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RESEARCH PAPER

Correlation of apparent affinity values from H_3-receptor binding assays with apparent affinity (pK_{app}) and intrinsic activity (α) from functional bioassays

EA Harper, NP Shankley[1] and JW Black

James Black Foundation, Dulwich, London, UK

Background and purpose: Agonist apparent affinities (pK_I') in histamine H_3-receptor binding assays were higher than expected from apparent affinity values (pK_{app}) estimated in bioassay. Here, we investigate whether the degree of pK_I' overestimation is related to agonist intrinsic efficacy, by studying the effect of buffer composition on the pK_I' of ligands with varying intrinsic activity.
Experimental approach: In the guinea-pig ileum bioassay, intrinsic activity (α) was determined from the maximal inhibition of the contraction produced by increasing agonist concentration. pK_{app} values were estimated using the method of Furchgott. The pK_L of [^{3}H]clobenpropit in guinea-pig cerebral cortex was estimated by saturation analysis in 20 mM HEPES-NaOH buffer (buffer $B_{(0,0,0)}$), or buffer $B_{(0,0,0)}$ containing 70 mM $CaCl_2$, 100 mM NaCl and 100 mM KCl (buffer $B_{(0.07,0.1,0.1)}$). PK_I values were determined in competition studies in both buffers.
Key results: [^{3}H]clobenpropit saturation isotherms had n_H values of unity in both buffers. In buffer $B_{(0.07,0.1,0.1)}$, agonist pK_I' values were closer to pK_{app} values than in buffer $B_{(0,0,0)}$ but were associated with n_H values <1. A two-site analysis of agonist data in buffer $B_{(0.07,\ 0.1,\ 0.1)}$ provided a better fit than a one-site fit and low affinity values (pK_{IL}) were comparable to pK_{app}. Differences between the pK_I' in buffer $B_{(0,0,0)}$ and pK_{IL} values in buffer $B_{(0.07,0.1,0.1)}$ (ΔpK) were correlated with α.
Conclusions and implications: H_3-receptor binding assays conducted in buffer $B_{(0,0,0)}$ and buffer $B_{(0.07,0.1,0.1)}$ can provide a measure of ligand affinity (pK_{app}) and intrinsic efficacy. The assay predicts that some ligands previously classified as H_3-receptor antagonists may possess residual intrinsic efficacy.
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Keywords: [^{3}H]clobenpropit, histamine H_3-receptors

Abbreviations: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1, 2-dihydroquinoline; *N*-α-MH, *N*-α-methylhistamine; PEI, polyethyleneimine; *R*-α-MH, *R*-α-methylhistamine; *S*-α-MH, S-α-methylhistamine

Introduction

Tissue- or assay-dependent expression of intrinsic efficacy is a well-recognized phenomenon and as such, ligands that act as antagonists or partial agonists in one tissue of a species may behave as full agonists in another tissue from the same species. For instance, Black (1988) found that dichloroisoprenaline was an agonist at β-adrenoceptors in the spontaneously beating guinea-pig heart preparation, but an antagonist in the guinea-pig cardiac papillary muscle. Similarly, Kenakin and Beek (1980) showed that prenalterol was almost a full agonist at β_2-adrenoceptors in guinea-pig trachea, a partial agonist in guinea-pig left atria and an antagonist in guinea-pig extensor digitorum longus.

Some years ago, before the histamine H_3-receptor was cloned (Lovenberg *et al*., 1999) and as part of a programme aimed at the development of high-affinity and selective H_3-receptor antagonists, we developed a radioligand binding assay using the H_3-receptor agonist, [^{3}H]*R*-α-methylhistamine ([^{3}H]*R*-α-MH; Harper *et al*., 1997a, b; 1999a). In the course of evaluating ligands in this assay, we found that the affinity estimates of ligands, previously characterized as competitive antagonists, were on the whole, comparable to those reported in histamine H_3-receptor isolated tissue bioassays. However, we also noticed that there were a number of ligands that expressed a considerably higher affinity in the radioligand binding assays than they

Correspondence: Dr EA Harper, James Black Foundation, 68 Half Moon Lane, Dulwich, London SE24 9JE, UK.
E-mail: elaine.harper@kcl.ac.uk
[1]Current address: Johnson & Johnson Pharmaceutical Research & Development, 3210 Merryfield Row, San Diego, CA 92121, USA.
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Table 1 Parameter estimates for histamine H_3-receptor ligands, obtained in buffer $B_{(0,0,0)}$ and buffer $B_{(0.07,0.1,0.1)}$, in guinea-pig cerebral cortex using [^{3}H]R-α-MH as radioligand

	Ligand	*Buffer* $B_{(0,0,0)}$		*Buffer* $B_{(0.07,0.1,0.1)}$		n
		pK_i'	n_H	pK_i'	n_H	
Antagonists	thioperamide	8.59±0.27	0.84±0.09	8.76±0.14	0.84±0.06	3
	clobenpropit	10.50±0.08	1.22±0.06	9.82±0.18	1.04±0.16	3
Agonist	iodoproxyfan	9.58±0.05	0.96±0.10	8.47±0.14	1.04±0.09	4
	proxyfan	8.89±0.34	0.95±0.02	7.40±0.05	1.11±0.06	3

Data are the mean±s.e.m. from the number of assays shown (*n*). Competition experiments were conducted as described by Harper *et al.* (1999a). A final assay tissue concentration of 6 mg and 0.1 nM concentration of [^{3}H]*R*-α-MH was used in buffer $B_{(0,0,0)}$. In buffer $B_{(0.07,0.1,0.1)}$, the concentration of [^{3}H]*R*-α-MH was 20 nM and a tissue concentration of 12 mg was used to achieve sufficient specific binding.

expressed in the isolated tissue bioassays. Moreover, for some of the ligands, the degree of affinity overestimation appeared to correlate with the ligand's intrinsic activity (α) measured in the functional assay, as though the overestimation provided a measure of agonist intrinsic efficacy. This observation raised the possibility that the H_3-receptor radioligand binding assay might be a sensitive method of detecting residual intrinsic efficacy of H_3-receptor ligands and, moreover, that it could allow identification of H_3-receptor partial agonism that had remained undetected in the isolated tissue bioassay.

To facilitate estimation of both the apparent affinity (pK_{app}) and intrinsic efficacy of H_3-receptor ligands, in the radioligand-binding assay, we considered that it would be necessary to manipulate the conditions of the assay to provide 'high' and 'low' affinity estimates for each ligand, the low affinity estimate being equivalent to the ligand's pK_{app} value estimated in isolated tissue bioassays and the difference between the 'low' and 'high' affinity estimates being a measure of the ligand's intrinsic efficacy.

We chose to try to manipulate the radioligand-binding assay, to provide the 'low' affinity (pK_{app}) estimates for each ligand, by adding salts to the assay buffer rather than by adding guanine nucleotide analogues or using the guinea-pig ileum Krebs–Henseleit (K–H) buffer. This was because it was noted that the affinity of agonists in H_3-receptor radioligand-binding assays, in the presence of high concentrations of guanine nucleotides or in Krebs buffer (e.g. Arrang *et al.*, 1990; [^{3}H]*R*-α-MH pK_L, 100 μM Gpp(NH) = 9.05, Krebs = 9.41) were still considerably higher than pEC_{50} values obtained in functional assays of the same tissue (pEC_{50} = 8.40) and therefore could not be equivalent to the agonist pK_{app}. In addition, in the process of developing the guinea-pig cortex H_3-receptor assay, we had found that increasing the buffer concentrations of a variety of salts (e.g. NaCl, $CaCl_2$, $MgSO_4$, NaH_2PO_4 and KCl) produced a concentration–dependent decrease in the specific binding of the agonist radioligand, [^{3}H]*R*-α-MH (data not shown). Saturation studies performed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES–NaOH) buffer (buffer B) containing 70 mM $CaCl_2$, 100 mM NaCl and 100 mM KCl, alone and in combination (buffer $B_{(0.07,0.1,0.1)}$), indicated that the salts reduced the specific binding of [^{3}H]*R*-α-MH by decreasing the pK_L without any change in B_{max} and the greatest decrease in pK_L was obtained in the presence of 100 mM NaCl, 100 mM KCl and 70 mM $CaCl_2$ (data not shown). Although competition studies performed in buffer B and in buffer $B_{(0.07,0.1,0.1)}$ indicated that antagonist affinity was unchanged by buffer composition (see Table 1) and that 'low' affinity estimates could be obtained for H_3-receptor agonists (see Table 1), which were comparable to those obtained in the guinea-pig ileum bioassay, we were concerned that these studies required high concentrations of radioligand (20 nM) and that it was necessary to increase the tissue concentration to obtain a suitable 'window' of specific binding. Consequently, we chose to try and use the H_3-receptor antagonist, [^{3}H]clobenpropit, to perform the studies on the assumption that an antagonist's affinity would not change appreciably, when the assay conditions were manipulated (see Table 1, Harper *et al.*, 1997c, 1999b).

In this study, we first report the effect that including NaCl, KCl and $CaCl_2$ (buffer $B_{(0.07,0.1,0.1)}$) in the assay buffer has on the affinity of [^{3}H]clobenpropit at H_3-receptors in guinea-pig cortex. We subsequently report the effect that this buffer has on the 'behaviour' of a series of H_3-receptor antagonists and agonists (see Figure 1), which had been described at the time, with varying α as defined by assays performed in the guinea-pig ileum bioassay. Some of these data were previously presented to the British Pharmacological Society (Harper *et al*, 1997d; Watt *et al.*, 1997).

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 was removed and immediately placed in ice-cold 20 mM HEPES–NaOH buffer (pH7. 4, 4°C). The cortex was dissected, weighed and homogenized in ice-cold 20 mM HEPES–NaOH buffer (pH7.4, 4°C) (1 g 15 ml^{-1}) using a polytron homogenizer (Kinematica AG, GmbH, Lucerne, Switzerland; PT-DA 3020/2TS; ~3 s × 3). The homogenate was centrifuged at 100 *g* for 5 min and the supernatants pooled and stored at 4°C. The pellets were rehomogenized in fresh ice-cold buffer (80 ml) and recentrifuged at 100 *g* for 5 min at 4°C. The supernatants were centrifuged at 39800 *g* for 12 min at 4°C and the final pellet was resuspended in 20 mM HEPES–NaOH buffer containing 3 mM metyrapone (21°C) (Harper *et al.*, 1997c), to the required tissue concentration, using a Teflon-in-glass homogenizer (setting 5, 3 ×).

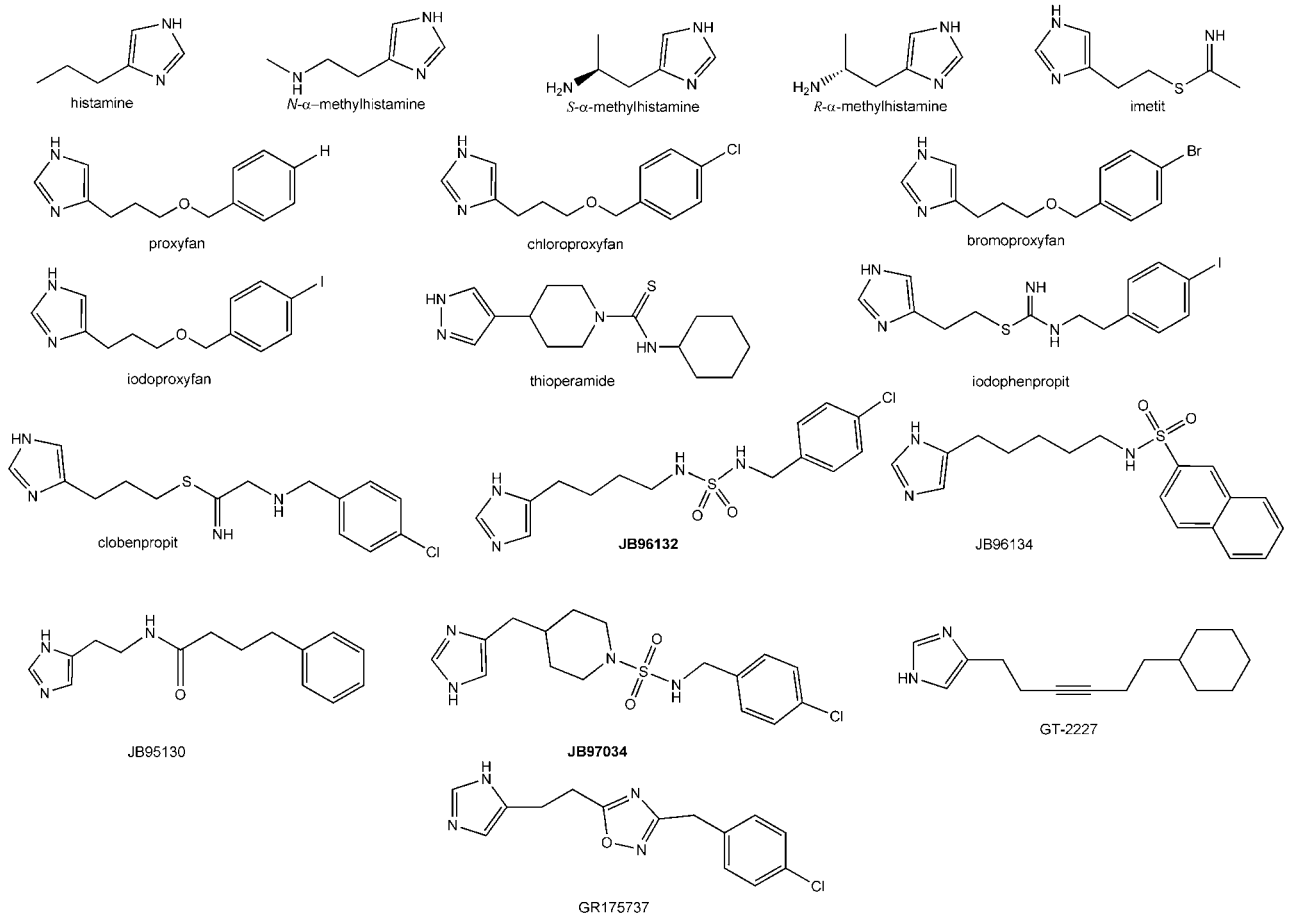

Figure 1 Chemical structures of histamine H_3-receptor ligands.

[^{3}H]clobenpropit – saturation studies

Guinea-pig cortical membranes (1.6 mg) were incubated for 165 min at 21±3°C, in a final volume of 0.5 ml with HEPES–NaOH buffer (buffer $B_{(0,0,0)}$) and 0.004 to 3 nM [^{3}H]clobenpropit. Total and nonspecific binding of [^{3}H]clobenpropit were defined using HEPES–NaOH buffer and 1μM thioperamide (pK_I at histamine H_3-receptors in guinea-pig cortex ~9.0, Harper *et al*., 1999a), respectively. The assay was terminated by rapid filtration through Whatman GF/B filters, presoaked in 0.3% polyethyleneimine, which were washed (3 × 3 ml) with ice-cold 50 mM Tris–HCl (pH7.4, 4°C) using a Brandell Cell Harvester (Brandell, Gaithersburg, MD, USA). 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.

To determine the effect of modifying the assay buffer on the binding of [^{3}H]clobenpropit, we performed saturation analysis in buffer $B_{(0,0,0)}$, and in this buffer containing final assay concentrations of 70 mM $CaCl_2$, 100 mM NaCl and 100 mM KCl (buffer $B_{(0.07,0.1,0.1)}$; with final ionic concentrations (M) Ca^{2+}, 0.07; Na^+, 0.1; K^+, 0.1; Cl^-, 0.27 = buffer $B_{(0.07,\ 0.10,\ 0.10,\ 0.27)}$). In a further series of experiments, [^{3}H]clobenpropit saturation analysis was performed in the presence of increasing (0, 30, 70, 100, 200, 300 mM) final assay concentrations of $CaCl_2$ (buffer $B_{(0,0,0)}$, $B_{(0.03,0,0)}$, $B_{(0.07,0,0)}$, $B_{(0.1,0,0)}$, $B_{(0.2,0,0)}$ and $B_{(0.3,0,0)}$, respectively).

[^{3}H]clobenpropit competition studies

Competition studies were conducted using a membrane concentration, which was previously found to result in zone A conditions for [^{3}H]clobenpropit binding at a 0.2 nM concentration (see Harper *et al*., 1999b). In addition, the competition assay incubation time was previously shown to be sufficient for equilibrium of both radioligand and competitor (see Harper *et al*., 1999b). Guinea-pig cerebral cortex membranes were resuspended in 20 mM HEPES–NaOH buffer containing 0.3 mM metyrapone. Membranes (1.6 mg) were incubated for 165 min at 21±3°C in a final volume of 0.5 ml with 20 mM HEPES–NaOH buffer containing [^{3}H]clobenpropit (0.2 nM), histamine H_3-receptor ligands and either 20 mM HEPES–NaOH, buffer $B_{(0.07,0.1,0.1)}$, buffer $B_{(0.03,0,0)}$, buffer $B_{(0.07,0,0)}$, buffer $B_{(0.1,0,0)}$, buffer $B_{(0.2,0,0)}$ or buffer $B_{(0.3,0,0)}$. Total and nonspecific binding of [^{3}H]clobenpropit were defined using HEPES–NaOH buffer and 1μM thioperamide, respectively.

Guinea-pig ileum assay

Measurement of intrinsic activity. Adult male Dunkin–Hartley guinea pigs (300–500 g) were killed by cervical dislocation.

The ileum was removed at a point 20 cm from the caecum and flushed with and placed in modified K–H buffer of the following mM composition: 118 NaCl, 5.9 KCl, 1.2 $CaCl_2$, 1.2 $MgSO_4$, 1, Na_2HPO_4, 25 $NaHCO_3$ and 10 D-glucose. Ileum segments (2.5–3 cm) were suspended in 20 ml organ baths containing K–H buffer maintained at $37 \pm 1^{\circ}C$ and gassed with 95% O_2/5% CO_2. The initial resting tension was adjusted to 1 g and the tissues field stimulated (0.1 Hz and 0.5 ms) at supramaximal voltage. A single cumulative concentration–effect curve ($E/(A)$) curve was obtained in each tissue. Decreases in tissue twitch tension (% inhibition) were recorded using isometric transducers (Grass FTO3). Mepyramine (3 μM) and famotidine (10 μM) were added to the K–H buffer to block postsynaptic H_1 and presynaptic H_2 receptors, respectively.

Measurement of antagonist affinity

Thioperamide, JB16132, JB96134, iodophenpropit, JB97034, GT-2227, JB95130 and GR175737 were preincubated with tissues for 1 h before the change in tension in response to increasing concentrations of the H_3-receptor agonist, *R*-α-MH, was determined. Clobenpropit was preincubated with tissues for 3 h.

Estimation of apparent agonist affinity (pK_{app})

Measurement of the pK_{app} of agonists at H_3-receptors in guinea-pig ileum was achieved using the method of Furchgott with the irreversible H_3-receptor antagonist, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, see Taylor and Kilpatrick, 1992). Agonist $E/(A)$ curves were constructed in untreated tissues and in tissues which had been incubated (15 min) with EEDQ (0.3 μM) and washed (six times at 10 min intervals) before use. In each experiment the effect of EEDQ treatment on agonist E/(A) curves was ascertained for *R*-α-MH and at least three other agonists.

Data analysis

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

Functional data–agonist concentration effect curves

To obtain estimates of pEC_{50}, and maximal asymptote (α), the Hill equation was fitted to agonist dose–response data, expressed as percentage inhibition of the electrically induced contraction of the ileum. To permit comparison of the agonist α values in different experiments, the α-values for each agonist, in each experiment were expressed as a percentage of the mean α-value obtained for *R*-α-MH in that experiment. *R*-α-MH was assigned a α-value of 1.0

Functional data – Schild analysis

When the minimum criteria for competitive antagonism were satisfied, that is, the antagonist produced a parallel, rightward shift in the *R*-α-MH concentration effect curve with no change in maximum asymptote, data were analysed according to the methods described by Black *et al*. (1985). pA_2 values were estimated by fitting the individual pEC_{50} values, obtained in the presence (pEC_{50}') and absence (pEC_{50}) of antagonist to the following derivative of the Schild equation

$$\log EC_{50}^{'} = \log EC_{50} + \log\left(1 + [B]^{b} 10^{\log K_B}\right) \qquad (1)$$

If the Schild slope parameter (b) was not significantly different from unity, it was constrained to unity and the data refitted to provide a pK_B estimate.

Functional data – estimation of apparent agonist affinity (pK_{app})

Mean data sets obtained for at least three agonists and *R*-α-MH, in control tissue and in EEDQ-pretreated tissues, were fitted to the operational model of agonism (see Black and Leff, 1983) with shared values of maximal effect (E_{max}) and transducer slope parameter (n). pK_{app} values were obtained by fitting all the data to the model using a derivative-free nonlinear, regression programme (BMDP Statistical Software, Module AR: Dixon, 1992).

Radioligand binding – saturation analysis

The Hill equation was fitted to saturation data (Eq. (2)) using Graph-Pad prism where the Hill slope (n_H) was permitted to vary and where this parameter was constrained to unity.

$$B = \left(\frac{B_{max}.[L]^{n_H}}{K_L^{n_H} + [L]^{n_H}}\right) \qquad (2)$$

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

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 Graph-Pad Prism software. When n_H parameter estimates were less than unity and the unconstrained Hill equation provided a significantly better fit of the data than the constrained equation, as determined using an F-test, a two-site model was also fitted to the data.

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) to correct for the different receptor occupancy of [^{3}H]clobenpropit in the different buffers. 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 that were used to correct pIC_{50} values obtained in buffer $B_{(0,0,0)}$ and Buffer $B_{(0.07,0.1,0.1)}$ were 10.36 and 9.82, respectively. pK_L values used to correct for the occupancy of [^{3}H]clobenpropit in the presence of increasing $CaCl_2$ concentrations are presented in Table 3.

Statistical analysis

The effect of antagonist treatment on pEC_{50} and α-values was assessed by analysis of variance (ANOVA) and the Bonferroni-modified *t*-test for multiple comparisons. Differences in [^{3}H]clobenpropit pK_L values were determined by ANOVA. *P*-values of less than 0.05 were considered significant.

Statistical comparison of ligand pK_I' values obtained in buffer $B_{(0,0,0)}$ and buffer $B_{(0.07,0.1,0.1)}$ were performed using Minitab version 13 (Mintab Inc.) by fitting a general linear model with corresponding ANOVA, according to Rosendaal and Stone (2003). This approach was used to avoid the situation that often occurs when dealing with comparisons of large complex data sets by the use of multiple *t*-tests, that is, by chance significant differences will be found.

Materials

[^{3}H]clobenpropit (VUF9153) was prepared to a specific activity of 1.67TBq mmol$^{-1.}$ by Amersham International plc. (Little Chalfont, Buckinghamshire, UK).

Iodophenpropit, proxyfan, 4-iodoproxyfan and the chloro and bromo derivatives, JB96132, JB96134, JB97034, GR175737, JB95130 and GT-2227 were synthesized by James Black Foundation chemists. Histamine, 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), HEPES, EEDQ and Trizma base were obtained from Sigma Chemical Co., Poole, Dorset, UK. *R*-α-MH, *S*-α-methylhistamine (*S*-α-MH), thioperamide and imetit were obtained from Research Biochemicals Inc. (Poole, Dorset, UK). *N*-α-methylhistamine (*N*-α-MH) was obtained from Tocris Cookson Ltd. (Bristol, UK). All other materials were obtained from Fisher Scientific (Loughborough, Leicestershire, UK).

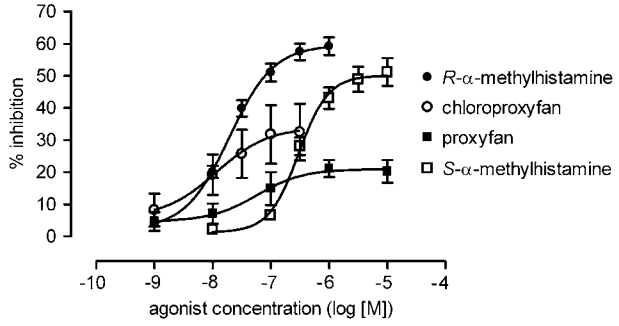

Figure 2 Effect of histamine H_3-receptor ligands on the electrically induced contraction of the guinea-pig ileum. Each point represents the mean ± s.e.m. of determinations in at least three separate preparations (see Table 2).

Results

Guinea-pig ileum bioassay

Histamine, *S*-α-MH, *R*-α-MH, *N*-α-MH, imetit, proxyfan, chloroproxyfan, iodoproxyfan and bromoproxyfan produced dose–dependent inhibition of the electrically induced twitch of the guinea-pig ileum (e.g. Figure 2). Agonist potency values (pEC_{50}) and maximal inhibitory effects (α) are shown in Table 2.

Thioperamide, clobenpropit, GR175737, iodophenpropit, JB95130, GT-2227, JB96132, JB96134 and JB97034 had no effect on the electrically induced twitch of the guinea-pig ileum but produced parallel rightward shifts in *R*-α-MH E/(*A*) curves without change in maximal response or slope (n_H) (Table 2). Antagonist pK_B or pA_2 values are shown in Table 2.

EEDQ treatment of tissues resulted in an increase in agonist pEC_{50} and decrease in the maximal inhibitory response to each agonist (Figure 3). The effect of EEDQ on agonist E/(*A*) curves was prevented following receptor protection with H_3-receptor antagonists (thioperamide 1 μM, $pA_2 = 8.53$; clobenpropit 30 nM, $pA_2 = 10.1$; data not shown). Agonist pK_{app} values, shown in Table 2, were obtained by global fitting of the data using the model of agonism (Black and Leff, 1983) and a derivative-free non-linear, regression programme (BMDP Statistical Software, Module AR: Dixon, 1992).

Table 2 pEC_{50}, α, pK_{app} and pK_B values for histamine H_3-receptor ligands in the guinea-pig ileum bioassay

H_3-receptor agonist	*pEC_{50}* (n)	α (n)	*pK_{app}*	*H_3-receptor antagonist*	*pA_2* (n)
imetit	7.95 ± 0.04 (12)	0.90 ± 0.09 (12)	7.61 ± 0.16	thioperamide	8.53 ± 0.08 (20)
proxyfan	7.29 ± 0.19 (4)	0.35 ± 0.07 (4)	7.66 ± 0.49 7.34 ± 0.10 (4)[a]	iodophenpropit	8.82 ± 0.34 (4)[c]
chloroproyfan	7.85 ± 0.14 (4)	0.45 ± 0.12 (4)	8.04 ± 0.25	JB96132	8.58 ± 0.10 (54)
bromoproxyfan	8.27 ± 0.10 (9)	0.69 ± 0.07 (9)	7.76 ± 0.33	JB96134	6.96 ± 0.10 (6)[c]
iodoproxyfan	8.33 ± 0.11 (10)	0.90 ± 0.05 (10)	8.11 ± 0.21	JB97034	7.22 ± 0.16 (5)[c]
R-α-MH	7.64 ± 0.06 (25)	1.00 ± 0.05 (25)	7.19 ± 0.20 (3)[b]	JB95130	5.44 ± 0.26 (7)[c]
N-α-MH	7.41 ± 0.07 (5)	0.93 ± 0.10 (5)	7.16[e]	GR175737	8.29 ± 0.28 (4)[c]
S-α-MH	6.52 ± 0.03 (10)	0.99 ± 0.08 (10)	5.59 ± 0.14	GT-2227	6.82 ± 0.11 (70)
histamine	7.40 ± 0.04 (6)	1.16 ± 0.11 (6)	6.37 ± 0.16	clobenpropit	9.93 ± 0.12 (3)[d]

Abbreviations: *N*-α-MH, *N*-α-methylhistamine; *R*-α-MH, *R*-α-methylhistamine; *S*-α-MH, S-α-methylhistamine.
Data shown are the mean ± s.e.m.; *n* = number of tissues used unless stated otherwise. The error on the pK_{app} values is the fitting error.
[a]The pA_2 of proxyfan determined in four separate experiments ± s.e.m.
[b]Mean pK_{app} from three separate experiments ± s.e.m.
[c]pA_2 determined from a single antagonist concentration.
[d]pA_2 determined in three separate experiments.
[e]See Taylor and Kilpatrick (1992).

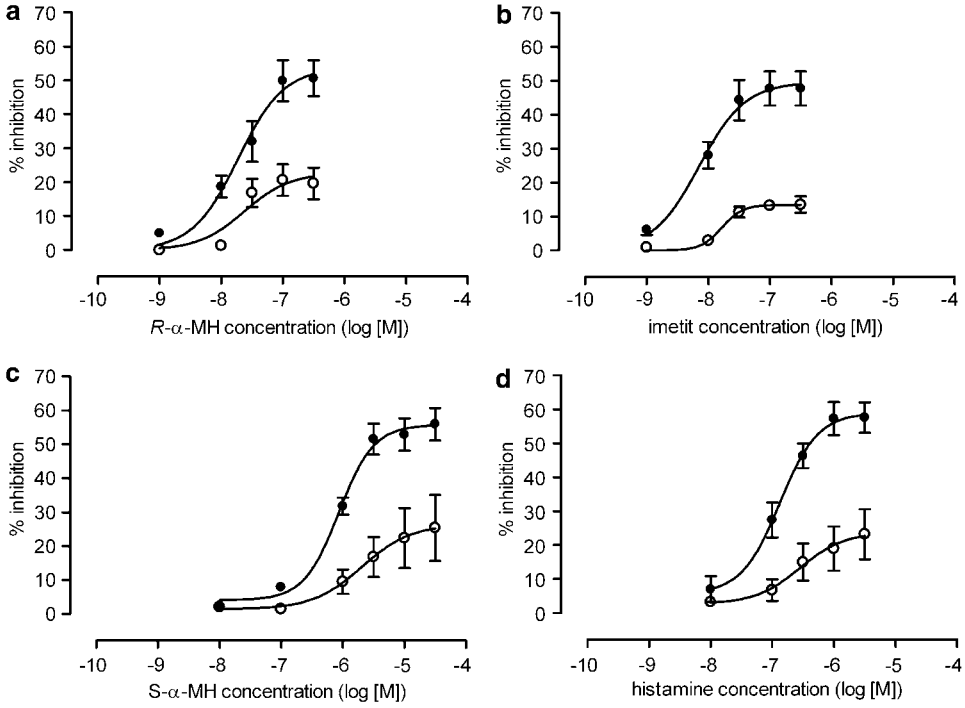

Figure 3 Effect of EEDQ (0.3 μM) on (**a**) R-α-MH, (**b**) imetit, (**c**) S-α-MH and (**d**) histamine-induced inhibition of the electrically induced contraction of the guinea-pig ileum. Each point is the mean ± s.e.m. of determinations in at least six separate preparations.

Effect of buffer composition on [^{3}H]clobenpropit saturation analyses

The effect of 70 mM $CaCl_2$, 100 mM KCl and 100 mM NaCl (buffer $B_{(0.07,0.1,0.1)}$) or the effect that increasing concentrations of $CaCl_2$ had on the binding of [^{3}H]clobenpropit to guinea-pig cerebral cortex membranes, was investigated. This was carried out so that it was possible to correct the H_3-receptor ligand pIC_{50} values, obtained in competition studies, to account for changes in the radioligand receptor occupancy that would result from a change in the radioligand's affinity (pK_L).

The binding of [^{3}H]clobenpropit to guinea-pig cerebral cortex membranes was saturable in buffer $B_{(0,0,0)}$ and buffer $B_{(0.07,0.1,0.1)}$ (Figure 4). Saturation isotherms were monophasic and n_H parameter estimates were not significantly different from unity in both buffers (buffer $B_{(0,0,0)}$ $n_H = 1.14 \pm 0.09$, buffer $B_{(0.07,\ 0.1,\ 0.1)}$ $n_H = 0.95 \pm 0.06$; $n = 4$, *t*-test $P > 0.05$). In addition, there was no significant difference between the goodness of fit to the Hill equation and that to the Hill equation with n_H constrained to unity (*F*-test, $P > 0.05$). The affinity (pK_L) of [^{3}H]clobenpropit was significantly lower in buffer $B_{(0.07,\ 0.1,\ 0.1)}$ ($pK_L = 9.82 \pm 0.07$; $n = 4$) than in buffer $B_{(0,0,0)}$ ($pK_L = 10.36 \pm 0.07$; $n = 4$; ANOVA $P < 0.002$) but there was no significant difference in the H_3-receptor density estimates in buffer $B_{(0,0,0)}$ ($B_{max} = 4.46 \pm 0.53$ fmol mg^{-1} original wet weight) and buffer $B_{(0.07,0.1,0.1)}$ ($B_{max} = 5.59 \pm 0.66$ fmol mg^{-1} original wet weight; ANOVA $P > 0.05$).

Binding of [^{3}H]clobenpropit to guinea-pig cerebral cortex membranes was also saturable in buffer containing concentrations of $CaCl_2$ up to and including 300 mM (buffers $B_{(0.03,0,0)}$, $B_{(0.07,0,0)}$, $B_{(0.1,0,0)}$, $B_{(0.2,0,0)}$ and $B_{(0.3,0,0)}$; Figure 5). Saturation isotherms were monophasic and n_H parameter estimates were not significantly different from unity at all $CaCl_2$ concentrations ($n = 3$; $P > 0.05$, *t*-test). There was no significant difference between the goodness of fit to the Hill equation and that to the Hill equation with n_H constrained to unity in all buffers (*F*-test, $P > 0.05$). $CaCl_2$ concentration had no significant effect on H_3-receptor density (Table 3, Figures 5 and 6; ANOVA $P > 0.05$). The pK_L of [^{3}H]clobenpropit was significantly decreased in the presence of increasing $CaCl_2$ concentration (Table 3, Figures 5 and 6; ANOVA $P < 0.01$). The decrease in pK_L appeared to be saturable (Figure 6) such that the greatest change in this parameter was obtained in the presence of 100 mM $CaCl_2$ (0.51 ± 0.10, $n = 3$) and increasing this concentration by a further 200 mM produced only a further 0.24 ± 0.19 decrease ($n = 3$).

Effect of buffer composition on specific binding of 0.2 nM [^{3}H]clobenpropit

In buffer $B_{(0,0,0)}$, the percentage specific binding of [^{3}H]clobenpropit ($35 \pm 1\%$, $n = 65$) was significantly less than that obtained in the presence of buffer $B_{(0.07,0.1,0,1)}$ ($73 \pm 1\%$, $n = 65$; ANOVA $P < 0.001$; see Figure 7). In contrast, the percentage coefficient of variation, calculated from all the triplicate data points forming each competition curve, was significantly greater in buffer $B_{(0.07,0.1,0.1)}$ (6.68 ± 0.24, $n = 65$) than in buffer $B_{(0,0,0)}$ (4.68 ± 0.14, $n = 65$; *t*-test, $P < 0.001$).

Changes in ligand pK_{app} obtained in buffer $B_{(0,0,0)}$ and buffer $B_{(0.07,0.1,0.1)}$

In both buffer $B_{(0,0,0)}$ and buffer $B_{(0.07,0.1,0.1)}$ each histamine H_3-receptor ligand produced a concentration–dependent

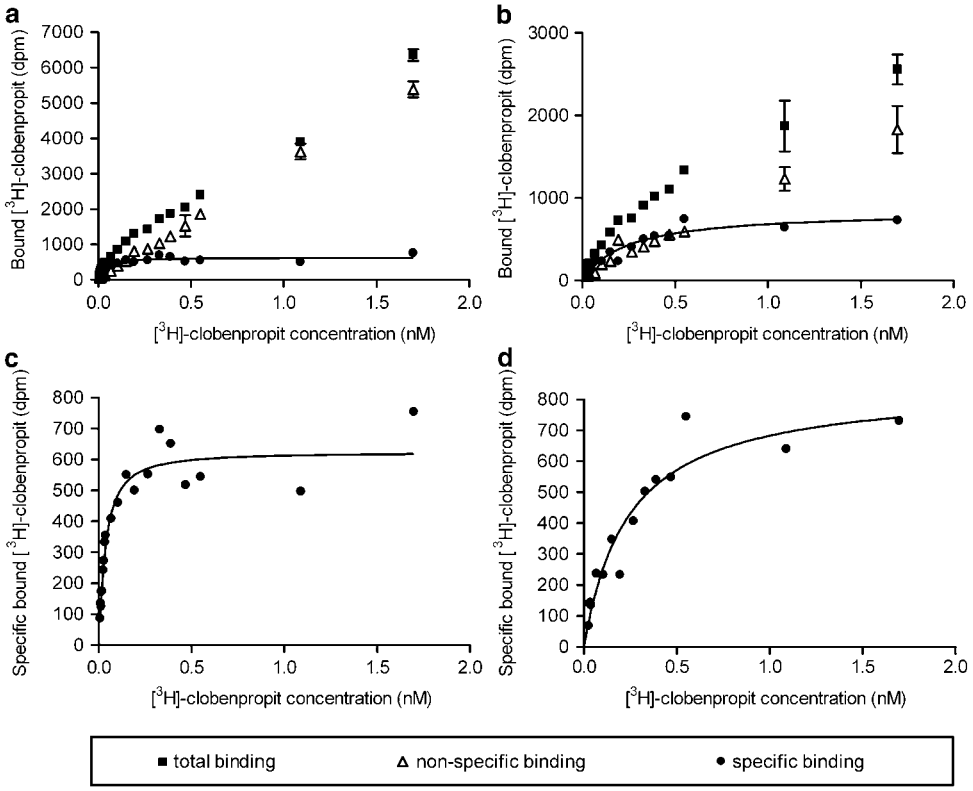

Figure 4 Representative saturation isotherms of [^{3}H]clobenpropit to sites in guinea-pig cerebral cortex membranes in buffer $B_{(0,0,0)}$ (**a** and **c**) and buffer $B_{(0.07,0.1,0.1)}$ (**b** and **d**). The lines shown superimposed on the data points are the saturation isotherm obtained by fitting the Hill equation with n_H constrained to unity to the data. Guinea-pig cortical membranes (1.6 mg) were incubated for 165 min at 21 ± 3°C in a final volume of 0.5 ml with HEPES–NaOH buffer and [^{3}H]clobenpropit. Total and nonspecific binding of [^{3}H]clobenpropit were defined using buffer $B_{(0,0,0)}$ or buffer $B_{(0.07,0.1,0.1)}$ and 1 μM thioperamide, respectively. All determinations were made in triplicate.

inhibition of the specific binding of [^{3}H]clobenpropit to H_3-receptors in guinea-pig cerebral cortex membranes (e.g. Figure 7). In buffer $B_{(0,0,0)}$, the estimated mid-point slope parameter estimates (n_H) for the agonist ligands; imetit, *N*-α-MH, *S*-α-MH, iodoproxyfan and chloroproxyfan were all significantly less than unity whereas in $B_{(0.07,0.1,0.1)}$ buffer, the n_H values were significantly less than unity for all the agonists (Table 4, *t*-test $P<0.05$). The n_H values, estimated from competition curves, for all the antagonist ligands obtained in both buffers were not significantly different from unity (Table 4, *t*-test, $P<0.05$).

Notwithstanding the non-unit n_H values, obtained with several agonist ligands in both buffer $B_{(0,0,0)}$ and buffer $B_{(0.07,0.1,0.1)}$, in the first instance, we investigated the relationship between the apparent affinity values (pK_I or pK_I'), obtained in the two buffers. The pK_I or pK_I' values for 14 of the 17 ligands, in buffer $B_{(0,0,0)}$, were higher than those obtained in buffer $B_{(0.07,0.1,0.1)}$ (Figure 8). All the agonist ligands expressed a lower pK_I or pK_I' in buffer $B_{(0.07,0.1,0.1)}$ than in buffer $B_{(0,0,0)}$. In addition, the difference between the estimated pK_I or pK_I' ($\Delta pK_I'$) of ligands in buffer $B_{(0,0,0)}$ and in buffer $B_{(0.07,0.1,0.1)}$, was significantly less ($P<0.0001$ *t*-test) for the antagonist ligands (see Figure 8 and Table 4, mean $\Delta pK_I' = 0.03 \pm 0.13$, $n=8$; for instance, JB97034 had $\Delta pK_I' = -0.06 \pm 0.05$) than for the ligands classified as agonists in the guinea-pig ileum bioassay (mean $\Delta pK_I' = 1.16 \pm 0.16$, $n=9$; *S*-α-MH and *R*-α-MH, $\Delta pK_I' = -1.37 \pm 0.15$ and $\Delta pK_I' = 1.94 \pm 0.11$, respectively).

There was a significant effect of tissue preparation on agonist affinity (pK_I or pK_I') ($P<0.001$) in buffer $B_{(0,0,0)}$ but not in buffer $B_{(0.7,0.1,0.1)}$ ($P>0.1$, ANOVA). ANOVA indicated that there was a significant effect of tissue preparation and agonist on the magnitude of the $\Delta pK_I'$ (tissue preparation $P<0.025$, agonist $P<0.001$). There was no significant effect of tissue preparation on antagonist affinity (pK_I) when determined in buffer $B_{(0,0,0)}$ or buffer $B_{(0.07,0.1,0.1)}$ (ANOVA, $P>0.1$). There was a significant effect of antagonist but no significant effect of tissue preparation on the magnitude of the $\Delta pK_I'$ (antagonist $P<0.001$, tissue preparation $P>0.1$ ANOVA).

Comparison of ligand pK_{app} values (pK_I'), obtained in buffer $B_{(0,0,0)}$ and buffer $B_{(0.07,0.1,0.1)}$, with pK_{app}, pA_2 or pK_B values obtained in the guinea-pig ileum bioassay

When the pK_I or pK_I' value, obtained for each ligand in buffer $B_{(0,0,0)}$, was compared with the pK_{app} or pA_2 value estimated in the guinea-pig ileum bioassay, the data points for the ligands appeared randomly scattered and 14 of the 17 data points lay below the expected line of identity ($y=x$), that is, the ligands expressed an affinity that was higher than expected from the pK_{app} or pA_2 value obtained in the

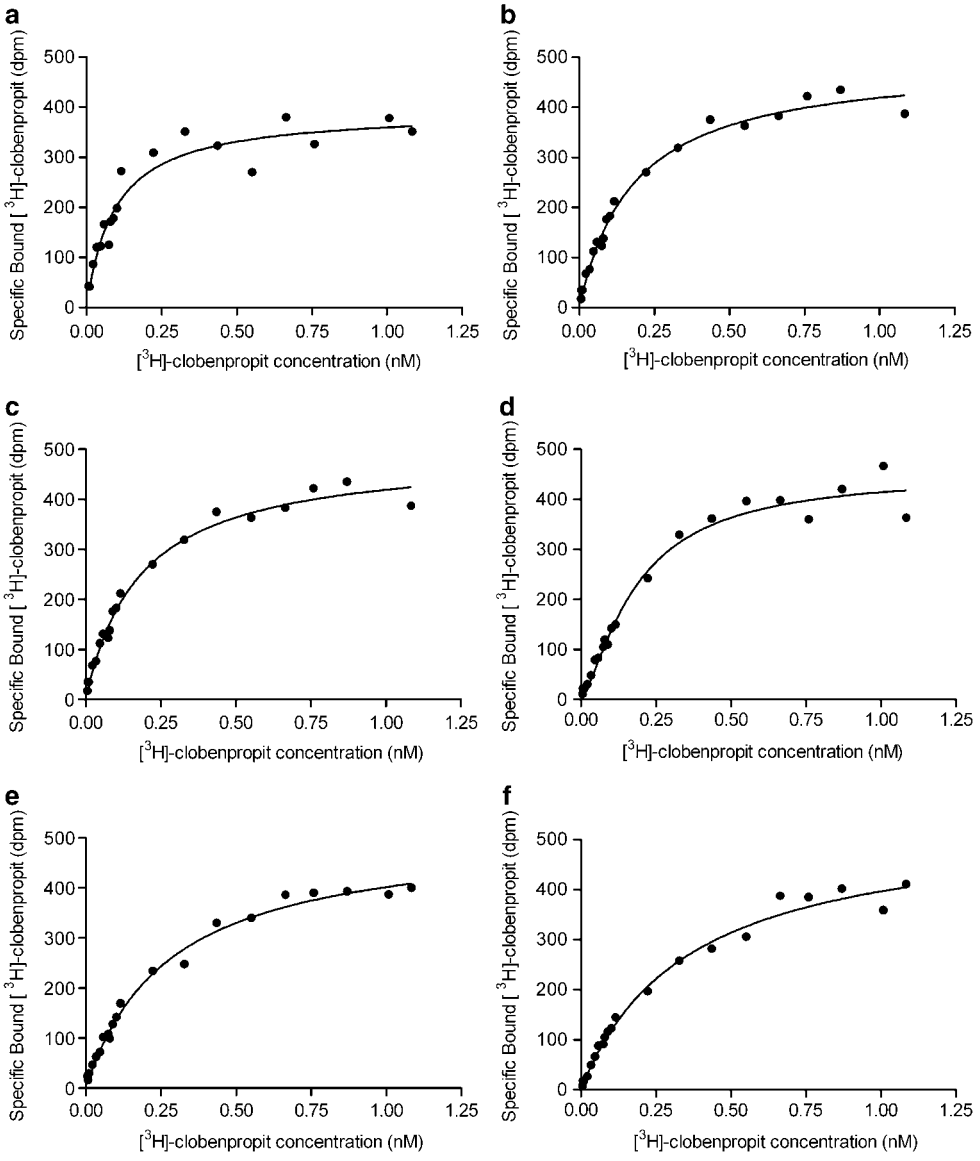

Figure 5 Representative saturation isotherms of [^{3}H]clobenpropit to sites in guinea-pig cerebral cortex membranes in (**a**) buffer $B_{(0,0,0)}$, (**b**) buffer $B_{(0.03,0,0)}$, (**c**) buffer $B_{(0.07,0,0)}$, (**d**) buffer $B_{(0.1,0,0)}$, (**e**) buffer $B_{(0.2,0,0)}$ and (**f**) buffer $B_{(0.3,0,0)}$. The lines shown superimposed on the data points are the saturation isotherm obtained by fitting the Hill equation with n_H constrained to unity to the data. Guinea-pig cortical membranes (1.6 mg) were incubated for 165 min at 21 ± 3°C in a final volume of 0.5 ml with HEPES–NaOH buffer and [^{3}H]clobenpropit. Total and nonspecific binding of [^{3}H]clobenpropit were defined using appropriate buffer and 1 μM thioperamide, respectively. All determinations were made in triplicate.

Table 3 Effect of increasing $CaCl_2$ concentration on the affinity (pK_L), n_H and Bmax of [^{3}H]clobenpropit at histamine H_3-receptors in guinea-pig cerebral cortex

Buffer	pK_L	B_{max} *(fmol mg^{-1})*	n_H
$B_{(0,0,0)}$	10.34 ± 0.10	3.19 ± 0.25	1.23 ± 0.11
$B_{(0.03,\ 0,0)}$	10.16 ± 0.22	3.54 ± 0.05	1.11 ± 0.05
$B_{(0.07,0,0)}$	10.05 ± 0.06	4.17 ± 0.38	1.08 ± 0.09
$B_{(0.1,0,0)}$	9.84 ± 0.15	4.14 ± 0.23	1.04 ± 0.14
$B_{(0.2,0,0)}$	9.70 ± 0.05	3.53 ± 0.32	1.06 ± 0.12
$B_{(0.3,0,0)}$	9.60 ± 0.02	4.10 ± 0.23	1.06 ± 0.02

Data are the mean ± s.e.m. of three separate experiments. The concentrations of $CaCl_2$ used in the buffers were (M): 0, 0.03, 0.07, 0.1, 0.2, 0.3. The s.e.m. of the n_H parameter from individual data sets was between 0.36 and 0.09.

functional bioassay (Figure 9a). When the same comparison was made using pK_I or pK_I' values, obtained in buffer $B_{(0.07,0.1,0.1)}$, the data points were more linearly distributed and closer to the line of identity (Figure 9b). The mean deviation from the line of identity ($\sum$(pK_i – pK_{app}, pA_2 or pK_B)/n) was greatest when the pK_I or pK_I' values were estimated in buffer $B_{(0,0,0)}$ rather than buffer $B_{(0.07,0.1,0.1)}$ ($B_{(0,0,0)}$, mean deviation from pK_{app}, pA_2 or $pK_B = 1.09 \pm 0.21$; $B_{(0.07,0.1,0.1)}$ mean difference from pK_{app}, pA_2 or $pK_B = 0.44 \pm 0.08$; Figure 9a and b).

When the antagonist pK_I values, were compared with pK_B or pA_2 values estimated in the guinea-pig ileum assay, there was no significant change in the deviation from $y = x$ when

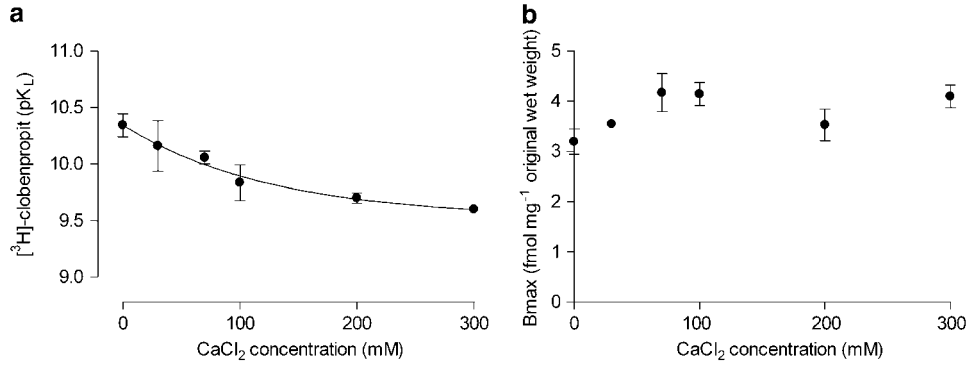

Figure 6 Effect of increasing concentration of $CaCl_2$ in buffer B on (**a**) pK_L and (**b**) B_{max} of [^{3}H]clobenpropit at sites in guinea-pig cerebral cortex membranes. The line shown superimposed on the data was obtained by fitting a hyperbolic function.

pK_I values were estimated in buffer $B_{(0,0,0)}$ or buffer $B_{(0.07,0.1,0.1)}$ (0.41±0.14 and 0.30±0.12, respectively; *t*-test, $P>0.05$; Figure 9e and f). In contrast, when only the agonist pK_I or pK_I' values, obtained in buffer $B_{(0,0,0)}$ were compared with pK_{app} values estimated in the guinea-pig ileum assay, the deviation from $y=x$ was 1.69±0.24. When the same comparison was performed using pK_I' values obtained in buffer $B_{(0.07,0.1,0.1)}$, the data points were significantly closer to $y=x$ (0.55±0.08; *t*-test, $P<0.0005$; Figure 9c and d) but 8 of the 9 data points still lay below the line of identity. In buffer $B_{(0,0,0)}$, the mean deviation from $y=x$, was significantly greater for agonists than antagonists (*t*-test, $P<0.005$) but there was no significant difference in the degree of deviation from $y=x$ in buffer $B_{(0.07,0.1,0.1)}$ (*t*-test, $P>0.05$).

In light of this finding and the observation that all the mean agonist n_H parameter estimates were significantly less than unity in buffer $B_{(0.07,0.1,0.1)}$, the agonist competition data were analysed further. The goodness of fit of the Hill equation and of the Hill equation with n_H constrained to unity, were compared. The Hill equation with unconstrained slope provided a significantly improved fit to the data for all agonists (*F*-test, $P<0.05$) and therefore a two-site model was fitted to the data. The two affinity values (pK_{IL} and pK_{IH}) that were obtained are presented in Table 4. When the pK_{IL} values, obtained from the two-site fit of the agonist competition curves in buffer $B_{(0.07,0.1,0.1)}$, were compared with pK_{app} values estimated in the guinea-pig ileum assay, the data points were evenly distributed about the line of identity and the deviation from $y=x$ (0.08±0.06) was significantly less than when pK_I' values were compared (0.55±0.08, Figure 10).

Comparison of α-values obtained in the guinea-pig ileum with ΔpK values from radioligand binding

There appeared to be a relationship between agonist ΔpK values and α-values estimated in the guinea-pig ileum bioassay such that agonists with higher α expressed higher ΔpK values (Figure 11).

Discussion

In this study, we have used a guinea-pig ileum bioassay to determine the affinity (pA_2) of histamine H_3-receptor antagonists and also the α and pK_{app} of a series of histamine H_3-receptor agonists at guinea pig H_3-receptors. In addition, we have investigated the effect of altering buffer composition on the apparent affinity (pK_I') that ligands, with known α, express in competition studies performed in the absence (buffer $B_{(0,0,0)}$) and presence of buffer salts (buffer $B_{(0.07,0.1,0.1)}$); with the aim of establishing whether assays of this type can be used to measure the pK_{app} and to detect residual intrinsic efficacy of ligands. We refer to ileum H_3-receptor affinity values estimated by the method of Furchgott, as pK_{app} values, in order to take account of the problem that defining this parameter as pK_A assumes that activation of the receptor has no effect on binding and this is not the case for currently proposed models of agonist action (see Colquhoun, 1998). The term pK_{app} is used to indicate that the measurement is a macroscopic equilibrium constant describing the overall constant for the binding of agonist to receptor and subsequent isomerization of this receptor to form AR* (see Neubig *et al*., 2003).

The pA_2 values of the previously described H_3-receptor antagonist ligands, thioperamide, clobenpropit and GR175737, in the guinea-pig ileum, were comparable to those reported previously (Arrang *et al*., 1987, 1990; Van der Goot *et al*., 1992; Barnes *et al*., 1993; Clitherow *et al*., 1996; Valentine *et al*., 1999). The affinity of iodophenpropit and GT-2227 were underestimated compared with those reported previously (iodophenpropit $pA_2=9.6$, Jansen *et al*., 1992; GT-2227, $pA_2=7.9$, Tedford *et al*., 1998); this underestimation may have been a consequence of the 1 h antagonist preincubation being insufficient for equilibration of these antagonists.

In the radioligand-binding studies, the competition curves for all the agonist ligands, with the exception of *R*-α-MH and histamine, in buffer $B_{(0,0,0)}$ and for all the agonists in buffer $B_{(0.07,0.1,0.1)}$, were associated with n_H parameter estimates that were significantly less than unity. This behaviour has been well described for the binding of agonists at many types of receptor and a number of models have been proposed to explain the phenomenon (e.g. Lefkowitz *et al*., 1993; Samama *et al*., 1993; Weiss *et al*., 1996). In light of n_H values less than unity, it could be argued that only the pIC_{50} values for *R*-α-MH and histamine should be corrected using the Cheng–Prusoff equation because the derivation of this correction relies on simple competition between two ligands

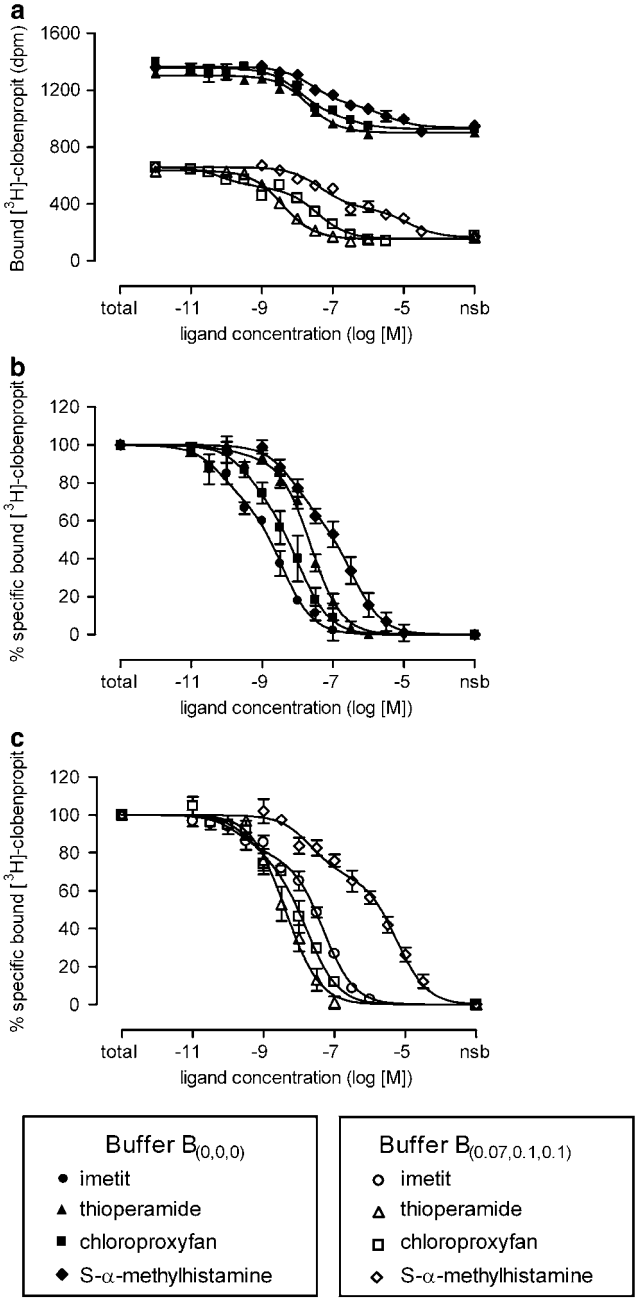

Figure 7 Competition curves for H_3-receptor agonists and antagonists at sites labelled with [^{3}H]clobenpropit in guinea-pig cerebral cortex. (**a**) Effect of increasing concentrations of ligands on [^{3}H]clobenpropit binding (dpm). Data were obtained in a single experiment in buffer $B_{(0,0,0)}$ and buffer $B_{(0.07,0.1,0.1)}$ and errors are the mean$\pm$s.e.m. of triplicates. (**b**) Mean competition curve data for ligands, expressed as percentage specific binding, in buffer $B_{(0,0,0)}$ or (**c**) buffer $B_{(0.07,0.1,0.1)}$. Guinea-pig cortical membranes (1.6 mg) were incubated for 165 min at 21$\pm$3°C in a final volume of 0.5 ml with HEPES–NaOH buffer, [^{3}H]clobenpropit (0.2 nM) and increasing concentrations of ligands. Total and nonspecific binding of [^{3}H]clobenpropit were defined using appropriate buffer and 1 μM thioperamide, respectively. Data are the mean$\pm$s.e.m. of between four and six experiments (see Table 4). The lines shown superimposed on the data for imetit, chloroproxyfan and *S*-α-methylhistamine were obtained using a two-site fit. The line shown superimposed on the thioperamide data was obtained using a one-site fit.

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 $\sim$0.2 nM [^{3}H]clobenpropit in the two buffers (buffer $B_{(0,0,0)}$, $pK_L = 10.36$ and buffer $B_{(0.07,0.1,0.1)}$, $pK_L = 9.82$). Ideally, in order for this not to be a confounding problem, we would have performed competition studies in both buffers at a concentration of [^{3}H]clobenpropit, which was equivalent to its pK_L. However, this was not possible because the low specific activity of the radioligand resulted in too small a specific-binding window in buffer $B_{(0,0,0)}$ at a $\sim$0.04 nM concentration. We also corrected all pIC_{50} values irrespective of n_H parameter because although *R*-α-MH and histamine had n_H values that were not different from unity, it appeared from functional pEC_{50} values that their pIC_{50} values were likely to have been overestimated by as much if not more than other agonists, where the n_H parameter was significantly less than unity (e.g. chloroproxyfan). Thus, the pIC_{50} value of *R*-α-MH in buffer $B_{(0,0,0)}$ was 8.96$\pm$0.16 and the pEC_{50} value in the functional assay was 7.64, whereas the pIC_{50} value of chloroproxyfan was 8.17$\pm$0.24 and the pEC_{50} value was 7.85. Clearly, if the same mechanism underlies the binding of all agonists it is equally inappropriate to correct an agonist pIC_{50} value when the n_H value is not different from unity as when the n_H is less than unity. To make a distinction between pIC_{50} values that have been corrected for [^{3}H]clobenpropit occupancy where n_H was equal to unity and those where n_H was less than unity and where strictly speaking the Cheng–Prusoff equation should not have been applied, we have assigned the latter the parameter pK_I'.

In the radioligand-binding studies, affinity values (pK_I or pK_I') for the agonist ligands, in the absence of buffer salts (buffer ($B_{(0,0,0)}$)), were higher than their estimated pK_{app} or pA_2 values obtained in bioassay studies (Figure 9a), which confirmed our previous observations where we used [^{3}H]*R*-α-MH as the radioligand (Harper *et al*., 1999a). This indicated that, despite using an antagonist radioligand, it was still possible to obtain overestimated 'high' affinity binding values for agonists. The finding that the apparent affinity values (pK_I') of ligands in buffer B containing salts (buffer $B_{(0.07,0.1,0.1)}$) were reduced relative to those obtained in buffer $B_{(0,0,0)}$ was also consistent with our preliminary studies in which [^{3}H]*R*-α-MH was used as the radioligand and indicated that it was also possible to obtain 'low' affinity estimates for agonist ligands when using the antagonist radioligand, [^{3}H]clobenpropit.

The observation that the change in pK_I' between that obtained in buffer $B_{(0,0,0)}$ and that obtained in buffer $B_{(0.07,0.1,0.1)}$ ($\Delta pK_I'$) was greater for agonists than antagonists (agonists = 1.16; antagonists = 0.03) and that there was a significant effect of agonist but not antagonist on the $\Delta pK_I'$ (agonist $P<0.001$, antagonist $P>0.1$) suggested that the change in pK_I' value, brought about by modification of the buffer composition, was related to a property of the ligands, which was only expressed by agonists. It is possible to explain the high- and low-affinity binding of agonists in H_3-receptor radioligand binding assays by considering the extended ternary complex model (TCM) developed by

Table 4 Parameter estimates for histamine H_3-receptor ligands obtained from analysis of competition experiments performed in buffer containing 3 mM metyrapone (buffer $B_{(0,0,0)}$) and in buffer $B_{(0.07,0.1,0.1)}$

Ligand	n	*Buffer B* $_{(0,0,0)}$			*Buffer B* $_{(0.07,0.1,0.1)}$			$\Delta pK_I'$	ΔpK
		pK_I *or* pK_I'	n_H	pK_{IH} *and* pK_{IL}	pK_I *or* pK_I'	n_H	pK_{IH} *and* pK_{IL}		
Histamine H_3-receptor agonists									
imetit	4	9.22±0.30	0.63±0.03*	10.20±0.31 8.04±0.34	8.06±0.07	0.66±0.07*	10.05±0.51 7.71±0.03	1.16±0.23	1.52±0.29
proxyfan	4	8.34±0.10	0.83±0.23		7.68±0.11	0.70±0.09*	9.53±0.11 7.36±0.11	0.66±0.06	0.98±0.21
chloroproxyfan	4	8.98±0.24	0.77±0.05*	9.47±0.23 8.22±0.36	8.41±0.02	0.72±0.06*	9.94±0.26 8.06±0.07	0.58±0.24	0.92±0.20
bromoproxyfan	3	8.93±0.25	0.82±0.22		8.37±0.09	0.63±0.03*	10.34±0.19 7.94±0.09	0.55±0.26	0.99±0.25
iodoproxyfan	5	9.54±0.22	0.71±0.04*	10.04±0.37 8.27±0.36	8.73±0.12	0.70±0.03*	10.21±0.22 8.37±0.10	1.21±0.23	1.16±0.13
R-α-MH	4	9.74±0.15	1.02±0.31		7.80±0.07	0.61±0.07*	9.07±0.37 7.24±0.15	1.94±0.11	2.51±0.29
N-α-MH	6	9.58±0.19	0.70±0.04*	10.08±0.10 8.19±0.30	8.09±0.29	0.51±0.04*	9.82±0.26 7.43±0.23	1.45±0.23	2.15±0.13
S-α-MH	6	7.67±0.14	0.64±0.03*	8.81±0.18 6.90±0.19	6.29±0.14	0.49±0.02*	8.08±0.22 5.68±0.12	1.37±0.15	1.98±0.19
histamine	4	8.87±0.32	0.83±0.14		7.04±0.15	0.54±0.02*	8.44±0.13 6.30±0.09	1.50±0.43	2.24±0.32
Histamine H_3-receptor antagonists									
thioperamide	4	8.41±0.06	0.99±0.08		8.93±0.06	0.92±0.04		−0.52±0.07	−0.52±0.07
iodophenpropit	4	9.72±0.05	1.08±0.09		9.62±0.14	1.03±0.08		0.10±0.14	0.10±0.14
JB96132	4	8.54±0.09	1.23±0.22		8.94±0.07	0.95±0.03		−0.40±0.13	−0.40±0.13
JB96134	4	7.79±0.12	1.15±0.30		7.44±0.03	0.91±0.15		0.35±0.16	0.35±0.16
JB97034	3	7.53±0.24	1.01±0.10		7.58±0.19	0.91±0.10		−0.06±0.05	−0.06±0.05
JB95130	3	6.16±0.31	1.02±0.25		5.97±0.11	1.08±0.03		−0.18±0.20	−0.18±0.20
GR175737	3	8.43±0.03	0.82±0.06		7.95±0.07	0.93±0.07		0.48±0.07	0.48±0.07
GT-2227	3	7.33±0.21	1.26±0.28		6.88±0.21	1.13±0.08		0.45±0.14	0.45±0.14

Abbreviations: *N*-α-MH, *N*-α-methylhistamine; *R*-α-MH, *R*-α-methylhistamine; *S*-α-MH, S-α-methylhistamine.
Data are the mean±s.e.m. from the number of assays shown (*n*). When the mean n_H parameter estimate for a ligand was not different from unity, an affinity (pK_I) value is provided. When n_H is significantly less than unity, pIC_{50} values were corrected using the Cheng–Prusoff equation but affinity values were assigned the parameter, pK_I'. pK_{IH} and pK_{IL} values were obtained by fitting a two-site model to the data. $\Delta pK_I'$ is the difference between pK_I or pK_I' values in buffer $B_{(0,0,0)}$ to pK_{IL} in buffer $B_{(0.07,0.1,0.1)}$. ΔpK is the difference between the pK_I or pK_I' in buffer $B_{(0,0,0)}$ to pK_{IL} in buffer $B_{(0.07,0.1,0.1)}$. *n_H significantly different from unity $P<0.05$, *t*-test.

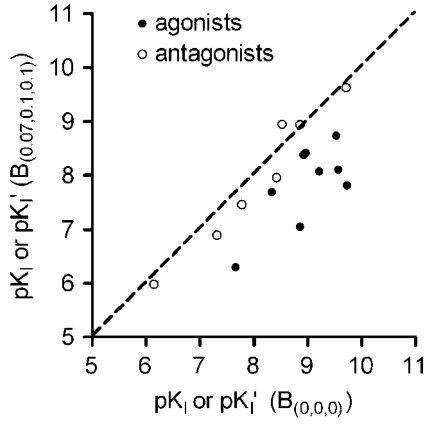

Figure 8 Comparison of the apparent affinities (pK_I or pK_I') of histamine H_3-receptor agonists and antagonists obtained in buffer $B_{(0,0,0)}$ and in buffer B containing 70 mM $CaCl_2$, 100 mM KCl and 100 mM NaCl (buffer $B_{(0.07,0.1,0.1)}$). The broken line represents the line of identity.

Lefkowitz and colleagues (Samama *et al*., 1993; Lefkowitz *et al*., 1993; see Figure 12a) and also by the cubic TCM that was described some years later (Weiss *et al*., 1996; see Figure 12b). In the extended TCM, it is proposed that the receptor can exist in a low-affinity agonist state (R) and a high-affinity state (R*), which can also interact with a G-protein (R*G) in the absence of agonist. Thus, the high-affinity H_3-receptor agonist binding may be a consequence of the agonist binding to R* or R*G. 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, high-affinity H_3-receptor agonist binding could result from either binding to preformed high-affinity receptor states (R* and R*G) or from the induction of these high-affinity states through binding to low-affinity receptors (R). Low-affinity agonist binding in buffer $B_{(0.07,0.1,0.1)}$ can be explained by considering that, under these conditions, the agonist binds only to the low-affinity receptor state (R) and cannot bind to or induce the formation of R* or R*G. Although, at the time these studies were performed it was not definitely known that the H_3-receptor was G-protein coupled because it had not yet been cloned, the possibility that agonists could induce H_3-receptor ternary complex formation was supported by studies suggesting that these

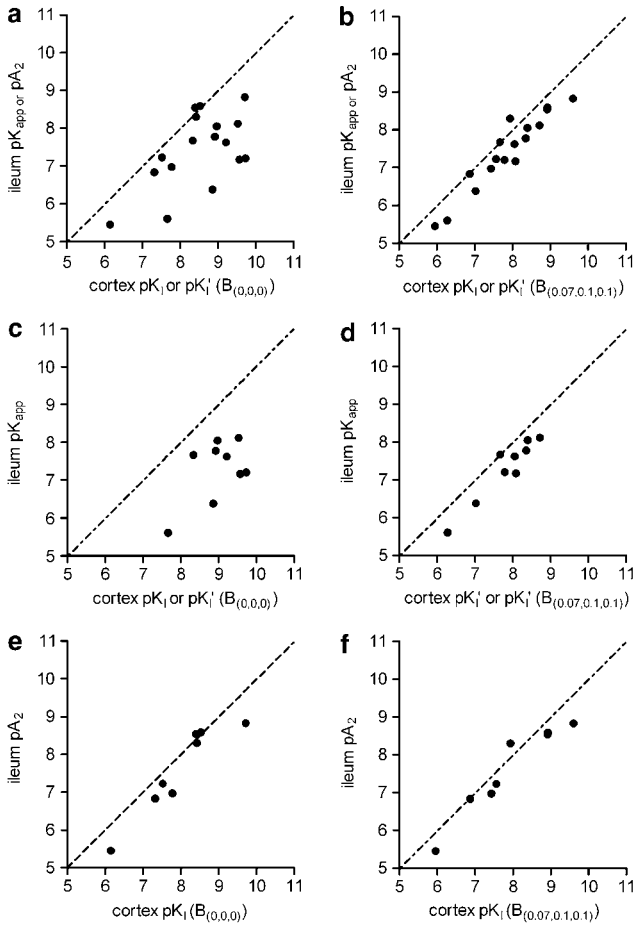

Figure 9 Comparison of the affinities of histamine H_3-receptor agonists (pK_{app}) and antagonists (pA_2 or pK_B) at H_3-receptors in the guinea-pig ileum bioassay (see Table 2) with those estimated in radioligand binding assays (see Table 4). pK_I or pK_I' values for (**a**) agonists and antagonists in standard buffer ($B_{(0,0,0)}$), (**b**) agonists and antagonists in $B_{(0.07,0.1,0.1)}$, (**c**) agonists in $B_{(0,0,0)}$, (**d**) agonists in $B_{(0.07,0.1,0.1)}$, (**e**) antagonists in $B_{(0.07,0.1,0.1)}$ and (**f**) antagonists in $B_{(0.07,0.1,0.1)}$. The broken line represents the line of identity ($y=x$).

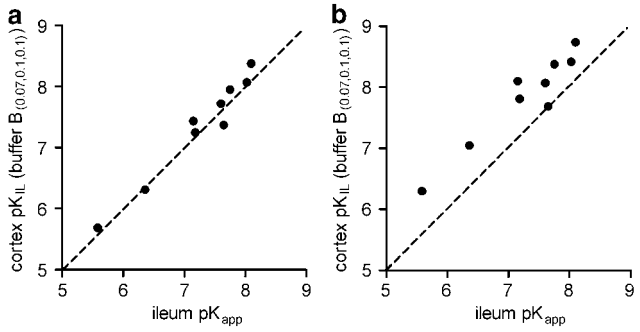

Figure 10 Comparison of (**a**) pK_I' and (**b**) pK_{IL} values for agonists in buffer $B_{(0.07,0.1,0.1)}$ with ileum pK_{app} estimates.

receptors were linked to effector systems through G-proteins (Arrang *et al*., 1990; West *et al*., 1990; Zweig *et al*., 1992; Clark *et al*., 1993; Litosch *et al*., 1993; Clark and Hill, 1995; Clark and Hill, 1996).

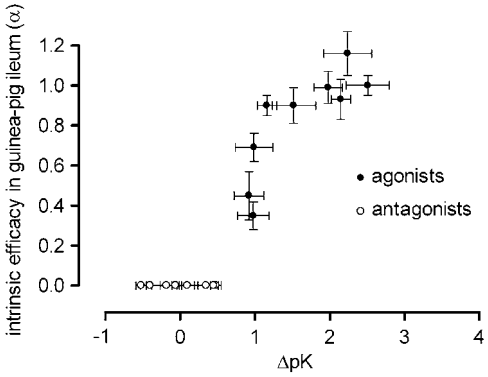

Figure 11 Comparison of ligand Δp*K* values and α measured in the guinea-pig ileum bioassay.

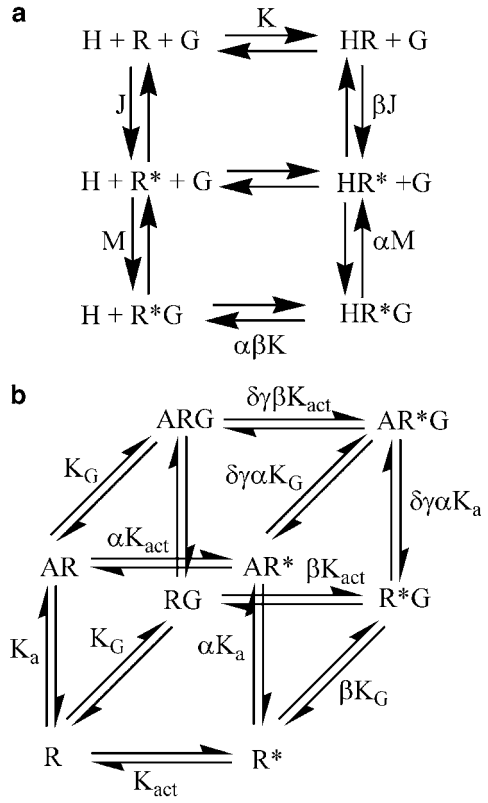

Figure 12 (**a**) The extended TCM as described by Samama *et al*. (1993). (**b**) The cubic TCM of Weiss *et al*. (1996). In both models, H is hormone, G is G protein and R is the inactive receptor state and R* the 'active' state. To allow simple comparison of the models, the terms used for 'inactive', R_i and 'active' receptor states (R_a) in the model of Weiss *et al*. (1996) have been modified to R and R*, respectively.

It seems unlikely that the apparent changes in ligand affinity obtained using buffer $B_{(0,0,0)}$ and buffer $B_{(0.07,0.1,0.1)}$ could have arisen simply as a consequence of highly variable data because although the percentage specific binding of [^{3}H]clobenpropit was higher in buffer $B_{(0.07,0.1,0.1)}$ ($73\pm1\%$) compared with that obtained in buffer $B_{(0,0,0)}$ ($35\pm1\%$), the % coefficient of variation of the data points in buffer $B_{(0,0,0)}$ was lower than that in buffer $B_{(0.07,0.1,0.1)}$. Furthermore, we previously found that although the percentage specific binding of [^{3}H]clobenpropit in buffer $B_{(0,0,0)}$ is low

(~40%), there was a close correlation between histamine H_3-receptor pK_I values estimated using this radioligand and a radioligand exhibiting considerably higher percentage specific binding ([^{3}H]*R*-α-MH with 89%; Harper *et al*., 1999a, b).

The pretext for performing competition studies in assay buffer containing salts, that is, that it could allow estimation of agonist affinity (pK_{app}), was supported by comparisons made between the radioligand binding pK_I' values and the pK_{app} or pA_2 values obtained in the guinea-pig ileum bioassay (Figure 9). Thus, the deviation from the expected $y=x$ for all the ligands, was significantly lower in modified buffer (buffer $B_{(0,0,0)}=1.09\pm0.21$; $B_{(0.07,0.1,0.1)}=0.44\pm0.08$; Figure 9a and b). In addition, when the same analysis was performed on subsets of ligands, characterized as agonists (Figure 9c and d) or antagonists (Figure 9e and f), in the guinea-pig ileum, the deviation from the expected $y=x$ was increased for the agonists (buffer $B_{(0,0,0)}=1.69\pm0.24$, Figure 9c) and unchanged for the antagonists (buffer $B_{(0,0,0)}=0.41\pm0.14$, Figure 9e) relative to the complete data set. In addition, the deviation from $y=x$ was significantly decreased when agonist but not antagonist pK_I values were determined in the buffer containing salts (buffer $B_{(0.07,0.1,0.1)}$ agonists $=0.55\pm0.08$, Figure 9d; antagonists $=0.30\pm0.12$, Figure 9e) and, furthermore, the mean deviation from $y=x$ was only significantly greater for agonists than that for antagonists when the pK_I' or pK_I values were estimated in buffer $B_{(0,0,0)}$.

Notwithstanding the finding that the deviation from the expected $y=x$ for the antagonists was unchanged by buffer, we noticed that for one antagonist, iodophenpropit, there was a large difference between the pA_2 and pK_I obtained in both buffers (~1 log unit; $pA_2=8.82\pm0.34$; pK_I buffer $B_{(0,0,0)}=9.72\pm0.05$, buffer $B_{(0.07,0.1,0.1)}=9.62\pm0.14$). This discrepancy could be explained by considering that the pA_2 value of iodophenpropit was underestimated because it had not been preincubated with tissue for long enough to reach equilibrium. In support of this possibility, Jansen *et al*. (1992) reported a pA_2 of 9.6 for iodophenpropit and we found that the pA_2 value of another H_3-receptor antagonist with similar structure, clobenpropit, was increased when preincubated with tissue for 3 h.

Despite the similarity between pK_{app} values in the ileum bioassay and the pK_I or pK_I' values of ligands in buffer $B_{(0.07,0.1,0.1)}$ (see Figure 9b), it was still apparent that eight of the nine agonist data points still lay below the expected line of identity ($y=x$, Figure 9d). It seems unlikely that we found that the ileum pK_{app} values were lower than the pK_I' values in buffer $B_{(0.07,0.1,0.1)}$ simply because of experimental error and because they were estimated, albeit with the exception of *R*-α-MH, in a single experiment. This is because, for one agonist, proxyfan, the pK_{app} (7.66 ± 0.49) from model fitting was comparable to the pA_2 determined by investigating the effect of this ligand on *R*-α-MH concentration effect curves (7.34 ± 0.10, $n=4$; Table 4 and Schlicker *et al*., 1996, $pA_2=7.12$). In addition, the pK_{app} values for *R*-α-MH and histamine were comparable to those previously reported by Taylor and Kilpatrick (1992) (*R*-α-MH = 7.01; histamine = 6.13).

The possibility that, even in buffer $B_{(0.07,0.1,0.1)}$, the agonist pK_I' values were not equivalent to the pK_{app} estimated in the ileum bioassay is supported by the observation that all the agonist competition curves in this buffer had n_H parameter estimates, which were significantly less than unity (Table 4). The cubic TCM (Weiss *et al*., 1996; see Figure 12b) predicts this behaviour if it is considered that the change in composition of the assay buffer (buffer $B_{(0.07,0.1,0.1)}$) is not sufficient to prevent the agonist inducing some high-affinity receptor states (ARG and/ or AR*G) or from binding to pre-existing high-affinity receptors (R* or RG). Therefore, according to this model, the flat competition curves result from competition for labelled R (low-affinity binding) and some induction or binding to R*G or RG (high-affinity binding component). The data cannot be explained by competition for labelled R and R* because in this situation competition curves would have unit slope. Interestingly, in support of this explanation, when the buffer $B_{(0.07,0.1,0.1)}$ agonist competition data were analysed using a two-site model, and the low-affinity estimates (pK_{IL}) were compared with the ileum pK_{app} values, the data points were closer to $y=x$ (Figure 10).

A possible explanation for the decrease in agonist affinity in buffer $B_{(0.07,0.1,0.1)}$ is that, in this buffer, the metal ions interfere in some way with the ability of agonists to induce high-affinity ternary complex receptor states. Indeed, many studies have demonstrated that agonist, but not neutral antagonist, binding is sensitive to metal ions (Limbird *et al*., 1982; Puttfarcken *et al*., 1986; Gowraganahalli *et al*., 1990) and have suggested that the effect that metal ions have on agonist binding is owing to them, in some way, preventing the formation of ternary complex between agonist, receptor and G-protein (Childers and La Riviere, 1984; Lambert and Childers, 1984; Kim and Neubig, 1985; Lynch *et al*., 1985; Demoliou-Mason and Barnard, 1986; Puttfarcken *et al*., 1986; Carraway *et al*., 1992). However, there are two other possible explanations to explain why the modified buffer reduces the high-affinity agonist binding and why some high-affinity binding remains in the modified buffer ($B_{(0.07,0.1,0.1)}$). This is because adding NaCl, $CaCl_2$ and KCl, also alters the osmotic and ionic strength of buffer $B_{(0,0,0}$ (buffer $B_{(0.07,0.1,0.1)}$ osmotic strength = 290 mM; ionic strength = 430 mM; buffer $B_{(0,0,0)}$ osmotic strength = 20 mM; ionic strength = 20 mM). In fact, buffer ionic strength has been previously shown to reduce agonist affinity (Arias, 1996). However, our previous observation that 70 mM $CaCl_2$ produced a greater reduction in [^{3}H]*R*-α-MH pK_L than 100 mM NaCl (data not shown) suggests that the osmotic strength of the buffer does not result in low-affinity agonist binding. Nonetheless, in retrospect, it would have been interesting to perform further competition studies to establish whether osmotic strength contributed to the reduction in agonist affinity, by adding glucose to buffer $B_{(0,0,0)}$.

To establish whether agonist competition curves with unit slope could be obtained, which at the same time had affinity estimates equal to ileum pK_{app} estimates and therefore to test the hypothesis that flat agonist competition curves, obtained in buffer $B_{(0.07,0.1,0.1)}$, resulted from some formation of ARG and/or AR*G, we performed further competition experiments in which the concentration of just one of the salts, $CaCl_2$, was increased. The results we obtained were consistent with this hypothesis. The pK_I' of *R*-α-MH decreased with

Table 5 Apparent affinity (pK_I') and n_H values expressed by *R*-α-MH and thioperamide at H_3-receptors in guinea-pig cortex in buffer containing increasing concentrations of $CaCl_2$

Buffer	*R*-α-*MH*		*Thioperamide*	
	pK_I'	n_H	pK_I	n_H
$B_{(0,0,0)}$	9.03±0.08	0.54±0.04*	8.28±0.08	1.04±0.28
$B_{(0.03,0,0)}$	8.59±0.21	0.52±0.04*	8.55±0.09	0.93±0.14
$B_{(0.07,0,0)}$	8.45±0.07	0.54±0.02*	8.46±0.08	0.90±0.04
$B_{(0.1,0,0)}$	8.09±0.13	0.60±0.06*	8.38±0.16	0.87±0.05
$B_{(0.2,0,0)}$	7.68±0.07	0.68±0.03*	8.44±0.08	1.14±0.13
$B_{(0.3,0,0)}$	7.24±0.06	0.88±0.08	8.39±0.06	0.95±0.02

Data are the mean±s.e.m. of three experiments. The concentrations of $CaCl_2$ used in the buffers were the same as those in the experiments described in Table 3.
*n_H significantly different from unity, $P<0.05$ *t*-test.

increasing $CaCl_2$ concentration, whereas the pK_I of thioperamide remained unchanged (Table 5), indicating that $CaCl_2$ alone had the same effect as the combination of NaCl, KCl and $CaCl_2$ in buffer $B_{(0.07,0.1,0.1)}$. In addition, the decrease in *R*-α-MH pK_I' was associated with an increase in n_H such that at the highest $CaCl_2$ concentration (300 mM) the n_H parameter estimate was not different from unity and, moreover, at this $CaCl_2$ concentration, the pK_I of *R*-α-MH (7.24±0.06, $n=3$) was not different to the pK_{IL} estimated from a two-site analysis of data obtained in buffer $B_{(0.07,0.1,0.1)}$ (7.24±0.15, $n=4$). An alternative approach to elucidate whether the nonunit agonist competition curves in buffer $B_{(0.07,0.1,0.1)}$ had resulted from the agonists binding to, or inducing the formation of ternary complex (ARG), would have been to add guanine nucleotides to the assay buffer. In addition, when these studies were performed, it had been suggested that the H_3-receptor was coupled to either Gi (Clark *et al*., 1993; Litosch *et al*., 1993; Clark and Hill, 1996) or Gs-proteins (Cherifi *et al*., 1992). Therefore, it would also have been interesting to establish if treatment of tissues with pertussis or cholera toxin resulted in unit n_H parameter estimates for agonists in buffer $B_{(0.07,0.1,0.1)}$.

The observation that $CaCl_2$ decreased the pK_L of [^{3}H]clobenpropit (Figure 6) was consistent with the effect that buffer containing 70 mM $CaCl_2$, 100 mM KCl and 100 mM NaCl (buffer $B_{(0.07,0.1,0.1)}$) had on this parameter. That this effect appeared saturable was consistent with the possibility that the increased buffer $CaCl_2$ concentration or increased buffer ionic strength prevented clobenpropit from binding to or inducing high-affinity receptor states. These states cannot be equivalent to R*G or RG because the n_H parameter estimates for clobenpropit at all $CaCl_2$ concentrations were not significantly different from unity. However, it is possible that the higher affinity of [^{3}H]clobenpropit, in buffer $B_{(0,0,0)}$, results from it inducing or binding to R*.

The finding of a relationship between α measured in the ileum bioassay and the Δp*K* (Tables 2 and 4 and Figure 11) suggests that H_3-receptor radioligand binding assays can be used to detect residual agonist efficacy. Thus, ligands with Δp*K* values of less than 1.00 were partial agonists ($\alpha=0.35$–0.90) in the guinea-pig ileum assay and those ligands with Δp*K* values of >1.1 were full agonists ($\alpha\sim1.00$). The finding that GR175737, an antagonist ligand as defined by the guinea-pig ileum bioassay, had a Δp*K* value of 0.48 can be accounted for by considering that the H_3-receptor radioligand binding assay detects intrinsic efficacy that has remained undetected in the bioassay. Thus, if a full agonist ($\alpha\sim1.0$, *R*-α-MH) has a Δp*K* of 2.51 and expresses a pK_I' value approximately 2.5 log units higher than its pK_{app}, a partial agonist ($\alpha\sim0.45$, chloroproxyfan) has a Δp*K* of 0.92 and expresses a pK_I' value approximately 1 log unit higher than its pK_{app}, then it is possible that a weaker partial agonist, which acts as a competitive antagonist in the functional assay, could express a pK_I' value that is still significantly higher than its pK_B. Interestingly, since these studies were conducted, GR175737 has been shown to be a partial agonist ($\alpha\sim0.4$) (Wulff *et al*., 2002) in a cAMP assay which also found that proxyfan, a partial agonist in the guinea-pig ileum assay ($\alpha=0.35$, $pEC_{50}=7.29$), was a full agonist with over 1 log unit higher potency ($pEC_{50}\sim8.4$).

Conclusion

We have manipulated the conditions of the H_3-receptor radioligand-binding assay to provide a method of obtaining a measure of both the pK_{app} and intrinsic efficacy of novel H_3-receptor ligands. The assay predicts that some ligands previously classified as H_3-receptor antagonists may possess residual agonist efficacy, so that under certain conditions radioligand-binding assays may be a more sensitive detector of agonist intrinsic efficacy than functional *in vitro* assays. The prospect that radioligand-binding assays can be used to detect intrinsic efficacy may be useful for the study of human receptors in native tissue, where it may not be possible to develop a functional assay. Studies of this type may be useful for excluding the possibility that, in human tissues, the receptor dimerizes with other receptor types or interacts with tissue-dependent factors (e.g. RAMP and scaffolding proteins) that modify the receptor pharmacology, such that ligands defined in recombinant systems as antagonists are found to express intrinsic efficacy.

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Conflict of interest

The authors state no conflict of interest.

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