British Journal of Pharmacology (1999) 128, 881–890
http://www.stockton-press.co.uk/bjp

Characterization of the binding of [^{3}H]-clobenpropit to histamine H_3-receptors in guinea-pig cerebral cortex membranes

*,[1]E.A. Harper, [1]N.P. Shankley & [1]J.W. Black

[1]James Black Foundation, 68 Half Moon Lane, Dulwich, London SE24 9JE

1 We have investigated the binding of a novel histamine H_3-receptor antagonist radioligand, [^{3}H]-clobenpropit ([^{3}H]-VUF9153), to guinea-pig cerebral cortex membranes.

2 Saturation isotherms for [^{3}H]-clobenpropit appeared biphasic. Scatchard plots were curvilinear and Hill plot slopes were significantly less than unity (0.63 ± 0.03; $n = 12 \pm$ s.e.mean). The radioligand appeared to label two sites in guinea-pig cerebral cortex membranes with apparent affinities (pK_D') of 10.91 ± 0.12 ($B_{max} = 5.34 \pm 0.85$ fmol mg^{-1} original wet weight) and 9.17 ± 0.16 ($B_{max} = 23.20 \pm 6.70$ fmol mg^{-1}).

3 In the presence of metyrapone (3 mM) or sodium chloride (100 mM), [^{3}H]-clobenpropit appeared to label a homogeneous receptor population ($B_{max} = 3.41 \pm 0.46$ fmol mg^{-1} and 3.49 ± 0.44 fmol mg^{-1}, $pK_D' = 10.59 \pm 0.17$ and 10.77 ± 0.02, respectively). Scatchard plots were linear and Hill slopes were not significantly different from unity (0.91 ± 0.04 and 0.99 ± 0.02, respectively). Granisetron (1 μM), rilmenidine (3 μM), idazoxan (0.3 μM), pentazocine (3 μM) and 1,3-di-(2-tolyl)guanidine (0.3 μM) had no effect on the binding of [^{3}H]-clobenpropit.

4 The specific binding of [^{3}H]-clobenpropit appeared to reach equilibrium after 25 min at 21 ± 3°C and remained constant for >180 min. The estimated pK_D' (10.27 ± 0.27; $n = 3 \pm$ s.e.mean) was not significantly different from that estimated by saturation analysis in the presence of metyrapone.

5 A series of histamine H_3-receptor ligands expressed affinity values for sites labelled with [^{3}H]-clobenpropit which were not significantly different from those estimated when [^{3}H]-*R*-α-MH was used to label histamine H_3-receptors in guinea-pig cerebral cortex membranes.

Keywords: Histamine; H_3; receptor; guinea-pig cerebral cortex; [^{3}H]-clobenpropit

Abbreviations: [^{3}H]-*R*-α-MH, [^{3}H]-*R*-α-methylhistamine; HEPES, (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]); *N*-α-MH, *N*-α-methylhistamine; PEI, polyethyleneimine

Introduction

Most radioligand binding studies characterizing histamine H_3-receptors have used receptor-selective agonist radioligands such as [^{3}H]-*R*-α-methylhistamine ([^{3}H]-*R*-α-MH, West *et al*., 1990a; Fujimoto *et al*., 1991; Pollard *et al*., 1993), [^{3}H]-*N*-α-MH (Korte *et al*., 1990; West *et al*., 1990b; Clark *et al*., 1993) and, more recently, [^{125}I]-iodoproxyfan (Ligneau *et al*., 1994; Stark *et al*., 1996). However, the classification and localization of receptors through characterization of the binding (pK_D and B_{max}) of agonist radioligands can be misleading because a number of assay-dependent factors can influence these parameters. These factors include tissue preparation (e.g. Childers & LaRiviere, 1984; Kim & Neubig, 1985), the ionic composition of the incubation buffer (e.g. Hamblin & Creese, 1982; Kilpatrick & Michel, 1991; Parkinson & Fredholm, 1992), incubation time (e.g. Insel *et al*., 1983; Convents *et al*., 1987) and the presence or absence of guanine nucleotides (e.g. Lynch *et al*., 1987; Kent *et al*., 1979). These same factors could also complicate studies aimed at investigating the relative distribution of receptors in central and peripheral tissues.

Although the binding of two radiolabelled histamine H_3-receptor antagonists, [^{125}I]-iodophenpropit (Jansen *et al*., 1992) and [^{3}H]-*S*-methylthioperamide (Yanai *et al*., 1994), had been described when this study was initiated there were no commercially-available radiolabelled histamine H_3-receptor antagonists. We therefore chose to radiolabel clobenpropit and characterize its binding to guinea-pig cerebral cortex membranes. Clobenpropit was selected because it was the highest affinity ligand that behaved as a simple competitive antagonist in both a functional histamine H_3-receptor bioassay (guinea-pig ileum; Van Der Goot *et al*., 1992) and in competition experiments in radioligand binding assays.

Whilst this study was being performed the binding of two more radiolabelled histamine H_3-receptor antagonists, [^{3}H]-thioperamide (Alves-Rodriguez *et al*., 1996) and [^{3}H]-GR168320 (Brown *et al*., 1996), were also reported.

A preliminary account of this study was presented to the British Pharmacological Society (Harper *et al*., 1997a).

Methods

Assessment of the radiochemical purity of [^{3}H]-clobenpropit

Fifty μl of a mixture of unlabelled clobenpropit (100 μl; 1 mM) and [^{3}H]-clobenpropit (10 μl; 35.7 μM) was injected onto a C-8 Techsphere column (250×4.6 mm; 5 μm). Material was eluted at a flow rate of 1 ml min^{-1} using a mobile phase comprising 65% acetonitrile in 0.1% triethylamine and 35% 0.1% triethylamine. Unlabelled and radiolabelled clobenpropit were detected using a u.v. detector (225 nm) and Flo-1β radiochemical detector, respectively.

*Author for correspondence.

Assessment of filter binding of [^{3}H]-clobenpropit

[^{3}H]-clobenpropit was diluted to a concentration of 2 nM with 20 mM HEPES-NaOH (pH 7.4 at 21±3°C) and 50 µl aliquots added to polypropylene tubes containing a further 450 µl of buffer. Samples were filtered through GF/B filters which had been soaked in Tris-HCl buffer or 0.3% polyethylenemine (PEI). Filters were washed (3×3 ml) with ice-cold 50 mM Tris-HCl (pH 7.4 at 21°C) and transferred into scintillation vials containing 4 ml Meridian Gold-Star liquid scintillation cocktail. After 3 h the bound radioactivity was determined by counting (3 min) in a Beckman liquid scintillation counter.

Preparation of guinea-pig cerebral cortex membranes

Guinea-pigs were killed by cervical dislocation and the whole brain removed and immediately placed in ice-cold 20 mM HEPES-NaOH buffer (pH 7.4 at 21±3°C). The cerebral cortex was dissected, weighed and homogenized in ice-cold 20 mM HEPES-NaOH buffer (pH 7.4 at 21±3°C) (1 g 15 ml^{-1}) using a polytron homogenizer (Kinematica AG; PT-DA 3020/2TS; ~3 s×3). The homogenate was centrifuged at 100×*g* for 5 min and the supernatants pooled and stored at 4°C. The pellets were rehomogenized in fresh ice-cold buffer (80 ml) and recentrifuged at 100×*g* for 5 min at 4°C. The supernatants were centrifuged at 39,800×*g* for 12 min at 4°C and the final pellet was resuspended in 20 mM HEPES-NaOH buffer (21°C), to the required tissue concentration, using a teflon-in-glass homogenizer.

[^{3}H]-clobenpropit – saturation studies

Guinea-pig cortical membranes (400 µl; 1.75 mg ml^{-1}) were incubated for 165 min at 21±3°C in a final volume of 0.5 ml with HEPES-NaOH buffer and 50 µl of 0.04 to 30 nM [^{3}H]-clobenpropit. Total and non-specific binding of [^{3}H]-clobenpropit were defined using 50 µl of HEPES-NaOH buffer and 50 µl thioperamide (pK_B at histamine H_3-receptors in guinea-pig ileum ~8.5), respectively. The assay was terminated by rapid filtration through Whatman GF/B filters, presoaked in 0.3% PEI, which were washed (3×3 ml) with ice-cold 50 mM Tris-HCl (pH 7.4 at 4°C) using a Brandell Cell Harvester. Filters were transferred into scintillation vials, 4 ml Meridian Gold-Star liquid scintillation cocktail added and after 3 h the bound radioactivity was determined by counting (3 min) in a Beckman liquid scintillation counter.

In a series of experiments to determine the specificity of the binding of [^{3}H]-clobenpropit and also to establish whether the high non-specific binding of [^{3}H]-clobenpropit could be reduced, we investigated whether [^{3}H]-clobenpropit labelled 5-HT_3 receptors, imidazoline I_2 sites, imidazoline I_1 sites, σ sites or cytochrome P450 enzymes under the assay conditions used. Accordingly, in initial experiments the apparent affinity (pK_i') of the selected ligands, granisetron (pK_i 5-HT_3 receptors ~9.7; Fletcher & Barnes, 1997), idazoxan (pK_i I_2 receptors ~8.8; MacKinnon *et al*., 1993), rilmenidine (pK_i I_1 receptors ~8.1; Gargalidis-Moudanos & Parini, 1995), pentazocine (pK_D σ_1 sites ~8.5; DeHaven-Hudkins *et al*., 1992),1,3-di-(2-tolyl)guanidine (pK_i σ_2 sites ~7.7; Bowen *et al*., 1988), metyrapone (P450 enzyme inhibitor) and debrisoquine sulphate (P450 enzyme substrate) at histamine H_3-receptors in guinea-pig cerebral cortex membranes was established by performing competition studies using [^{3}H]-*R*-α-MH as the radioligand (Table 1). These results were used to select concentrations of each ligand that would produce significant block of the target receptors without significant occupancy of histamine H_3-receptors in a [^{3}H]-clobenpropit saturation analysis. Thus, the saturation analysis was performed in the presence of either granisetron (1 µM), idazoxan (0.3 µM), rilmenidine (3 µM), 1,3-di-(2-tolyl)guanidine (0.3 µM), pentazocine (3 µM), metyrapone, (3 mM) and debrisoquine sulphate (10 µM).

Table 1 Estimated pK_i and Hill slope parameters (n_H) for $\sigma1$, $\sigma2$, I_1, I_2 and 5-HT_3 receptor ligands at histamine H_3-receptors labelled with [^{3}H]-*R*-α-MH

Compound	*Target site*	$pK_i' \pm s.e.mean$	$n_H \pm s.e.mean$	n
Metyrapone	cytochrome P450	>1		3
Debrisoquine sulphate	cytochrome P450	4.3±0.06	0.78±0.06	3
Pentazocine	σ	4.64±0.12	0.98±0.04	3
DTG	σ	4.54±0.06	1.25±0.06	4
Rilmenidine	I_1	4.95±0.04	0.72±0.06	4
Granisetron	5-HT_3	4.53±0.09	1.35±0.13	3
Idazoxan	I_2	5.53±0.21	0.81±0.10	3

In another series of experiments, we investigated the sensitivity of [^{3}H]-clobenpropit binding in the presence of high concentrations of monovalent cations by performing a saturation analysis in the presence of 100 mM NaCl.

[^{3}H]-clobenpropit – kinetic studies

The observed association rate was determined at 21±3°C by incubating [^{3}H]-clobenpropit (50 µl; 2 nM) for increasing time intervals (0.5–150 min) in 12 tubes containing membranes (400 µl; 4 mg ml^{-1} containing 3 mM metyrapone) and either 50 µl HEPES-NaOH buffer or 50 µl 10 µM thioperamide. The specific binding was ascertained from 150–250 min by defining total and non-specific binding at each time point in triplicate.

The $t_{1/2}$ was determined by adding 10 µl of 50 µM thioperamide to six tubes which had been incubated (150 min at 21±3°C) with [^{3}H]-clobenpropit and 50 µl HEPES-NaOH buffer. The bound radioligand was ascertained at times from 0.5–100 min.

Competition studies

Guinea-pig cerebral cortex membranes (400 µl; 4 mg ml^{-1} [^{3}H]-clobenpropit assay; 7.5 mg ml^{-1} [^{3}H]-*R*-α-methylhistamine assay ([^{3}H]-*R*-α-MH)) were incubated for 165 min at 21±3°C in a final volume of 500 µl with 20 mM HEPES-NaOH buffer containing [^{3}H]-clobenpropit or [^{3}H]-*R*-α-MH (50 µl; 2 or 1 nM, respectively) and competing compound. Total and non-specific binding of [^{3}H]-clobenpropit or [^{3}H]-*R*-α-MH were defined using 50 µl of HEPES-NaOH buffer and 50 µl 10 µM thioperamide, respectively. When [^{3}H]-clobenpropit was used as the radioligand the buffer also contained 3 mM metyrapone.

Data analysis

Saturation data were analysed using the non-linear curve fitting programme, LIGAND (Munson & Rodbard, 1980) Elsevier-BIOSOFT. Association and dissociation data were analysed using a non-linear regression analysis programme, Enzfitter (Leatherbarrow, 1987: Elsevier-BIOSOFT).

Competition curve data were fitted to the Hill equation using Graph-Pad Prism software.

$$B = \text{non} - \text{specific}\frac{\text{total} - \text{non} - \text{specific}}{1 + 10^{(\log IC_{50} - \log[C]^{nH})}}$$

Dissociation constants (K_i) were determined using the Cheng & Prusoff equation (1973) to correct for the receptor occupancy by the radioligand:

$$K_i = \frac{IC_{50}}{1 + [L]/K_D}$$

In this equation, IC_{50} is the concentration of competitor required for half-maximal displacement of the radioligand (concentration, [L]) and K_D is the equilibrium dissociation constant of the radioligand.

All data are presented as the mean ± s.e. mean.

Materials

[^{3}H]-clobenpropit ([^{3}H]-VUF9153) was prepared by Amersham International plc, Little Chalfont, Buckinghamshire, U.K. to a specific activity of 45 Ci mmol^{-1}. [^{3}H]-*R*-α-MH (specific activity ~38 Ci mmol^{-1} was also obtained from Amersham International plc.

Iodophenpropit, proxyfan (3-(1*H*-imidazol-4-yl)propyl-benzyl ether), iodoproxyfan, GR175737, JB96132 (*N*-[4-(1*H*-imidazol-4-yl)butyl]-*N'*-(4-chlorobenzyl)-sulphamide) and JB96134 (*N*-[5-(1*H*-imidazol-4-yl)pentyl]-2-napthalenesulphonamide) were synthesized by James Black Foundation chemists.

2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), idazoxan, HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]), PEI (polyethyleneimine) and Trizma base® were obtained from Sigma Chemical Co., Poole, Dorset, U.K. DTG (1,3-di-(2-tolyl)guanidine), pentazocine, *R*-α-MH and thioperamide were obtained from Research Biochemicals Inc., Poole, Dorset, U.K. Rilmenidine and *N*-α-methylhistamine (*N*-α-MH) were obtained from Tocris Cookson Ltd., Bristol, U.K. Granisetron was synthesized and kindly supplied by chemists at Janssen Pharmaceuticals, Beerse, Belgium. All other materials were obtained from Fisher Scientific, Loughborough, Leicestershire, U.K.

Results

Chemical properties of [^{3}H]-clobenpropit

[^{3}H]-clobenpropit was estimated to be >95% radiochemically pure by RP-HPLC. In the absence of membranes, 56.7 ± 3.7% of the added [^{3}H]-clobenpropit was bound to GF/B filters. When the filters were pre-soaked in 0.3% PEI, the filter binding of [^{3}H]-clobenpropit was reduced to 0.3 ± 0.1% (n = 6).

Characterization of binding in guinea-pig cerebral cortex membranes

Tissue concentration curve Total binding, non-specific binding and apparent specific binding of [^{3}H]-clobenpropit increased with guinea-pig cerebral cortex membrane concentration (Figure 1). The apparent specific binding increased linearly with increasing added cerebral cortex concentration up to 2.5 mg ml^{-1} (Figure 1). At a 1.75 mg ml^{-1} added tissue concentration 6.6 ± 1.9% of the added [^{3}H]-clobenpropit (0.2 nM) was bound and the apparent specific binding was 52.6 ± 7.7% (n = 5). This tissue concentration was used for subsequent saturation analysis experiments.

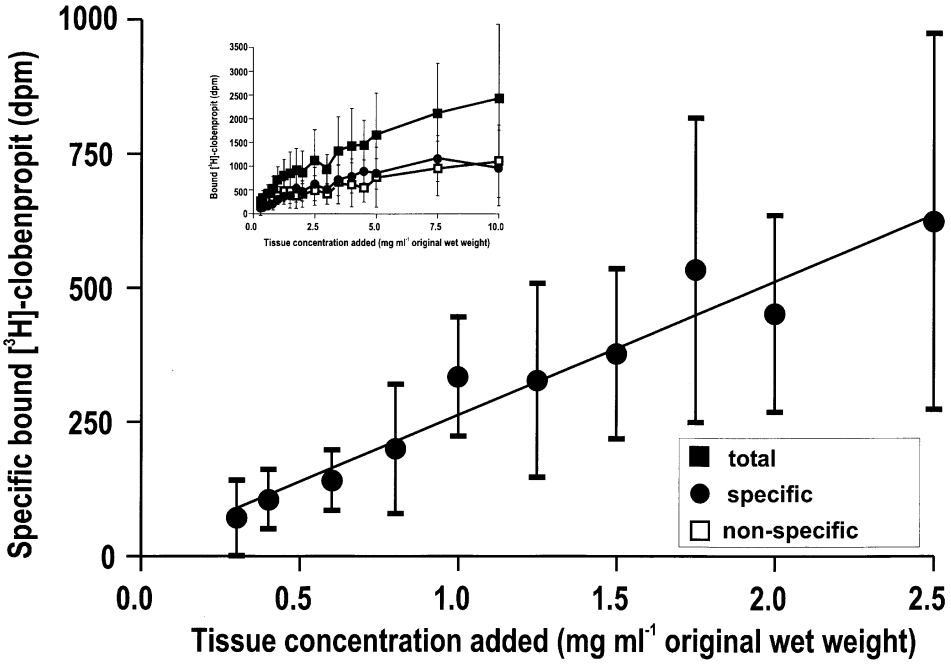

Figure 1 Linearity of the relationship between guinea-pig cerebral cortex membrane concentration and the specific binding of [^{3}H]-clobenpropit (0.2 nM; ~9500 d.p.m.). Data represents the mean ± s.e.mean of five experiments. The line shown superimposed on the data was obtained by linear regression. The inset shows the total binding, non-specific binding and specific binding of [^{3}H]-clobenpropit (0.2 nM) as a function of increasing guinea-pig cerebral cortex membrane tissue concentration. Increasing concentrations (0.3–15 mg ml^{-1}) of guinea-pig cortical membranes (400 μl) were incubated in triplicate with 0.2 nM (50 μl; 2 nM) [^{3}H]-clobenpropit for 165 min at 21 ± 3°C. Total binding and non-specific binding were defined with 50 μl buffer and 50 μl of thioperamide, respectively. Data represents the mean ± s.e.mean of five experiments.

Saturation analysis

The saturation isotherm for the binding of [^{3}H]-clobenpropit to guinea-pig cerebral cortex membranes appeared biphasic (Figure 2A). Scatchard plots were curvilinear (Figure 2B) and Hill plots had slopes which were significantly less than unity ($n_H = 0.63 \pm 0.03$; $n = 12$) ($P < 0.05$). The apparent affinity (pK_D') of [^{3}H]-clobenpropit at sites in guinea-pig cerebral cortex was 9.73 ± 0.29 ($n = 12$) and the estimated B_{max} was 29.86 ± 4.72 fmol mg^{-1} (original wet weight).

When the data was analysed using a two site model [^{3}H]-clobenpropit was estimated to express affinities (pK_D) of

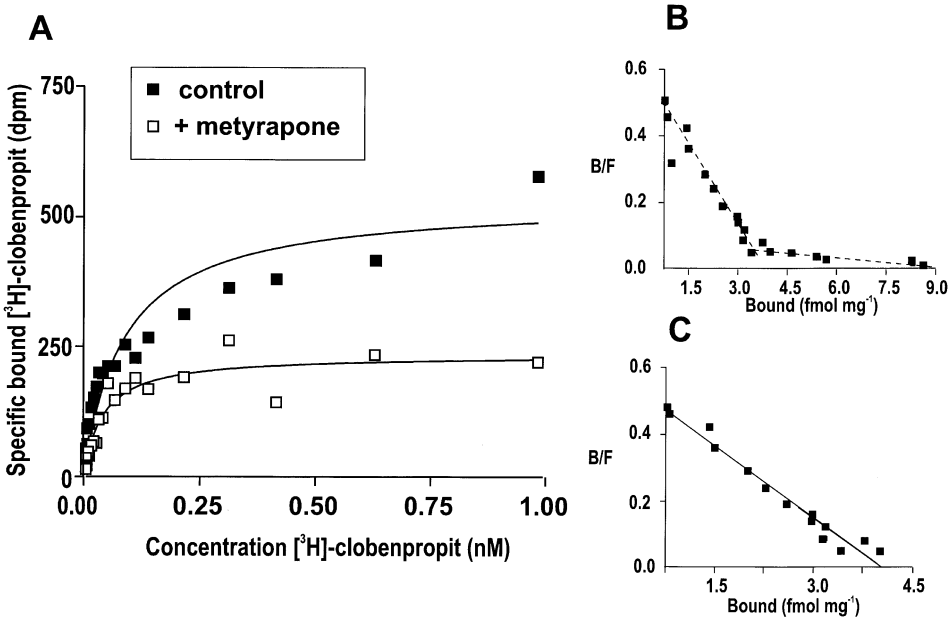

Figure 2 (A) Saturation analysis of the binding of [^{3}H]-clobenpropit to sites in guinea-pig cerebral cortex membranes in the absence and presence of 3 mM metyrapone. The line shown superimposed on the data points is the saturation isotherm obtained from fitting the data to a one-site model. Tissue (400 μl; 4 mg ml^{-1}) was incubated in triplicate with increasing concentrations of [^{3}H]-clobenpropit (50 μl; 0.04–30 nM) and 50 μl of buffer or 50 μl thioperamide to define total and non-specific binding, respectively. The incubation was terminated after 165 min at 21 ± 3°C. Data is representative of six experiments. (B) Corresponding Scatchard plot of data obtained in the absence of metyrapone. For details of data analysis see text. (C) Corresponding Scatchard plot of data obtained in the presence of metyrapone (3 mM).

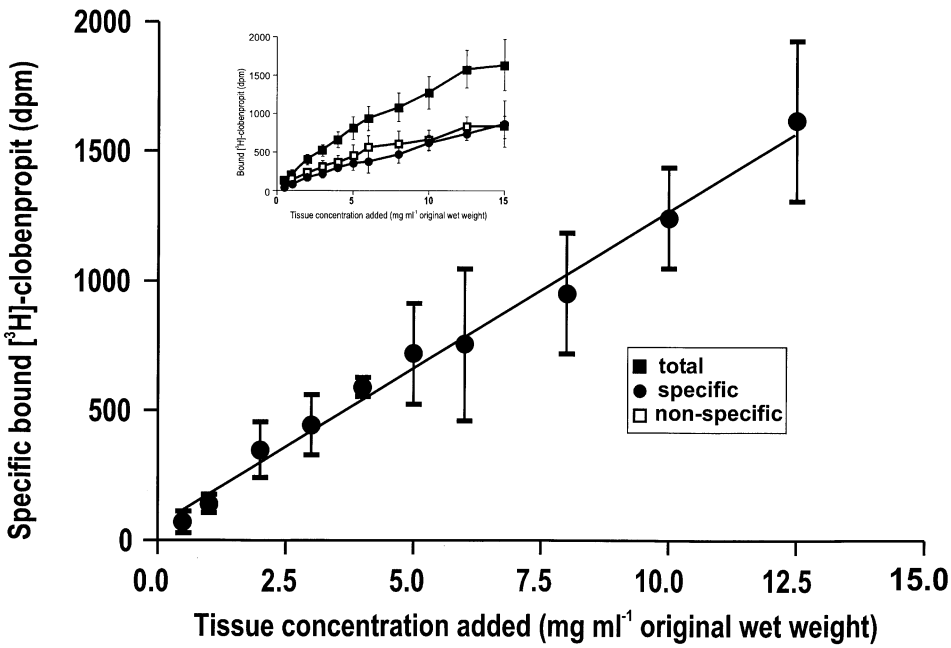

Figure 3 Linearity of the relationship between guinea-pig cerebral cortex membrane concentration and the specific binding of [^{3}H]-clobenpropit (0.2 nM; ~9500 d.p.m.) in the presence of 3 mM metyrapone. Data represents the mean ± s.e.mean of five experiments. The line shown superimposed on the data was obtained by linear regression. The inset shows the total binding, non-specific binding and specific binding of [^{3}H]-clobenpropit (0.2 nM) in the presence of 3 mM metyrapone. Data represents the mean ± s.e.mean of five experiments.

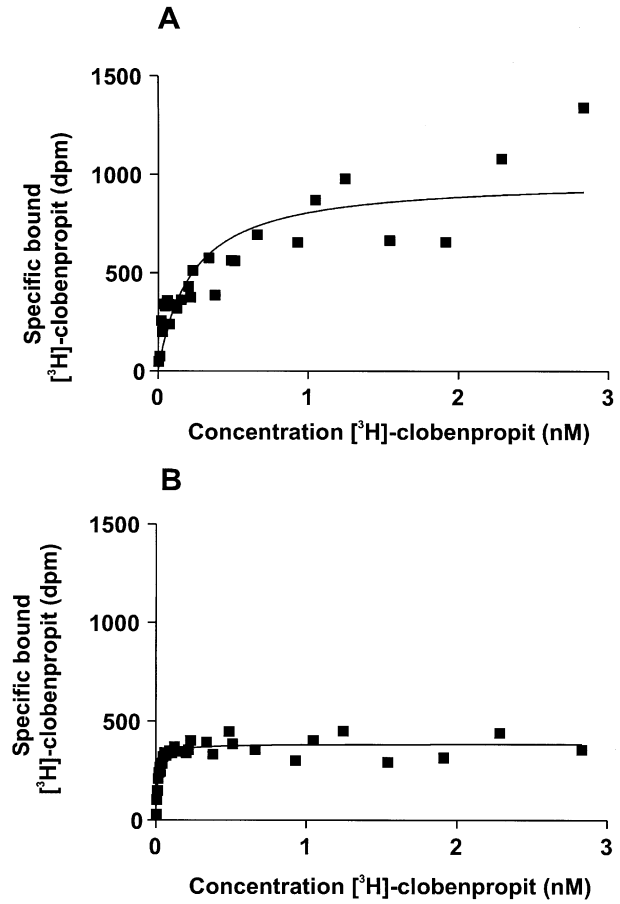

Figure 4 Saturation analysis of the binding of [^{3}H]-clobenpropit to sites in guinea-pig cerebral cortex membranes in the absence (A) and presence (B) of 100 mM NaCl. The line shown superimposed on the data points is the saturation isotherm obtained from fitting the data to a one-site model. Tissue (400 μl; 4 mg ml^{-1}) was incubated in triplicate with increasing concentrations of [^{3}H]-clobenpropit (50 μl; 0.04 – 30 nM) and 50 μl of buffer or 50 μl thioperamide to define total and non-specific binding, respectively. The incubation was terminated after 165 min at 21 $\pm$ 3°C. Data is representative of four experiments.

10.91 ± 0.12 and 9.17 ± 0.16 for the two sites. The corresponding B_{max} estimates were 5.34 ± 0.85 and 23.20 ± 6.70 fmol mg^{-1} (original wet weight).

The presence of granisetron (1 μM), rilmenidine (3 μM), idazoxan (0.3 μM), DTG (0.3 μM) and pentazocine (3 μM) (see Methods; saturation studies) had no effect on the [^{3}H]-clobenpropit saturation isotherm or on the apparent non-specific binding. However, the presence of metyrapone (3 mM) normalized the complex [^{3}H]-clobenpropit saturation isotherm so that it appeared monophasic. Therefore, tissue concentration experiments were repeated in the presence of the cytochrome P450 enzyme inhibitor metyrapone (3 mM).

Tissue concentration curve in the presence of 3 mM metyrapone

Total binding, non-specific binding and specific binding of [^{3}H]-clobenpropit increased with guinea-pig cerebral cortex membrane concentration (Figure 3). Specific binding increased linearly with increasing added cerebral cortex membrane concentration up to at least a 4 mg ml^{-1} concentration in each replicate experiment (Figure 3) although in some of these experiments the specific binding was linear up 12.5 mg ml^{-1}. At a 4 mg ml^{-1} added membrane concentration, and the tissue concentration used for subsequent saturation, competition and kinetic studies, 12.0 ± 1.0% of the added [^{3}H]-clobenpropit was bound and the specific binding was 45.4 ± 2.5% ($n = 5$).

Saturation analysis in the presence of metyrapone or sodium chloride

The saturation isotherm for the binding of [^{3}H]-clobenpropit to guinea-pig cerebral cortex membranes, in the presence of metyrapone, was monophasic (Figure 2A). Scatchard plots were linear (Figure 2C) and Hill plot slopes were not significantly different from unity (+ metyrapone $n_H = 0.91 \pm 0.04$; control $n_H = 0.70 \pm 0.03$; $n = 6$). [^{3}H]-clobenpropit was estimated to express an affinity (pK_D) of 10.59 ± 0.17. The estimated receptor density (B_{max}) was 3.41 ± 0.46 fmol mg^{-1} (original wet weight) tissue.

In the presence of sodium chloride (100 mM), the saturation isotherm for the binding of [^{3}H]-clobenpropit to guinea-pig cerebral cortex membranes appeared monophasic (Figure 4). Scatchard plots were linear and Hill plots had slopes which were not significantly different from unity (+ NaCl $n_H = 0.99 \pm 0.02$; control $n_H = 0.53 \pm 0.10$; $n = 4$). The estimated pK_D was 10.77 ± 0.02 and B_{max} 3.49 ± 0.44 fmol mg^{-1} (original wet weight) tissue ($n = 4$).

There was no significant difference between the B_{max}, n_H and pK_D estimates for [^{3}H]-clobenpropit in either the presence of metyrapone (3 mM) or NaCl (100 mM).

Kinetic studies

A 0.2 nM concentration of [^{3}H]-clobenpropit was used to study association and dissociation profiles in the presence of metyrapone (3 mM). The specific binding of [^{3}H]-clobenpropit appeared to reach equilibrium after a 25 min incubation at room temperature (21 $\pm$ 3°C) and remained constant for at least a further 180 min ($n = 3$; Figure 5). Association data was transformed according to the pseudo-first-order rate equation on the basis that only $\sim$10% of added ligand was bound at equilibrium. The data obtained for binding to guinea-pig cerebral cortex membranes could be fitted by a mono-exponential function. The association rate constant (k_{+1}) for the binding of [^{3}H]-clobenpropit to histamine H_3-receptors on guinea-pig cerebral cortex membranes was $7.49 \pm 1.32 \times 10^8$ M^{-1} min^{-1} ($n = 3$).

The dissociation data obtained with [^{3}H]-clobenpropit, following the addition of an excess concentration of thioperamide, could also be fitted to a mono-exponential function. The dissociation rate constant (k_{-1}) was 0.044 ± 0.006 min^{-1} ($n = 3$). The pK_D calculated from these kinetic studies was 10.27 ± 0.27 ($n = 3$) which was not significantly different from the value estimated by saturation analysis.

Competition studies

Each of the histamine H_3-receptor ligands produced a concentration-dependent inhibition of the specific binding of either [^{3}H]-clobenpropit or [^{3}H]-*R*-α-MH to sites in guinea-pig cerebral cortex membranes (Table 2). Examples of competition curve data are shown in Figure 6. When [^{3}H]-clobenpropit was used to label histamine H_3-receptors in guinea-pig cerebral cortex, and the data obtained were fitted to the Hill equation, the mid-point slope parameter estimates (n_H) for thioperamide, GR175737 (Clitherow *et al.*, 1996), *N*-α-MH, JB96134 and iodoproxyfan were significantly different from unity. In contrast, when [^{3}H]-*R*-α-MH was used as the radiolabel the mid-point slope parameter estimates obtained for JB96132,

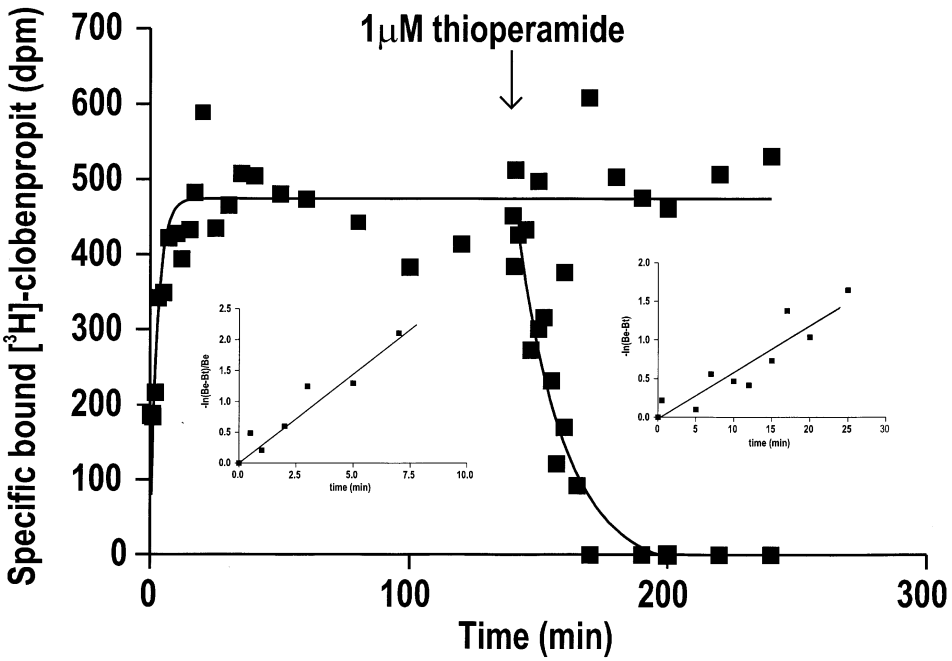

Figure 5 Representative association-dissociation analysis of [^{3}H]-clobenpropit binding to guinea-pig cerebral cortex membranes, in the presence of 3 mM metyrapone. The association rate was measured under pseudo-first order conditions (Weiland & Molinoff, 1981) at 21±3°C by incubating [^{3}H]-clobenpropit (50 μl; 2 nM) for increasing times (0.5–250 min) in triplicate tubes containing membranes (400 μl; 4 mg ml^{-1} original wet weight) and 50 μl of buffer or 10 μM thioperamide to define the total and non-specific binding, respectively. The dissociation rate for [^{3}H]-clobenpropit from sites on membranes was determined by incubating [^{3}H]-clobenpropit (50 μl; 2 nM), in nine tubes with 50 μl of buffer (total binding) and in triplicate with 50 μl 10 μM thioperamide (non-specific binding), for 150 min at 21±3°C. At this time 10 μl of 50 μM thioperamide was added to six tubes defining total binding and the bound radioligand was determined at increasing times (0.5–100 min). Semilogarithmic plots of association and dissociation data are provided as inserts.

Table 2 Analysis of multiple data sets from competition experiments between [^{3}H]-clobenpropit or [^{3}H]-*R*-α-MH and histamine H_3-receptor ligands (see text for details)

		[^{3}H]-clobenpropit			*[^{3}H]*-R-α-*MH*		
Compound	*pIC_{50}±s.e.mean*	*n_H±s.e.mean*	*pK_i'±s.e.mean*	n	*n_H±s.e.mean*	*pK_i'±s.e.mean*	n
Thioperamide†	7.58±0.06	0.86±0.03*	8.69±0.05	9	0.66±0.03*	9.08±0.13	17
Iodophenpropit†	9.03±0.13	0.95±0.06	10.08±0.11	10	0.94±0.12	9.96±0.23	6
JB96134	6.80±0.24	1.48±0.12*	7.86±0.18	9	1.00±0.09	7.83±0.05	5
JB96132	7.77±0.08	1.02±0.13	8.84±0.08	10	0.82±0.04*	9.16±0.12	6
GR175737	7.72±0.05	0.74±0.10*	8.76±0.05	3	0.80±0.10*	8.79±0.22	3
R-α-MH†	8.41±0.17	0.71±0.09	9.48±0.16	12	0.91±0.20	10.07±0.16	8
N-α-MH	8.92±0.23	0.63±0.07*	9.99±0.24	7	0.77±0.05*	10.31±0.24	4
Proxyfan†	7.27±0.12	0.89±0.14	8.32±0.13	9	0.79±0.05*	8.80±0.23	6
Iodoproxyfan†	8.68±0.23	0.76±0.07*	9.76±0.23	10	0.94±0.08	10.00±0.09	10

pK_i' were calculated using the Cheng Prusoff equation (1973) (*$P<0.05$). †data presented previously for competition with [^{3}H]-*R*-α-MH (Harper *et al.*, 1999).

GR175737 and thioperamide (Jansen *et al.*, 1992) were significantly different from unity.

The apparent affinity estimates (pK_i') obtained for histamine H_3-receptor ligands when [^{3}H]-clobenpropit was used as the radioligand were not significantly different from those obtained using [^{3}H]-*R*-α-MH (Table 2) and regression analysis of the data indicated that there was a good correlation between the pK_i' values obtained using the two radioligands ($r=0.95$, slope = 1.00; Figure 7).

Discussion

We decided to radiolabel clobenpropit because it has been classified as a high affinity, competitive, histamine H_3-receptor antagonist (Van der Goot *et al.*, 1992). In this study, the replicate saturation isotherms for [^{3}H]-clobenpropit in guinea-pig cerebral cortex membranes were biphasic and, consequently, the corresponding Scatchard plots were curvilinear and Hill plots had slopes that were significantly different from unity. There are a number of possible explanations for these data. However, because there is no evidence from functional *in vitro* assays to suggest that the low Hill slope parameter may have resulted from negative co-operativity (Van der Goot *et al.*, 1992), we considered that the most plausible explanation for the data was that [^{3}H]-clobenpropit was labelling a heterogeneous population of sites in guinea-pig cerebral cortex membranes.

We considered that there were two possible explanations for the heterogeneous population of sites which [^{3}H]-clobenpropit appeared to label. Either, that [^{3}H]-clobenpropit was labelling two histamine H_3-receptor subtypes or that in addition to a

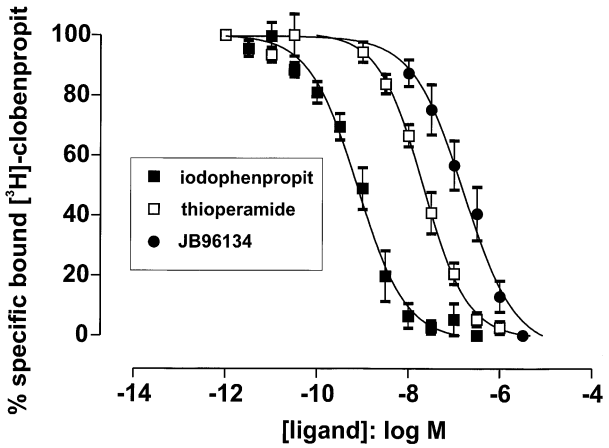

Figure 6 Competition between [^{3}H]-clobenpropit (0.2 nM) and increasing concentrations of iodophenpropit, thioperamide and JB96134 for histamine H_3-receptors in guinea-pig cerebral cortical membranes, in the presence of 3 mM metyrapone. Data represent the mean ± s.e.mean of nine or ten experiments where each point was determined in triplicate. The curves shown superimposed on the mean experimental data points were obtained by simulation using the Hill equation where the parameters were set at the mean values estimated by fitting each replicate curve to that equation. The parameters used in the simulations are presented in Table 2.

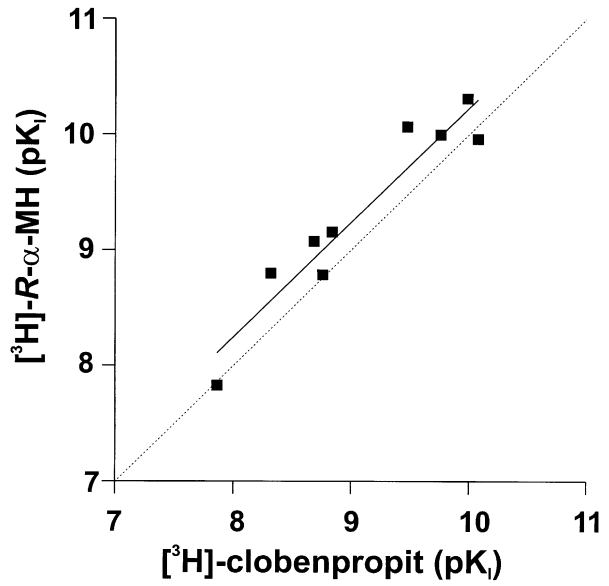

Figure 7 Comparison of the estimated affinities (pK_i') of histamine H_3-receptor ligands at H_3-receptors labelled with [^{3}H]-clobenpropit or [^{3}H]-*R*-α-MH. The hatched line represents the line of identity.

single histamine H_3-receptor population, [^{3}H]-clobenpropit labelled a further population of independent sites.

The first possibility, that [^{3}H]-clobenpropit could pharmacologically distinguish between two histamine H_3-receptors, appeared a plausible explanation for the data because it has been suggested that histamine, in addition to its action at membrane-bound receptors, has an additional intracellular site of action (Brandes *et al*., 1985, 1986; Brandes & Bogdanovic, 1986). Moreover, a number of studies have suggested that there may be two histamine H_3-receptors (West *et al*., 1990b; Clapham & Kilpatrick, 1992; Leurs *et al*., 1996; Schlicker *et al*. 1996). However, we obtained no evidence for histamine H_3-receptor heterogeneity from replicate [^{3}H]-*R*-α-MH saturation analyses because Scatchard plots were linear and the corresponding Hill plots had slopes which were not significantly different from unity (Harper *et al*., 1997b, 1999 [accompanying paper]). Nevertheless, the lack of evidence for histamine H_3-receptor heterogeneity could be accounted for by [^{3}H]-*R*-α-MH also expressing a low affinity (>10 nM) for the putative 'low affinity' [^{3}H]-clobenpropit binding site. Thus, only concentrations of [^{3}H]-*R*-α-MH concentrations above those used in the saturation analysis (0.01 – 20 nM) would have labelled a significant number of these other sites. We could have also failed to detect histamine H_3-receptor heterogeneity in the [^{3}H]-*R*-α-MH saturation analysis if the label expressed similar affinity for both the 'high and low affinity' [^{3}H]-clobenpropit binding sites. However, this would appear not to be the case because the mid-point slope parameter (n_H) estimate for clobenpropit when competing with [^{3}H]-*R*-α-MH in guinea-pig cerebral cortex was not significantly different from unity (Harper *et al*., 1997c).

The hypothesis, that at least one of the populations of [^{3}H]-clobenpropit binding sites corresponded to the previously characterized population of histamine H_3-receptors in the guinea-pig cerebral cortex, was supported by the finding that the estimated affinity (pK_D) of clobenpropit at the 'high affinity' site was indistinguishable from the estimated affinity (pK_i) of clobenpropit at receptors labelled with [^{3}H]-*R*-α-MH (Harper *et al*., 1997b, 1999 [accompanying paper]). This hypothesis was further supported by the observation that the estimated density of the 'high affinity' [^{3}H]-clobenpropit binding sites (3.41 ± 0.46) was similar to the density of H_3-receptors in guinea-pig cerebral cortex (3.91 ± 0.37) determined under the same assay conditions when [^{3}H]-*R*-α-MH was used as the radioligand (Harper *et al*., 1997b).

The second possibility, that [^{3}H]-clobenpropit was labelling both histamine H_3-receptors and a population of non-histamine H_3 sites, also appears a plausible explanation for the data because several radioligands, initially considered to be highly receptor selective ligands, have been found to bind with high affinity to other receptors/sites. For instance, the histamine H_2-receptor antagonist, cimetidine, has been found to label imidazoline recognition sites (Rising *et al*., 1980; Warrander *et al*., 1983) while it has been suggested that the histamine H_1-receptor antagonist, pyrilamine, binds to a cytochrome P450 type enzyme in liver and brain (Liu *et al*., 1994; Hiroi *et al*., 1995). However, the possibility that [^{3}H]-clobenpropit is labelling non-histamine H_3-receptors can only explain the data if thioperamide, the ligand that was used to define the non-specific binding, also expresses high affinity for the second population of [^{3}H]-clobenpropit binding sites. This seemed feasible in view of the structural similarities between thioperamide and clobenpropit. Indeed, the majority of the histamine H_3-receptor antagonist ligands described to date share sufficient structural similarities for some common non-histamine H_3-receptor binding. Thus, several histamine H_3-receptor ligands have been shown to bind to other receptors/sites. For example, thioperamide and iodophenpropit both bind to 5-HT_3-, 5-HT_4-, Ach M_1-, sigma- and α_2-receptors (Leurs *et al*., 1995).

The possibility that, under the current assay conditions, [^{3}H]-clobenpropit was labelling 5-HT_3 receptors appeared unlikely because the 'low affinity' [^{3}H]-clobenpropit binding was eliminated in the presence of 100 mM NaCl whilst the binding of the 5-HT_3 receptor selective ligand, [^{3}H]-zacopride, has been shown to be insensitive to salts (Pinkus *et al*., 1989). Moreover, in this study, the selective 5-HT_3 receptor ligand, granisetron (1 μM) (pK_i 5-HT_3 receptors ~9.7; Fletcher & Barnes, 1997) did not modify the complex saturation isotherm of [^{3}H]-clobenpropit. The prospect that [^{3}H]-clobenpropit was

labelling imidazoline binding sites was also excluded by the finding that curvilinear Scatchard plots and non-unit Hill slopes were still obtained in the presence of the imidazoline I_1 ligand, rilmenidine (3 μM) (pK_i I_1 receptors $\sim$8.1; Gargalidis-Moudanos & Parini, 1995) and the imidazoline I_2 ligand, idazoxan (0.3 μM) (pK_i I_2 receptors $\sim$8.8; MacKinnon *et al*., 1993).

The lack of effect of pentazocine (3 μM; pK_D σ_1 sites $\sim$8.5; DeHaven-Hudkins *et al*., 1992) or 1,3-di-(2-tolyl)guanidine (DTG) (0.3 μM; pK_i σ_2 sites $\sim$7.7; Bowen *et al*., 1988) on the complex saturation isotherm of [^{3}H]-clobenpropit suggested that the second population of [^{3}H]-clobenpropit binding sites were not σ sites. However, a number of studies have suggested that there are multiple binding sites on the σ 'receptor' (e.g. Bowen *et al*., 1989) such as the site labelled by [^{3}H]-emopamil (Moebius *et al*., 1993) which is distinct from that to which pentazocine binds. Therefore, [^{3}H]-clobenpropit could be labelling independent allosterically-coupled sites on the 'sigma receptor' which are similar or identical to those labelled by [^{3}H]-emopamil. Such an independent allosteric interaction would explain why [^{3}H]-pentazocine binding is insensitive to sodium ions whilst the 'low affinity' binding of [^{3}H]-clobenpropit and the binding of [^{3}H]-emopamil are both sensitive to sodium ions (Zech *et al*., 1991).

The observation that in the presence of the cytochrome P450 inhibitor, metyrapone, the complex [^{3}H]-clobenpropit saturation binding isotherm was rectified, Scatchard plots were linear and Hill plot slopes were not significantly different from unity suggested that the second population of [^{3}H]-clobenpropit binding sites were cytochrome P450 isoenzyme(s). This was not surprising in view of previous studies with a close-structural histamine H_3-receptor analogue, thioperamide. Thus, thioperamide has been shown to interact with cytochrome P450 enzymes in adrenal microsomes (LaBella *et al*., 1992) and the binding of [^{3}H]-thioperamide has been shown to be partly inhibited by cytochrome P450 inhibitors (Alves-Rodriguez *et al*., 1996).

The hypothesis that, in the presence of metyrapone, [^{3}H]-clobenpropit labelled a homogeneous population of 'high affinity' binding sites corresponding to histamine H_3-receptors was supported by the saturation and the kinetic data. Thus, in saturation studies, in the presence of metyrapone, [^{3}H]-clobenpropit appeared to label a homogeneous population of sites with a pK_D value of 10.59 ± 0.17. This affinity value was not significantly different from the pK_i of clobenpropit when competing with [^{3}H]-*R*-α-MH under identical assay conditions (10.49 ± 0.16; Harper *et al*., 1997c; 1999) or to the estimated affinity of [^{3}H]-clobenpropit at the 'high affinity' sites in the absence of metyrapone ($pK_D = 10.91 \pm 0.12$). Furthermore, the B_{max} of [^{3}H]-clobenpropit in the presence of metyrapone (3.41 ± 0.46) was not significantly different from that estimated at the 'high affinity' [^{3}H]-clobenpropit binding sites (5.34 ± 0.85) in the absence of metyrapone. In kinetic studies, performed in the presence of metyrapone, both the association and dissociation data could be fitted by pseudo-first order and first order rate equations, respectively. In addition, the estimated pK_D of [^{3}H]-clobenpropit (10.27 ± 0.27) was not significantly different from that estimated by saturation analysis in the presence of metyrapone (10.59 ± 0.17) or to the estimated affinity of [^{3}H]-clobenpropit at the 'high affinity' binding sites in the absence of metyrapone (10.91 ± 0.12).

The data obtained in competition studies, performed in the presence of metyrapone, also supported the hypothesis that the high affinity [^{3}H]-clobenpropit binding sites corresponded to histamine H_3-receptors. Consequently, notwithstanding the fact that the Hill slopes of some ligands were significantly different from unity in both assays (Table 2), there was a good correlation between the ligand affinity estimates (pK_i') obtained for a chemically diverse series of histamine H_3-receptor ligands and those estimated when [^{3}H]-*R*-α-MH was used as the radioligand under the same assay conditions ($r = 0.95$, slope $= 1.00$). In addition, the affinity estimates obtained for GR175737, thioperamide, iodophenpropit, JB96132, JB96134, proxyfan, iodoproxyfan, *N*-α-MH and *R*-α-MH were all comparable to those previously estimated at histamine H_3-receptors in either cerebral cortex radioligand binding assays, or in functional *in vitro* assays such as the guinea-pig ileum or cerebral cortical slice release assays (Clark *et al*., 1992; West *et al*., 1990b; Jansen *et al*., 1992; Schlicker *et al*., 1994; 1996; Clitherow *et al*., 1996; James Black Foundation patent, 1997).

Conclusion

In this study we have established that [^{3}H]-clobenpropit labels histamine H_3-receptors and 'metyrapone-sensitive' site(s) which may correspond to cytochrome P450 enzyme(s). In the presence of metyrapone, [^{3}H]-clobenpropit can be used as a suitable alternative to agonist radioligands such as [^{3}H]-*R*-α-MH and [^{3}H]-*N*-α-MH to label histamine H_3-receptors in central nervous tissues. The potential utility of this radioligand for labelling histamine H_3-receptors in peripheral tissues has yet to be determined.

This work was funded by Johnson & Johnson.

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(Received May 4, 1999
Revised July 22, 1999
Accepted July 29, 1999)