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Evidence that histamine homologues discriminate between H_3-receptors in guinea-pig cerebral cortex and ileum longitudinal muscle myenteric plexus

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1 The binding of the selective histamine H_3-receptor agonist ([^{3}H]-*R*-α-methylhistamine) to sites in guinea-pig cerebral cortex and ileum longitudinal muscle myenteric plexus has been characterized and a comparison made of the apparent affinities of a series of H_3-receptor ligands.

2 Saturation analysis suggested that [^{3}H]-*R*-α-methylhistamine labelled a homogeneous population of histamine H_3-receptors in guinea-pig cerebral cortex ($pK_D = 9.91 \pm 0.07$; $n_H = 1.07 \pm 0.03$; $n = 5$) and ileum longitudinal muscle myenteric plexus ($pK_D = 9.75 \pm 0.21$; $n_H = 0.97 \pm 0.02$; $n = 5$). There was no significant difference in the estimated affinity of [^{3}H]-*R*-α-methylhistamine in the two tissues. The cerebral cortex had a significantly higher receptor density (3.91 ± 0.37 fmol mg^{-1} tissue) than the ileum longitudinal muscle myenteric plexus (0.39 ± 0.11 fmol mg^{-1}).

3 Overall, the apparent affinities of compounds, classified as H_3-receptor ligands, in cerebral cortex and ileum longitudinal muscle myenteric plexus were well correlated ($r = 0.91$, $P < 0.0001$) and consistent with the cerebral cortex and ileum longitudinal muscle myenteric plexus expressing histamine H_3-receptor population(s) that are pharmacologically indistinguishable by the majority of histamine H_3-receptor ligands. However, it was evident that the homologues of histamine within this group of compounds could discriminate between the receptor populations in the two tissues. Thus, the estimated affinity of five imidazole unbranched alkylamines (histamine, homohistamine, VUF4701, VUF4732 and impentamine) were significantly higher in the guinea-pig cerebral cortex than in the ileum longitudinal muscle myenteric plexus assay.

Keywords: Guinea-pig cerebral cortex; longitudinal muscle myenteric plexus; [^{3}H]-*R*-α-methylhistamine; histamine H_3-receptors

Abbreviations: [^{3}H]-*R*-α-MH, [^{3}H]-*R*-α-methylhistamine; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]; LMMP, ileum longitudinal muscle myenteric plexus

Introduction

Radioligand binding studies have been used extensively (e.g. Arrang *et al*., 1990; Jansen *et al*., 1994; Clark & Hill, 1995), together with functional bioassay studies (e.g. Arrang *et al*., 1985; Clapham & Kilpatrick, 1992; Schlicker *et al*., 1996), to characterize histamine H_3-receptors in central nervous system tissue. In contrast, characterization of histamine-H_3 receptors in peripheral tissues has largely been accomplished by the use of 'functional' *in-vitro* bioassay studies (e.g. Coruzzi *et al*., 1991; Hew *et al*., 1990; Poli *et al*., 1991; Schworer *et al*., 1994) and only a few studies have attempted to characterize these receptors using radioligand binding assays (e.g. Korte *et al*., 1990; Courillonmallet *et al*., 1995).

A number of histamine H_3-receptor ligands (VUF8326, VUF4701 and VUF4702) have been reported to express different pharmacological behaviour at histamine H_3-receptors in the central nervous system to those receptors present in the gastrointestinal tract (Leurs *et al*., 1996; Schlicker *et al*., 1996). It is possible that some of these differences could be explained by speciation of histamine H_3-receptors or to tissue-dependent differences in the efficiency of receptor coupling. However, they could also be accounted for by subtypes of histamine H_3-receptors. Indeed, West and colleagues (1990a) postulated the existence of H_{3A} and H_{3B} receptors in the cerebral cortex in order to account for the flat competition curves obtained with two H_3-receptor antagonists (thioperamide and burimamide) in radioligand binding assays.

There were two aims of this study. First, to label histamine H_3-receptors under identical assay conditions, with [^{3}H]-R-α-MH, in both a central nervous system tissue (cerebral cortex) and a peripheral tissue (ileum longitudinal muscle myenteric plexus) from the same species (guinea-pig). Second, to determine whether we could obtain any further evidence for histamine H_3-receptor heterogeneity by characterizing the behaviour of a series of H_3-receptor agonists and antagonists in these tissues. The ileum longitudinal muscle myenteric plexus (LMMP) was selected as the peripheral tissue because we had obtained data on the same ligands in a functional bioassay of this tissue Watt *et al*. (1997b).

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

Methods

Assessment of the filter binding of [^{3}H]-R-α-methylhistamine

[^{3}H]-*R*-α-methylhistamine ([^{3}H]-R-α-MH) was diluted to a concentration of 1 nM with 20 mM HEPES-NaOH buffer (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 pre-soaked GF/B filter circles mounted on a

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Millipore filter block. Filters were washed (3 × 3 ml) with ice-cold 50 mM Tris HCl, transferred into scintillation vials, 5 ml Beckman Ready-Solv HP liquid scintillation cocktail added and, after a further 4 h, the bound radioactivity determined by counting (3 min) in a Beckman liquid scintillation counter.

Tissue preparation – guinea-pig cerebral cortex

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 from each centrifugation were pooled and centrifuged at 39,800 × *g* for 12 min at 4°C. The final pellet was resuspended in 20 mM HEPES-NaOH buffer (pH 7.4 at 21 ± 3°C), to the required tissue concentration, using a teflon-in-glass homogenizer (setting 5; 3 ×).

Tissue preparation – guinea-pig ileum longitudinal muscle myenteric plexus (LMMP)

Guinea-pigs were killed by cervical dislocation and the small intestine rapidly removed and placed in ice-cold 20 mM HEPES-NaOH buffer (pH 7.4 at 21 ± 3°C). The tissue was cut into ~10 cm segments, flushed through with ice-cold buffer and placed in fresh HEPES-NaOH buffer at 4°C. Segments of ileum (10 cm) were threaded onto a glass pipette and the longitudinal muscle teased away from the circular muscle using damp cotton-wool. Thus the prepared tissue was identical to that used in our functional histamine H_3-receptor bioassay (Watt *et al*., 1997a). The LMMP was immediately placed in ice-cold Viaspan® solution (1 g 15 ml^{-1}) and incubated at 4°C. After 24 h the tissue was homogenized (Polytron Kinematica AG; PT-DA 3020/2TS; 3 × ~1–2 s), diluted to a final concentration of 50 mM Tris-HCl (pH 6.9 at 4°C) and centrifuged at 39,800 × *g* for 12 min at 4°C. The supernatants were discarded and pellets rehomogenized in 100 ml ice-cold HEPES-NaOH buffer (pH 7.4 at 21 ± 3°C) using a teflon-in-glass homogenizer. The homogenate was recentrifuged at 39,800 × *g* and the pellet resuspended in 20 mM HEPES-NaOH (pH 7.4 at 21 ± 3°C) to the required tissue concentration, using a polytron homogenizer (Brinkman, PT10, 3 × ~1 s).

Incubation conditions – saturation studies

Membranes from guinea-pig cerebral cortex (400 μl; 7.5 mg ml^{-1}; original wet weight) or the LMMP (400 μl; 50 mg ml^{-1}; original wet weight) 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 0.1 to 200 nM [^{3}H]-R-α-MH. Total and non-specific binding of [^{3}H]-R-α-MH were defined using 50 μl of HEPES-NaOH buffer and 50 μl of 10 μM 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, pre-soaked in 0.1% 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, 5 ml Beckman Ready-Solv HP liquid scintillation cocktail added and after 4 h the bound radioactivity was determined by counting (3 min) in a Beckman liquid scintillation counter.

Incubation conditions – kinetic studies

To ascertain the time course of the association, [^{3}H]-R-α-MH (50 μl; 1 nM cortex and 3 nM LMMP) was incubated in triplicate in tubes containing membranes (400 μl; 7.5 mg ml cortex or 50 mg ml^{-1} LMMP) and 50 μl of HEPES-NaOH buffer or 50 μl of 10 μM thioperamide for increasing times (1–320 min). The incubations were terminated by rapid filtration through pre-soaked Whatman GF/B filter circles.

For dissociation experiments, [^{3}H]-R-α-MH was incubated (50 μl; 1 nM cortex and 3 nM LMMP), in sextruplicate with 50 μl of HEPES-NaOH buffer (total binding) and in triplicate with 50 μl of 10 μM thioperamide (non-specific binding), for 165 min at 21 ± 3°C. At this time dissociation was initiated by addition of an excess concentration (10 μl of 50 μM) of unlabelled thioperamide, to a triplicate group of tubes defining total binding. The bound [^{3}H]-R-α-MH was determined at increasing times (1–180 min) by rapid filtration through pre-soaked Whatman GF/B filter circles.

Incubation conditions – competition studies

Membranes from guinea-pig cerebral cortex (400 μl; 7.5 mg ml^{-1}; original wet weight) or the LMMP (400 μl; 50 mg ml^{-1}; original wet weight) were incubated for 165 min at 21 ± 3°C in a final volume of 500 μl with 20 mM HEPES-NaOH containing [^{3}H]-R-α-MH (50 μl; 1 nM cortex and 3 nM LMMP) and competing compound. Total and non-specific binding of [^{3}H]-R-α-MH were defined using 50 μl of HEPES-NaOH buffer and 50 μl of 10 μM thioperamide, respectively. The assay was terminated by rapid filtration through pre-soaked Whatman GF/B filters using a Brandell Cell Harvester. Bound radioactivity was determined by liquid scintillation counting.

Data analysis

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

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

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

Dissociation constants (pK_I) were determined using the Cheng & Prusoff equation (1973),

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

In this equation, [L] is the radioligand concentration and the K_D is the equilibrium dissociation constant of [^{3}H]-*R*-α-MH determined by saturation analysis in each tissue (cortex $pK_D = 9.91 \pm 0.07$; LMMP $pK_D = 9.75 \pm 0.21$). All data is presented as the mean value ± s.e.mean unless otherwise indicated.

Materials

[^{3}H]-R-α-methylhistamine (specific activity of ~38 Ci.m-mol^{-1})([^{3}H]-*R*-α-MH) was supplied by Amersham Interna-

tional plc., Little Chalfont, Buckinghamshire, U.K. Iodophenpropit, proxyfan (3-(1*H*-imidazol-4-yl)propyl-benzyl ether), chloroproxyfan (3-(1*H*-imidazol-4-yl)propyl-(4-chlorobenzyl) ether), bromoproxyfan (3-(1*H*-imidazol-4-yl)propyl-(4-bromobenzyl) ether), iodoproxyfan, JB96085 (trans-[3-(1*H*-imidazol-4-yl)prop-2-enyl](4-iodobenzyl)ether, JB96183 ((±)-trans-1-(1*H*-imidazol-4-yl)-2-(4-iodobenzyloxymethyl)-cyclopropane), JB96180 (3-(1*H*-imidazol-4-yl)propyl-(4-trifluoromethylbenzyl)ether), clobenpropit (*S*-[3-(1*H*-imidazol-4-yl)propyl]-*N*-(4-chlorobenzyl)-isothiourea), JB96156 (*S*[3-(1*H*-imidazol-4-yl)propyl]-*N*-benzyl-isothiourea), JB96157 (*S*-[3-(1*H*-imidazol-4-yl)propyl]-*N*-(4-fluorobenzyl)-isothiourea), JB96158 (*S*-[3-(1*H*-imidazol-4-yl)propyl]-*N*-(4-bromobenzyl)-iso-thiourea), JB96088 (trans-[3-(1*H*-imidazol-4-yl)prop-2-enyl] benzylether), JB95130 (*N*-[2-(1*H*-imidazol-4-yl)ethyl]-4-phenylbutyramide), impentamine (VUF4702), homohistamine (VUF 8326), imbutamine (VUF4701), imhexamine (VUF4732) and GT-2016 (4-(1*H*-imidazol-4-yl)-1-(5-cyclohexyl-butylcarbamyl) piperidine) were synthesized by James Black Foundation chemists.

HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]), histamine, Trizma base®, pyrilamine and cimetidine were obtained from Sigma Chemical Co., Poole, Dorset, U.K. *R*-α-methylhistamine, *S*-α-methylhistamine, thioperamide and imetit were obtained from Research Biochemicals Inc., Poole, Dorset, U.K. All other materials were obtained from Fisher Scientific, Loughborough, Leicestershire, U.K.

Results

Chemical properties of [^{3}H]-R-α-MH

In excess of 19% of the added [^{3}H]-R-α-MH (0.1 nM) bound to GF/B filters in the absence of tissue. When the filters were pre-soaked in 0.1% polyethyleneimine the amount of [^{3}H]-R-α-MH bound to the filters was reduced to 0.5±0.1% ($n=6$).

Characterization of radioligand binding assays in the guinea-pig cerebral cortex and the LMMP membranes

Tissue concentration curves Total binding, non-specific binding and specific binding of [^{3}H]-R-α-MH increased with increasing concentration of both cerebral cortex (Figure 1A) and LMMP membranes (Figure 1B). There was a linear relationship between specific binding and added tissue concentration up to 12.5 mg ml^{-1} cortex (Figure 1C) and 70 mg ml^{-1} (original wet weight) LMMP tissue (Figure 1D). When tubes contained 3 mg of cerebral cortex tissue, 9.6±1.1% ($n=5$) of the added [^{3}H]-R-α-MH bound to the membranes and the specific binding was 88.6±1.1%. When 20 mg of LMMP tissue was used, 2.8±0.2% ($n=5$) of the added [^{3}H]-R-α-MH bound and the specific binding was 69.0±3.6%. These tissue concentrations were used for all subsequent experiments.

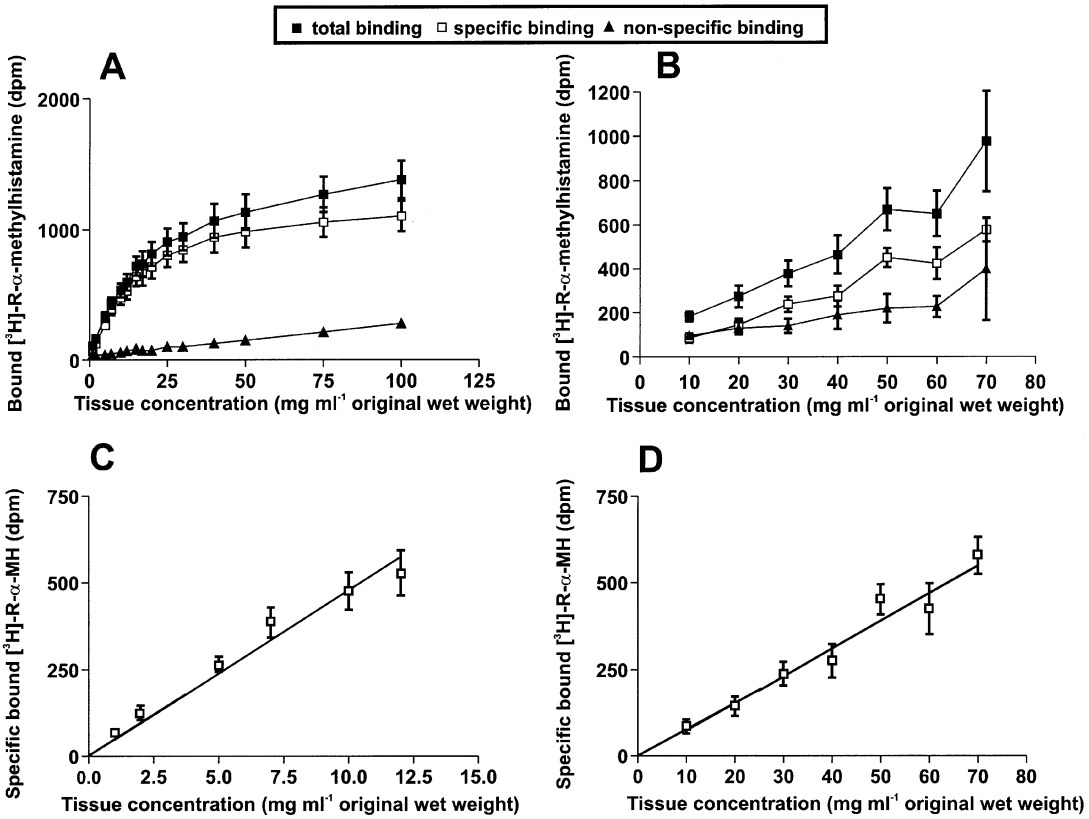

Figure 1 (A) Total binding, non-specific binding and specific binding of [^{3}H]-R-α-MH (0.1 nM; ~4500 d.p.m.) as a function of increasing concentration of guinea-pig cerebral cortex membranes. Increasing concentrations (0.5–100 mg ml^{-1}) of guinea-pig cerebral cortex membranes (400 μl) were incubated in triplicate with 0.1 nM (50 μl; 1 nM) [^{3}H]-R-α-MH for 165 min at 21±3°C. Total binding and non-specific binding were defined with 50 μl buffer and 50 μl of 10 μM thioperamide, respectively. Data represent the mean±s.e.mean of five experiments. (B) Total binding, non-specific binding and specific binding of [^{3}H]-R-α-MH (0.3 nM) as a function of increasing concentration of guinea-pig longitudinal muscle myenteric plexus membranes. Data represent the mean±s.e.mean of five experiments. (C) Pseudo-linear region of the relationship between guinea-pig cerebral cortex membrane concentration and the specific binding of [^{3}H]-R-α-MH (0.1 nM). Data represent the mean±s.e.mean of five experiments. (D) Pseudo-linear region of the relationship between guinea-pig LMMP membrane concentration and the specific binding of [^{3}H]-R-α-MH (0.3 nM). Data represent the mean±s.e.mean of five experiments.

Saturation analysis

The binding of [^{3}H]-R-α-MH to cerebral cortex and LMMP membranes was saturable (Figure 2). Scatchard and Hill plots were linear in the cerebral cortex ($pK_D = 9.91 \pm 0.07$, $n_H = 1.07 \pm 0.03$; $n = 5 \pm$ s.e.mean) and the LMMP ($pK_D = 9.75 \pm 0.21$, $n_H = 0.97 \pm 120.02$; $n = 5 \pm$ s.e.mean). There was no significant difference between the pK_D of [^{3}H]-R-α-MH estimated in the cerebral cortex and LMMP assays. The estimated receptor density in the cerebral cortex ($B_{max} = 3.91 \pm 0.37$ fmol mg^{-1} original wet weight) was significantly higher than that in the LMMP ($B_{max} = 0.39 \pm 0.11$ fmol mg^{-1} original wet weight).

Kinetic studies

The specific binding of [^{3}H]-R-α-MH to cerebral cortex (Figure 3A) and LMMP (Figure 3B) membranes reached equilibrium after approximately 150 min incubation at room temperature (21 ± 3°C) and remained constant for at least a further 150 min. In the cerebral cortex and LMMP the specific bound [^{3}H]-R-α-MH could be dissociated by the addition of an excess of thioperamide (1 μM). The $t_{½}$ for the dissociation of [^{3}H]-R-α-MH from cerebral cortex (35 ± 11 min) was not significantly different from that estimated in the LMMP (26 ± 4 min; $n = 3 \pm$ s.e.mean). Inspection of the data from the individual experiments (Figure 3) suggested that the association and dissociation curves were not monophasic in either tissue. This deviation from monoexponential behaviour was confirmed by the finding that a biexponential function provided a better fit of the dissociation data in both the cerebral cortex ($F_{(20,22)} = 80.1$, $P < 0.05$) and LMMP assays ($F_{(21,23)} = 31.9$, $P < 0.05$) as assessed by comparison of the residual variance of the fits to the data using the 'extra sum of squares' principle (De Lean *et al*., 1980). Therefore, pK_D values were not calculated from these kinetic data.

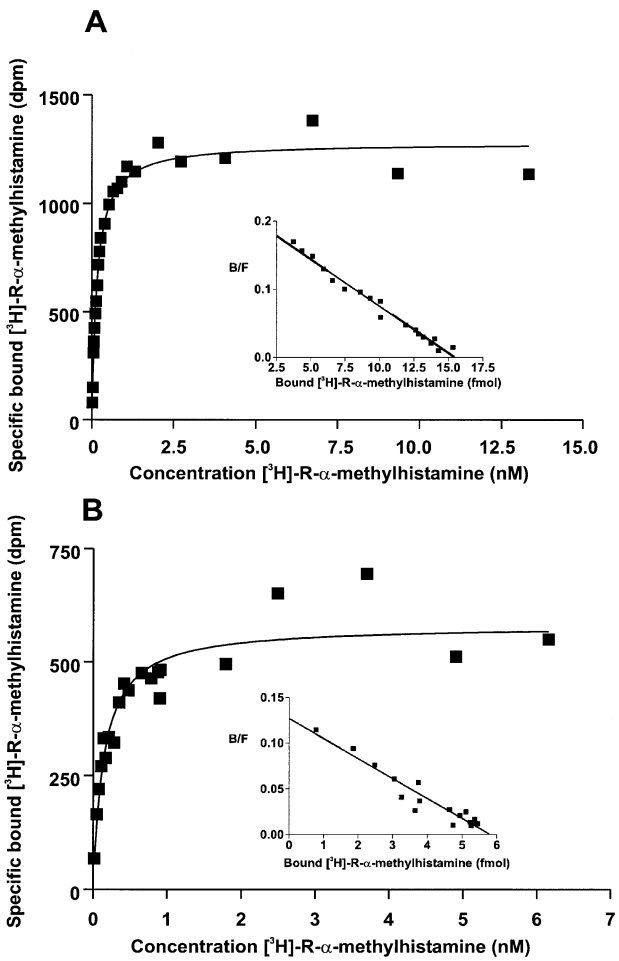

Figure 2 Saturation analysis of the binding of [^{3}H]-R-α-MH to receptors in the guinea-pig cerebral cortex (A) and the LMMP membranes (B). Tissue (400 μl; 7.5 mg ml^{-1} cortex or 50 mg ml^{-1} LMMP) was incubated in triplicate with increasing concentrations of [^{3}H]R-α-MH (50 μl; 0.1–200 nM) and 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 five experiments in each tissue. The corresponding Scatchard plots of the same data are provided as insets.

Competition studies

Histamine H_1- and histamine H_2- receptor ligands The histamine H_1-receptor antagonist, pyrilamine, produced a concentration-dependent inhibition of the specific binding of [^{3}H]-R-α-MH to both guinea-pig cerebral cortex and the LMMP membranes. In the cerebral cortex and the LMMP assays the mean Hill slope parameter estimate (n_H) obtained from the competition curve was significantly less than unity ($P < 0.05$) (Table 1). The estimated affinity (pK_I') of pyrilamine at sites labelled with [^{3}H]-R-α-MH was 5.12 ± 0.12 ($n = 5$) in the cerebral cortex assay and 5.47 ± 0.06 ($n = 4$) in the LMMP assay.

The histamine H_2-receptor antagonist, cimetidine, also produced a concentration-dependent inhibition of the specific binding of [^{3}H]-R-α-MH to sites in guinea-pig cerebral cortex and the LMMP (cortex $pK_I' = 4.03 \pm 0.10$, $n = 3$; ileum LMMP $pK_I' = 4.82 \pm 0.11$, $n = 4$). In both the cerebral cortex and the LMMP the competition curves were monophasic and the mean mid-point slope parameter estimates were not significantly different from unity (Table 1).

These results indicate that a significant population of histamine H_1 and H_2-receptors were not labelled by [^{3}H]-R-α-MH at 0.1 or 0.3 nM concentrations.

H3 receptor 'antagonists'

The histamine H_3-receptor ligands, previously characterized as antagonists, all produced a concentration-dependent inhibition of the specific binding of [^{3}H]-*R*-α-MH to sites in guinea-pig cerebral cortex and the LMMP.

In the cerebral cortex assay, the competition curves for thioperamide, JB96157 and JB95130 were flat and the mean mid-point slope parameter estimates (n_H) were significantly less than unity ($P < 0.05$) (Table 1). In the LMMP assay only the n_H for thioperamide and JB96156 were significantly less than unity ($P < 0.05$; Table 1).

In the cerebral cortex assay, the histamine H_3-receptor 'antagonist' ligands expressed apparent affinities (expressed as pK_I' values, that is, pIC_{50} values corrected for the radiolabel occupancy regardless of the slope of the competition curve), for sites labelled with [^{3}H]-R-α-MH, ranging from 6.48 ± 0.24 ($n = 5$) to 10.51 ± 0.22 ($n = 6$). In the LMMP assay, the antagonists expressed pK_I' values, for sites labelled with [^{3}H]-R-α-MH, ranging from 6.62 ± 0.08 ($n = 4$) to 10.09 ± 0.11 ($n = 4$) (Table 1). In the guinea-pig cerebral cortex assay the estimated affinities of eight of the nine 'antagonist' ligands (thioperamide, clobenpropit, iodophenpropit, JB96156, JB96158, JB96088, JB95130 and GT-2016) were not significantly different from those estimated in the LMMP assay (Table 1).

Table 1 Comparison of the estimated pK_I' and n_H parameters for histamine H_3-receptor ligands in guinea-pig cerebral cortex and LMMP membranes

	LMMP			*Cortex*		
Ligand	$pK_I' \pm$ s.e.mean	$n_H' \pm$ s.e.mean	n	$pK_I' \pm$ s.e.mean	$n_H' \pm$ s.e.mean	n
Histamine H_1- and H_2-receptor 'antagonists'						
Pyrilamine	5.47±0.06	0.77±0.02*	4	5.12±0.12	0.69±0.08*	5
Cimetidine	4.82±0.11◆	0.80±0.08	4	4.03±0.10◆	0.83±0.04	3
Histamine H_3-receptor 'antagonists'						
Thioperamide	8.64±0.06	0.83±0.05*	16	9.08±0.13	0.66±0.03*	17
Iodophenpropit	9.69±0.16	1.40±0.23	5	9.96±0.23	0.94±0.12	6
JB96156	9.85±0.08	0.77±0.05*	4	10.31±0.29	0.87±0.08	5
Clobenpropit	10.07±0.13	1.20±0.10	7	10.49±0.16	1.03±0.09	10
JB96157	9.80±0.05◆	1.09±0.11	4	10.51±0.22◆	0.87±0.04*	6
JB96158	10.09±0.11	1.05±0.12	4	10.24±0.09	1.30±0.09*	5
JB96088	7.29±0.06	0.95±0.13	5	7.32±0.06	0.86±0.07	5
JB95130	6.62±0.08	0.91±0.13	4	6.48±0.24	0.74±0.05*	5
GT2016	7.33±0.05	1.24±0.14	4	7.40±0.08	1.18±0.28	4
Histamine H_3-receptor 'agonists'						
R-α-methylhistamine	9.82±0.09	0.95±0.15	9	10.07±0.16	0.91±0.20	8
S-α-methylhistamine	8.52±0.11◆	0.76±0.07*	7	9.11±0.18◆	0.76±0.08*	6
Histamine	7.98±0.16◆	0.74±0.07*	7	9.84±0.14◆	0.66±0.04*	6
Imetit	10.01±0.12	1.31±0.35	5	10.07±0.13	0.79±0.08	6
Proxyfan	8.74±0.10	1.01±0.18	5	8.80±0.23	0.79±0.05*	7
Chloroproxyfan	9.45±0.08◆	1.34±0.11*	5	10.01±0.13◆	0.87±0.06	6
Iodoproxyfan	9.73±0.20	1.02±0.11	6	10.00±0.09	0.94±0.08	10
Bromoproxyfan	9.57±0.08	1.18±0.18	5	9.91±0.19	0.94±0.08	6
JB96085	7.38±0.27	1.23±0.20	6	7.42±0.20	0.99±0.09	7
JB96183	8.77±0.06	1.14±0.02*	3	8.75±0.14	0.84±0.04*	4
JB96180	9.50±0.12	0.99±0.16	4	9.51±0.17	0.87±0.03*	5
Homohistamine (VUF8326)	7.59±0.07◆	1.18±0.04	4	8.47±0.14◆	0.97±0.14	6
Imbutamine (VUF4701)	8.37±0.09◆	0.85±0.06	4	10.38±0.09◆	0.80±0.04*	6
Impentamine (VUF4702)	8.98±0.14◆	0.88±0.06	6	10.31±0.15◆	0.72±0.04*	6
Imhexamine (VUF4732)	7.67±0.21◆	1.19±0.35	6	9.88±0.09◆	0.57±0.03*	6

◆$P<0.05$ ANOVA, n_H value significantly different from unity, *$P<0.05$.

Histamine H_3-receptor 'agonists'

The histamine H_3-receptor ligands, previously characterized as agonists, all produced concentration-dependent inhibition of the specific binding of [^{3}H]-R-α-MH to sites in guinea-pig cerebral cortex and the LMMP.

In the cerebral cortex assay the mid-point slope parameter estimates (n_H) for histamine, S-α-methylhistamine, proxyfan, JB96085, JB96183, VUF4702, VUF4732 and impentamine were significantly less than unity ($P<0.05$). In the LMMP assay only the mid-point slope parameter estimates for histamine and S-α-methylhistamine were significantly less than unity ($P<0.05$; Table 1) whilst those for chloroproxyfan, JB96183 and homohistamine were significantly greater than unity ($P<0.05$).

In the cerebral cortex assay, the histamine H_3-receptor 'agonists' expressed apparent affinities (pK_I') for sites labelled with [^{3}H]-R-α-MH, ranging from 7.42±0.20 ($n=7$) to 10.38±0.09 ($n=6$). In the LMMP assay the same 'agonists' expressed pK_I' values, for sites labelled with [^{3}H]-R-α-MH, ranging from 7.38±0.27 ($n=6$) to 10.01±0.12 ($n=5$). The estimated affinities of eight of the fourteen agonist ligands in the cerebral cortex assay (imetit, R-α-methylhistamine, *S*-α-methylhistamine, iodoproxyfan, proxyfan, bromoproxyfan, JB96085, JB96183 and JB96180) were not significantly different from those estimated in the LMMP assay (Table 1). However, the estimated affinities of the five H_3-receptor 'agonists' that are imidazole unbranched alkylamines (histamine, homohistamine, imbutamine (VUF4702), imhexamine (VUF4732) and impentamine) were significantly higher in the guinea-pig cerebral cortex than in the LMMP assay (ANOVA $P<0.05$; Table 1). In addition, the estimated affinities of chloroproxyfan and *S*-α-methylhistamine were also significantly higher in the cerebral cortex than in the LMMP assay.

Comparison of ligand pK_I values between cerebral cortex and the LMMP

Notwithstanding the non-unit Hill slopes obtained with several of the ligands, we investigated the relationship between the pK_I' values estimated in the two tissues (Figure 4). Although there was a significant correlation between the values ($r=0.91$, $P<0.0001$), the data deviated significantly ($F_{(1,260)}=12.3$, $P<0.01$) from the line of identity (i.e. $y=x$) as tested by a principal components analysis applied to all the data (for details see Meester *et al*., 1998). When the same analysis was applied just to those compounds previously described as 'antagonists', the data did not deviate significantly from the line of identity ($F_{(1,110)}=3.81$, $P>0.05$).

Discussion

In this study we have characterized the binding of the histamine H_3-receptor agonist, [^{3}H]-R-α-MH, to sites in guinea-pig cerebral cortex and ileum longitudinal muscle myenteric plexus (LMMP) membranes. The binding in both the cerebral cortex and the LMMP was tissue concentration-dependent, reversible and saturable. The saturation analysis suggested that [^{3}H]-R-α-MH expressed high affinity for a homogeneous population of histamine H_3-receptors in both guinea-pig cerebral cortex ($pK_D=9.91$) and the LMMP ($pK_D=9.75$). Thus, in both tissues saturation isotherms were hyperbolic, Scatchard plots were linear and Hill plots had slopes (n_H) which were not significantly different from unity.

The affinity estimates of [^{3}H]-R-α-MH (pK_D) and other histamine H_3-receptor agonists (expressed as pK_I' values) were higher than the $p[A]_{50}$ values determined for the same ligands in functional *in-vitro* bioassay studies (e.g. R-α-MH

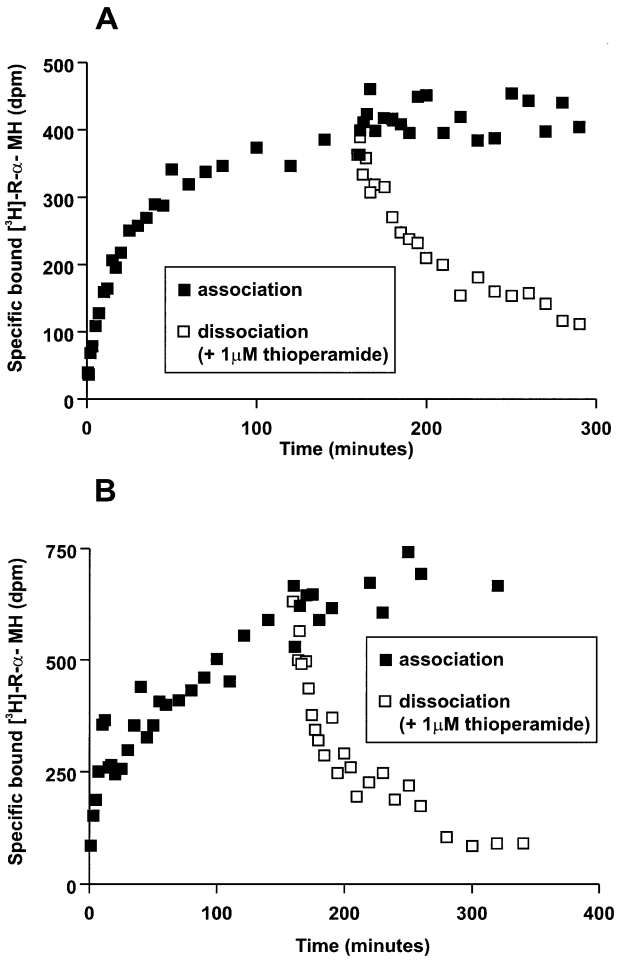

Figure 3 Representative association and dissociation analysis (one of three replicate experiments performed) of [^{3}H]-R-α-MH binding to histamine H_3-receptors on (A) guinea-pig cerebral cortex membranes and (B) LMMP membranes. The dissociation of [^{3}H]-R-α-MH from sites on membranes was initiated by addition of an excess of thioperamide (1 μM).

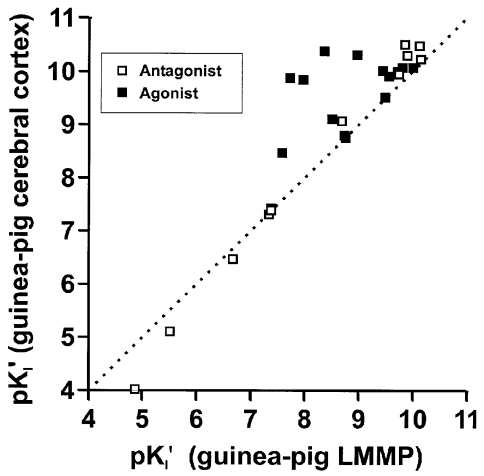

Figure 4 Comparison of apparent affinity values (pK_I') obtained for histamine H_3-receptor agonists and antagonists in the guinea-pig cerebral cortex and LMMP (see Table 1). A comparison of the data was made by fitting the line of identity ($y = x$) to the data by least squares. The analysis indicated that the data deviates from the line of identity ($F_{(1,260)} = 12.34$, $P < 0.01$). The line shown superimposed on the experimental data represents the line of identity.

$p[A]_{50} = 7.15$; Watt *et al*., 1997b). These results are consistent with existing explanations for the behaviour of agonists in radioligand binding assays in which it is assumed that the agonist can bind to, or induce the formation of, high affinity states of the receptor (e.g. Burt *et al*., 1976; Jacobs & Cuatrecasas, 1976; Jarv *et al*., 1979; Kent *et al*., 1980; Spain & Coscia, 1987; Werling *et al*., 1988; Samama *et al*., 1992; Lefkowitz *et al*., 1993; Leff, 1995).

The estimated pK_D value for [^{3}H]-R-α-MH in the cerebral cortex (9.91) was higher than reported previously ($pK_D = 8.92$, West *et al*., 1990b; $pK_D = 9.37$, Arrang *et al*., 1990) and may have resulted, in part, from the ionic composition of the buffer used in our study. In our study the buffer contained negligible sodium and calcium ions. Previously, millimolar concentrations of sodium (Clark & Hill, 1995) and calcium ions have been reported to inhibit the specific binding of [^{3}H]-R-α-MH to histamine H_3 binding sites in rat cerebral cortex (Arrang *et al*., 1990). Accordingly, in preliminary studies (data not shown), we did find that millimolar concentrations of sodium, calcium, magnesium, and potassium salts all inhibited the specific binding of [^{3}H]-R-α-MH to sites in cerebral cortex membranes.

In contrast to the saturation analysis data, the association and dissociation kinetics for the binding of [^{3}H]-R-α-MH to histamine H_3- receptors in guinea-pig cerebral cortex and the LMMP membranes were complex, although similar to those previously reported for the binding of this radioligand to sites in guinea-pig cerebral cortex (West *et al*., 1990a). The apparently biphasic dissociation profiles could be interpreted as providing evidence for histamine H_3-receptor heterogeneity. However, the data could also be indicative of [^{3}H]-R-α-MH binding to high and low affinity states of the same receptor, either a consequence of ternary complex formation or, alternatively, induction of a second conformational state of the receptor. Another explanation is that [^{3}H]-R-α-MH accessed multiple compartments of H_3-receptors at different diffusion rates. The likelihood of the high affinity binding being due to the formation of a G-protein ternary complex has been enhanced by the recent discovery that the cloned human H_3-receptor is G-protein coupled (Lovenberg *et al*., 1999).

The probability that the guinea-pig cerebral cortex and the LMMP express identical and homogeneous population(s) of histamine H_3-receptors, when labelled by [^{3}H]-R-α-MH, was supported by the finding that there was no significant difference between the estimated apparent affinities (pK_I') of 16 of the 24 H_3 ligands in the two tissues and, moreover, by the significant correlation ($r = 0.91$ $P < 0.0001$) between the pK_I' estimates obtained in the two tissues. However, the results from the principal components analysis (Figure 4), that provided a formal test of whether overall the values could be considered indistinguishable, raises the possibility that these tissues do express different or heterogeneous populations of histamine H_3-receptors. The observation that seven of the eight ligands, that expressed a tissue-dependent apparent affinity, were compounds previously described as 'agonists' and also that six of the eight compounds, classified as histamine H_3-receptor ligands, were unbranched homologues of histamine (histamine, homohistamine (VUF8326), imbutamine (VUF4701), impentamine (VUF4702) and imhexamine (VUF4732)), suggests that a discrete chemical class of ligands can discriminate between these sub-populations.

Interestingly, within the series of unbranched histamine homologues, those compounds with an even number of carbon side-chain atoms expressed approximately two log units higher apparent affinity for the cerebral cortex assay (C2 histamine = 1.86, C4 imbutamine = 2.01, C6 imhexamine = 2.21) whereas those compounds with an odd number expressed

approximately a one log unit higher apparent affinity for the cerebral cortex assay (C3 homohistamine = 0.88, C5 impentamine = 1.33). This effect with odd and even chain lengths may be due to the different folding conformations which would be required to allow the imidazole and amine to access their corresponding binding sites. As a result of this, the odd and even dispositions would lead to the access of different hydrophobic regions within the putative sub-type receptor binding sites, thus leading to a possible selectivity. A preliminary consideration of these conformational preferences has been discussed by Vollinga *et al*. (1995).

The possibility that [^{3}H]-R-α-MH labels a heterogeneous population of receptors in both tissues is also supported by the observation that the previously characterized histamine H_3-receptor antagonist, thioperamide, (Arrang *et al*., 1990; Jansen *et al*., 1994) had a mean Hill slope parameter (n_H) which was significantly less than unity in both the cerebral cortex and the LMMP, in agreement with the original observations by West *et al*. (1990b). However, within the data set some of the ligands were also associated with significantly steep Hill slopes. Therefore, although the affinity estimates for all the ligands appeared to be relatively robust, as judged by the values from replicate experiments within assays, it seems inappropriate to rely on the Hill slope value estimates as indicators of receptor heterogeneity.

We considered whether there were other possible explanations for the tissue-dependent apparent affinity of the homologues of histamine. One difference between the cortex and LMMP tissue preparation was the inclusion of 24 h incubation with Viaspan® for the LMMP. We have not examined the effect of this treatment on the cortex H_3 receptor assay and so we have not formally controlled for any effects of Viaspan® on the subsequent estimates of ligand affinity. However, Viaspan® is routinely used for transporting organs for transplant and we have also found that its inclusion does not affect pharmacological binding profiles at a number of receptor systems in a variety of tissues. The possibility that the homologue affinity differences could be attributed to non-equilibrium binding of these ligands also seemed unlikely because the incubation time in both tissues was identical and, furthermore, there was no obvious difference in the time it took to for [^{3}H]-*R*-α-MH to dissociate from sites in cerebral cortex and from those in the LMMP (see Figure 3). The prospect that [^{3}H]-*R*-α-MH was labelling different amounts of either histamine H_1 or histamine H_2-receptors also appeared an unlikely explanation. The selective histamine H_1-receptor antagonist pyrilamine (pK_I H_1-receptors ~9; Hill & Young, 1977; Dickenson & Hill, 1994) and histamine H_2-receptor selective antagonist, cimetidine (pK_B histamine H_2-receptors ~6.1; Shankley *et al*., 1988), both expressed low affinities at sites labelled with [^{3}H]-*R*-α-MH (Table 1). The apparent affinity values for both histamine H_3-receptor agonists and antagonists have been shown to be affected by the ionic composition of the assay buffer (Clark & Hill, 1995) and therefore we considered that this might explain the between-tissue differences. However, both the cerebral cortex and LMMP assays were performed in the same assay buffer. Assay incubation temperature has also been shown to influence the apparent affinity of agonists (Weiland *et al*., 1979; Dalpiaz *et al*., 1996, Borea *et al*., 1996) and antagonists (Zahniser & Molinoff, 1982) at a number of different receptors. Nevertheless, it seems unlikely that assay incubation temperature could account for any differences in the data obtained in the LMMP and cerebral cortex because both assays were incubated at the same temperature. Similarly, the LMMP and cerebral cortex tissues used in this study were both obtained from the guinea-pig, thus negating species-dependent variation in the amino acid sequence of receptors as the source of affinity variation as reported for other receptor systems (e.g. Lotti & Chang, 1989; Beinborn *et al*., 1993).

Recently, Lovenberg *et al*. (1999) reported the cloning and characterization of a human G-protein-coupled H_3-receptor in agreement with some previous evidence to suggest that this receptor could be G-protein-coupled (Arrang *et al*., 1990; Zweig *et al*., 1992; Clark & Hill, 1995; 1996). Some compounds have been shown to directly activate G-proteins (Detert *et al*., 1995; 1996; Leschke *et al*., 1997) and therefore we considered whether this action could explain the discrepancies observed for the homologues of histamine (VUF8326, VUF4701, VUF4702 and VUF4732). However, again this possibility seemed unlikely because such substituted histamines and alkyl substituted di- and triamines have only been shown to activate G-proteins at concentrations (μM to mM) far higher than their apparent affinity in this study.

Previously, some homologues of histamine (homohistamine, imbutamine and impentamine) have been reported to express partial agonist activity at histamine H_3-receptors in a mouse cerebral cortex slice, [^{3}H]-noradrenaline release, assay (Leurs *et al*., 1996). Therefore, we considered the possibility that the between-tissue differences in the apparent affinity of these ligands had resulted from the formation of different proportions of a ternary complex of agonist (A), receptor (R) and G-protein (G) (Kent *et al*., 1980) in the two tissues rather than to the presence of different receptor populations. Accordingly, we examined whether the data obtained in each experiment in the cerebral cortex and the LMMP, for each of the histamine homologues, could be matched to theoretical competition curves generated using the simple ternary complex model.

In the simple ternary complex model there are four parameters which can be varied; the absolute concentration and ratio of R and G, the ligand affinity for R (K_L) and the affinity of the agonist-receptor complex (LR) for G (K_{LR}). In model simulations, the pA_2 estimate previously reported for each ligand in the guinea-pig ileum (impentamine ~7.7, VUF4701 ~8.4, homohistamine ~5.9 and VUF4732 ~7.8; Leurs *et al*., 1996) was used as the pK_L value on the basis that this parameter should be constant if the receptors in the ileum are identical to those in the cerebral cortex. In addition, the concentration and ratio of R and G in the cerebral cortex and ileum were fixed for each experimental data set obtained for the histamine homologues. It seemed reasonable to fix both these parameters because the same tissue homogenate was used to assay all the histamine homologues in each experiment. It was possible to account for the data when the K_{LR} value for each ligand was allowed to vary between tissues. However, it was not possible to account for the cerebral cortex and ileum data when the K_{LR} value for each ligand was fixed, as is necessary according to the model, to maintain the ratio of the agonist activity of the compounds observed in the guinea-pig ileum (intrinsic activity, α, as a fraction of that expressed by R-α-MH, impentamine = 0.6, VUF4701 = 0.6, homohistamine = 0.3, VUF4732 = 0.3; Leurs *et al*., 1996). Therefore, it was not possible to account for the tissue differences on the basis that there was a different proportion of ternary complex made up of the same R and G in each tissue.

The data obtained in this study are consistent with the cerebral cortex and LMMP expressing histamine H_3-receptor population(s), labelled by [^{3}H]-*R*-α-MH, which seem to be pharmacologically indistinguishable by the majority of histamine H_3-receptor ligands. Further studies are required in order to account for the complex binding kinetics of [^{3}H]-*R*-α-

MH, the apparent affinity differences expressed by homologues of histamine between the two tissues and the significantly low Hill slope parameter for thioperamide. The development of assays in guinea-pig cerebral cortex and ileum using a radiolabelled histamine H_3-receptor antagonist may help to clarify these points (Harper *et al*., 1999, this issue).

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