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RESEARCH PAPER

Histamine H_3-receptor agonists and imidazole-based H_3-receptor antagonists can be thermodynamically discriminated

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Background and purpose: Studies suggest that measurement of thermodynamic parameters can allow discrimination of agonists and antagonists. Here we investigate whether agonists and antagonists can be thermodynamically discriminated at histamine H_3 receptors.
Experimental approach: The pK_L of the antagonist radioligand, [^{3}H]-clobenpropit, in guinea-pig cortex membranes was estimated at 4, 12, 21 and 30°C in 20mM HEPES-NaOH buffer (buffer A), or buffer A containing 300mM $CaCl_2$, (buffer A_{Ca}). pK_I' values for ligands with varying intrinsic activity were determined in buffer A and A_{Ca} at 4, 12, 21 and 30°C.
Key results: In buffer A, the pK_L of [^{3}H]-clobenpropit increased with decreasing temperature while it did not change in buffer A_{Ca}. The Bmax was not affected by temperature or buffer and n_H values were not different from unity. In buffer A, pK_I' values for agonists remained unchanged or decreased with decreasing temperature, while antagonist pK_I values increased with decreasing temperature; agonist binding was entropy-driven while antagonist binding was enthalpy and entropy-driven. In buffer A_{Ca}, temperature had no effect on antagonist and agonist pK_I values; both agonist and antagonist binding were enthalpy and entropy-driven.
Conclusions and implications: The binding of H_3-receptor agonists and antagonists can be thermodynamically discriminated under conditions where agonist pK_I' values are over-estimated ($pK_I' \neq pK_{app}$). However, under conditions when agonist $pK_I \sim pK_{app}$, the thermodynamics underlying the binding of agonists are not different to those of antagonists.
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Keywords: [^{3}H]clobenpropit; histamine H_3-receptors

Abbreviations: PEI, polyethyleneimine; *R*-α-MH, *R*-α-methylhistamine

Introduction

Radioligand-binding assays, second messenger assays and functional *in vitro* bioassays are used to provide receptor-affinity estimates (pK_I and pK_b) for novel ligands. Although these estimates are valuable in the development of high affinity, selective receptor antagonists, they provide little information about the molecular mechanisms underlying the ligand–receptor interaction. However, it is possible to obtain information about these interactions by performing thermodynamic studies in which the receptor affinity of the ligand is determined at a number of different temperatures (see Hitzeman, 1988; Raffa and Porreca, 1989).

The value of thermodynamics for investigating receptor–ligand interactions has been demonstrated in numerous studies (Weiland *et al*., 1979; Mohler and Richards, 1981; Zahniser and Molinoff, 1983; Reith *et al*., 1984; Kilpatrick *et al*., 1986; Testa *et al*., 1987; Todd and Babinski, 1987; Aronstam and Narayanan, 1988; Duarte *et al*., 1988; Borea *et al*., 1996a, b; Dalpiaz *et al*., 1996; Maguire and Loew, 1996; Li *et al*., 1998). In addition, some of these studies have suggested that measurement of thermodynamic parameters can allow the discrimination of agonist and antagonist ligands (Weiland *et al*., 1979; Zahniser and Molinoff, 1983; Borea *et al*., 1996a).

There has been no thermodynamic analysis of ligand binding at the histamine H_3-receptor. Therefore, the primary aim of this study was to determine whether the binding of ligands, characterised as agonists and antagonists at histamine H_3-receptor in a functional bioassay of the guinea-pig ileum, could be discriminated thermodynamically.

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However, in previous studies, we have shown that agonist affinity values are overestimated in H_3-receptor radioligand-binding assays conducted in the absence of buffer salts by an amount which is related to the ligand's intrinsic activity (see Harper *et al*., 2007) and, additionally, that when radioligand-binding assays are conducted in the presence of salts, pK_I estimates are closer to pK_{app} values estimated by the method of Furchgott in a guinea-pig ileum bioassay. Therefore, an additional aim of this study was to establish whether the thermodynamic parameters underlying the binding interaction of agonists paralleled those of the antagonists, when the assay was conducted under conditions where their pK_I values were similar to pK_{app} values and where the agonist n_H values were not different from unity.

The agonist ligands that were selected for this study had varying intrinsic activity (α) as defined by bioassay on the guinea-pig ileum myenteric plexus longitudinal muscle (see Figure 1 and Harper *et al*., 2007) (immepip, $\alpha = 1.0$; imetit, $\alpha = 0.90$; *R*-α-methylhistamine (*R*-α-MH), $\alpha = 1.0$; proxyfan, $\alpha = 0.35$; chloroproxyfan $\alpha = 0.45$; bromoproxyfan $\alpha = 0.65$; and iodoproxyfan, $\alpha = 0.90$). The antagonist ligands, as defined by bioassay (thioperamide, JB96132 and [^{3}H]clobenpropit), all contained an imidazole moiety (Figure 1 and Harper *et al*., 2007).

A preliminary account of some of these data was presented to the British Pharmacological Society (Harper *et al*., 2002).

Methods

Preparation of guinea-pig cerebral cortex membranes

Adult male Dunkin–Hartley guinea-pigs (200–300 g) were killed by cervical dislocation and the whole brain removed and immediately placed in ice-cold 20 mM HEPES–NaOH buffer (buffer A; pH 7.4 at 21±3°C). The cortex was dissected, weighed and homogenised in ice-cold buffer A (1 g 15 ml^{-1}) using a polytron homogeniser (Kinematica AG, Gmbh, Lucerne, Switzerland; PT-DA 3020/2TS; ~3 s × 3). The homogenate was centrifuged (100 *g*, 5 min at 4°C) and the supernatants pooled and stored at 4°C. The pellets were rehomogenised in ice-cold buffer A (80 ml) and re-centrifuged (100 *g*, 5 min at 4°C). The supernatants were centrifuged (39 800 *g*, 12 min at 4°C) and the final pellet was resuspended in buffer A (containing 3 mM metyrapone; at 4, 12, 21 or 30°C) to the required tissue concentration using a Teflon-in-glass homogeniser. Metyrapone was included in the assay buffer because this prevents [^{3}H]clobenpropit binding to cytochrome P450 isoenzymes (see Harper *et al*., 1999b).

[^{3}H]clobenpropit: tissue-concentration studies

Previous studies have indicated that, when using [^{3}H]-clobenpropit to label histamine H_3-receptors in guinea-pig cerebral cortex membranes at 21°C, 1.6 mg of membranes is optimal (Harper *et al*., 1999b). However, before investigating whether incubation temperature had any effect on the estimated affinity (pK_L) of [^{3}H]-clobenpropit, it was necessary to confirm that this was also the optimal membrane concentration for studies at 4, 12 and 30°C.

Guinea-pig cerebral cortex membranes (0.2–4 mg) were incubated (2.75 h at 21 and 30°C; 24 h at 4 and 12°C) with [^{3}H]-clobenpropit (0.2 nM) and buffer A in a final assay volume of 0.5 ml. Total binding was defined with buffer A and non-specific binding with 1 μM thioperamide (pK_I at histamine H_3-receptors in guinea-pig cortex ~9.1, Harper *et al*., 1999a). The assay was terminated by rapid filtration through Whatman GF/B filters, pre-soaked in 0.3% polyethyleneimine (PEI), that were washed (3 × 3 ml) with ice-cold 50mM 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.

[^{3}H]clobenpropit: saturation studies

Guinea-pig cerebral cortex membranes (1.6 mg) were incubated (2.75 h at 21 and 30°C; 24 h at 4 and 12°C) in a final assay volume of 0.5 ml with buffer A and 0.004 to 3 nM [^{3}H]clobenpropit. Total and non-specific binding were defined using buffer A and thioperamide (1 μM).

In an additional series of experiments, we investigated the effect of temperature (4, 12, 21 and 30°C) on the binding of

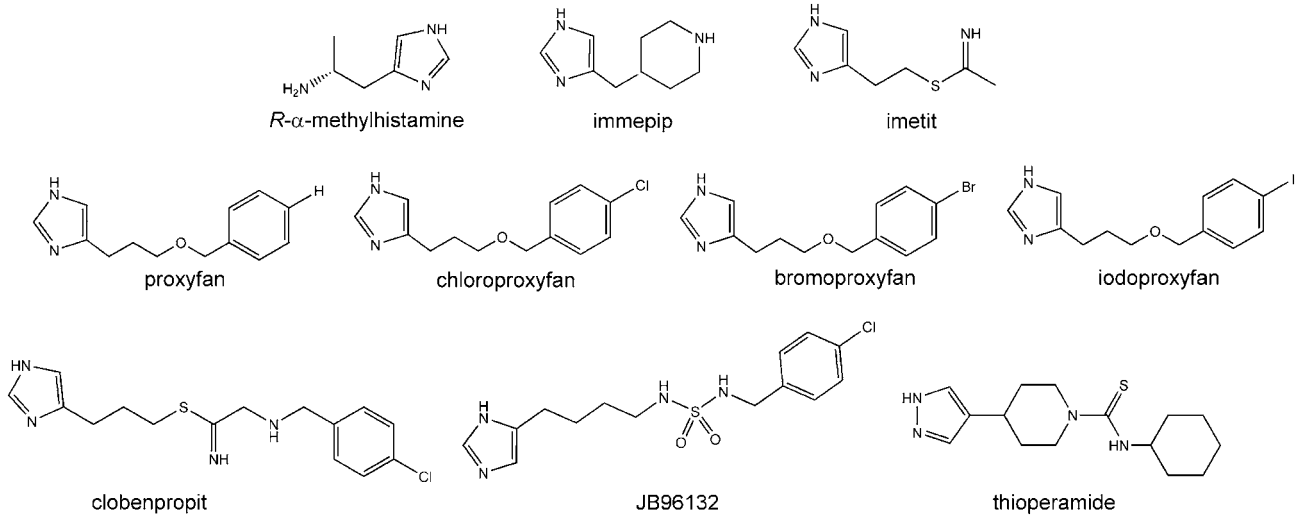

Figure 1 Structure of histamine H_3-receptor agonist and antagonist ligands.

[^{3}H]clobenpropit in the presence of 300 mM $CaCl_2$ (buffer A_{Ca}). This was because in previous studies, we found that this buffer was more effective than 100 mM NaCl, 100 mM KCl and 70 mM $CaCl_2$ at reducing *R*-α-MH pK_I values to those equivalent to pK_{app} values estimated in a functional bioassay; see Harper *et al*., 2007).

[^{3}H]clobenpropit: kinetic studies

The observed association rate was determined at 4, 12, 21 and 30°C by incubating [^{3}H]clobenpropit (0.2 nM) for increasing time intervals (0.25–150 min), in a final assay volume of 0.5 ml, in six tubes containing membranes (1.6 mg) and either buffer A or 1 μM thioperamide.

The dissociation rate and $t_{1/2}$ for [^{3}H]clobenpropit was ascertained by adding 10 μl of 50 μM thioperamide to three tubes in which membranes (1.6 mg) had been incubated (4°C, 220 min; 12°C, 150 min; 21°C, 150 min; 30°C, 120 min) with [^{3}H]clobenpropit (0.2 nM). The bound radioligand was determined at increasing incubation times (0.5–400 min).

Competition studies

Guinea-pig cerebral cortex membranes, resuspended in either buffer A or buffer A_{Ca}, (1.6 mg) were incubated with [^{3}H]clobenpropit (0.2 nM) and competing compound, in a final assay volume of 0.5 ml, for 2.75 h at 30 and 21°C and for 24 h at 12 and 4°C (at least $5 \times t_{1/2}$; [^{3}H]clobenpropit at each temperature). Total and non-specific binding of [^{3}H]clobenpropit were defined using buffer A or A_{Ca} and 1 μM thioperamide, respectively.

Data analysis

All data are presented as the mean ± s.e.m. unless stated otherwise.

Saturation data

The Hill equation was fitted to saturation data (equation (2)) using GraphPad prism software with the Hill slope (n_H) constrained to unity and with n_H unconstrained.

$$B = \left(\frac{B_{max}[L]^{n_H}}{K_L^{n_H} + [L]^{n_H}}\right) \quad (1)$$

In this equation, L is the radioligand concentration, B_{max} is the receptor density and K_L is the equilibrium dissociation constant of the radioligand.

Kinetic data

Association and dissociation data were analysed using GraphPad prism software.

Radioligand binding: competition curve data

To obtain pIC_{50} and n_H parameter estimates, competition data were fitted to the Hill equation and to the Hill equation with n_H constrained to unity, using GraphPad Prism software. Notwithstanding the finding of n_H values that were significantly less than unity, dissociation constants (pK_I) were subsequently determined from pIC_{50} values using the Cheng and Prusoff (1973) equation to correct for the different receptor occupancy of [^{3}H]clobenpropit in the different buffers at the different temperatures. The parameter pK_I' has been assigned to dissociation constants which were derived from pIC_{50} values, where competition curve n_H parameter estimates were significantly less than unity. The pK_L values which were used to correct pIC_{50} values obtained in buffer A at 4, 12 21 and 30°C are shown in Table 1 and were 10.57, 10.38, 10.40 and 10.15, respectively. The pK_L values that were used to correct pIC_{50} values obtained in buffer A_{Ca} at 4, 12 and 21°C are shown in Table 1 and were 9.66, 9.69 and 9.77, respectively.

Calculation of thermodynamic parameters

The change in the standard Gibbs free energy (ΔG°) was calculated using the Gibbs–Helmholz thermodynamic equation (equation (2)).

$$\Delta G^\circ = -RT \ln KA \quad (2)$$

where R is the ideal gas constant (8.31 J^{-1} mol^{-1} K), T is the temperature in degrees Kelvin and K_A is the apparent association constant of the ligand at 294 K ($1/K_I$).

Equation (2) is combined with the Gibbs free energy equation (3), to form the integrated van't Hoff equation (4).

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (3)$$

where ΔS° is the entropy change of binding.

$$\ln K_A = -\Delta H^\circ/RT + \Delta S^\circ/R \quad (4)$$

A plot, therefore, of $\ln K_A$ versus $1/T$ allows the estimation of the enthalpy of binding (ΔH°) because the slope is $-\Delta H^\circ/R$. In addition, the entropy change (ΔS°) can be estimated as the *y*-intercept ($-\Delta S^\circ/R$) (see Borea *et al*., 1998). In these studies, ΔG°, ΔH° and ΔS° have been designated by their primed counterparts

Table 1 Effect of temperature and assay buffer on the estimated pK_L, n_H and B_{max} of [^{3}H]clobenpropit in guinea-pig cerebral cortex membranes

Temperature (°C)	*Buffer A*			*Buffer A_{Ca}*		
	pK_L	B_{max}	n_H	pK_L	B_{max}	n_H
30	10.15 ± 0.04	4.00 ± 1.17	0.93 ± 0.02	ND	ND	ND
21	10.40 ± 0.08	3.80 ± 0.97	1.08 ± 0.09	9.77 ± 0.20	3.28 ± 0.65	1.20 ± 0.05
12	10.38 ± 0.07	4.06 ± 0.55	0.90 ± 0.03	9.69 ± 0.18	4.02 ± 0.83	1.05 ± 0.09
4	10.57 ± 0.03	4.00 ± 0.48	1.11 ± 0.06	9.66 ± 0.27	4.04 ± 0.68	1.13 ± 0.12

Abbreviations: ND, not determined.
pK_L, B_{max} (fmol mg^{-1}) and n_H values were obtained by fitting saturation data to the Hill equation.
Data are the mean ± s.e.m. of three experiments.

($\Delta G^{\circ\prime}$, $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$) because the studies were conducted at pH 7.4 (although it is common to see the primes omitted). This is because the terms ΔG°, ΔH° and ΔS°, only apply to measurements made under standard state conditions of 1 atmosphere and unit activity (sometimes stated as 1 M concentration) and at a 1 M hydrogen ion concentration (pH 0).

Statistical analysis

Differences in pK_L and B_{max} values of [^{3}H]clobenpropit were determined using analysis of variance (ANOVA) with Bonferroni *post hoc* test or paired *t*-test. An *F*-test was used to establish whether saturation data was best fitted by the Hill equation or by the Hill equation with n_H constrained to unity. The significance of differences in pK_I values obtained at different temperatures, in replicate experiments, was determined by paired *t*-test. r^2 values obtained from linear regression were used to determine whether there was a significant linear relationship between temperature ($1/T$) and $\ln K_A$ values. An *F*-test was used to establish whether the slope was significantly different from zero. *P*-values of less than 0.05 were considered significant.

Materials

[^{3}H]clobenpropit (VUF9153) was prepared by Amersham International plc, (Little Chalfont, Buckinghamshire, UK) to a specific activity of 45 Ci mmol^{-1}.

Proxyfan, chloroproxyfan, bromoproxyfan, iodoproxyfan, immepip and JB96132 were synthesised by James Black Foundation Chemists. 2-Methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]) and Trizma base were obtained from Sigma Chemical Co., (Poole, Dorset, UK). *R*-α-MH, thioperamide and imetit were obtained from Research Biochemicals Inc., (Poole, Dorset, UK). All other materials were obtained from Fisher Scientific, Loughborough (Leicestershire, UK).

Results

Effect of incubation temperature on the optimal tissue concentration

At 4, 12, 21 and 30°C (277, 285, 294 and 303 K), there was a linear relationship between the specific binding of 0.2 nM [^{3}H]clobenpropit and membrane concentration up to 4 mg (see Figure 2). In buffer A, at 4, 12, 21 and 30°C, and a membrane concentration of 1.6 mg, 14.0±1.7; 13.1±1.3; 13.3±1.0 and 11.9±0.9% of the added [^{3}H]clobenpropit was bound, respectively ($n=3$). There was no significant difference between the specific binding of [^{3}H]clobenpropit as a percent of total binding (percentage specific binding) at each temperature in buffer A (4°C = 41.5±3.4; 12°C = 38.0±4.9; 21°C = 38.8±5.0; 30°C = 42.9±8.6%; $n=3$, ANOVA).

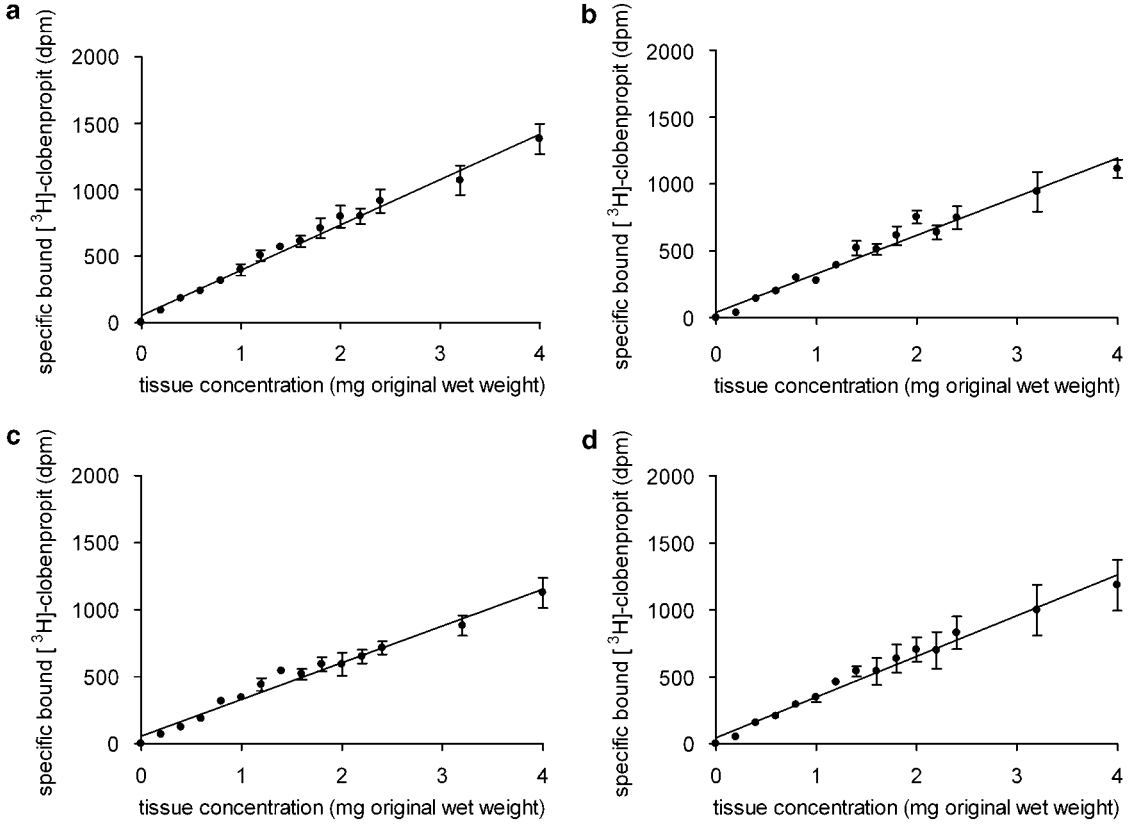

Figure 2 Linearity of the relationship between specific binding of [^{3}H]clobenpropit (0.2 nM) and added guinea-pig cerebral cortex membrane concentration at (**a**) 4°C, (**b**) 12°C, (**c**) 21°C and (**d**) 30°C. Increasing concentrations of guinea-pig cerebral cortex membranes (0.2–4 mg) were incubated in triplicate with [^{3}H]clobenpropit (0.2 nM) and buffer A in a final assay volume of 0.5 ml. The incubation was terminated after 2.75 h at 21 and 30°C and after 24 h at 4 and 12°C. Total binding was defined with buffer A and non-specific binding with 1 μM thioperamide. Data represent the mean±s.e.m. of three experiments. The line shown superimposed on the data was obtained by linear regression.

In buffer A_{Ca}, at 4, 12 and 21°C, and a membrane concentration of 1.6 mg, 5.2±0.8; 4.5±0.8 and 5.2±0.8% of the added 0.2 nM [^{3}H]clobenpropit was bound, respectively ($n=8$). The percentage specific binding of [^{3}H]clobenpropit at 21°C was significantly lower than that obtained at 4 and 12°C (4°C = 77.9±2.6; 12°C = 76.3±2.5 and 21°C = 66.3±2.8%; $n=4$, $P<0.05$, ANOVA and Bonferroni *post hoc* test) and, at all temperatures, the non-specific binding was lower than that obtained in buffer A.

A membrane concentration of 1.6 mg was used for all subsequent experiments in both buffers.

Effect of incubation temperature on [^{3}H]clobenpropit saturation isotherms

In buffer A, the binding of [^{3}H]clobenpropit was saturable at all temperatures (Figure 3). The mean Hill slope parameter estimates (n_H), at the four temperatures, were not significantly different from unity (Table 1, *t*-test) and in each experiment, for each temperature, there was no significant difference between the fit to the Hill equation and the fit to the Hill equation with n_H constrained to unity ($P>0.05$, *F*-test). The incubation temperature had no significant effect on the estimated histamine H_3-receptor density (B_{max}, Table 1, ANOVA).

In buffer A_{Ca}, at 30°C and over the concentration range of [^{3}H]clobenpropit used (0.004–3 nM), the specific binding was lower and more variable than that obtained at 4, 12 and 21°C and, as a result, it was not possible to obtain accurate B_{max} or pK_L estimates. At 21, 12 and 4°C, the specific binding of [^{3}H]clobenpropit was saturable (Figure 4), mean Hill slope parameter estimates (n_H), were not significantly different from unity (Table 1, *t*-test) and in each experiment for each temperature, there was no significant difference between the fit to the Hill equation and the fit to the Hill equation with n_H constrained to unity ($P>0.05$, *F*-test). The incubation temperature did not significantly change the estimated H_3-receptor B_{max} (Table 1, ANOVA).

In buffer A, the pK_L of [^{3}H]clobenpropit increased significantly with decreasing incubation temperature (Table 1, ANOVA and Bonferroni *post hoc* test, $P<0.05$;) and the pK_L was significantly higher at 4°C than at 30°C (paired *t*-test, $P<0.05$). A van't Hoff plot of $\ln K_A$ versus $1/T$ was linear with a positive slope which was significantly different from zero (*F*-test, $P<0.05$; Figure 5). In contrast, in buffer A_{Ca}, there was no relationship between assay incubation temperature and pK_L, and there was no significant difference in the pK_L of [^{3}H]clobenpropit at the three temperatures (Table 1, paired *t*-test). The slope of the van't Hoff plot for

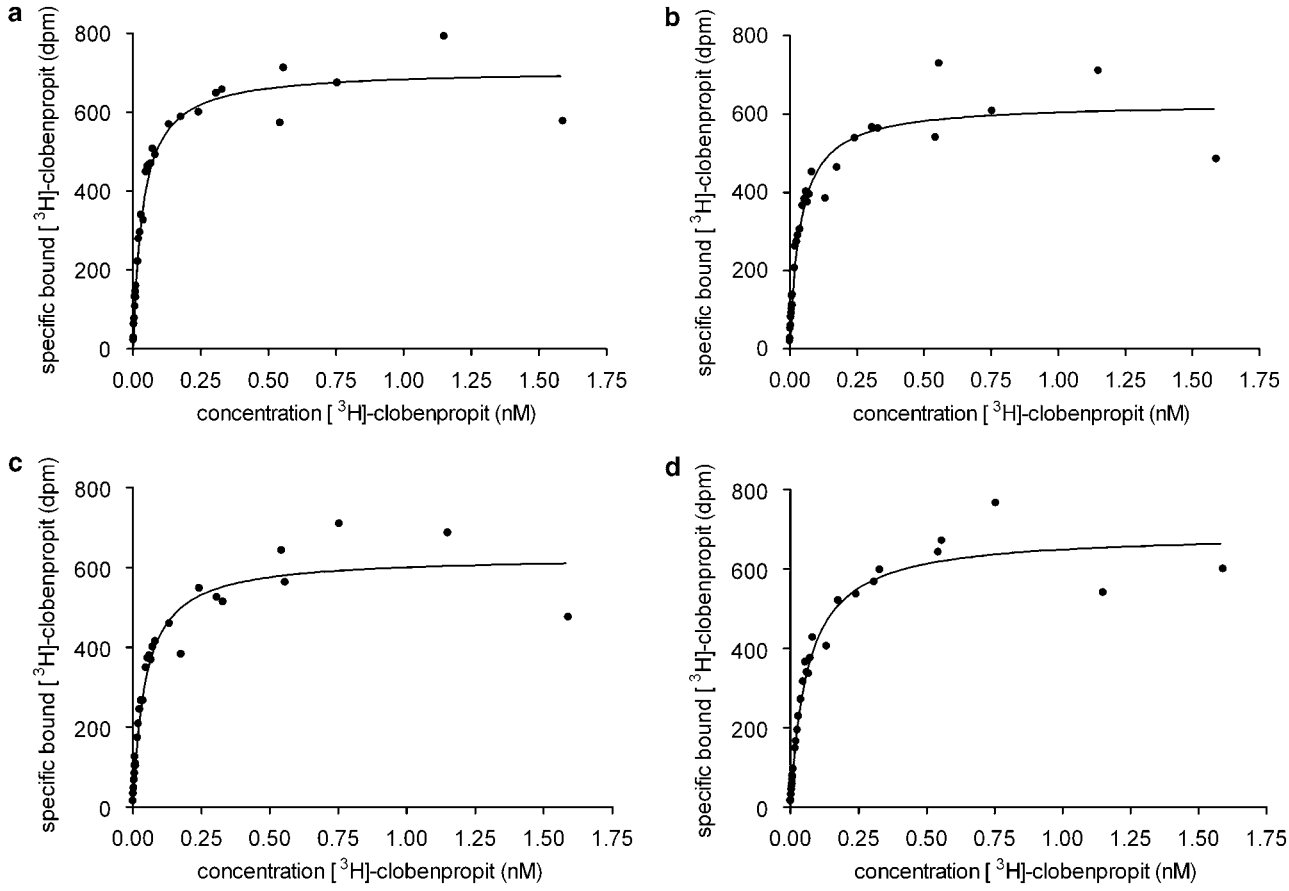

Figure 3 Saturation analysis of the binding of [^{3}H]clobenpropit to guinea-pig cerebral cortex H_3-receptors at (**a**) 4°C, (**b**) 12°C, (**c**) 21°C and (**d**) 30°C, in buffer A. Tissue (1.6 mg) was incubated in triplicate with increasing concentrations of [^{3}H]clobenpropit (0.004–3 nM) in a final assay volume of 0.5 ml. Total and non-specific binding were defined with buffer A or 1 μM thioperamide, respectively. The incubation was terminated after 2.75 h at 21 and 30°C and after 24 h at 4 and 12°C. Data are representative of three experiments. The line shown superimposed through the data are the fit to the Hill equation.

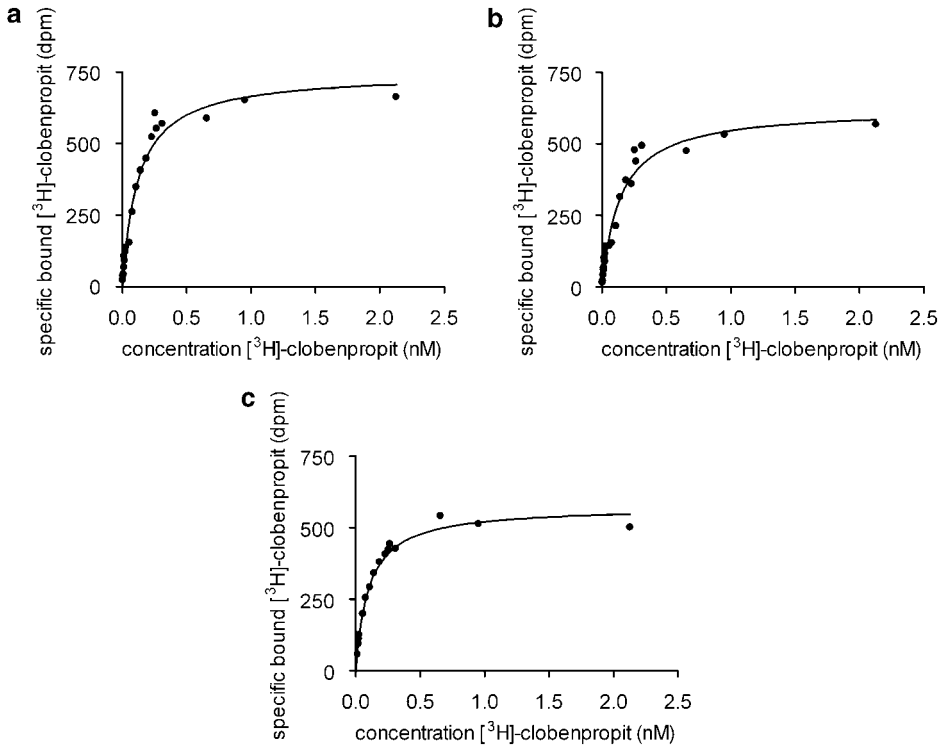

Figure 4 Saturation analysis of the binding of [^{3}H]clobenpropit to guinea-pig cerebral cortex H_3-receptors at (**a**) 4°C, (**b**) 12°C and (**c**) 21°C, in buffer A_{Ca}. Tissue (1.6 mg) was incubated in triplicate with increasing concentrations of [^{3}H]clobenpropit (0.004–3 nM). Total and non-specific binding were defined with buffer A_{Ca} or 1 μM thioperamide, respectively. The incubation was terminated after 2.75 h at 21°C and after 24 h at 4 and 12°C. Data is representative of three experiments.

[^{3}H]clobenpropit, in buffer A_{Ca}, was not different from zero (*F*-test, Figure 5).

Effect of incubation temperature on the association and dissociation rates of [^{3}H]clobenpropit

In buffer A, the specific binding of 0.2 nM [^{3}H]clobenpropit reached equilibrium after approximately 80, 30, 25 and 3 min incubations at 4, 12, 21 and 30°C, respectively ($n=3$, Figure 6). The [^{3}H]clobenpropit association data obtained at all four temperatures could be fitted by a pseudo-first-order rate equation. The association rate constants (k_{+1}) for the binding of [^{3}H]clobenpropit to histamine H_3-receptors on guinea-pig cortex membranes at 4, 12, 21 and 30°C are given in Table 2.

The dissociation data for [^{3}H]clobenpropit could also be fitted by a first-order rate equation. The dissociation rate constants (k_{-1}) at 4, 12, 21 and 30°C are shown in Table 2, along with the calculated pK_L values; the latter increased with decreasing temperature (Table 2). These pK_L values were not significantly different from those obtained using saturation analysis at 4, 12, 21 and 30°C (compare pK_L values in Tables 1 and 2, *t*-test).

Effect of incubation temperature on ligand pK_I values in buffer A and buffer A_{Ca}

In both buffer A and buffer A_{Ca}, each histamine H_3-receptor ligand produced a concentration-dependent inhibition of

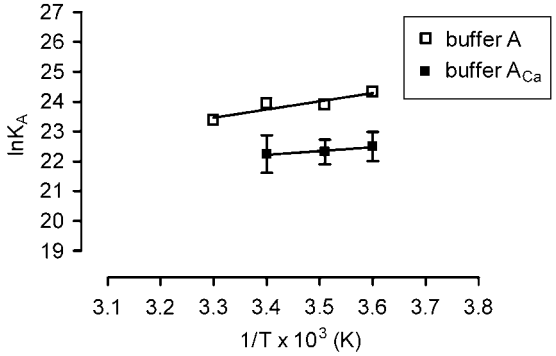

Figure 5 Van't Hoff plot showing the effect of temperature on the equilibrium association constants (K_A) of [^{3}H]clobenpropit in 20 mM HEPES buffer (buffer A) and in buffer A with 300 mM $CaCl_2$ (buffer A_{Ca}). Data are the mean ± s.e.m. of three replicate experiments. The lines shown superimposed through the data were obtained by linear regression.

the specific binding of [^{3}H]clobenpropit to H_3-receptors in guinea-pig cerebral cortex membranes at all temperatures, as illustrated in Figure 7 for *R*-α-MH and thioperamide. In buffer A, the mean mid-point slope parameter estimates (n_H) for all the agonists, except proxyfan and imetit (see Table 3), at most incubation temperatures were significantly less than unity (*t*-test, $P<0.05$). The mean mid-point slope parameter estimate for thioperamide was significantly less than unity at 21 and 4°C (Table 3, *t*-test, $P<0.05$).

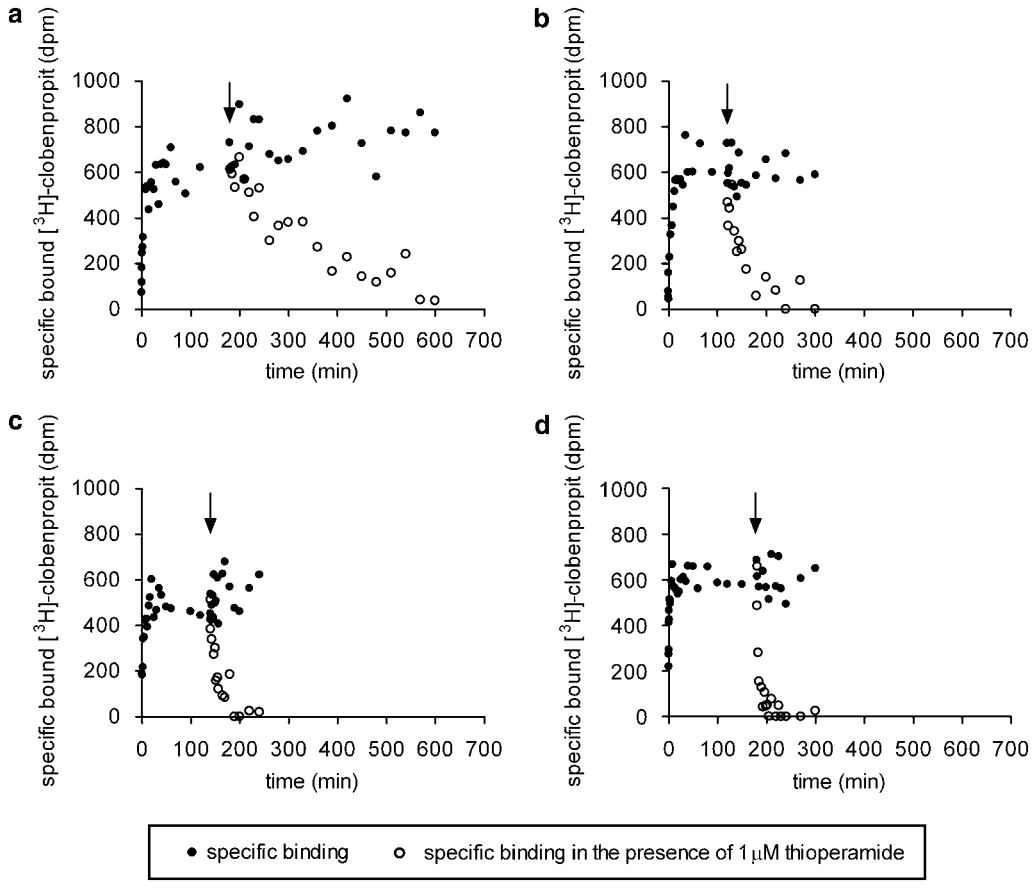

Figure 6 Representative association–dissociation analysis of [^{3}H]clobenpropit binding to H_3-receptors in guinea-pig cerebral cortex membranes at (**a**) 4°C, (**b**) 12°C, (**c**) 21°C and (**d**)30°C. The association rate was determined under pseudo-first-order conditions because only ~10% of the added [^{3}H]clobenpropit was bound. [^{3}H]clobenpropit (0.2 nM) was incubated, in a final assay volume of 0.5 ml for increasing times with membranes (1.6 mg at 4, 12, 21 or 30°C). Total and non-specific binding were defined with buffer A and 1 μM thioperamide, respectively. The dissociation rate for [^{3}H]clobenpropit from H_3-receptors was determined by incubating [^{3}H]clobenpropit (0.2 nM) with membranes and buffer A and then adding 10 μl of 50 μM thioperamide (arrow). The bound radioligand was determined at increasing incubation times.

Table 2 Effect of temperature on the association rate, dissociation rate and pK_L of [^{3}H]clobenpropit at histamine H_3-receptors in guinea-pig cerebral cortex membranes

Temperature (°C)	*Temperature (K)*	k_{+1} *(M^{-1} min^{-1})*	k_{-1} *(min^{-1})*	pK_L
30	303	$77.26 \pm 5.29 \times 10^8$	$3.32 \pm 0.50 \times 10^{-1}$	10.37 ± 0.10
21	294	$7.49 \pm 1.32 \times 10^8$	$0.44 \pm 0.06 \times 10^{-1}$	10.27 ± 0.27
12	285	$10.60 \pm 0.84 \times 10^8$	$0.33 \pm 0.05 \times 10^{-1}$	10.60 ± 0.12
4	277	$6.37 \pm 3.3 \times 10^8$	$0.047 \pm 0.001 \times 10^{-1}$	11.01 ± 0.20

Data are the mean ± s.e.m. of three experiments.

In buffer A_{Ca}, the mean n_H parameter estimates for the agonists were not significantly different from unity with the exception of iodoproxyfan and *R*-α-MH at 12°C and immepip at 4 and 12°C. The mean n_H value for thioperamide was significantly less than unity at 4°C (*t*-test, $P<0.05$).

Notwithstanding the finding of n_H values that were significantly less than unity, dissociation constants were subsequently determined from pIC_{50} values using the Cheng and Prusoff (1973) equation to correct for the different receptor occupancy of [^{3}H]clobenpropit in the different buffers at the different temperatures. The parameter pK_I' has been assigned to dissociation constants which were derived from pIC_{50} values, where mean n_H parameter estimates were significantly less than unity. The pK_L values that were used to correct pIC_{50} values obtained in buffer A and buffer A_{Ca} are presented in Table 1.

The effect of temperature on pK_I or pK_I' values of all ligands in buffer A and buffer A_{Ca} are listed in Table 3. In buffer A, the pK_I values of the two antagonist ligands, thioperamide and JB96132 were significantly higher at 4°C than at 30°C ($P<0.05$, paired *t*-test). The pK_I' values for the H_3-receptor agonists, proxyfan, chloroproxyfan, iodoprox-

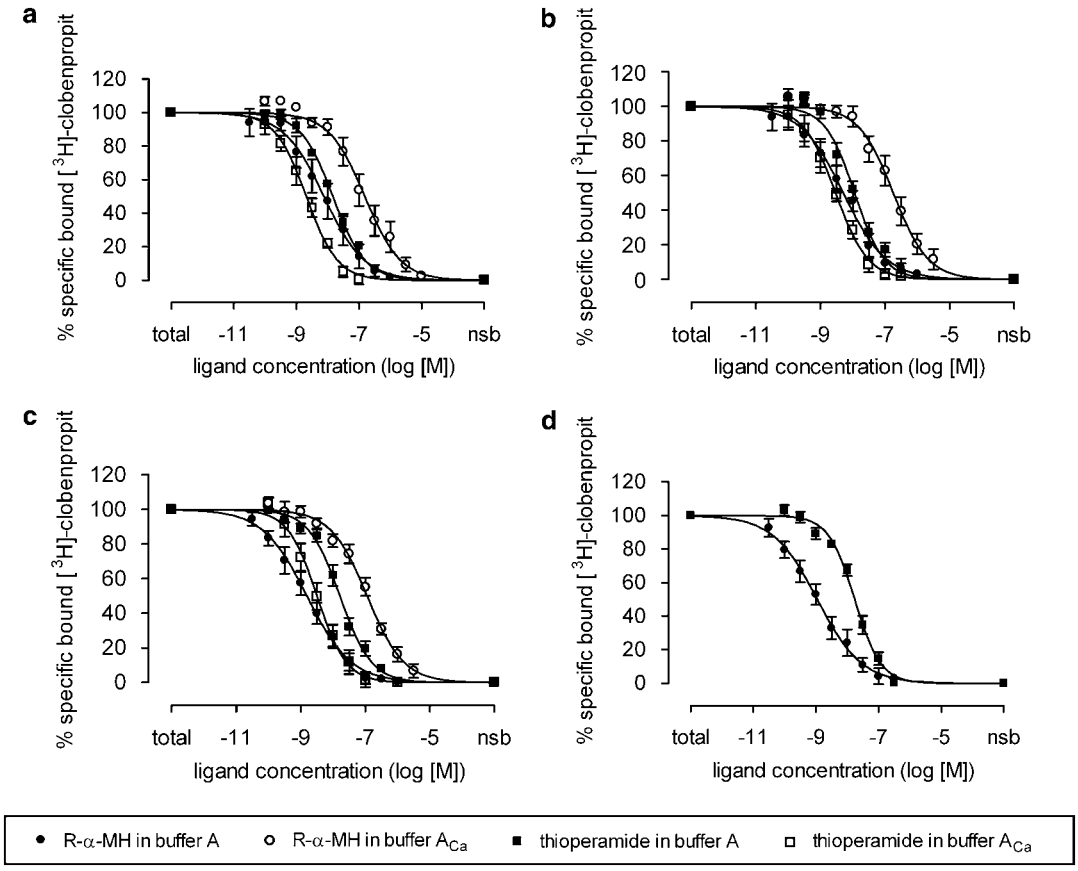

Figure 7 Competition curves for an H_3-receptor agonist, *R*-α-MH, and antagonist, thioperamide, at sites labelled with 0.2 nM [^{3}H]clobenpropit in guinea-pig cerebral cortex. (**a**–**c**) Mean % specific binding of *R*-α-MH and thioperamide at 4°C, 12 and 21°C in buffer A and buffer A_{Ca} (**d**) mean % specific binding of *R*-α-MH and thioperamide at 30°C in buffer A. Guinea-pig cerebral cortex membranes (1.6 mg) were incubated in a final volume of 0.5 ml with HEPES–NaOH buffer, [^{3}H]clobenpropit (0.2 nM) and increasing concentrations of ligands for 2.75 h at 30 and 21°C and for 24 h at 12 and 4°C. Total and non-specific binding of [^{3}H]clobenpropit were defined using appropriate buffer and 1 μM thioperamide, respectively. Data are the mean ± s.e.m. of between five and six experiments (see Table 3). The lines shown superimposed on the data were obtained using the fit to the Hill equation.

yfan and *R*-α-MH were significantly lower at 4°C than at 30°C ($P<0.05$, paired *t*-test) and there was no significant difference between pK_I' values obtained for immepip, imetit and bromoproxyfan at these temperatures (paired *t*-test). In buffer A_{Ca}, there was no significant difference between pK_I values at 4 and 21°C for all ligands (Table 3, paired *t*-test).

Thermodynamic parameters of ligand binding

Van't Hoff plots of $\ln K_A$ versus $1/T$ were constructed for all ligands using the affinity values ($1/K_I$) obtained in buffer A (4, 12, 21 and 30°C) and buffer A_{Ca} (4, 12, 21°C) (see, for e.g., Figures 8 and 9). The van't Hoff plots, constructed for thioperamide and JB96132, using pK_I values obtained in buffer A, had positive slopes which were significantly different from zero (Figure 8, *F*-test, $P<0.05$). The van't Hoff plots, constructed for proxyfan, chloroproxyfan and iodoproxyfan using pK_I values obtained in buffer A, had negative slopes which were significantly different from zero (*F*-test, $P<0.05$). The van't Hoff plots, constructed for all ligands using pK_I values obtained in buffer A_{Ca}, had slopes which were not significantly different from zero (*F*-test, $P<0.05$).

Mean values of enthalpy ($\Delta H^{\circ\prime}$), entropy ($\Delta S^{\circ\prime}$), $-T\Delta S^{\circ\prime}$ and the Gibb's free energy ($\Delta G^{\circ\prime}$) of ligands in buffer A and A_{Ca}, were obtained from van't Hoff plots and are presented in Tables 4 and 5. $\Delta G^{\circ\prime}$ was also calculated at 21°C (294 K) using the Gibbs–Helmholz equation.

In buffer A, the mean $\Delta H^{\circ\prime}$ values for JB96132, thioperamide and [^{3}H]clobenpropit were negative (Table 4) and mean $\Delta S^{\circ\prime}$ values were positive (Table 4). The mean $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ values for the ligands classified as agonists in the guinea-pig ileum longitudinal muscle myenteric plexus (see Harper *et al.*, 2007) were also positive (Table 4).

In buffer A_{Ca}, the mean $\Delta H^{\circ\prime}$ values for the antagonists were all negative, over a fourfold range, while the mean $\Delta S^{\circ\prime}$ values were positive (Table 5) The mean $\Delta H^{\circ\prime}$ values for the agonist ligands, with the exception of immepip and imetit, were also negative (Table 5) and the mean $\Delta S^{\circ\prime}$ values for the agonists in buffer A_{Ca} were positive (Table 5).

Extrathermodynamic plots

A plot of the $\Delta H^{\circ\prime}$ and $-T\Delta S^{\circ\prime}$ for all ligands, obtained in buffer A and shown in Table 3, indicated that the binding of the antagonists was enthalpy- and entropy-driven (Figure 10a).

Table 3 pK_I and n_H values for histamine H_3-receptor agonists and antagonists at 4, 12, 21 and 30°C (277, 285, 294 and 300 K)

Ligand	*Temperature (K)*	*Buffer A*			*Buffer A_{Ca}*		
		pK_I *or* pK_I'	n_H	n	pK_I *or* pK_I'	n_H	n
Histamine H_3-receptor agonists							
proxyfan	303	8.51±0.13	0.97±0.21	3	ND	ND	
	294	8.61±0.17	0.73±0.06		ND	ND	
	285	8.05±0.08	0.89±0.06		ND	ND	
	277	7.89±0.07	1.07±0.12		ND	ND	
chloroproxyfan	303	9.04±0.15	0.81±0.04[a]	5	ND	ND	4
	294	9.00±0.22	0.77±0.04[a]		7.76±0.12	1.04±0.05	
	285	8.78±0.14	0.71±0.07[a]		7.68±0.14	0.88±0.04	
	277	8.66±0.10	0.90±0.12		7.82±0.20	0.94±0.07	
bromoproxyfan	303	9.32±0.05	0.77±0.07[a]	3	ND	ND	4
	294	9.28±0.02	0.83±0.09		8.13±0.26	0.99±0.04	
	285	9.04±0.14	0.81±0.04[a]		8.04±0.30	0.91±0.06	
	277	9.13±0.15	0.88±0.16		8.16±0.29	0.95±0.08	
iodoproxyfan	303	9.82±0.07	0.65±0.10[a]	4	ND	ND	4
	294	9.79±0.05	0.67±0.03[a]		8.10±0.36	0.94±0.08	
	285	9.45±0.10	0.62±0.08[a]		8.26±0.26	0.88±0.02	
	277	9.27±0.08	0.94±0.16		8.31±0.31	0.91±0.03	
imetit	303	9.17±0.19	0.76±0.09	4	ND	ND	6
	294	9.33±0.12	0.80±0.12		7.93±0.17	0.86±0.05	
	285	9.03±0.07	0.85±0.08		7.87±0.12	0.89±0.05	
	277	9.16±0.12	0.87±0.08		7.89±0.14	0.94±0.07	
R-α-MH	303	9.13±0.22	0.63±0.06[a]	5	ND	ND	5
	294	9.15±0.18	0.63±0.02[a]		7.30±0.14	0.83±0.07	
	285	8.89±0.21	0.66±0.05[a]		7.09±0.20	0.81±0.05[a]	
	277	8.87±0.22	0.62±0.03[a]		7.21±0.22	0.86±0.04	
immepip	303	9.47±0.03	0.71±0.06[a]	5	ND	ND	5
	294	9.53±0.04	0.88±0.05		8.07±0.11	0.86±0.05	
	285	9.39±0.13	0.82±0.05[a]		7.91±0.12	0.91±0.03[a]	
	277	9.34±0.14	0.73±0.03[a]		7.99±0.19	0.90±0.02[a]	
Histamine H_3-receptor antagonists							
JB96132	303	8.95±0.07	1.08±0.14	3	ND	ND	4
	294	9.17±0.13	0.99±0.06		8.97±0.14	1.06±0.12	
	285	9.11±0.09	0.85±0.05		8.82±0.17	0.95±0.04	
	277	9.49±0.05	0.97±0.09		9.20±0.24	0.94±0.03	
thioperamide	303	8.35±0.06	0.90±0.08	6	ND	ND	5
	294	8.50±0.05	0.82±0.04[a]		9.00±0.15	0.96±0.11	
	285	8.66±0.09	0.96±0.06		8.84±0.12	0.88±0.05	
	277	8.75±0.02	0.81±0.04[a]		9.00±0.13	0.86±0.03[a]	

Abbreviation: ND, not determined.
Data are the mean±s.e.m. pK_I' is the ligand affinity when n_H is significantly less than unity.
[a]n_H significantly different from unity, $P<0.05$ *t*-test.

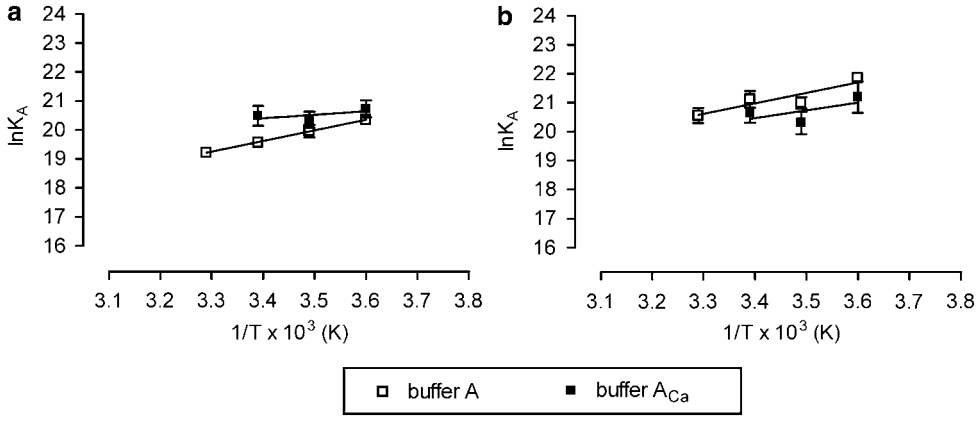

Figure 8 Van't Hoff plots showing the effect of temperature on the equilibrium association constants (K_A) of (**a**) thioperamide and (**b**) JB96132 in buffer A and buffer A_{Ca}. Data are the mean±s.e.m. of between three and six replicate experiments. The lines shown superimposed through the data were obtained by linear regression.

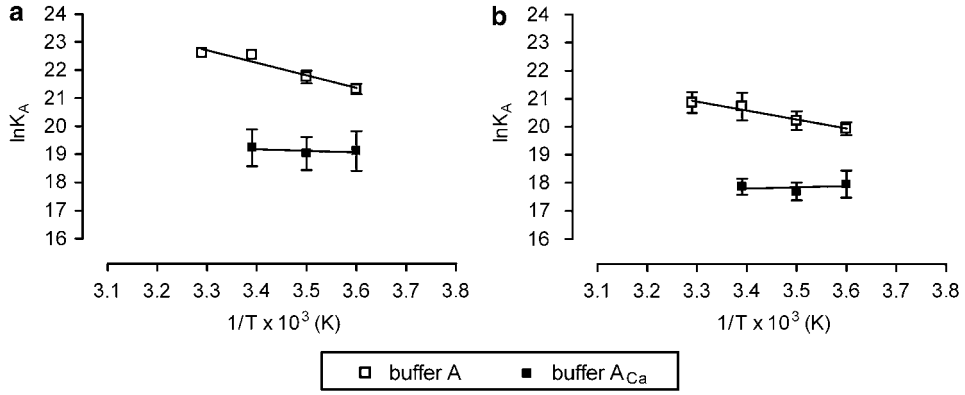

Figure 9 Van't Hoff plots showing the effect of temperature on the equilibrium association constants (K_A) of (**a**) iodoproxyfan and (**b**) chloroproxyfan in buffers A and A_{Ca}. Data are the mean ± s.e.m. of between four and five replicate experiments. The lines shown superimposed through the data were obtained by linear regression.

Table 4 Thermodynamic parameters of binding to histamine H_3-receptors in guinea-pig cerebral cortex membranes in buffer A

Ligand	*Intrinsic activity (α)*	*ΔG°′ calculated (kJ mol⁻¹)*[a]	*ΔG°′ (kJ mol⁻¹)*	*ΔH°′ (kJ mol⁻¹)*	*−TΔS°′ (kJ mol⁻¹)*	*ΔS°′ (J mol⁻¹ K⁻¹)*	n
Histamine H_3-receptor agonists							
immepip	1.00[b]	−53.8±0.3	−53.4±0.2	9.1±10.0	−62.5±9.9	211.7±33.3	5
imetit	0.90[b,c]	−52.6±0.7	−51.9±0.8	6.4±6.2	−58.3±6.9	197.8±23.3	4
R-α-MH	1.00[c]	−51.6±1.0	−51.2±1.1	19.1±3.9	−70.3±4.0	218.3±14.2	5
proxyfan	0.35[c]	−48.6±1.0	−47.4±0.7	44.5±9.2	−91.8±9.8	311.3±33.1	3
chloroproxyfan	0.45[c]	−46.9±2.9	−50.5±0.9	26.6±4.9	−77.1±5.8	261.4±19.7	5
bromoproxyfan	0.69[c]	−51.6±0.9	−52.2±0.2	14.2±13.4	−66.3±13.3	224.9±45.0	3
iodoproxyfan	0.90[c]	−55.3±0.3	−54.7±0.3	37.3±7.3	−92.0±7.3	311.0±24.4	4
Histamine H_3-receptor antagonists							
JB96132	0[c]	−51.8±0.7	−51.0±0.5	−34.1±6.5	−16.9±6.8	57.3±23.2	3
thioperamide	0[c]	−47.8±0.3	−48.0±0.3	−30.5±6.1	−17.5±6.1	59.3±20.6	6
[^{3}H]clobenpropit	0[c]	−58.5±0.4	−58.0±0.3	−22.9±1.2	−35.1±1.3	119.5±4.4	3

Data are the mean ± s.e.m.
[a]$\Delta G^{o\prime}$ at 21°C, calculated using equation 2.
[b]*a* values from Alves-Rodrigues *et al.*, 2001
[c]Intrinsic activity in guinea-pig ileum bioassay, see Harper *et al.* (2007).

Table 5 Thermodynamic parameters of binding to histamine H_3-receptors in guinea-pig cerebral cortex membranes in buffer A_{Ca}

Ligand	*ΔG°′ calc (kJ mol⁻¹)*[a]	*ΔG°′ (kJ mol⁻¹)*	*ΔH°′ (kJ mol⁻¹)*	*−TΔS°′ (kJ mol⁻¹)*	*ΔS°′ (J mol⁻¹ K⁻¹)*	n
Histamine H_3-receptor agonists						
immepip	−45.6±0.6	−45.6±0.7	15.1±3.4	−60.7±3.3	205.7±11.3	5
imetit	−44.8±1.0	−44.7±0.8	3.4±11.1	−50.0±9.6	169.6±32.6	6
R-α-MH	−39.2±2.2	−40.9±0.7	−9.2±17.6	−50.2±17.6	170.0±59.9	5
chloroproxyfan	−45.1±1.5	−43.7±0.7	−3.3±8.9	−38.1±10.4	136.8±28.7	4
bromoproxyfan	−45.9±1.4	−45.7±1.5	−2.8±6.6	−42.9±6.1	145.5±20.8	4
iodoproxyfan	−47.1±1.5	−47.2±1.6	−4.7±4.2	−51.8±3.9	175.5±13.1	4
Histamine H_3-receptor antagonists						
JB96132	−50.6±0.8	−50.1±0.8	−20.9±9.4	−29.3±8.8	99.1±29.9	4
thioperamide	−50.1±0.8	−51.3±0.8	−40.7±6.4	−10.3±6.8	137.9±21.8	5
[^{3}H]clobenpropit	−54.4±1.5	−54.6±1.1	−10.5±8.5	−43.8±9.7	149.0±32.9	3

Data are the mean ± s.e.mean.
[a]$\Delta G^{o\prime}$ at 21°C, calculated using equation 2.

In contrast, the extrathermodynamic plot indicated that the agonist binding was driven by an increase in entropy. There was a significant linear relationship between the two parameters ($r=0.99$, $P<0.01$, slope $=0.98\pm0.03$, *y*-intercept $=-51.9$, *x*-intercept $=-52.8$; Figure 10a).

The extrathermodynamic plot of $\Delta H^{o\prime}$ versus $-T\Delta S^{o\prime}$ for all ligands, obtained in buffer A_{Ca}, indicated that the binding of all ligands, except imetit and immepip, was enthalpy- and entropy-driven (Figure 10b). There was a significant linear relationship between $\Delta H^{o\prime}$ and $-T\Delta S^{o\prime}$ ($r=0.99$, $P<0.01$,

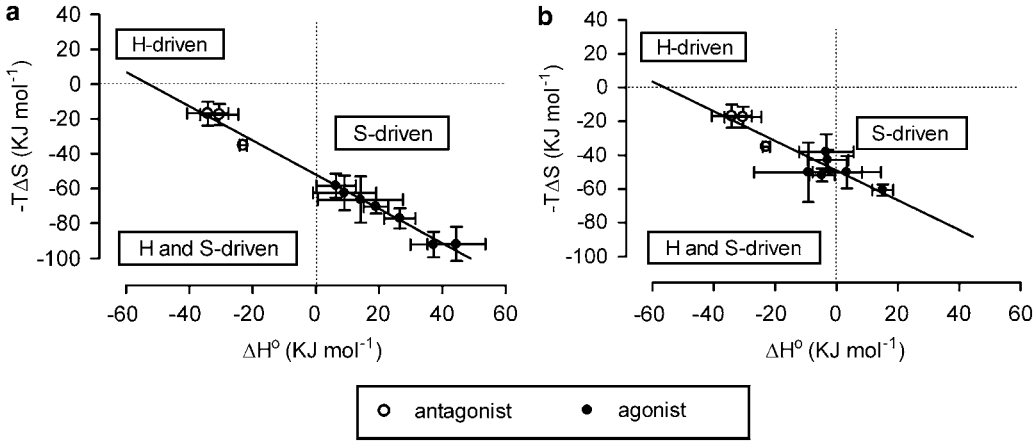

Figure 10 An extrathermodynamic plot for agonist and antagonist ligands in (**a**) buffer A and (**b**) buffer A_{Ca}. The lines shown superimposed through the data were obtained by linear regression.

slope = 0.88 ± 0.13, *y*-intercept = −53.9, *x*-intercept = 49.5; Figure 10b).

Discussion

In this study, we have determined the thermodynamic parameters underlying the binding of 10 ligands (seven agonists and three antagonists) at histamine H_3-receptors of guinea-pig cortex. In previous studies, we have shown that agonist affinity values are overestimated in H_3-receptor radioligand-binding assays, when buffer does not contain salts, and that the degree of affinity overestimation is correlated with the ligand's intrinsic activity (α) (see Harper *et al.*, 2007). In addition, we have shown that when H_3-receptor radioligand-binding assays are conducted in the presence of salts, pK_I estimates are closer to pK_{app} values estimated by the method of Furchgott in the guinea-pig ileum bioassay (see Harper *et al.*, 2007). Therefore, to establish whether the thermodynamic parameters underlying the binding of agonists is dependent on whether agonist pK_I values are equivalent to pK_{app} values, we have determined agonist pK_I values in the presence and absence of buffer salts (buffer A_{Ca} and buffer A).

Both kinetic studies and saturation studies of [^{3}H]clobenpropit binding were performed at each assay temperature, in order to satisfy criteria which should be met when performing thermodynamic analysis of ligand binding, that is, that the binding should be to a homogeneous receptor population and that the binding of radioligand and ligand should reach equilibrium at each temperature. Saturation analysis was also performed at each temperature so that pIC_{50} values obtained in competition assays could be corrected for by any change in the occupancy of [^{3}H]clobenpropit resulting from temperature-dependence of the pK_L. Saturation analysis confirmed that [^{3}H]clobenpropit labelled a homogeneous population of H_3-receptors in guinea-pig cerebral cortex in both the presence (buffer A_{Ca}) and absence of buffer salt (300 mM $CaCl_2$) (buffer A; Figures 2 and 3). Thus, at all incubation temperatures, the mean n_H parameter estimate was not significantly different from unity and the H_3-receptor density (B_{max}) was not significantly changed (Figure 2, 3 and Table 1). Kinetic studies confirmed that the incubation time used for the saturation analysis (4 and 12°C = 24 h; 21 and 30°C = 2.75 h; Figure 5) was sufficient for equilibrium binding of the radioligand to have been achieved (3, 25, 30 and 80 min at 30, 21, 12 and 4°C) so that the pK_L values estimated at each temperature were correct. The dissociation rate ($t_{1/2}$) of [^{3}H]clobenpropit, determined in the kinetic studies at each temperature, indicated that the incubation times of 2.75 h at 21 and 30°C and of 24 h at 4 and 12°C, used for competition studies, were sufficient for equilibrium binding of agonist and antagonist ligands and thus for the accurate determination of pIC_{50} values. This was because in each case the incubation time was in excess of five times the $t_{1/2}$ (Figure 6; see Motulsky and Mahan, 1983).

In the competition studies performed in buffer A, the finding that mean n_H parameter estimates for some of the agonist ligands at 21°C were significantly less than unity was consistent with our previous studies (Harper *et al.*, 2007). This agonist behaviour at 21°C and also that detected for some agonists at 4, 12 and 30°C, can be explained by the extended ternary complex or cubic ternary complex model (TCM) (Samama *et al.*, 1993; Lefkowitz *et al.*, 1993; Weiss *et al.*, 1996). In the extended TCM, it is proposed that the receptor can exist in a low-affinity state (R) and a high-affinity state (R*) which can also interact with a G-protein (R*G) in the absence of the agonist. Thus, flat competition curves are observed because the agonist binds with high affinity to R* or R*G and with lower affinity to R. In the cubic TCM, it is postulated that the receptor can exist in a low-affinity state (R_i equivalent to R in the TCM) and a high-affinity state (R_a, equivalent to R* in the TCM) and that both R and R* can exist as high-affinity states as a consequence of interaction with a G-protein (RG and R*G). Therefore, according to this model, it is possible to obtain flat competition curves because the agonist binds with low affinity to R and with high affinity to preformed high-affinity receptor states (R* and R*G). However, flat competition curves could also arise because the agonist induces varying amounts of receptor to form a

high-affinity state (AR*G or ARG) through binding to low-affinity receptors (R).

The finding of mean n_H values significantly less than unity for the antagonist thioperamide at 4 and 21°C in buffer A and at 4°C in buffer A_{Ca}, may be a result of type 1 error. This is because at 21°C, at least, this contrasts with our previous studies where we found that n_H was not different from unity (Harper *et al*., 2007). In addition, we found that there was no significant difference between the fit of individual replicate thioperamide competition curves, obtained at 4 and 21°C, to the Hill equation and to the Hill equation with n_H constrained to unity, as judged by an *F*-test. Type 1 error could also explain the finding of n_H values significantly less than unity for iodoproxyfan and *R*-α-MH at 12°C and immepip at 4 and 12°C, under assay conditions (buffer-containing salts, buffer A_{Ca}) which we have previously suggested, provide a measure of the agonist pK_{app} (Harper *et al*., 2007) and therefore under conditions where we would expect n_H not to be different from unity. In support of this, when the mean agonist n_H values were significantly less than unity, there was no significant difference between the fit of the individual replicate competition curves to the Hill equation and to the Hill equation with n_H constrained to unity, as judged by an *F*-test.

In light of the finding of n_H values which were significantly less than unity for some ligands at some of the temperatures, it could be argued that we should not have derived pK_I values from pIC_{50} values using the Cheng–Prusoff equation. This is because the derivation of this correction relies on simple competition between two ligands at a homogenous receptor population and, therefore, should be applied only when n_H is not different from unity. However, in this study, we have corrected all pIC_{50} values using the Cheng–Prusoff equation to correct for the differential occupancy of ~0.2 nM [^{3}H]clobenpropit in the two buffers (buffer A and buffer A_{Ca}) at the different incubation temperatures. Ideally, for this not to be a confounding problem, we would have performed competition studies in both buffers at each temperature at [^{3}H]clobenpropit concentrations equivalent to the K_L. However, this was not possible because the low specific activity of the radioligand resulted in too small a specific binding window in buffer A at the ligand concentration ideally required for studies at 4°C (~0.03 nM).

It could also be reasoned that the data could have been analysed using a two-site model and then the pIC_{50} values corrected using the Cheng–Prusoff equation to provide pK_H and pK_L parameters. However, we chose not to analyse the data in this way because it would not have been possible to obtain these parameters for all the agonists at all the temperatures. This is because for some agonists at some temperatures, the mean n_H value was not different from unity and for individual replicate competition data, the fit to the Hill equation was not significantly different to the fit to the Hill equation with n_H constrained to unity (e.g. proxyfan at 4, 12, 21 and 30°C; bromoproxyfan at 4 and 21°C, Table 3).

It is noteworthy that the affinity values obtained for all the ligands in buffer A at 21°C were consistent with observations made in a previous study using the same assay buffer and incubation temperature, as was the pK_I value for *R*-α-MH at 21°C in buffer A_{Ca} (Harper *et al*., 2007). In addition, it is notable that the pK_I values for all the agonists, at 21°C in buffer A_{Ca}, were associated with n_H values that were not different from unity and, moreover, that were not different from agonist pK_{app} values estimated previously in the guinea-pig ileum (see Harper *et al*., 2007).

The discovery of thermodynamic discrimination of agonists and antagonists, at the H_3-receptor (Figure 10a, antagonist binding $= \Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$-driven; agonist binding $= \Delta S^{\circ\prime}$-driven) mirrors that reported for binding of agonist and antagonist ligands to the adenosine A_1 receptor (Dalpiaz *et al*., 2000), adenosine A_{2A} receptor (Borea *et al*., 1996b), $GABA_A$ receptor (Maksay, 1994) and serotonin 5-HT_3 receptor (Borea *et al*., 1996a). The finding that the binding of all the H_3-receptor ligands, investigated in this study was, at least in part, entropy driven could be explained by disorganisation of a solvation sphere around the ligands as they bind to the receptor; this is because all the ligands contain an imidazole moiety and this would be protonated at the assay pH of 7.4. It would have been interesting to determine whether the binding of some of the recently described non-imidazole histamine H_3-receptor antagonists (e.g. Linney *et al*., 2000; Lazewska *et al*., 2002; Meier *et al*., 2002; Shah *et al*., 2002; Apodaca *et al*., 2003; Chai *et al*., 2003; Miko *et al*., 2003, 2004; Turner *et al*., 2003; Zaragoza *et al*., 2004, 2005; Sun *et al*., 2005; Lazewska *et al*., 2006; Rivara *et al*., 2006) are also, in part, entropy driven.

It has been suggested for the ligand-gated ion channels, where the thermodynamic discrimination of agonists and antagonists mirrors that of the H_3-receptor, that this phenomenon can be explained by both interaction of the ligand with the receptor and variation in the water-accessible surface which occurs when the agonist induces channel opening (Borea *et al*., 1998). Therefore, a possible explanation for the thermodynamic discrimination of H_3-receptor agonists and antagonists is that the agonists induce a change in receptor conformation, perhaps into a less-constrained state, which in turn, leads to the formation of a ternary complex with a G-protein and this consequently results in a decrease in the solvation of the cytosolic side of the receptor. The finding of a decrease in enthalpy associated with antagonist binding may be explained by hydrogen bond formation and van der Waals interactions occurring between the ligands and the binding pocket which cannot be compensated for by changes in entropy that result from agonist-induced conformational changes in the receptor.

The hypothesis that agonist binding at H_3-receptors in guinea-pig cerebral cortex induces ternary complex formation (ARG or AR*G) and this brings about the large increase in entropy, is supported by the finding that under conditions (buffer A_{Ca}) where the agonists express pK_I values that are closely correlated with their pK_{app} values (see Harper *et al*., 2007), agonist and antagonists cannot be discriminated thermodynamically (Figure 10b). Thus, in buffer A_{Ca}, the agonist pK_I values remained unchanged with decreasing temperature in the same manner as the antagonist ligands (Table 3). In addition, van't Hoff plots constructed from agonist thermodynamic data obtained in buffer A_{Ca} (Figure 9), are similar to those of the antagonists [^{3}H]clobenpropit (Figure 5), thioperamide and JB96132 (Figure 8).

Despite the gross thermodynamic discrimination between the agonist and antagonist ligands at H_3-receptors (Figure 10a), it was surprising that there was no relationship between agonist intrinsic activity (α), measured previously in a guinea-pig ileum bioassay (see Harper *et al.*, 2007) and either mean $\Delta H^{\circ\prime}$ or mean $\Delta S^{\circ\prime}$ (see Table 4; e.g. *R*-α-MH, $\Delta H^{\circ\prime} = 19.1$, $\Delta S^{\circ\prime} = 218.3$, $\alpha = 1.0$; proxyfan, $\Delta H^{\circ\prime} = 44.5$, $\Delta S^{\circ\prime} = 311.3$, $\alpha = 0.35$) even for the analogues of proxyfan which differ only in the halogen substitution at the *para* position of the benzene ring (Figure 1) (Table 4; proxyfan $\Delta S^{\circ\prime} = 311.3$, $\Delta H^{\circ\prime} = 44.5$, $\alpha = 0.35$; iodoproxyfan $\Delta S^{\circ\prime} = 311.0$, $\Delta H^{\circ\prime} = 37.3$, $\alpha = 0.90$). Failure to find a relationship between intrinsic activity and thermodynamic parameters may have been a consequence of the experimental design. This is because we have previously found a significant effect of tissue preparation on the agonist pK_I' values under conditions where this parameter is not equivalent to the pK_{app} (see Harper *et al.*, 2007). Ideally, we would have generated competition curve data for all the ligands at each temperature on the same experimental day, however, this was not possible due to restrictions in the availability of equipment required to accurately maintain temperatures of 4, 12, 21 and 30°C.

It is perplexing that the entropy associated with the binding of antagonists is increased in the presence of buffer salts (buffer A_{Ca}) despite an overall decrease in the entropy of agonist binding (Tables 4 and 5; e.g. clobenpropit buffer A $\Delta S^{\circ\prime} = 119.5$; buffer ACa, $\Delta S^{\circ\prime} = 149.0$; iodoproxyfan buffer A $\Delta S^{\circ\prime} = 311.0$, buffer ACa, $\Delta S^{\circ\prime} = 175.5$). This may be a consequence of the buffer salts increasing the hydration of the ligands so that it is necessary for more water to be stripped away upon receptor binding. This possibility may explain why although the overall entropy associated with agonist binding is reduced in the presence of salts and in conditions where the agonist affinity is similar to pK_{app}, it is not as low as that of antagonist binding in the absence of buffer salts (buffer A). It would be interesting to repeat these thermodynamic studies to establish whether agonist $\Delta S^{\circ\prime}$ values are lower when G-protein coupling of the H_3-receptor is prevented, perhaps by *Pertussis* toxin treatment, but where there is not the potential complication of changes in ligand hydration.

The linear relationship between mean $\Delta H^{\circ\prime}$ and $T\Delta S^{\circ\prime}$ for the H_3-receptor was not unexpected and has been reported for numerous G-protein-coupled receptors (GPCRs) and ligand-gated ion channels (e.g. β-adrenoceptor, adenosine A_1, adenosine A_{2A}, dopamine D_2, serotonin 5-HT_{1A}, glycine, $GABA_A$, and nicotinic receptor; see Borea *et al.*, 1998). The linear relationship indicates that enthalpy–entropy compensation exists for the H_3-receptor, that is, changes in enthalpy are compensated for by changes in entropy (or vice versa) such that the free-energy change $\Delta G^{\circ\prime}$ is constant.

Conclusion

The binding of agonists and antagonists at the histamine H_3-receptor can be thermodynamically discriminated when agonist pK_I values are not equivalent to pK_{app} values; agonist binding is entropy-driven and antagonist binding enthalpy- and entropy-driven. In the presence of buffer salts, where the ligand pK_I values are more closely correlated with their pK_{app} values estimated in a functional bioassay, the thermodynamic parameters underlying agonist binding are changed and are not different from those of antagonists.

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Conflict of interest

The authors state no conflict of interest.

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