding studies using radiolabelled agonists, the dissociation constants of histamine H₃-receptor agonists reported are approximately 10 fold lower than corresponding pD_2-values. Hence, the pK_D-values observed in these receptor binding studies may not reflect their affinity for the functional histamine H₃-receptor.

Recently, a detailed description of the distribution of histamine H₃-receptors in rat brain was published using [³H]-(R)α-methylhistamine as a radioligand (Pollard et al., 1993). The distribution of [¹²⁵I]-iodophenpropit binding sites we described in the present study was essentially the same as for [³H]-(R)α-methylhistamine observed by Pollard et al. (1993). Histamine H₃-receptors showed a clearly distinct distribution compared to histamine H₁- and H₂-receptors. Moreover, the distribution of histamine H₃-receptors seemed to be distinct from the distribution of histaminergic nerve endings in rat brain (Watanabe et al., 1984). This observation may be explained by the occurrence of the histamine H₃-receptor as a heteroreceptor. The histamine H₃-receptor was first characterized as an autoreceptor inhibiting both synthesis and release of neuronal histamine (Arrang et al., 1983; Van Der Werf et al., 1987). However, several studies have indicated the involvement of histamine H₃-receptors in modulation of the release of other neurotransmitters such as 5-hydroxy-tryptamine (Fink et al., 1990), noradrenaline (Schlicker et al., 1989) and acetylcholine (Clapham & Kilpatrick, 1992; Vollinga et al., 1992).

In conclusion, [¹²⁵I]-iodophenpropit binds to rat cerebral cortex membranes with a high affinity, saturability and reversibility. [¹²⁵I]-iodophenpropit binding sites in rat brain are likely to represent histamine H₃-receptors as the binding is displaced by selective histamine H₃-receptor ligands, in contrast to ligands selective for other receptors. Biphasic displacement of [¹²⁵I]-iodophenpropit by burimamide and dimaprit may be indicative of the existence of histamine H₃-receptor subtypes. The sensitivity of histamine H₃-receptor agonist competition binding curves to guanine nucleotides confirms the results of previous experiments indicating the interaction of histamine H₃-receptors with G-proteins. The distribution of [¹²⁵I]-iodophenpropit binding sites in rat brain is heterogeneous and essentially the same as previously reported for the selective histamine H₃-receptor agonist [³H]-(R)α-methylhistamine. [¹²⁵I]-iodophenpropit is a valuable new tool for studying histamine H₃-receptors.

References

ARRANG, J.-M., GARBARG, M. & SCHWARTZ, J.-C. (1983). Auto-inhibition of brain histamine release mediated by a novel class (H₃) of histamine receptor. Nature, **302**, 832–837.

BARBIN, G., PALACIOS, J.M., RODERGAS, E., SCHWARTZ, J.C. & GARBARG, M. (1980). Characterization of the high-affinity binding sites of [³H]histamine in rat brain. Mol. Pharmacol., **18**, 1–10.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem., **72**, 248–254.

CLAPHAM, J. & KILPATRICK, G.J. (1992). Histamine H₃-receptors modulate the release of [³H]-acetylcholine from slices of rat entorhinal cortex – evidence for the possible existence of H₃-receptor subtypes. Br. J. Pharmacol., **107**, 919–923.

CUMMING, P., SHAW, C. & VINCENT, S.R. (1991). High affinity histamine binding site is the H₃ Receptor – characterization and autoradiographic localization in rat brain. Synapse, **8**, 144–151.

DRAPER, N.R. & SMITH, H. (1966). In Applied Regression Analysis. New York: Wiley.

FINK, K., SCHLICKER, E., NEISE, A. & GOTHERT, M. (1990). Involvement of presynaptic H₃ receptors in the inhibitory effect of histamine on serotonin release in the rat brain cortex. Naunyn-Schmied. Arch. Pharmacol., **342**, 513–519.

HATTORI, Y., ENDOU, M., GANDO, S. & KANNO, M. (1991). Identification and characterization of histamine H₁- and H₂-receptors in guinea-pig left atrial membranes by [³H]-mepyramine and [³H]-tiotidine binding. Br. J. Pharmacol., **103**, 1573–1579.

JANSEN, F.P., RADEMAKER, B., BAST, A. & TIMMERMAN, H. (1992). The first radiolabeled histamine H₃ receptor antagonist. [¹²⁵I]iodophenpropit: saturable and reversible binding to rat cortex membranes. Eur. J. Pharmacol., **217**, 203–205.

LEURS, R. & TIMMERMAN, H. (1992). The histamine H₃-receptor: a target for developing new drugs. Prog. Drug Res., **39**, 127–165.

MENGE, W.M.P.B., VAN DER GOOT, H., TIMMERMAN, H., EERSELS, J.L.H. & HERSCHEID, J.D.M. (1992). Synthesis of S-<3-(4(5)-imidazolyl)propyl>,N-<2-(4-(I-125))-iodophenyl)ethyl> isothiourea sulfate (I-125-Iodophenpropit), a new probe for histamine-H₃-receptor binding sites. J. Label. Compd. Radiopharm., **31**, 781–786.

MUNSON, P.J. & RODBARD, D. (1980). A versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem., **107**, 220–239.

POLLARD, H., MOREAU, J., ARRANG, J.-M. & SCHWARTZ, J.-C. (1993). A detailed autoradiographic mapping of histamine-H-3 receptors in rat brain areas. Neuroscience, **52**, 169–189.

RUAT, M., TRAIFFORT, E., BOUTHENET, L., SCHWARTZ, J.C., HIRS-CHFELD, J., BUSCHAUER, A. & SCHUNACK, W. (1990). Reversible and irreversible labeling and autoradiographic localization of the cerebral histamine H_2-receptor using [^{125}I]iodinated probes. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 1658–1662.

SCHLICKER, E., FINK, K., HINTERHANER, M. & GOTHERT, M. (1989). Inhibition of noradrenaline release in the rat brain cortex via presynaptic H_3-receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 633–638.

VAN DER GOOT, H., SCHEPERS, M.J.P., STERK, G.J. & TIMMERMAN, H. (1992). Isothiourea analogues of histamine as potent agonists or antagonists of the histamine H_3 receptor. *Eur. J. Med. Chem.*, **27**, 511–517.

VAN DER WERF, J.F., BAST, A., BIJLOO, G.J., VAN DER VLIET, A. & TIMMERMAN, H. (1987). HA autoreceptor assay with superfused slices of rat brain cortex and electrical stimulation. *Eur. J. Pharmacol.*, **138**, 199–206.

VOLLINGA, R.C., DE KONING, J.T., JANSEN, F.P., LEURS, R., MENGE, W.M.P.B. & TIMMERMAN, H. (1994). A new potent and selective histamine H_3-receptor agonist, 4-(1*H*-imidazol-4-ylmethyl)piperidine. *J. Med. Chem.*, **37**, 332–333.

VOLLINGA, R.C., ZUIDERVELD, O.P., SCHEERENS, H., BAST, A. & TIMMERMAN, H. (1992). A simple and rapid in vitro test system for the screening of histamine H_3 ligands. *Methods Find. Exp. Clin. Pharmacol.*, **14**, 747–751.

VOSS, H.-P., DONNELL, D. & BAST, A. (1992). Atypical molecular pharmacology of a new long-acting β_2-adrenoceptor agonist. TA2005. *Eur. J. Pharmacol.*, **227**, 403–409.

WATANABE, T., TAGUCHI, Y., SHIOSAKA, S., TANAKA, J., KUBOTA, H., TERANO, Y., TOHYAMA, M. & WADA, H. (1984). Distribution of the histaminergic neuron system in the central nervous system of rats; a fluorescent immunohistochemical analysis with histidine decarboxylase as a marker. *Brain Res.*, **295**, 13–25.

WEINSHANK, R.L., ZGOMBICK, J.M., MACCHI, M., ADHAM, N., LICHTBLAU, T., BRANCHEK, A. & HARTIG, P.R. (1990). Cloning, expression, and pharmacological characterization of a human α_{2B}-adrenergic receptor. *Mol. Pharmacol.*, **38**, 681–688.

WEST, R.E., ZWEIG, A., GRANZOW, R.T., SIEGEL, M.I. & EGAN, R.W. (1990a). Biexponential kinetics of (R)-α-[^{3}H]methylhistamine binding to the rat brain histamine receptor. *J. Neurochem.*, **55**, 1612–1616.

WEST, R.E., ZWEIG, S., SHIH, N.-Y., SIEGEL, M.I., EGAN, R.W. & CLARCK, M.A. (1990b). Identification of two H_3-histamine receptor subtypes. *Mol. Pharmacol.*, **38**, 610–613.