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[^{3}H]-thioperamide as a radioligand for the histamine H_3 receptor in rat cerebral cortex

Alexandra Alves-Rodrigues, [1]Rob Leurs, Tin-Seng Wu, *George D. Prell, †Christian Foged & Henk Timmerman

Leiden/Amsterdam Center for Drug Research, Department of Pharmacochemistry, Faculty of Chemistry, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands; *Dept. Pharmacology, Mount Sinai School of Medicine of the City University of New York, New York, N.Y. 10029, U.S.A.; and †Isotope Chemistry Department, Novo Nordisk A/S, Novo Nordisk Park, 2760 Måløv, Denmark

1 The purpose of the present study was to characterize the binding of the histamine H_3 receptor antagonist, [^{3}H]-thioperamide, to rat cerebral cortical membranes.

2 The binding of [^{3}H]-thioperamide to rat cerebral cortical membranes reached equilibrium after incubation with [^{3}H]-thioperamide after 8-10 h at 4°C. Equilibrium was maintained for up to 18 h of incubation. Addition of 1 μM **(R)**-α-methylhistamine rapidly dissociated [^{3}H]-thioperamide from its binding sites. From these kinetic experiments a dissociation constant of 0.3 nM was obtained for [^{3}H]-thioperamide.

3 Saturation experiments with [^{3}H]-thioperamide using 1 μM **(R)**-α-methylhistamine to define nonspecific binding were best analysed according to a single site model. A dissociation constant (K_D) of 0.80 ± 0.06 nM ($n=3$) and a maximal number of binding sites (B_{max}) of 73 ± 20 fmol mg^{-1} protein ($n=3$) were obtained for the binding of [^{3}H]-thioperamide to rat cerebral cortical membranes.

4 Saturation experiments with [^{3}H]-thioperamide using 0.3 μM iodophenpropit to define nonspecific binding were best analysed according to a two site model. For the high affinity [^{3}H]-thioperamide site a K_D value of 1.1 ± 0.3 nM ($n=3$) and B_{max} value of 162 ± 108 fmol mg^{-1} protein ($n=3$) were obtained whereas K_D and B_{max} values for the low affinity site were 96 ± 19 nM and 4346 ± 3092 fmol mg^{-1} protein ($n=3$), respectively.

5 Using 5 nM [^{3}H]-thioperamide, the binding was hardly displaced by H_3 agonists within concentration-ranges expected to bind to the histamine H_3 receptor. Under these conditions, [^{3}H]-thioperamide binding was fully displaced by various H_3-antagonists, yet most H_3 antagonists showed K_i values different from those expected for the histamine H_3 receptor.

6 Using 0.3 nM [^{3}H]-thioperamide, 50-60% of the total binding was potently displaced by the H_3 agonists histamine, **(R)**-α-methylhistamine, **(S)**-α-methylhistamine, imetit and immepip. Displacement of the binding of 0.3 nM [^{3}H]-thioperamide binding exhibited clear stereoselectivity for the **R** and **S** isomers of α-methylhistamine.

7 Binding of 0.3 nM [^{3}H]-thioperamide was completely displaced by several H_3 antagonists (thioperamide, iodophenpropit, iodoproxyfan, and burimamide) and biphasic displacement curves were obtained; the K_i values for the high affinity site corresponded well with the expected values for the H_3 receptor. Antagonists fully displaced the binding of 5 nM [^{3}H]-thioperamide with affinities comparable to the low affinity site found with 0.3 nM [^{3}H]-thioperamide.

8 Ondansetron and haloperidol did not displace binding of 5 nM [^{3}H]-thioperamide at concentrations at which the former are known to bind to 5-HT_3 or σ receptors, respectively. On the other hand, nonselective cytochrome P_{450} inhibitors displaced the binding of 5 nM [^{3}H]-thioperamide from both rat cerebral cortical membranes and rat liver microsomes.

9 It is concluded that the histamine H_3 antagonist, [^{3}H]-thioperamide, can be used as a radioligand to study the histamine H_3 receptor in rat brain, provided that subnanomolar concentrations are used in displacement studies. Moreover, the specific binding should be defined with an H_3 agonist, since most H_3 antagonists share with [^{3}H]-thioperamide a low affinity, high density, non-H_3 receptor binding site(s) in rat brain. The latter is probably due to binding to cytochrome P_{450} isoenzymes.

Keywords: Histamine H_3-receptor; H_3 antagonist; [^{3}H]-thioperamide; stereoselectivity; rat cerebral cortex; cytochrome P_{450} isoenzymes

Introduction

The histamine H_3 receptor was originally identified as a presynaptic receptor that regulated the synthesis and release of histamine in the CNS (Arrang *et al.*, 1983; 1987; Van der Werf *et al.*, 1987). This receptor is also present as a heteroreceptor on nonhistaminergic neurones and is known to regulate the release of several other neurotransmitters such as 5-hydroxytryptamine (Schlicker *et al.*, 1988; Alves Rodrigues *et al.*, 1995), noradrenaline (Schlicker *et al.*, 1989), dopamine (Schlicker *et al.*, 1993) and acetylcholine (Clapham & Kilpatrick, 1992) in both the central and peripheral nervous systems (Bertaccini *et al.*, 1991; Barnes *et al.*, 1993). Concomitant with the initial pharmacological definition of this new histamine receptor subtype, Arrang *et al.* (1987) described **(R)**-α-methylhistamine and thioperamide as the first selective H_3 agonist and antagonist, respectively. Although many other potent and selective H_3-receptor agonists and antagonists have since been introduced (Leurs *et al.*, 1995b), the early availability of thioperamide made this compound one of the most used and

[1] Author for correspondence.

best characterized H_3 antagonists. In H_3 receptor binding studies, thioperamide shows an affinity for the H_3 receptor in the low nanomolar range (Arrang *et al.*, 1990; Jansen *et al.*, 1994). Yet, its interactions with 5-HT_3 and σ receptors have been observed at higher concentrations (Leurs *et al.*, 1995a). Furthermore, thioperamide has been shown to inhibit steroidogenesis by an interaction with cytochrome P_{450} isoenzymes (Labella *et al.*, 1992).

Acting as an H_3 antagonist, thioperamide has been reported to affect central and peripheral functions *in vivo*. In the CNS of laboratory animals thioperamide shows anxiolytic (Imaizumi & Onodera, 1993) and anticonvulsant (Yokoyama *et al.*, 1993) properties; it also increases locomotor activity (Sakai *et al.*, 1991), improves learning and memory skills (Meguro *et al.*, 1995), increases wakefulness (Monti, 1993), inhibits amphetamine-induced hyperactivity (Clapham & Kilpatrick, 1994), and decreases food intake (Oohara *et al.*, 1994; Sakata *et al.*, 1994). Based on these pharmacological activities, there has been increasing interest in the development of H_3 antagonists as potential therapeutical tools particularly for CNS disorders.

Despite the early availability and high affinity of thioperamide for the H_3 receptor, its labelling and use as a radioligand for the H_3 receptor was reported as unsuccessful (Yanai *et al.*, 1994). Yanai *et al.* (1994) briefly described the use of the related (**S**)-[^{3}H]-methylthioperamide as a radioligand for the histamine H_3 receptor. Although early results regarding its affinity and autoradiographic tissue distribution in the rat brain appear promising, the pharmacological definition of its binding site(s) is not yet clear. For example, H_3 receptor density observed in the rat forebrain (Yanai *et al.*, 1994) was 5 fold higher than values reported for [^{3}H]-(**R**)-α-methylhistamine binding.

In the present study we established experimental conditions under which [^{3}H]-thioperamide (Figure 1) can be used as a radioligand to study the histamine H_3 receptor *in vitro*. New insights on the definition of H_3 receptor specific binding using radiolabelled H_3 antagonists are also presented. Despite its low specific activity, when compared to most of the labelled H_3 antagonists described (Jansen *et al.*, 1994; Ligneau *et al.*, 1994; Yanai *et al.*, 1994), [^{3}H]-thioperamide can be used as radioligand to study the histamine H_3 receptor in the rat brain. As such, thioperamide, a brain penetrating compound, may be of importance for the development of radioligands with short half-lives for use in PET studies in CNS disorders where the histamine H_3 receptor is suspected to be involved (Onodera *et al.*, 1994; Smith *et al.*, 1994; Yokoyama *et al.*, 1994; Alves Rodrigues *et al.*, 1995).

Methods

Synthesis of [^{3}H]-thioperamide

Cyclohexylisothiocyanate (2.5 mg) was dissolved in 250 μl of ethanol. [^{3}H]4-(4-piperidyl) imidazole (50 mCi in 0.6 ml ethanol, 6 Ci mmol^{-1}) was added to this solution together with triethylamine (2.2 mg). The mixture was heated in a sealed vial at 55–60°C for 5 h and then separated on a preparative C-18 h.p.l.c. column (Novo Nordisk A/S, 16 × 250 mm, 7 μm), using as eluent a mixture of triethylamine (0.2%, pH 6.0 using phosphoric acid) and acetonitrile 70:30 (v:v). The flow rate was 6.0 ml min^{-1}. The collected fractions (R_t = 30–35 min) of the product were concentrated and extracted with dichloromethane. The organic layer was dried ($MgSO_4$), filtered, and solvents were eliminated under vacuum to yield a yellow oil. The final product was stored at −20°C in methanol.

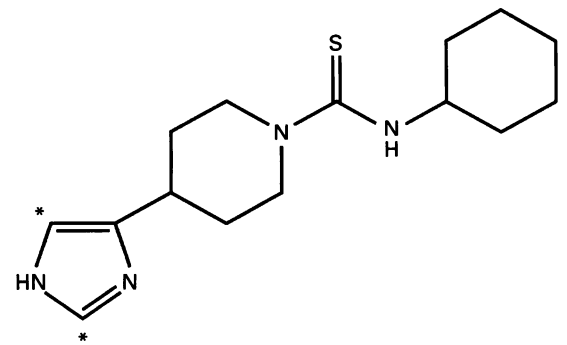

Figure 1 Structure of [^{3}H]-thioperamide; asterisks indicate the positions labelled with tritium.

H.p.l.c. analysis of the radiolabelled product was performed using a S5 phenyl column (250 × 4.6 mm, 5 μm, Phase Sep) with as eluent a mixture of triethylamine (0.2%, pH 6.0 adjusted with phosphoric acid) and acetonitrile 85:15 (v:v). The flow rate was 2.0 ml min^{-1}; u.v. absorption (215 nm) and radioactivity (Radiomatic/Canberra Flo-One beta detector A-200) were monitored.

The radiochemical yield was 45%. The final product had a specific activity of 6.0 Ci mmol^{-1} (determined by h.p.l.c. with non-labelled thioperamide as a reference standard). The radiochemical purity was higher than 98%.

Preparation of membranes from rat cerebral cortex

Male Wistar rats (200–220 g) were decapitated and the brains were rapidly removed. Whole cortices were dissected and homogenized in 10 volumes (v:w) of ice-cold 50 mM Tris-HCl buffer (containing 5 mM $MgCl_2$, 145 mM NaCl, pH 7.4 at 4°C) using a Ultra-Turrax blender and a Potter-Elvhjem homogenizer. This homogenate was centrifuged at 800 *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 40,000 *g* for 40 min. The resulting pellet was rinsed twice under the same conditions. The final pellet was resuspended in 1.5 volumes (v:w) of the Tris-HCl buffer described above and stored at −80°C until the day of the experiment when it was diluted 2.5 fold (v:w) in the same solution.

[^{3}H]-thioperamide binding assays

[^{3}H]-thioperamide binding assays were based on the procedure described by Jansen *et al.* (1994) for the binding of [^{125}I]-iodophenpropit. Kinetic (using 0.3 nM [^{3}H]-thioperamide, Figure 2), saturation (using either 1 μM **R**-(α)-methylhistamine, Figure 3b; or 0.3 μM iodophenpropit, Figure 3a), and displacement experiments (both using 0.3 nM and 5 nM [^{3}H]-thioperamide; Figures 4 and 5) were performed at 4°C in 50 mM Tris-HCl buffer described above in a total incubation volume of 0.25 or 0.5 ml, in polyethylene tubes. Determinations were performed in duplicate. Drugs were prepared in the same buffer. Rat cerebral cortical membranes and rat liver microsomes (Jefcoate, 1978), previously prepared and kept at −80°C, were incubated for 14 h to reach equilibrium. Incubations were started by the addition of membranes and were terminated by the addition of 3 ml ice-cold Tris-HCl buffer (pH 7.4, at 4°C) followed immediately by filtration through Whatman GF/B filters using a Brandel filtration apparatus. Filter binding was less than 1% of total radioactivity added. After filtration of the membranes, filters were washed once with 3 ml ice-cold Tris-HCl buffer then transferred to vials. Scintillation fluid was added and the radioactivity bound to the filters were measured in a Wallac beta scintillation counter. Samples were left to equilibrate for 24 h before counting for 5 min per sample. The standard error of the mean of the d.p.m. counted did not exceed 5% both when the experiments were performed in triplicate or in duplicate. Counting efficiency was 60%.

Data analysis

Saturation and competition binding experiments were evaluated on a Macintosh computer using the non-linear curve fitting programme LIGAND (Munson & Rodbard, 1980). With the aid of this programme binding curves were, respec-

tively, fitted (unweighted) to a one and two independent sites models. The quality of the fit for each model with additional parameters was evaluated based on the 'extra sum of squares' principal (Draper & Smith, 1966) using a confidence interval of 95%.

Protein determination

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

Chemicals

[^{3}H]-4-(4-piperidyl) imidazole with a specific activity of 6 Ci mmol^{-1} was obtained from Amersham and used without further purification. Cyclohexylisothiocyanate was synthesized at Novo Nordisk A/S. All other reagents and solvents used in the synthesis of [^{3}H]-thioperamide were of analytical grade.

For the binding studies the following drugs were used: thioperamide maleate, iodophenpropit dihydrobromide, imetit dihydrobromide, immepip dihydrobromide, (all synthesized at the Department of Pharmacochemistry, Vrije Universiteit, Amsterdam, The Netherlands); iodoproxyfan, (**R**)-α-methylhistamine maleate (gifts from Dr W. Schunack, Berlin), (*S*)-α-methylhistamine dihydrobromide (Cookson Chemicals), burimamide (gift from Smith Kline Beecham), haloperidol (RBI, Natick, U.S.A.), ondansetron (gift from Solvay Duphar), histamine dihydrochloride (Sigma), imidazole (Merck), SKF 525A hydrochloride (proadifen hydrochloride RBI) and metyrapone (Aldrich-Chemie).

Results

Time course of the [^{3}H]-thioperamide binding to rat cortical membranes

Our initial binding studies with [^{3}H]-thioperamide were performed at 37°C as described in the methods of Jansen *et al.* (1994). A very slow association (>8 h to reach equilibrium) of 5 nM [^{3}H]-thioperamide to rat brain cortical membranes was observed. In parallel experiments with 600 μg ml^{-1} membrane protein we observed at 5 nM [^{3}H]-thioperamide, 2921±152 and 1342±43 d.p.m. total binding (mean±s.d., n=3) at 4°C and 37°C, respectively. At 37°C almost no total binding of 0.3 nM [^{3}H]-thioperamide could be detected (100±20 d.p.m.), whereas at 4°C 1430±50 d.p.m. total binding was found. Therefore, subsequent studies were performed at 4°C. At this temperature, binding of 0.3 nM [^{3}H]-thioperamide to rat brain cortical membranes reached equilibrium after 8–10 h (Figure 2) resulting in an association constant (K_{on}) of 0.2±0.1 10^9 mol min^{-1} (mean±s.d., n=3). The binding of [^{3}H]-thioperamide was readily reversible by the addition of 1 μM (**R**)-α-methylhistamine which displaced 80–85% of the specific binding with a dissociation constant (K_{off}) of 0.065±0.056 min^{-1} (mean±s.d., n=3). Based on these kinetic data the K_d value ($K_{off/on}$) of [^{3}H]-thioperamide was calculated to be 0.3±0.4 nM (mean±s.d., n=3).

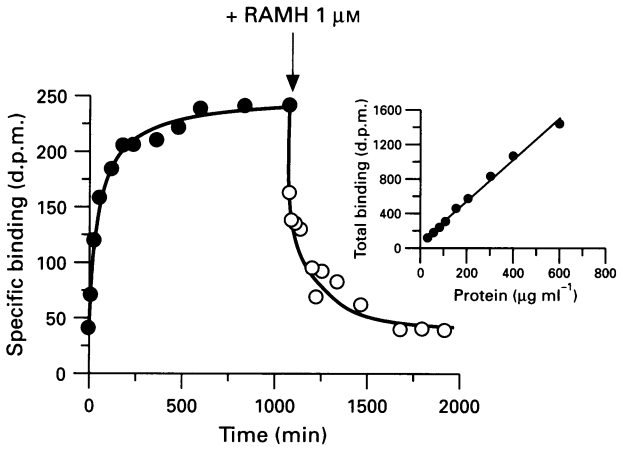

Figure 2 Association and dissociation curves of the [^{3}H]-thioperamide binding to rat brain cortical membranes. Membranes (50 μg/tube) were incubated at 4°C with 0.3 nM [^{3}H]-thioperamide in a final volume of 250 μl. Nonspecific binding was measured in the presence of 1 μM (**R**)-α-methylhistamine (RAMH). The specific binding at equilibrium represented 50–60% of the total binding. For the dissociation of [^{3}H]-thioperamide binding, (**R**)-α-methylhistamine (1 μM) was added, after an 18 h incubation. One typical experiment of three is shown. K_{off}/K_{on} = 0.3 nM. Inset shows the binding of 0.3 nM [^{3}H]-thioperamide at different protein concentrations. A typical experiment performed in triplicate is shown. Similar data were obtained in two other independent experiments.

At 4°C the binding of 0.3 nM (inset Figure 2) and 5 nM [^{3}H]-thioperamide (data not shown) to rat cortical membranes increased linearly with increasing concentrations of protein up to 400 μg ml^{-1}. Based on these characteristics, saturation and displacement experiments were performed using an incubation period of 14 h, at 4°C, with 200–400 μg ml^{-1} of protein.

[^{3}H]-thioperamide saturation binding experiments

Incubation of rat cerebral cortical membranes with increasing concentrations of [^{3}H]-thioperamide showed binding to be saturable. When specific binding was determined with 0.3 μM iodophenpropit, biphasic Scatchard plots were obtained (Figure 3a); analysis of the [^{3}H]-thioperamide saturation curves (0.03–120 nM) revealed two populations of binding sites (Table 1). When specific binding was determined using 1 μM (**R**)-α-methylhistamine (Figure 3b), a linear Scatchard plot was obtained and [^{3}H]-thioperamide binding (0.03–7 nM) was best fitted according to a single site model (Table 1). Specific binding represented 50 to 55% of the total binding at [^{3}H]-thioperamide concentrations below 0.5 nM. Specific binding decreased significantly when the concentration of [^{3}H]-thioperamide was increased.

Displacement curves using H_3-antagonists

Total binding of [^{3}H]-thioperamide to rat cerebral cortical membranes was fully displaced by H_3 antagonists. Figure 4 shows displacement of [^{3}H]-thioperamide by the two H_3 antagonists, iodophenpropit (Figure 4a) and iodoproxyfan (Figure 4b) each at 0.3 nM and 5 nM [^{3}H]-thioperamide. When 0.3 nM [^{3}H]-thioperamide was used in competition assays, the H_3-receptor antagonists clearly distinguished between high and low affinity components. K_i values obtained for the high affinity site were consistent with those reported for the H_3-receptor (Table 2). Using 5 nM [^{3}H]-thioperamide, the displacement curves were all fitted best to a single site model. The K_i values obtained, under these conditions, differed from those expected for the H_3 receptor and were comparable to K_i values for the low affinity site found at 0.3 nM (Table 2).

Displacement curves using H_3-agonists

Figure 5 shows the displacement of the binding of 5 nM and 0.3 nM [^{3}H]-thioperamide by (**R**)-α-methylhistamine, histamine, and (**S**)-α-methylhistamine. When 5 nM [^{3}H]-thioperamide was used, less than 15% of the total binding was displaced by the tested H_3 agonists applying a concentration-range expected to bind to the H_3 receptor. Even under these conditions it was possible to observe stereoselectivity of the (**R**) and (S) isomers of α-methylhistamine (Figure 5a). Nevertheless, the small amount of binding displaced did not allow proper fittings of the agonist curves. On the other hand, when experiments were performed with 0.3 nM [^{3}H]-thioperamide, up to 60% of total binding was displaced by the H_3 agonists (Figure 5b). Also in these experiments, stereoselectivity be-

tween the (**R**) and (**S**)-isomers of α-methylhistamine was evident up to micromolar concentrations. K_i values obtained for these compounds and for other well established H_3 agonists (imetit and immepip) are shown in Table 2. Biphasic curves were obtained only for the displacement of 0.3 nM [^{3}H]-thioperamide by (**R**)-α-methylhistamine. Nevertheless, this displacement was not affected by the presence of 10 μM GTPγS (data not shown). For the other agonists LIGAND was unable to discriminate two independent sites, although at the higher agonists concentrations the agonists started to displace a second site.

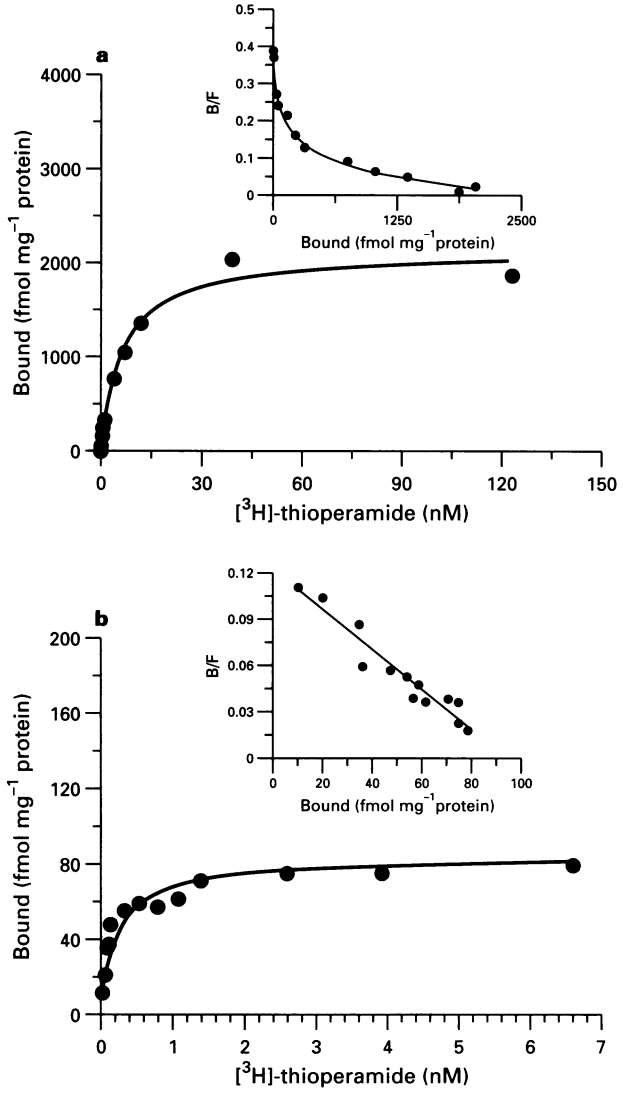

Figure 3 Saturation binding of [^{3}H]-thioperamide to membranes (200 μg/tube) from rat cerebral cortex. The insets show the transformation of the data into Scatchard plots. (a) The nonspecific binding was defined in the presence of 0.3 μM iodophenpropit. (b) The nonspecific binding was defined in the presence of 1 μM (**R**)-α-methylhistamine. Results shown are from one representative experiment of three performed in duplicate.

Displacement of [^{3}H]-thioperamide by other ligands

Thioperamide displays high to moderate affinity for 5-HT_3 and for σ receptors (Leurs *et al.*, 1995a). Hence ondansetron, a 5-HT_3 selective ligand, and haloperidol, which shows high affinity for the σ receptor, were tested in displacement assays. Neither haloperidol ($K_i > 10$ μM, $n = 3$) nor ondansetron

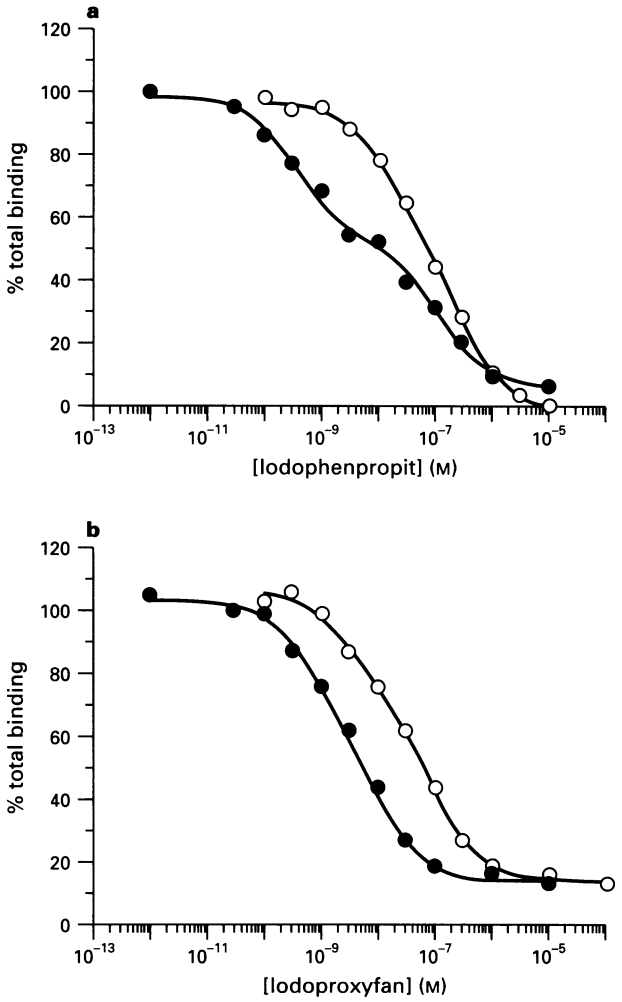

Figure 4 Displacement of the total binding of 0.3 nM (●) and 5 nM (○) [^{3}H]-thioperamide to rat brain cortical membranes (100 μg/tube) at 4°C by iodophenpropit (a) or iodoproxyfan (b). Data represent a typical experiment of at least three independent experiments performed in duplicate.

Table 1 Affinity constants (K_D) and number of binding sites of [^{3}H]-thioperamide to membranes of rat brain cortex

Nonspecific binding defined with:	K_D *high* (nM)	B_{max} *high* (fmol mg^{-1} protein)	K_D *low* (nM)	B_{max} *low* (fmol mg^{-1} protein)
Iodophenpropit 0.3 μM	1.1±0.3	162±108	96±19	4,346±3,092
(**R**)-α-methylhistamine 1 μM	0.8±0.1	73±20	–	–

Radioligand binding studies were performed as described in Methods. Data represent mean±s.d. of three independent experiments performed in duplicate. Saturation curves were fitted using the non-linear fitting programme LIGAND.

Table 2 Affinity of different ligands towards [^{3}H]-thioperamide binding sites on rat cerebral cortex membranes

Ligand	K_i high/K_i low (nM) [^{125}I]-iodophenpropit	[^{3}H]-thioperamide (0.3 nM)	[^{3}H]-thioperamide (5 nM)
H_3-agonists:			
Histamine	38±10/2,500±600[a]	143±29	ND
(R)-α-methylhistamine	3.5±1.2/1,200±300[a]	9±3/180±15	ND
(S)-α-methylhistamine	230±97/9,500±1,800[a]	250±156	ND
Imetit	2.7±0.8/40,000±12,000[a]	8.1±5.6	ND
Immepip	2.7±0.8/1,000±200[a]	2.8±0.7	ND
H_3-antagonists			
Iodophenpropit	0.97±0.06[a]	0.36±0.14/80±19	9.5±1 nM
Thioperamide	4.3±1.6[a]	0.66±0.64/14±9	20±12 nM
Burimamide	18±9/725±392[a]	11.4±4.1/454±93	103±13 nM
Iodoproxyfan	2.4±0.2	0.33±0.16/6.3±0.8	6±2 nM

[a]Determined on rat cerebral cortex (Jansen *et al.*, 1994). The compounds that show high and low affinity K_i values fitted best to a two site model ($P<0.05$). Values are given as mean±s.d. of at least three independent experiments performed in duplicate. ND; not determined.

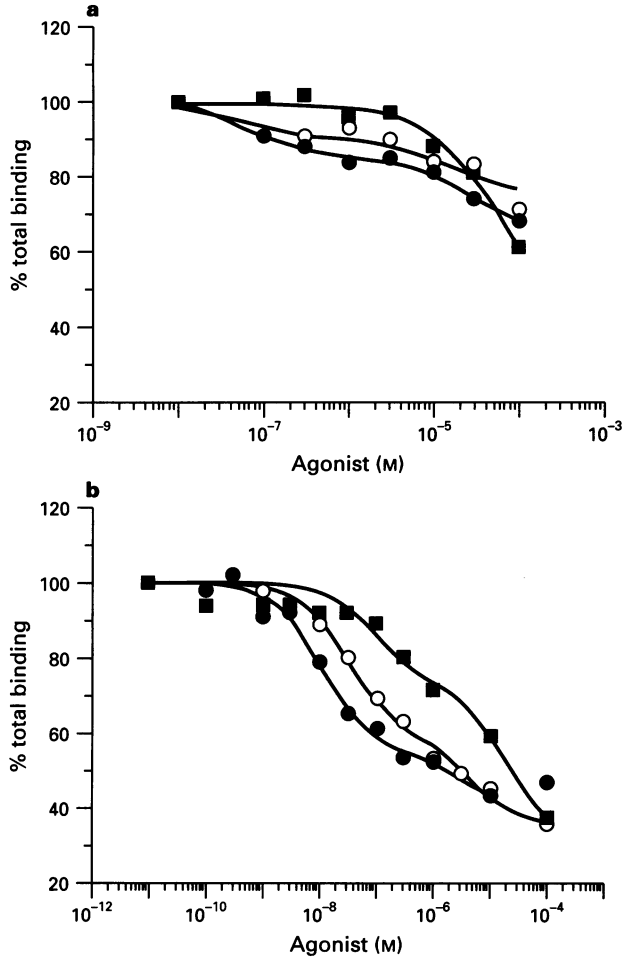

Figure 5 Displacement of the total binding of 5 nM (a) and 0.3 nM (b) [^{3}H]-thioperamide to rat brain cortical membranes (100 μg/tube) at 4°C by (R)-α-methylhistamine (●), (S)-α-methylhistamine (■) and histamine (○). Data represent one of at least three independent experiments performed in duplicate.

($K_i>10$ μM, $n=3$) displaced the binding of 5 nM [^{3}H]-thioperamide at concentrations selective for the σ or 5-HT_3-receptors, respectively.

Imidazole, metyrapone, and SKF 525A are known to inhibit cytochrome P_{450} isoenzymes nonselectively (Halpert *et al.*, 1994). These compounds displaced up to 80% of the total binding of 5 nM [^{3}H]-thioperamide in rat cerebral cortex membranes (Figure 6) at concentrations known to inhibit

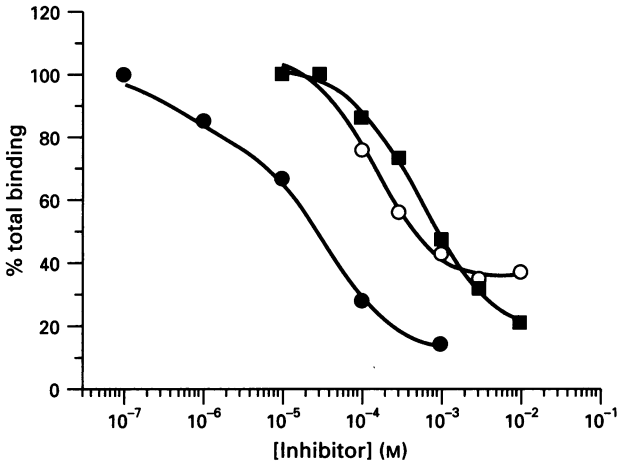

Figure 6 Displacement of the total binding of 5 nM [^{3}H]-thioperamide to rat brain cortical membranes (100 μg/tube) at 4°C by imidazole (●), metyrapon (○) and SKF 525A (■). Data represent one of at least three independent experiments performed in duplicate.

binding to cytochrome P_{450} isoenzymes (Halpert *et al.*, 1994). The IC_{50} values obtained for imidazole, metyrapone, and SKF 525A were, respectively: 11±1 μM (means±s.d., $n=3$), 522±92 μM ($n=3$), and 481±18 μM ($n=3$).

Finally, we investigated the binding of 5 nM [^{3}H]-thioperamide to rat liver microsomes. Total binding of 5 nM [^{3}H]-thioperamide to 950 μg ml^{-1} rat liver microsomal proteins amounted to 61014±431 d.p.m. This binding was inhibited by 12.1±6.3%; 88.9±0.4%; 93.1±0.1% and 90.5±0.1% (mean±s.d., $n=2$) respectively by 1 μM R-α-methylhistamine, 100 μM imidazole, 1 mM metyrapone and 1 mM SKF 525A.

Discussion

Until recently, the highly selective H_3 agonists, [^{3}H]-(R)-α-methylhistamine and [^{3}H]-N^{α}-methylhistamine, were the only tools available to label the H_3 receptor and they have been of great use in the elucidation of the pharmacological characteristics of the H_3 receptor (Arrang *et al.*, 1988; West *et al.*, 1990). Nevertheless, the binding properties of radiolabelled agonists are known to be complex and often difficult to interpret (West *et al.*, 1990; Clark & Hill, 1995). In an attempt to solve some of the difficulties related to the use of labelled agonists, new H_3 antagonists were synthesized, radiolabelled and pharmacologically characterized. The first radiolabelled H_3 antagonist was [^{125}I]-iodophenpropit (Menge *et al.*, 1992) an iodinated analogue of clobenpropit, the most potent H_3

antagonist available to date (Leurs *et al.*, 1995b). This radioligand is suitable for characterization of histamine H_3 receptors in rat cerebral cortex (Jansen *et al.*, 1992; 1994). Yet, competition experiments with the two H_3 antagonists, burimamide and dimaprit, showed biphasic displacement curves suggesting that this radioligand interacts with two binding sites (Jansen *et al.*, 1994). [^{125}I]-iodoproxyfan, another iodinated H_3 antagonist, was used to label H_3 receptors in rat striatum (Ligneau *et al.*, 1994). However, agonists displaced a maximum of 60% of the total binding whereas antagonists fully displaced the binding of [^{125}I]-iodoproxyfan. Apparently [^{125}I]-iodoproxyfan labels an additional binding site with high affinity. The labelling of this secondary site(s) by [^{125}I]-iodoproxyfan was potently inhibited by H_3 antagonists, sometimes resulting in complex displacement curves with Hill coefficients significantly higher than one (Ligneau *et al.*, 1994).

In the present study we describe the synthesis of the radiolabelled H_3 antagonist [^{3}H]-thioperamide as well as the pharmacological characterization of its binding to rat cerebral cortical membranes. Under the experimental conditions used, [^{3}H]-thioperamide binding was saturable and reversible. When saturation experiments were performed (0.01 – 7 nM [^{3}H]-thioperamide) using the H_3-agonist, (**R**)-α-methylhistamine to define nonspecific binding, linear Scatchard plots were obtained consistent with labelling of a single class of binding site (Table 1). When competition experiments were performed with 0.3 nM [^{3}H]-thioperamide, H_3 agonists displaced up to 60% of the total binding. Stereoselectivity for the (**R**) and (**S**)-isomers of the H_3 agonist, α-methylhistamine, was observed within the concentration interval expected to bind to the H_3 receptor. In contrast to previous studies with [^{125}I]-iodophenpropit at 37°C (Jansen *et al.*, 1994) agonist displacement of [^{3}H]-thioperamide was not affected by GTPγS at 4°C. Since recently Clark & Hill (1996) presented conclusive evidence on the interaction of the H_3 receptor to pertussis toxin-sensitive G proteins in the rat cerebral cortex, the lack of GTPγS effect in the present study is probably due to differences in the thermodynamic/thermokinetic behaviour of G-protein coupled receptors. For example, it has been shown that for agonist binding to β_2-adrenoceptors the high affinity dissociation constant decreases much more with decreasing temperature than the low affinity constant (Miklavc *et al.*, 1990).

At higher concentrations of [^{3}H]-thioperamide H_3 agonists also displaced the [^{3}H]-thioperamide binding, but stereoselectivity for the isomers of α-methylhistamine was almost totally lost. We, therefore, consider 60% of the total binding of 0.3 nM [^{3}H]-thioperamide to rat brain cortical membranes to represent the H_3 receptor. Displacement studies with several H_3 antagonists confirmed this conclusion. For these ligands displacement studies with 0.3 nM [^{3}H]-thioperamide resulted in biphasic displacement curves. The K_i values for the high affinity [^{3}H]-thioperamide binding site (approximately 50 – 60% of total binding) corresponded well with the known affinities for the H_3 receptor (Jansen *et al.*, 1994) for the tested H_3 antagonists.

When 5 nM [^{3}H]-thioperamide was used, only a small part (< 15%) of the total binding was displaced by H_3 agonists. Yet, under these conditions, the H_3 antagonists fully displaced [^{3}H]-thioperamide binding monophasically at concentrations which mostly did not correspond with their observed affinities for the H_3 receptor. Our findings indicate that at 5 nM [^{3}H]-thioperamide binds mostly to a secondary, non-H_3, binding site(s). Furthermore, the H_3 antagonists we tested showed moderate to high affinity for this secondary site(s), albeit at concentrations more than 10 fold higher than their affinities for the H_3 receptor. The relatively high affinity of iodophenpropit for the secondary [^{3}H]-thioperamide site(s) (K_i = 80 nM) appears to account for the observed curvilinear Scatchard plots of the [^{3}H]-thioperamide saturation curves, when iodophenpropit was used to define the nonspecific binding (Figure 3a). Although the estimates of the density of the binding obtained were relatively inaccurate for the secondary site(s) (Table 1), it is evident that this low affinity [^{3}H]-thioperamide binding site(s) is present in large excess over the H_3 receptor binding site.

Previously Leurs *et al.* (1995a) extensively characterized the receptor selectivity of iodophenpropit and thioperamide. In those radioligand binding studies, displacement with thioperamide showed that the latter had relatively high affinities for the 5-HT_3 (120 ± 30 nM) and σ (180 ± 90 nM) receptors. Present results with the selective σ ligand, haloperidol and the selective 5-HT_3 ligand, ondansetron, indicated that these two receptors do not contribute significantly to the total binding of [^{3}H]-thioperamide.

Thioperamide has also been shown to interact with the cytochrome P_{450} isoenzymes (Labella *et al.*, 1992). Imidazole, metyrapone, and SKF 525A, all nonselective inhibitors of cytochrome P_{450} isoenzymes (Halpert *et al.*, 1994), showed almost full displacement of the binding of 5 nM [^{3}H]-thioperamide in rat cerebral cortical membranes strongly indicating that those enzymes may be a major source for the non-H_3 receptor component [^{3}H]-thioperamide binding. Since cytochrome P_{450} enzymes are present in relatively high amounts in the brain cortex (74 pmol mg^{-1} protein) (Ravindranath, 1995) the binding to cytochrome P_{450} isoenzymes may explain the high density of the binding observed at higher concentrations of [^{3}H]-thioperamide (Table 1, Figure 3). This suggestion is supported by our observation that [^{3}H]-thioperamide also binds to rat liver microsomes. Labelling of a cytochrome P_{450} isoenzyme has also been reported to complicate histamine H_1 receptor binding studies with [^{3}H]-mepyramine (Leurs *et al.*, 1989; 1990; Fukui *et al.*, 1995).

Considering the results obtained from this study, the following picture regarding the available radiolabelled H_3 antagonists emerges. [^{125}I]-iodophenpropit (Jansen *et al.*, 1994) can be considered a suitable radioligand for labelling H_3 receptors as it markedly discriminates between high and low affinity binding sites of H_3 antagonists in the rat cerebral cortex (selectivity ratio 222). Yet, it would be advisable to use agonists to define nonspecific binding, in saturation studies in particular where the nonspecific binding increases markedly with increasing concentrations of radioligand. [^{125}I]-iodoproxyfan (Ligneau *et al.*, 1994) should be used with more care when labelling the H_3 receptor. This compound does not discriminate well between high and low affinity binding sites of H_3 antagonists in the rat cerebral cortex (selectivity ratio 19). This observation is consistent with the finding that only 60% of the total binding of this ligand to rat striatal membranes is displaced by H_3 agonists while several antagonists fully displace the binding of [^{125}I]-iodoproxyfan (Ligneau *et al.*, 1994). [^{3}H]-(**S**)-methylthioperamide (Yanai *et al.*, 1994) binding was characterized only with (**R**)- and (**S**)-methylhistamine, and thioperamide. Moreover, nonspecific binding was defined with 10 μM thioperamide. In view of our recent results this procedure appears to be inadequate for the selective labelling of the H_3 receptor. Indeed, Yanai *et al.* (1994) reported relatively high densities of (**S**)-[^{3}H]-methylthioperamide binding in the rat brain. In various peripheral tissues, such as the liver and the lung, where the H_3 receptor is not expected to be present (Korte *et al.*, 1990) the density of the binding sites for (**S**)-[^{3}H]-methylthioperamide was even higher (Yanai *et al.*, 1994). This may be due to binding of this radioligand to cytochrome P_{450} isoenzymes present in high densities in these tissues (Ravindranath, 1995).

In conclusion, [^{3}H]-thioperamide binds to rat brain cortical membranes in a saturable and reversible manner showing high and low affinity components. The high affinity site is likely to represent the histamine H_3 receptor as the binding is displaced by (**R**)- and (**S**)-α-methylhistamine in a stereoselective manner and by several other H_3 ligands with a pharmacological profile of the H_3 receptor. At nanomolar concentrations, binding of [^{3}H]-thioperamide to a low affinity non-H_3 receptor binding site(s) increases steeply, reaching, at saturation, a density 30 fold higher than the number of H_3 receptors. Also other H_3

antagonists we tested show high affinity for the non-H_3 receptor sites. Therefore, based on this study, it would be judicious to use H_3 agonists rather than H_3 antagonists to delineate specific (and nonspecific) binding of radioactivity labelled H_3 antagonists.

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