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RESEARCH PAPER

Thermodynamic analysis of ligands at cholecystokinin CCK_2 receptors in rat cerebral cortex

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Background and purpose: Several studies using radioligand binding assays, have shown that measurement of thermodynamic parameters can allow discrimination of agonists and antagonists (Weiland *et al*., 1979; Borea *et al*., 1996a). Here we investigate whether agonists and antagonists can be thermodynamically discriminated at CCK_2 receptors in rat cerebral cortex.
Experimental approach: The pK_L of [^{3}H]-JB93182 in rat cerebral cortex membranes was determined at 4, 12, 21 and 37°C in 50 mM Tris-HCl buffer (buffer B pH 6.96; containing 0.089 mM bacitracin). pK_I values of ligands of diverse chemical structure and with differing intrinsic activity (α), as defined by the lumen-perfused rat and mouse stomach bioassays, were determined in buffer B at 4, 12, 21 and 37°C.
Key results: [^{3}H]-JB93182 labelled a homogeneous population of receptors in rat cerebral cortex at 4, 12, 21 and 37°C and the pK_L and B_{max} were not altered by incubation temperature. [^{3}H]-JB93182 binding reached equilibrium after 10, 50, 90 and 220 min at 37, 21, 12 and 4°C, respectively. pK_I values for *R*-L-365,260, *R*-L-740,093, YM220, PD134,308 and JB95008 were higher at 4°C than at 37°C. There was no effect of temperature on pK_I values for pentagastrin, CCK-8S, *S*-L-365,260, YM022, PD140,376 and JB93242.
Conclusions and implications: CCK_2 receptor agonists and antagonists at rat CCK_2 receptors cannot be discriminated by thermodynamic analysis using [^{3}H]-JB93182 as the radioligand.
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Abbreviations: CCK-8S, cholecystokinin sulphated octapeptide; pentagastrin, boc-pentagastrin

Introduction

The CCK_2 receptor is a G-protein-coupled receptor (GPCR) and one of two receptors that bind cholecystokinin (CCK_2 and CCK_1) and which were previously designated CCK_B/gastrin and CCK_A, respectively (see Noble *et al*., 1999). The CCK_2 receptor is widely expressed in the gastrointestinal tract and the central nervous system (CNS). In the gastrointestinal tract this receptor regulates acid secretion and cellular proliferation, while in the CNS, it is associated with the control of pain and anxiety (see McDonald, 2001). The CCK_2 receptor has been cloned from a number of species (Kopin *et al*., 1992; Nakata *et al*., 1992; Pisegna *et al*., 1992; Wank *et al*., 1992; Lee *et al*., 1993; Denyer *et al*., 1994; Kopin *et al*., 1997; Lay *et al*., 2000) and agonist stimulation of this receptor has been shown to lead to activation of both *Pertussis* toxin-sensitive and -insensitive G-proteins with resulting activation of phospholipase C (PLC), phospholipase A_2 and adenylate cyclase (Detjen *et al*., 1997; Pommier *et al*., 1999; Mazzocchi *et al*., 2004).

Four splice variants of the CCK_2 receptor have also been identified; CCK_{2L} and CCK_{2S} which arise from alternate splicing at a site in exon 4 (Song *et al*., 1993); an N-terminally truncated receptor generated by alternative usage of exon Ib (Miyake, 1995; Monstein *et al*., 1998) and the CCK_{2i4sv} which results from complete retention of intron 4 (Hellmich *et al*., 2000; Schmitz *et al*., 2001). To date, radioligand-binding studies suggest that synthetic ligands express only small differences in affinity ($pK_I < 0.4$ log units) for these receptor splice variants (Harper *et al*., 2000; Hellmich *et al*., 2000; Morton *et al*., 2003). However, interspecies polymorphisms have been shown to affect the affinity and intrinsic efficacy of a number of ligands, including *R*-L-365,260, L-364,718, *S*-L-740,093 and PD135,158 (Beinborn *et al*., 1993; Kopin *et al*., 1997).

A number of years ago and despite evidence indicating that interspecies polymorphisms could affect the affinity of ligands for the CCK_2 receptor (Beinborn *et al*., 1993), we

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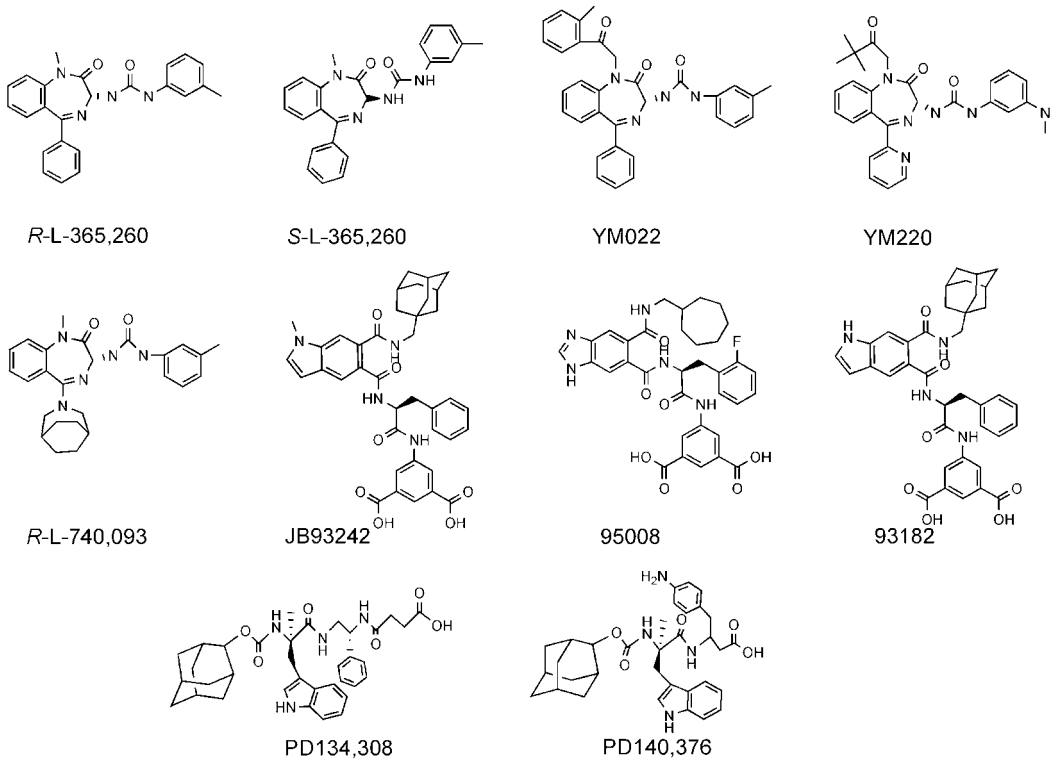

Figure 1 Structure of CCK_2 receptor ligands.

provided evidence, from both radioligand-binding and functional bioassays, to suggest the existence of two endogenous CCK_2 receptors in and between native tissues of a variety of species (gastrin-G_1 and -G_2) (Harper *et al*., 1996a; Roberts *et al*., 1996a; Shankley *et al*., 1997; Harper *et al*., 1999b). The bioassay studies suggested that the rat stomach possessed a homogenous population of gastrin-G_1 receptors while the mouse stomach possessed both gastrin-G_1 and G_2 receptors. In addition, these studies suggested that some ligands (for example PD134,308), previously characterized as CCK_2 receptor antagonists, possessed intrinsic efficacy at gastrin-G_1 receptors which could only be detected in the mouse stomach assay, where the receptor–effector coupling was high and manifested by a higher pEC_{50} for pentagastrin (see Roberts *et al*., 1996a). Radioligand-binding and bioassay studies suggested that JB93182 was a high-affinity and selective gastrin-G_1 receptor antagonist (G_1 $pK_B = 9.8 \pm 0.18$; G_1 $pK_I = 9.94 \pm 0.16$; G_2 $pK_I = 8.57 \pm 0.15$, Roberts *et al*., 1996b; Harper *et al*., 1999a) and that [^{3}H]JB93182 could be used to selectively label gastrin-G_1 receptors in rat cerebral cortex (Harper *et al*., 1999b).

For some receptors such as the β-adrenoceptor, 5-HT_3 receptor, adenosine A_1, A_{2A} and A_3 receptors, radioligand-binding studies performed at a number of temperatures have shown that agonists and antagonists can be discriminated by the thermodynamic parameters (entropy, ΔS° and enthalpy, ΔH°; see Hitzeman, 1988; Raffa and Porreca, 1989) that underlie their binding (see Weiland *et al*., 1979; Borea *et al*., 1996a, b; Lorenzen *et al*., 2000; Merighi *et al*., 2002). However, there are also studies that have found no thermodynamic discrimination of agonists and antagonists (Kilpatrick *et al*., 1986; Dalpiaz *et al*., 1996; Li *et al*., 1998).

In light of the potential for thermodynamic studies to allow agonists and antagonists to be distinguished, we wished to establish whether this technique could be used as an alternative to time-consuming tissue bioassays, such as the lumen-perfused mouse stomach assay, to establish whether novel ligands express intrinsic efficacy at the CCK_2 receptor. Here, we have used [^{3}H]JB93182 to investigate the thermodynamics of binding at CCK_2 receptors (gastrin-G_1) in rat cerebral cortex using selected ligands with varying intrinsic activities (for example PD134,308, pentagastrin and compound 4 (JB93242, see Kalindjian *et al*., 2001)) at gastrin-G_1 receptors as measured with the lumen-perfused mouse or rat stomach bioassays (see Shankley *et al*., 1997). In addition, these ligands were chemically diverse and included peptides (cholecystokinin sulphated octapeptide (CCK-8S) and pentagastrin), peptoids (PD134,308 and PD140,376), benzodiazepines (*R*-L-365,260, *S*-L-365,260, L-740,093, YM022 and YM220), indole-based (JB93242 and JB93182) and benzimidazole-based (JB95008) ligands (Figure 1).

Methods

Preparation of rat cerebral cortex membranes

Male rats (Wistar 250–500 g) were fed on standard laboratory chow and stunned and killed by cervical dislocation. The whole brain was removed and placed in ice-cold HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid])-NaOH (buffer A) (7.2 at $21 \pm 3^\circ$C) of the following composition (mM) 130 NaCl, 4.7 KCl, 5 $MgCl_2$, 10 HEPES, 1 EGTA (ethyleneglycol-bis(β-aminoethylether)*N*,*N*,*N*′,*N*′-tetraacetic acid),

0.089 bacitracin. The cortex was immediately dissected, weighed and added to 10 volumes of ice-cold buffer B of the following composition (mM): 250 sucrose, 5 ethylenediamine tetraacetic acid, 0.1 phenylmethylsulphonyl fluoride, 25 imidazole; pH 7.4 at 21±3°C. The tissue was homogenized using a Polytron PT-10 (setting 10; 1 min) and centrifuged (800 *g*, 12 min at 4°C). The supernatants were pooled and stored at 4°C, while the pellets were re-homogenized in 10 volumes of the original tissue weight of buffer B and re-centrifuged (800 *g*, 12 min at 4°C). This procedure was repeated, all supernatants were pooled, filtered through six layers of gauze, diluted to give a final concentration of 50 mM Tris–HCl using 500 mM Tris–HCl and centrifuged (39 800 *g*, 20 min at 4°C). The final pellet was re-suspended in 50 mM Tris–HCl buffer (buffer C; adjusted to pH 6.96 at 4, 12, 21 and 37°C containing 0.089 mM bacitracin).

Tissue concentration studies
Increasing concentrations of rat cerebral cortex membranes (0.2–40 mg original wet weight) were incubated in triplicate with [^{3}H]JB93182 (0.3 nM) in a final assay volume of 0.5 ml. Total and non-specific bindings were defined using buffer C and YM022 (1 μM; pK_I rat CCK_2 receptor = 10.19; Harper *et al*., 1999a), respectively. Assays were terminated after 2.5 h (37, 21°C) or 6.5 h (12, 4°C) by rapid filtration (Brandell Cell Harvester) through pre-soaked filters (Whatman GF/B), which were washed with ice-cold 50 mM Tris–HCl (pH 7.4 at 4°C). Filters were transferred into scintillation vials and 5 ml Ready-Solv HP liquid scintillation cocktail was added. Bound radioactivity was determined after 4 h by counting (5 min) in a Beckman liquid scintillation counter. This procedure for terminating the assay was also used for all assays.

Incubation conditions – kinetic studies
Kinetic studies were performed to establish that saturation studies were incubated for a sufficient time for equilibrium binding of [^{3}H]JB93182 to be achieved and to establish the optimal incubation time for equilibrium of radioligand and competitor in competition studies. To ascertain the time required for equilibrium binding of [^{3}H]JB93182 (0.3 nM) at 4, 12, 21 and 37°C, the radioligand was incubated in triplicate with rat cortex membranes (8 mg original wet weight) and buffer C or YM022 (1 μM) for increasing time intervals in a final assay volume of 0.5 ml. The incubations were terminated by rapid filtration through Whatman GF/B filter circles. To determine the time course of the dissociation, YM022 (10 μl; 50 μM) was added after the binding of [^{3}H]JB93182 (1 nM) had reached equilibrium (220 min at 4°C, 160 min at 12°C, 100 min at 21°C and 80 min at 37°C). Bound [^{3}H]JB93182 was determined at increasing times by rapid filtration through Whatman GF/B filter circles.

Incubation conditions – saturation studies
Rat cortex membranes (8 mg original wet weight) were incubated for 2.5 h. (37, 21°C) or 6.5 h (12, 4°C) in a final assay volume of 0.5 ml with 0.02–6 nM [^{3}H]JB93182. Total and non-specific bindings were defined using buffer C and YM022 (1 μM), respectively.

Incubation conditions – competition studies
Rat cortex membranes (8 mg original wet weight) were incubated in a final assay volume of 0.5 ml for 24 h (4 and 12°C) or 2.5 h (21 and 37°C) with competing ligand, diluted in buffer C, and 0.3 nM [^{3}H]JB93182. Total and non-specific bindings were defined using buffer C and 1 μM YM022, respectively.

Immature rat and mouse isolated, lumen-perfused stomach assays
Gastric acid secretion was measured in isolated, lumen-perfused stomachs from male mice (Charles River CDI, 22–26 g, 18 h fasted, water *ad libitum*) and pre-weaned rat pups (Wistar, 28–52 g). The tissues were prepared essentially as described previously (Black and Shankley, 1985a; Roberts *et al*., 1996a). The abdomen was opened and the stomach cannulated via the duodenal sphincter. The oesophagus was ligated at the level of the cardiac sphincter and the stomach excised from the abdomen. A small incision was made in the fundic region, a cannula ligated tightly into the incision and the stomach flushed through with mucosal solution (mM: 118 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 31.6 glucose) to remove any remaining food. The stomach was placed in an organ bath containing 40 ml of buffered serosal solution (mM: 118 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.14 KH_2PO_4, 15.9 $NaHPO_4$, 0.65 $CaCl_2$, 31.6 glucose). The serosal solution was maintained at 37±1°C and gassed with 95% O_2 and 5% CO_2. Then, 30 μM 3-isobutyl-methylxanthine was added to the serosal solution in the mouse stomach assay, and in the rat stomach assay the serosal solution contained 30 nM 5-methylfurmethide. The stomachs were perfused with mucosal solution gassed with 100% O_2 at a rate of 1 ml min^{-1} and the perfusate passed over an internally referenced pH electrode which was placed 12 cm above the stomach. A continuous record of pH was obtained from chart recorders coupled to the pH electrode. The preparations were allowed to stabilize for 90 min before addition of ligands, which were dosed at log unit intervals. Agonist responses were expressed as the change in pH (ΔpH) of the lumen-perfusate from the basal pH immediately before addition of ligand. For ligand affinity estimation, the ligands were equilibrated with the tissue for 60 min before the decrease in pH produced by increasing concentrations of pentagastrin (PG) was determined.

Data analysis
Data are presented as the mean±s.e.m. unless indicated otherwise.

Radioligand binding – saturation data
The Hill equation was fitted to saturation data (Equation 1) using Graph-Pad 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.

Radioligand binding – kinetic data
First-order rate equations were fitted to association and dissociation data to obtain estimates of the observed association rate (k_{obs}) and dissociation rate (k_{-1}), respectively. The K_L of [^{3}H]JB93182 was determined from k_{obs} and k_{-1}, using Equations (2) and (3)

$$k_{+1} = \frac{k_{obs} - k_{-1}}{[L]} \quad (2)$$

$$K_L = \frac{k_{-1}}{k_{+1}} \quad (3)$$

Radioligand binding – competition data
In order 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.

Dissociation constants (pK_I) were subsequently determined from pIC_{50} values using the Cheng and Prusoff equation (1973) to correct for the occupancy of [^{3}H]JB93182. A global pK_L value for [^{3}H]JB93182 ($pK_L = 9.51$) was used to correct pIC_{50} values obtained at 4, 12, 21 and 37°C because there was no significant effect of temperature on the pK_L of [^{3}H]JB93182 (analysis of variance (ANOVA)).

Calculation of thermodynamic parameters
The change in the standard Gibbs free energy ($\Delta G^{\circ\prime}$) was calculated using the Gibbs–Helmholz thermodynamic equation (Equation 4).

$$\Delta G^\circ = -RT \ln K_A \quad (4)$$

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

Equation 4 is combined with the Gibbs free energy (Equation (5)) to form the integrated van't Hoff (Equation (6)).

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (5)$$

where, ΔS° is the entropy change of binding and ΔH° the enthalpy change.

$$\ln K_A = -\Delta H^\circ/RT + \Delta S^\circ/R \quad (6)$$

A plot, therefore, of $\ln K_A$ versus $1/T$ allows 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 ($\Delta G^{\circ\prime}$, $\Delta H^{\circ\prime}$ 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).

Functional data – agonist concentration effect curves
In order to obtain estimates of pEC_{50}, and maximal response (α), the Hill equation was fitted to agonist dose–response data, expressed as ΔpH. To permit comparison of the α-values for different ligands, the maximal response of each ligand, in each stomach, was expressed as a fraction of the mean maximum response produced by a full agonist. CCK-8S was assigned an α-value of 1.0 in the rat stomach and pentagastrin an α-value of 1.0 in the mouse stomach.

Functional data – pA_2 estimation
When the minimum criteria for competitive antagonism were satisfied, that is, the antagonist produced parallel, rightward shift in the pentagastrin 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 (7).

$$\log EC_{50} = \log EC_{50} + \log\left(1 + \frac{[B]^b}{10^{\log K_B}}\right) \quad (7)$$

Statistical analysis
Differences in [^{3}H]JB93182 pK_L and B_{max} values were determined by ANOVA. An F-test was used to establish whether saturation data were 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 and ANOVA. 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 of the van't Hoff plot was significantly different from zero. The effect of antagonist treatment on pEC_{50} and α-values was assessed by ANOVA and the Bonferroni modified t-test for multiple comparisons. Comparison of ligand affinity values obtained in the rat cortex and rat stomach assays was made by fitting straight line models to the data by least squares (see Meester *et al*., 1998). With the exception of linear regression, where P-values of 0.1 were considered significant, P-values of less than 0.05 were considered significant.

Materials
[^{3}H]JB93182 (5-[[[(1*S*)-[[(3,5-dicarboxyphenyl)amino]carbonyl]-2-[4-^{3}H-phenyl]ethyl]amino]-carbonyl]-6-[[(1-adamantylmethyl)amino]carbonyl]-indole) with specific activity ~1036 GBq mmol^{-1} (28 Ci mmol^{-1}) was supplied as a custom synthesis by Amersham International, UK (Kalindjian *et al*., 1996). *R*-L-365,260, *S*-L-365,260, *R*-L-740,093, YM022, YM220, PD134,308, PD140,376, JB93242 (compound 4, Kalindjian *et al*., 2001) and JB95008 were synthesized by James Black Foundation chemists. Cholecystokinin-8S (CCK-8S) and boc-pentagastrin (PG) were obtained from Cambridge Research Biochemicals Inc. Trizma base, HEPES,

EGTA, 3-isobutyl-methylxanthine, 5-methyl furmethide and bacitracin were obtained from the Sigma Chemical Co., Poole, Dorset, UK. All other materials were obtained from Fisher, Loughborough, Leics, UK.

Results

Effect of incubation temperature on [^{3}H]JB93182 specific binding
The specific binding of 0.3 nM [^{3}H]JB93182 increased with increasing rat cerebral cortex membranes up to a concentration of 12 mg tube^{-1} (original wet weight) at 4, 12, 21 and 37°C (277, 285, 294 and 310 K). At a tissue concentration of 8 mg per tube, there was no significant effect of temperature on the added 0.3 nM [^{3}H]JB93182 bound (4°C = 12.98 ± 2.09, 12°C = 11.95 ± 0.59, 21°C = 10.98 ± 1.10 and 37°C = 11.30 ± 0.54%; $n = 4$, ANOVA). Incubation temperature had no significant effect on the specific binding expressed as a percentage of total binding (37°C = 43.4 ± 6.6%; 21°C = 52.6 ± 2.1%; 12°C = 56.7 ± 1.80%; 4°C = 56.8 ± 7.0%, $n = 4$, ANOVA). In total, 8 mg of membranes was added to all tubes for subsequent characterization of [^{3}H]JB93182 binding at each temperature.

Effect of incubation temperature on the kinetics of binding of [^{3}H]JB93182
At all temperatures, the specific binding of 0.3 nM [^{3}H]JB93182 to CCK_2 receptors in rat cerebral cortex increased with increasing incubation time and the data at all temperatures could be fitted by a first-order rate equation. Equilibrium was reached after ~10 min at 37°C ($t_{1/2} = 1.7 \pm 0.2$ min, $n = 4$, Figure 2a) and ~50 min at 21°C ($t_{1/2} = 11.9 \pm 1.1$ min, $n = 7$, Figure 2b) and remained constant for at least a further 240 min. At 12 and 4°C, the specific 0.3 nM [^{3}H]JB93182 binding reached equilibrium by ~90 min (12°C $t_{1/2} = 23.8 \pm 3.0$ min, $n = 6$, Figure 2c) and ~220 min (4°C $t_{1/2} = 62.6 \pm \pm 7.4$ min, $n = 4$, Figure 2d), respectively, and remained constant for at least 24 h.

[^{3}H]JB93182-specific binding was fully dissociated after ~20 and ~100 min at 37 and 21°C, respectively. The $t_{1/2}$ of dissociation of the radioligand was determined by fitting a first-order rate equation to the data (37°C $5 \times t_{1/2} = 15.3 \pm 2.1$ min, $n = 4$; 21°C $5 \times t_{1/2} = 115.0 \pm 19.5$ min, $n = 4$). During the time course of the assays at 12 and 4°C, the specific binding of [^{3}H]JB93182 was not completely dissociated (12°C = 84 ± 6% at 220 min; 4°C = 64.7 ± 5.2% at 260 min). A first-order rate equation was fitted to the 12°C data ($5 \times t_{1/2} = 378.5 \pm 15.8$ min, $n = 3$) but because only ~65% of the radioligand was dissociated after 260 min at 4°C, it was not possible to accurately estimate the $t_{1/2}$ at this temperature (Figure 2d) by fitting a first-order rate equation.

The affinities (pK_L) of [^{3}H]JB93182, determined by kinetic analysis, were 10.34 ± 0.06 ($n = 4$), 9.54 ± 0.06 ($n = 4$) and 9.83 ± 0.06 ($n = 3$), at 37, 21 and 12°C, respectively. A pK_L was not calculated at 4°C because a k_{-1} value could not be determined since only ~65% of the radioligand was dissociated.

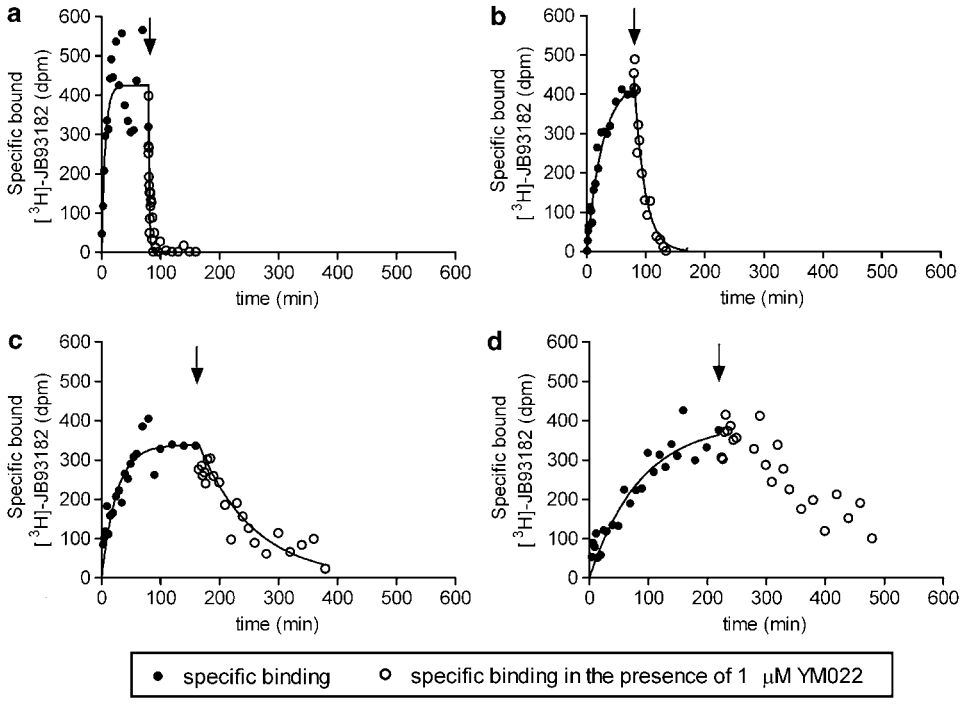

Figure 2 Representative association–dissociation analysis of [^{3}H]JB93182 binding to CCK_2 receptors in rat cerebral cortex membranes at (**a**) 37°C, (**b**) 21°C, (**c**) 12°C and (**d**) 4°C. The association rate was determined under pseudo-first-order conditions because only ~10% of the added [^{3}H]JB93182 was bound. [^{3}H]JB93182 (0.3 nM) was incubated, in a final assay volume of 0.5 ml for increasing times with membranes (8 mg at 4, 12, 21 or 37°C). Total and non-specific bindings were defined with buffer C and 1 μM YM022, respectively. The dissociation rate for [^{3}H]JB93182 from CCK_2 receptors was determined by incubating [^{3}H]JB93182 (0.3 nM) with membranes and buffer B for increasing incubation times (4°C 220 min; 12°C 160 min; 21°C 100 min; 37°C 80 min), and then adding 10 μl of 50 μM YM022 (arrow). The bound radioligand was ascertained at increasing incubation times. The solid lines superimposed through the data represent the fit to first-order rate equations.

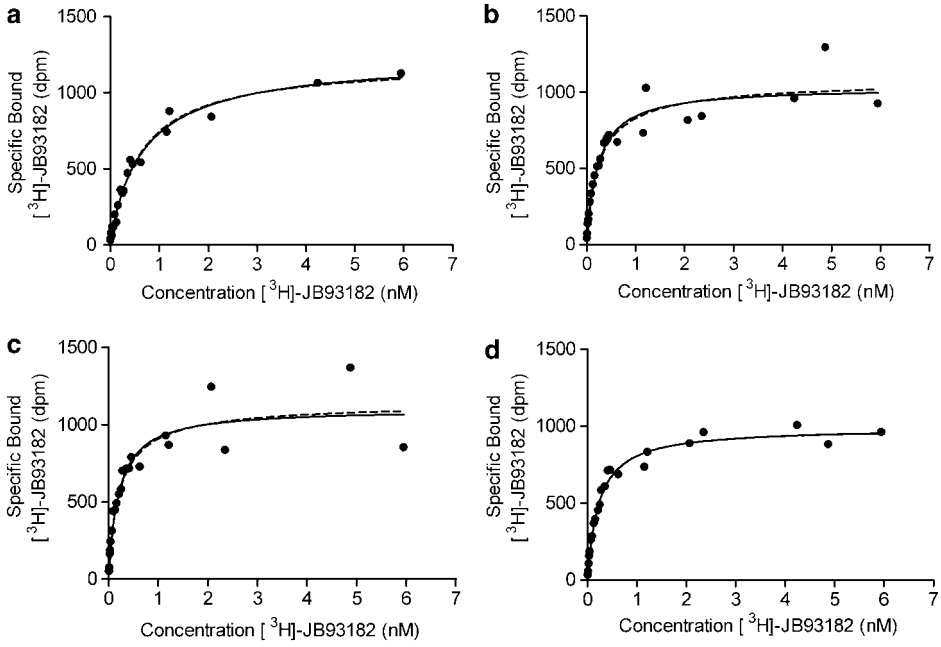

Figure 3 Representative saturation analysis of the binding of [^{3}H]JB93182 to CCK_2 receptors in rat cerebral cortex at (**a**) 37, (**b**) 21, (**c**) 12 and (**d**) 4°C. Rat cerebral cortex membranes (8 mg original wet weight) were incubated with increasing concentrations of [^{3}H]JB93182 (0.02–6 nM) in a final assay volume of 0.5 ml. Total and non-specific bindings were defined using buffer C and YM022 (1 μM), respectively. Assays were terminated after 2.5 h (37, 21°C) or 6.5 h (12, 4°C). The solid line shown superimposed on each graph represents the saturation isotherm obtained by fitting the data to the Hill equation with Hill slope (n_H) constrained to unity and the broken line is that obtained by fitting the data to the Hill equation with unconstrained n_H.

Effect of incubation temperature on [^{3}H]JB93182 saturation isotherms

The specific binding of [^{3}H]JB93182 to CCK_2 receptors of rat cerebral cortex 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, at 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 ($n=5$, F-test). The incubation temperature had no significant effect on the estimated CCK_2 receptor density (B_{max}; Table 1, ANOVA). Temperature had no significant effect on the affinity (pK_L) of [^{3}H]JB93182 (ANOVA, Table 1) and there was no significant difference between the pK_L at 4 and 37°C ($n=5$, paired *t*-test).

At 21 and 12°C, the pK_L values for [^{3}H]JB93182, estimated by saturation analysis, were not significantly different from those estimated using kinetic analysis (*t*-test). The pK_L determined using saturation analysis at 37°C was significantly lower than that determined using kinetic analysis ($P<0.05$, *t*-test).

Effect of temperature on ligand pK_I values

All the CCK_2-receptor ligands produced concentration-dependent inhibition of [^{3}H]JB93182 binding in rat cerebral cortex membranes at 4, 12, 21 and 37°C (for example Figures 4e, f, g and h). The mean percentage coefficient of variation (%cv), calculated from all the triplicate data points forming each competition curve, was less than 3% at all temperatures (4°C = 2.75 ± 0.17%, 12°C = 2.87 ± 0.20%, 21°C = 2.84 ± 0.20%, 37°C = 2.89 ± 0.18%; $n=13$; e.g. Figures 4a, b, c and d). The mean n_H parameter estimates for all ligands, with the exception of *S*-L-365,260 at 4°C, were not significantly different from unity (Table 2, *t*-test).

Table 1 Effect of temperature on the estimated pK_L, n_H and B_{max} of [^{3}H]JB93182 in rat cerebral cortex membranes

Temperature (°C)	B_{max} *(pmol g^{-1})*	pK_L	n_H
37	2.52 ± 0.18	9.31 ± 0.06	1.09 ± 0.09
21	2.49 ± 0.06	9.56 ± 0.08	1.05 ± 0.11
12	2.72 ± 0.30	9.66 ± 0.07	1.07 ± 0.06
4	3.00 ± 0.35	9.52 ± 0.06	1.08 ± 0.05

Data represents the mean ± s.e.m. of five experiments.

The effects of temperature on pK_I values of all ligands are presented in Table 2. There was a significant effect of temperature on ligand pK_I values, as judged by ANOVA, for JB95008, *R*-L-365,260 and YM220 ($P<0.05$). There was no significant effect of temperature on pK_I values of JB93242, PD134,308, PD140,376, *R*-L-740,093, *S*-L-365,260, YM022, pentagastrin and CCK-8S as judged by ANOVA. The pK_I values of *R*-L-365,260, *R*-L-740,093, YM220, PD134,308 and JB95008 were significantly higher at 4°C than at 37°C ($P<0.05$, paired *t*-test). There was no significant difference between pK_I values obtained for *S*-L-365,260, YM022, PD140,376, JB93242, pentagastrin and CCK-8S at 4 and 37°C (paired *t*-test).

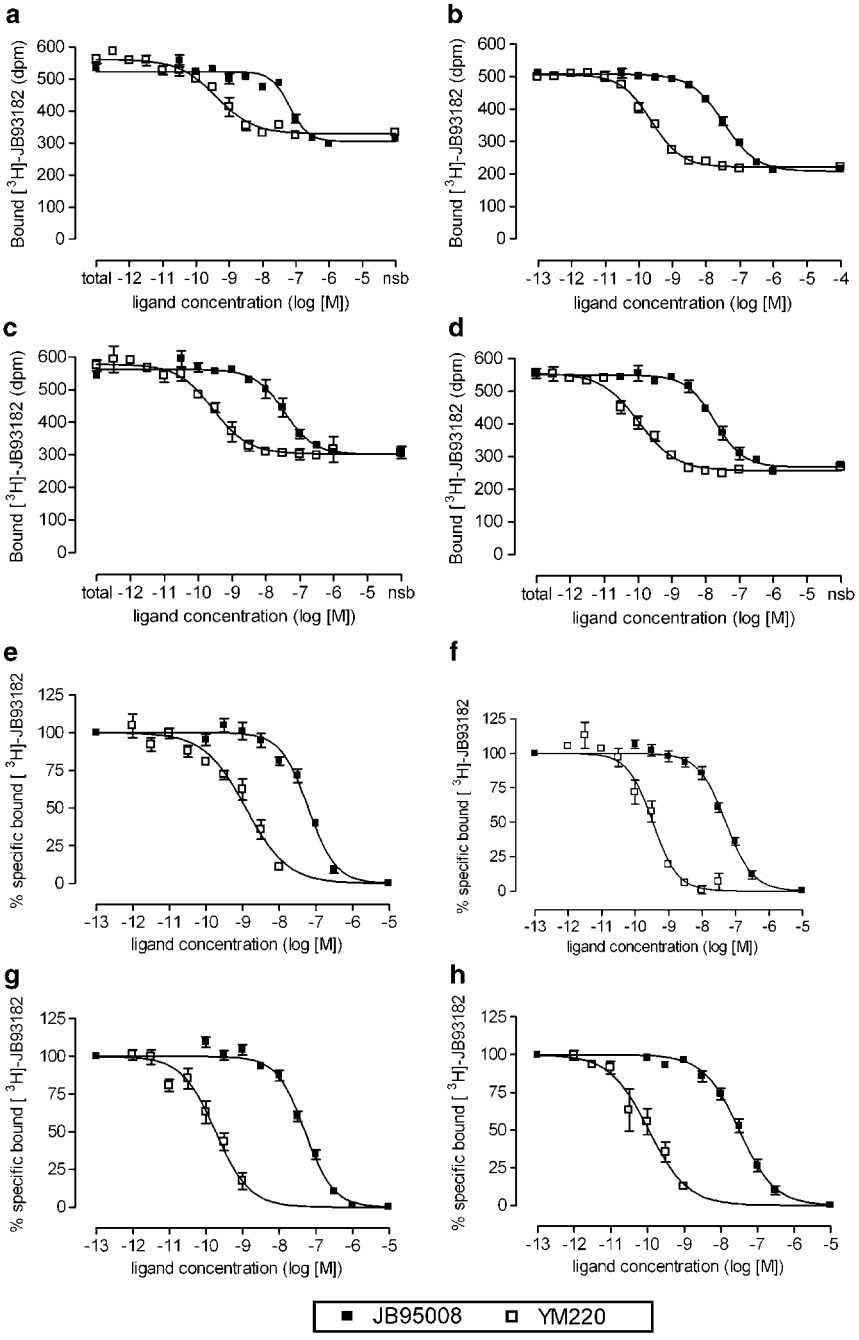

Figure 4 Competition curves for CCK_2 receptor ligands at CCK_2 receptors in rat cerebral cortex. (**a–d**) Competition data obtained for YM220 and JB95008 at (**a**) 37°C (**b**), 21°C (**c**), 12°C and (**d**) 4°C in a single experiment. Data are the mean ± s.e.m. of triplicates. (**e–h**) Competition data for YM220 and JB95008, expressed as % specific bound [^{3}H]JB93182, at (**e**) 37°C, (**f**) 21°C, (**g**) 12°C and (**h**) 4°C. Data are the mean ± s.e.m. of between four and six experiments (see Table 2). Rat cerebral cortex membranes (8 mg) were incubated in a final volume of 0.5 ml with buffer B, [^{3}H]JB93182 (0.3 nM) and increasing concentrations of JB95008 or YM220 for 2.5 h at 30 and 21°C and for 24 h at 12 and 4°C. Total and non-specific bindings of [^{3}H]JB93182 were defined using buffer B and 1 μM YM022, respectively. The lines shown superimposed on the data were obtained by fitting to the Hill equation.

Intrinsic activity and affinity measurement in lumen-perfused stomach assays

In the rat stomach, both pentagastrin and CCK-8S produced a concentration-dependent decrease in pH (pEC_{50}, pentagastrin = 7.33 ± 0. 22, $n_H = 0.98 \pm 0.12$, $n = 5$; CCK-8S = 6.74 ± 0.24, $n_H = 1.09 \pm 0.13$, $n = 5$; Figure 5). There was no significant difference between the maximal response produced by CCK-8S ($\Delta pH = 0.40 \pm 0.05$, $\alpha = 1.00 \pm 0.13$, $n = 5$, Table 4) and that produced by pentagastrin ($\Delta pH = 0.38 \pm 0.09$, $\alpha = 0.95 \pm 0.23$, $n = 5$, Table 4; *t*-test).

Table 2 Temperature-dependence of ligand pK_I values when competing with the binding of [^{3}H]JB93182 at CCK_2 receptors in rat cerebral cortex

Temperature (°C)	*4*		*12*		*21*		*37*		n
	*p*K$_I$	n$_H$	*p*K$_I$	n$_H$	*p*K$_I$	n$_H$	*p*K$_I$	*n$_H$*	
CCK-8S	6.99±0.12	0.86±0.06	6.65±0.18	1.05±0.18	7.28±0.13	1.01±0.09	6.87±0.16	0.98±0.20	5
Pentagastrin	6.77±0.15	0.99±0.10	6.73±0.08	0.85±0.09	6.75±0.09	1.06±0.06	6.70±0.09	1.13±0.10	4
PD134,308[a]	8.27±0.08	1.01±0.07	8.13±0.06	1.06±0.09	8.17±0.07	1.05±0.07	7.98±0.09	1.24±0.17	10
PD140,376	8.53±0.11	0.86±0.10	8.48±0.03	0.94±0.07	8.40±0.09	0.98±0.07	8.36±0.06	0.92±0.06	4
YM022	10.11±0.11	1.07±0.06	10.27±0.10	1.32±0.14	9.98±0.08	1.15±0.05	10.02±0.09	0.91±0.08	6
YM220[a]	10.21±0.21	0.86±0.11	9.96±0.13	0.90±0.08	9.84±0.11	1.09±0.14	9.65±0.14	0.90±0.17	3
R-L-365,260[a]	8.34±0.12	0.93±0.04	8.10±0.06	1.02±0.08	7.89±0.08	1.01±0.06	7.69±0.04	0.96±0.11	9
S-L-365,260	6.58±0.13	1.22±0.03[b]	6.40±0.07	1.31±0.22	6.42±0.04	1.21±0.25	6.38±0.11	1.21±0.20	4
R-L-740,093[a]	9.96±0.17	1.23±0.14	9.92±0.16	1.10±0.10	9.62±0.14	1.16±0.07	9.34±0.17	1.10±0.15	5
JB93242	9.08±0.19	1.20±0.17	9.00±0.19	0.86±0.10	9.08±0.16	1.22±0.30	8.96±0.17	0.93±0.09	5
JB95008[a]	7.69±0.07	0.96±0.03	7.58±0.04	0.97±0.02	7.61±0.04	0.99±0.04	7.42±0.04	1.21±0.10	6

Abbreviation: CCK-8S, cholecystokinin sulphated octapeptide.
pK_I values were calculated from pIC$_{50}$ values using the Cheng and Prusoff equation (1973) and a global pK_L for [^{3}H]JB93182 of 9.51. Data are the mean±s.e.m.
[a]pK_I at 4°C significantly higher than 37°C.
[b]n_H significantly greater than unity ($P<0.05$, *t*-test).

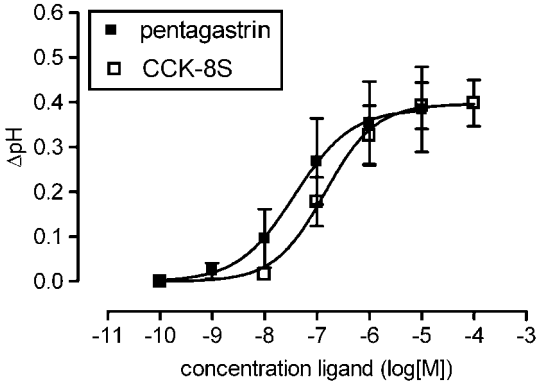

Figure 5 Effect of pentagastrin and cholecystokinin sulphated octapeptide (CCK-8S) on acid secretion in the lumen-perfused immature rat stomach assay. Each point represents the mean± s.e.m. of determinations in five stomachs.

Table 3 Comparsion of pA_2 values for ligands in the immature rat stomach with pK_I values obtained at 37°C in the rat cerebral cortex using [^{3}H]JB93182 as the radioligand

Ligand	*pA$_2$*	*p*K$_I$	n
PD134,308	7.85±0.20 (37)[a]	7.98±0.09	10
PD140,376	7.89±0.28 (5)	8.36±0.06	4
YM220	9.88±0.31 (5)	9.65±0.14	4
R-L-365,260	7.54±0.03 (153)[b]	7.69±0.04	9
S-L-365,260	6.42±0.34 (12)	6.38±0.11	4
JB93242	8.99±0.18 (5)[c]	8.96±0.17	5
JB95008	7.57±0.14 (6)	7.42±0.04	6
JB93182	9.42±0.25 (14)	9.31±0.06[d]	5

Data are the mean±s.e.m. Numbers in parentheses after pA_2 and pK_B values represent the number of immature rat stomachs used for parameter determination.
[a]pK_B determined by Schild analysis.
[b]pK_B determined by Schild analysis; see Roberts *et al.* (1996a).
[c]See Kalindjian *et al.* (2001).
[d]pK_L of [^{3}H]JB93182.

JB93242, JB95008, JB93182, PD134,308, PD140,376, *R*-L-365,260, *S*-L-365,260, *R*-L-740,093, YM022 and YM220 had no effect on basal acid secretion.

JB93242, JB95008, JB93182, PD134,308, PD140,376, *R*-L-365,260, *S*-l365,260 and YM220 produced parallel rightward shifts in the pentagastrin dose–response (E/[A]) curves without change in maximal response (α) or slope (n_H). The affinity values (pA_2 or pK_B) obtained for ligands are presented in Table 3. pA_2 values could not be calculated for *R*-L-740 093 (10 nM) and YM022 (10 nM) because at the concentrations tested, they produced a significant decrease in the maximal response to pentagastrin.

When pA_2 values were compared to pK_I values determined in the rat cerebral cortex radioligand binding assay at 37°C, the data were found not to deviate from the line of identity ($y=x$, $F_{(1,80)}=0.07$, $P>0.01$), that is, affinity values in the rat stomach were not different from those in the rat cortex (Figure 6).

In the mouse stomach assay, JB95008, JB93182, *R*-L-365,260, *S*-L-365,260, *R*-L-740,093, YM022 and YM220 had no effect on basal acid secretion. Pentagastrin, CCK-8S, JB93242, PD134,308 and PD140,376 all produced a concentration-dependent decrease in pH (pentagastrin pEC$_{50}$=8.53±0.10, n_H=0.48±0.08, n=4; CCK-8S pEC$_{50}$=8.69±0.32, n_H=0.60±0.14, n=5; JB93242 pEC$_{50}$=8.74±0.12, n=13; PD134,308 pEC$_{50}$=8.71±0.25, n=5; PD140,376 pEC$_{50}$=8.84±0.12, n=5; Figure 7). The slopes of pentagastrin and CCK-8S E/[A] curves were significantly less than those determined in the rat stomach assay (*t*-test, $P<0.05$). The maximal response produced by pentagastrin (ΔpH=0.49±0.04, α=1.00±0.09, n=4, Table 4) was not significantly different from that of CCK-8S (see Table 4, ANOVA and Bonferroni *post hoc* test). The maximal responses of JB93242, PD140,376 and PD134,308 (JB93242 ΔpH=0.23±0.02, n=13; PD140, 376 ΔpH=0.17±0.05; PD134,308 ΔpH=0.26±0.04, n=5) were significantly lower than for CCK-8S or pentagastrin ($P<0.05$, ANOVA and Bonferroni *post hoc* test).

R-L-365,260 (1 μM) produced a parallel rightward shift of the PD134,308 and PD140,376 dose–response (E/[A]) curves (data not shown) and also of the JB93242 E/[A] curves consistent with their action being mediated through gastrin-G_1 type CCK_2 receptors (see Kalindjian *et al*., 2001).

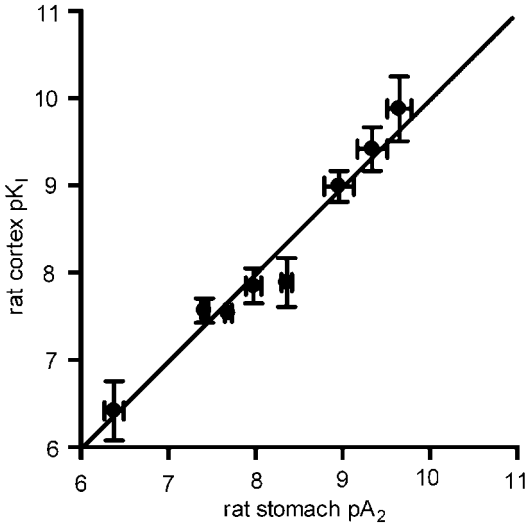

Figure 6 Comparison of ligand pK_I values determined at 37°C in the rat cerebral cortex, using [^{3}H]JB93182 as the radioligand, with pA_2 values estimated in the lumen-perfused rat stomach assay. Comparison of the data was made by fitting a nested set of straight line models to the data (see Meester *et al*., 1998). The data did not deviate significantly from $y=x$. F-test for linearity ($y=m.x+c$), $F(6,80)=0.81$, $P>0.01$; F-test for unit slope ($y=x+c$), $F(1,80)=0.44$, $P>0.01$; F-test for zero intercept ($y=x$), $F(1,80)=0.07$, $P>0.01$. The line shown superimposed on the data represents the best fit to a straight line model with unit slope and intercept where $y=x$.

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 ($1/K_I = K_A$) obtained at 4, 12, 21 and 37°C (see for example, Figures 8a and b). The van't Hoff plots, for JB95008, *R*-L740,093, *R*-L-365,260, YM220 and PD134,308 had positive slopes that were significantly different from zero (F-test, $P<0.10$). The van't Hoff plots constructed for [^{3}H]JB93182, *S*-L-365,260, JB93242, CCK-8S, PD140,376, pentagastrin and YM022 had slopes that were not significantly different from zero (F-test, $P>0.10$).

The thermodynamic parameters derived from van't Hoff analysis are shown in Table 4. The change in free energy ($\Delta G^{\circ\prime} = \Delta H^{\circ\prime} - T\Delta S^{\circ\prime}$ at 294 K) associated with the binding of the 12 ligands ranged from -35.1 ± 0.4 kJ mol^{-1}, for *S*-L-365,260 ($n=4$), to -56.6 ± 0.4 kJ mol^{-1} for YM022 ($n=6$). The $\Delta H^{\circ\prime}$ values for all ligands except CCK-8S ($\Delta H^{\circ\prime} = 9.2\pm$

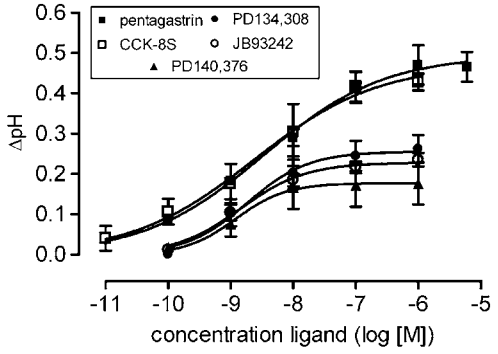

Figure 7 Effect of pentagastrin, cholecystokinin sulphated octapeptide (CCK-8S), JB93242, PD134,308 and PD140,376 on acid secretion in the lumen-perfused mouse stomach assay. Each point represents the mean$\pm$s.e.m. of determinations between four and 13 stomachs.

Table 4 Thermodynamic binding parameters for structurally diverse ligands at CCK_2 receptors in rat cerebral cortex membranes

Ligand	n	$\Delta G^{\circ\prime}$ (kJ mol^{-1})	$\Delta H^{\circ\prime}$ (kJ mol^{-1})	$\Delta S^{\circ\prime}$ (J mol^{-1} K^{-1})	*Rat stomach* α	*Mouse stomach* α
Peptide						
CCK-8S	6	-37.3 ± 2.4	9.2 ± 4.5	158.4 ± 21.3	1.00 ± 0.13 (5)	0.93 ± 0.06(5)
Pentagastrin	4	-37.9 ± 0.4	-1.3 ± 6.1	124.4 ± 20.5	0.95 ± 0.23 (5)	1.00 ± 0.09 (4)
Peptoid						
PD134,308	10	-45.8 ± 0.4	-17.2 ± 5.1	97.3 ± 17.5	0	0.52 ± 0.07 (5)[a]
PD140,376	4	-47.4 ± 0.4	-8.9 ± 2.0	130.9 ± 6.0	0	0.35 ± 0.11 (5)[a]
Benzodiazepine						
YM022	6	-56.6 ± 0.4	-13.0 ± 4.4	148.4 ± 13.8	0	0
YM220	3	-55.6 ± 0.8	-26.7 ± 4.7	97.3 ± 15.7	0	0
R-L-365,260	9	-44.8 ± 0.3	-32.6 ± 4.6	41.5 ± 14.6	0	0
S-L-365,260	4	-35.1 ± 0.4	-3.5 ± 4.8	107.4 ± 15.9	0	0
R-L-740,093	5	-54.4 ± 0.9	-32.4 ± 3.6	74.5 ± 12.1	0	0
Indole and benzimidazole						
JB93242	5	-50.8 ± 1.0	-4.4 ± 4.8	157.6 ± 13.5	0	0.46 ± 0.03 (13)[a]
JB95008	6	-42.5 ± 0.2	-12.3 ± 3.7	103.0 ± 12.3	0	0
JB93182	5	-52.9 ± 0.7[b]	-6.4 ± 3.9[b]	158.2 ± 11.6[b]	0	0

Abbreviation: CCK-8S, cholecystokinin sulphated octapeptide.
Data represent the mean$\pm$s.e.m. Numbers in parentheses after α-values are the number of stomach preparations used for parameter determination.
[a]Intrinsic activity significantly different to pentagastrin.
[b]Values are those for [^{3}H]JB93182.

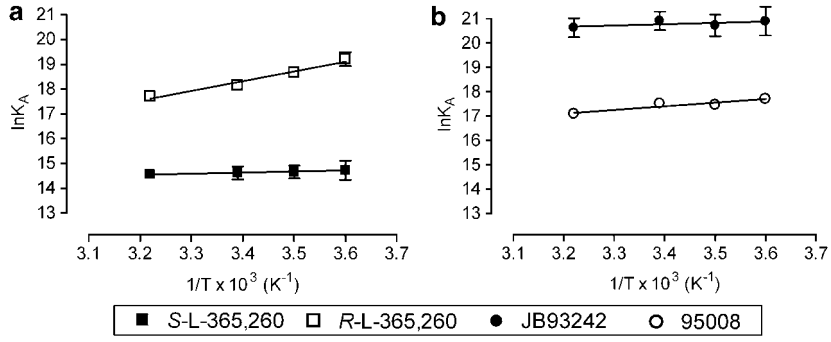

Figure 8 Van't Hoff plots showing the effect of temperature on the equilibrium association constants (K_a) of (**a**) *R*-L-365,260 and *S*-L-365,260; (**b**) JB95008 and JB93242. Data are the mean ± s.e.m. of four and 10 replicate experiments. The lines shown superimposed through the data were obtained by linear regression. CCK-8S, cholecystokinin sulphated octapeptide.

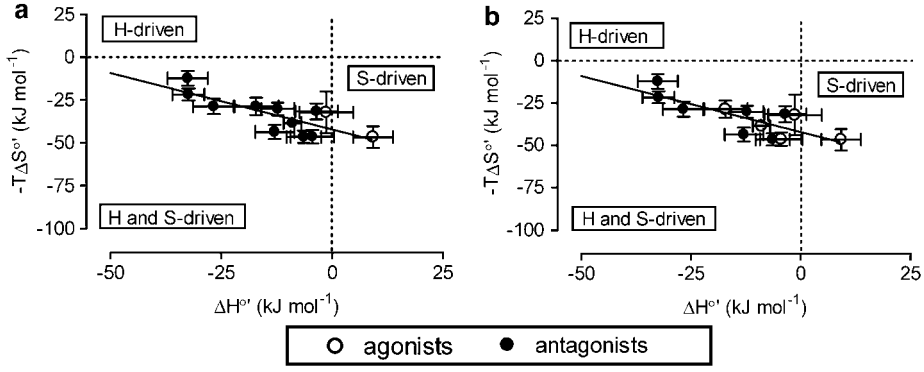

Figure 9 An extrathermodynamic plot showing the relationship between $-T\Delta S^{\circ\prime}$ and $\Delta H^{\circ\prime}$ values for gastrin-G1 receptor agonists and antagonists, as defined by (**a**) the lumen-perfused rat or (**b**) mouse-stomach bioassays (see Table 4). The lines shown superimposed through the data were obtained by linear regression.

4.5 kJ mol^{-1}, $n=5$) were negative and ranged from -1.3 ± 6.1 kJ mol^{-1} ($n=4$), for pentagastrin, to -32.6 ± 4.5 kJ mol^{-1}, for *R*-L-365,260 ($n=9$) (Table 4). The $\Delta S^{\circ\prime}$ values for all ligands were positive and ranged from 41.5 ± 14.6 J mol^{-1} K^{-1}, for *R*-L-365,260 ($n=9$), to 158.4 ± 21.3 J mol^{-1} K^{-1}, for CCK-8S ($n=5$) (Table 4).

Extrathermodynamic plot

A plot of the $\Delta H^{\circ\prime}$ and $-T\Delta S^{\circ\prime}$ for all ligands indicated that with the exception of CCK-8S, their binding was both enthalpy and entropy-driven ($\Delta H^{\circ\prime}<0$; $-T\Delta S^{\circ\prime}$ ranged from 12.2 ± 4.3 to 46.6 ± 6.3 J mol^{-1} K^{-1}). The binding of CCK-8S was entropy-driven. There was a significant linear relationship between the $\Delta H^{\circ\prime}$ and $-T\Delta S^{\circ\prime}$ ($r=0.79$, $P<0.01$, slope $=-0.66\pm0.16$, *y*-intercept $=-42.1\pm2.8$, *x*-intercept $=-63.7$; Figure 9). The binding of ligands found to express intrinsic efficacy in the lumen-perfused rat or mouse stomach assays (see Table 4) were not discriminated from those of ligands classified as antagonists in these tissues (Figures 9a and b).

Comparison of ΔH$^{\circ\prime}$ or ΔS$^{\circ\prime}$ values and intrinsic activity (α)

There was no relationship between $\Delta H^{\circ\prime}$ or $\Delta S^{\circ\prime}$ parameters and intrinsic activity (α) measured in the mouse stomach assay (Figures 10a and c). The $\Delta S^{\circ\prime}$ values for ligands defined as antagonists in the lumen-perfused rat stomach assay (Figures 10a and c) were positive and not different from the two ligands, pentagastrin and CCK-8S, which had intrinsic activity.

Discussion

In this study we have investigated whether agonists and antagonists can be thermodynamically discriminated at CCK_2 receptors (gastrin-G_1) in rat cerebral cortex. We have used the radiolabelled antagonist, [^{3}H]JB93182, which has previously been shown to selectively label rat gastrin-G_1/CCK_2 receptors with high affinity (G_1 p$K_I=9.94\pm0.16$, G_2 p$K_I=8.57\pm0.15$; Harper *et al*., 1999a) and a series of structurally diverse ligands with varying intrinsic activities at gastrin-G_1 receptors as detected by the lumen-perfused mouse or rat stomach bioassays.

Both kinetic studies and saturation studies of [^{3}H]JB93182 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

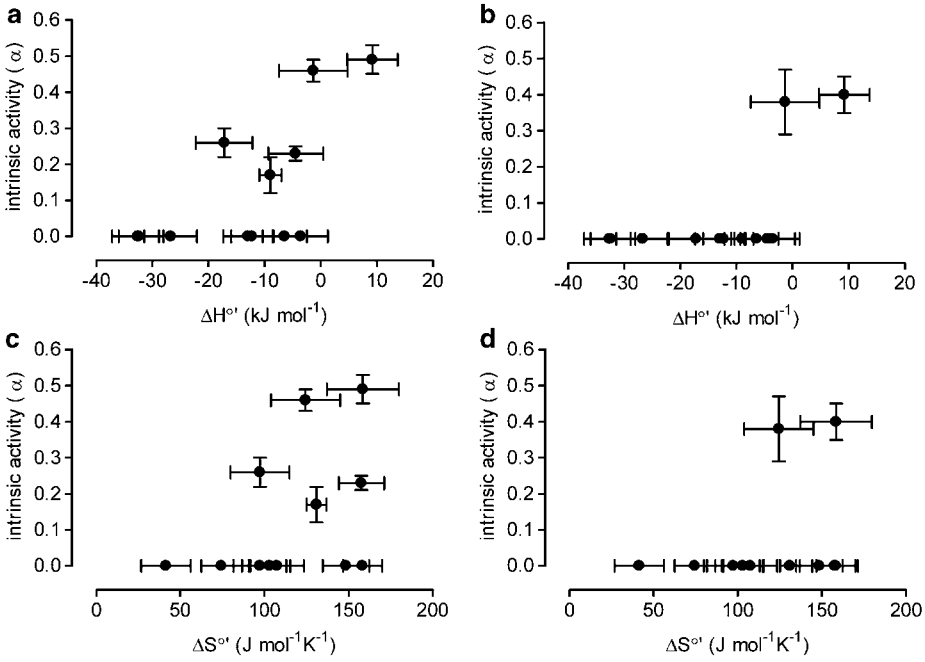

Figure 10 Relationship between $\Delta H^{\circ\prime}$ (**a, b**) and $\Delta S^{\circ\prime}$ (**c, d**) parameters and intrinsic activity (α) determined in the lumen-perfused mouse (**a, c**) and rat stomach (**b, d**) assays.

performed at each temperature so that pIC_{50} values, obtained in competition assays, could be corrected for any change in the occupancy of [^{3}H]JB93182 resulting from temperature-dependence of the pK_L. Saturation analysis indicated that [^{3}H]JB93182 labelled a single population of non-interacting rat CCK_2 receptors. Thus, at all temperatures, specific binding was saturable, and n_H values were not significantly different from unity and there was no significant change in the CCK_2 receptor density (B_{max}) (Figure 3, Table 1).

Kinetic studies confirmed that the incubation time used for the saturation analysis (4 and 12°C = 6.5 h; 21 and 37°C = 2.5 h; Figure 2) was sufficient for equilibrium binding of the radioligand to have been achieved (10, 50, 90 and 220 min at 37, 21, 12 and 4°C) so that the pK_L values estimated at each temperature were accurate. The dissociation rate ($t_{1/2}$) of [^{3}H]JB93182, determined in the kinetic studies at 37, 21 and 12°C, indicated that the incubation times used for competition studies (2.5 h at 21 and 30°C and 24 h at 12°C) were sufficient for equilibrium binding of ligands and thus for accurate determination of pIC_{50} values. This is because they were in excess of five times the dissociation $t_{1/2}$ of [^{3}H]JB93182 ($5 \times t_{1/2}$ 37°C = 15.3 min, 21°C = 96.5 min, 12°C = 378.5 min; Figures 2a, b and c; see Motulsky and Mahan, 1983). At 4°C, it was not possible to determine the $t_{1/2}$ of [^{3}H]JB93182 by fitting a first-order rate equation to the data because only 65% of the [^{3}H]JB93182 was dissociated after 260 min (Figure 2d). However, despite this, the 24 h incubation time used for competition studies should have been sufficient for equilibrium binding of ligands, because more than 50% of the radioligand was dissociated after 260 min. In light of the approximate fourfold decrease in the $t_{1/2}$ of [^{3}H]JB93182 on reducing the incubation temperature from 21 to 12°C, it seems the most likely explanation for our failure to observe complete dissociation of [^{3}H]JB93182 at 4°C is that we were unable to monitor the time course of the dissociation for a sufficient period of time at this temperature. Obviously, it would have been ideal to have continued to monitor the specific binding at increasing time points beyond 260 min, but because the duration of the experiment at this stage was already in excess of 10 h, this was impracticable.

It is notable that the [^{3}H]JB93182 pK_L values estimated at 12 and 21°C in kinetic assays were not different from those determined by saturation analysis. The finding of a significantly higher pK_L value for [^{3}H]JB983182 in the kinetic experiments at 37°C could have resulted from inaccuracy in the estimation of the observed association rate at this temperature (see Figure 2a).

The data obtained in competition studies, for ligands characterized as antagonists in the lumen-perfused rat stomach assay, supported the conclusion drawn from saturation studies; that is, that at all temperatures in rat cerebral cortex membranes, [^{3}H]JB93182 labelled a homogenous receptor population. Thus, with the exception of *S*-L-365,260 at 4°C, the mean n_H parameter estimates for all the antagonist ligands at all temperatures were not significantly different from unity. The finding of a mean n_H value significantly greater than unity for the *S*-L-365,260 at 4°C may be a type 1 error. Thus, although the mean n_H value was significantly greater than unity, for each individual competition curve, the fit to the Hill equation was not better than that to the Hill equation with n_H constrained to unity as judged by an F-test.

pK_L and pK_I values obtained in [^{3}H]JB93182 saturation studies and competition studies performed at 4, 12, 21 and

37°C indicated temperature-dependence of the affinity of some of the CCK_2 receptor ligands (Tables 1 and 2). Thus, the van't Hoff plots for JB95008, *R*-L-740,093, *R*-L-365,260, YM220 and PD134,308 had positive slopes which were significantly different from zero, while those for [^{3}H]JB93182, YM022, pentagastrin, CCK-8S, *S*-L-365 260, PD140,376 and JB93242 had slopes that were not different from zero. An extrathermodynamic plot showed that the binding of all the ligands with the exception of CCK-8S were both enthalpy and entropy-driven (Figures 9a and b) and there was no apparent thermodynamic distinction between agonists and antagonists (Figures 10a and b) or between peptides, peptoids, benzodiazepines, benzimidazole-based or indole-based ligands (Table 4).

It is possible that agonists and antagonists are indeed not thermodynamically discriminated at the CCK_2 receptor, in the same way as the 5-HT_{1A} receptor (Dalpiaz *et al*., 1996), dopamine D_2 receptor (Zahniser and Molinoff, 1983; Duarte *et al*., 1988) and μ-opioid receptor (Li *et al*., 1998). However, it is also possible that binding of CCK_2 receptor agonists and antagonists are discriminated and that we failed to detect this.

It seems unlikely that our failure to find thermodynamic discrimination of agonists and antagonists was because the competition data were too variable because of the relatively low percentage-specific binding of [^{3}H]JB93182 in rat cerebral cortex (43.4 ± 6.6 to 56.8 ± 7.0%). This is because the percentage coefficient of variation of competition data, at each temperature, was less than 3% (see Figures 4a–d) and the data obtained in replicate experiments was highly reproducible (Figures 4e–h). In addition, it seems improbable that radioligand depletion (see Wells *et al*., 1980; Goldstein and Barrett, 1987) resulted in our inability to detect thermodynamic discrimination because only $\sim$10% of the added radioligand was bound at each temperature.

It is noteworthy that the slope of pentagastrin and CCK-8S E/[A] curves in the mouse stomach assay are significantly lower than those obtained in the rat stomach. It is possible to explain this behaviour by considering that there is better receptor–effector coupling of CCK_2 receptors in the mouse than in the rat stomach assay (Black and Leff, 1983). It is also possible to explain this by considering that pentagastrin and CCK-8S express operational selectivity for one of the two putative receptors (G_1 and G_2) in the mouse stomach assay. However, we do not believe that the partial agonism, relative to pentagastrin and CCK-85, of PD134,308, PD140,376 and JB93242 in the mouse stomach was because these ligands only express intrinsic efficacy at the G_1 receptor subtype and that activation of the G_1 receptor by a full agonist (intrinsic activity = 1.0) cannot produce the maximal response elicited by a full agonist activating both G_2 and G_1. This is because our previous studies using the antagonist *R*-L-365,260 (see Roberts *et al*., 1996a) were consistent with the ability of pentagastrin to produce a maximal response through either receptor in isolation.

A potential explanation for not finding thermodynamic discrimination of ligands, defined as agonists and antagonists in the mouse stomach bioassay, could be because our original classification of gastrin-G_1 receptors was incorrect. To our knowledge, there is still no molecular pharmacological evidence for an additional CCK_2 receptor type and our studies suggest that gastrin-G_1 and G_2 receptors do not correspond to the currently identified isoforms of the CCK_2 receptor (CCK_{2S} and CCK_{2L}, Ito *et al*., 1994; see Harper *et al*., 2000; Morton *et al*., 2003 or CCK_{2i4v}, Hellmich *et al*., 2000; data not shown). However, the gastrin-G_1 and G_2 receptor classification was based on the behaviour of a number of structurally different CCK_2 receptor ligands (Harper *et al*., 1996a; Roberts *et al*., 1996a, b; Shankley *et al*., 1997; Harper *et al*., 1999a, b). In addition, the recent demonstration that *R*-L-365,260 expresses lower affinity at human CCK_2 receptors dimerized with CCK_1 receptors ($pK_I \sim 7.1$) than at the CCK_2 receptor homodimer ($pK_I \sim 7.8$; Cheng *et al*., 2003) raises the possibility that the pharmacological definition of G_1 receptors may be correct and result from heterodimerization of CCK_1 and CCK_2 receptors.

If our classification of gastrin-G_1 receptors and G_2 receptors is incorrect, then the detection of intrinsic efficacy of JB93242, PD134,308 and PD140,376, in the mouse stomach, was not because this tissue has higher receptor–effector coupling of gastrin-G_1 receptors compared to the rat, but because these ligands are agonists at mouse, and not rat CCK_2 receptors. Therefore, we would not expect these ligands to show thermodynamic discrimination at the rat receptor. This possibility is supported by studies that have shown that as a consequence of differences in the amino-acid sequence of the CCK_2 receptor, some ligands such as PD135,158 and *S*-L-740,093 are agonists at mouse but not at human CCK_2 receptors (Kopin *et al*., 1997). In addition, although species polymorphisms have not been shown to have an effect on the affinity and efficacy of endogenous CCK_2 receptor ligands (Kopin *et al*., 1997; Blaker *et al*., 2000; Kopin *et al*., 2000), recent studies by Langer *et al*. (2005) suggest that the absolute amino acids of the receptor that interact with the agonist are different in rat and human CCK_2 receptors. The consequence of this finding is that the thermodynamic parameters underlying the binding of synthetic or endogenous agonists may be different for rat and mouse CCK_2 receptors irrespective of their intrinsic efficacy.

Regardless of whether the gastrin-G_1 receptor nomenclature is correct, our failure to find that the binding of the only two ligands with intrinsic activity (α) in the rat stomach assay (pentagastrin and CCK-8S; Figure 5) were not both entropy-driven is difficult to explain when it is considered that the intrinsic activity (α) measurements made at these CCK_2 receptors are from the same species used for the radioligand-binding studies, the rat. Thus, genetic polymorphism across species cannot explain the discrepancy. In addition, we found no evidence to suggest that the rat stomach CCK_2 receptors mediating the actions of pentagastrin and CCK-8S were different from those in the rat cerebral cortex labelled with [^{3}H]JB93182. Thus, when the pA_2 or pK_B values, obtained for eight of the 10 antagonists in the rat stomach, were compared to pK_I values in the rat cortex, they were found not to deviate from a line of identity ($y = x$; Figure 6). In light of this finding, it seems unlikely that we failed to find thermodynamic discrimination of ligands because the buffer conditions used for the rat stomach assay and radioligand-binding assays were different and as such

changed the environment of the amino-acid residues in the binding pocket.

The finding that *R*-L-740,093 and YM022 produced a depression of the maximal pentagastrin response in the rat stomach assay so that pA_2 values could not be estimated is consistent with these ligands acting as insurmountable antagonists (see Dunlop *et al*., 1997, 1998).

Thermodynamic discrimination of agonists and antagonists, at some receptors, has been shown to be dependent on the presence of high-affinity agonist binding such that it is ablated when high-affinity agonist binding is prevented by including guanine nucleotides in the assay buffer (for example Contreras *et al*., 1986; Fraeyman *et al*., 1994; Lorenzen *et al*., 2000). In previous studies we have shown that high affinity agonist binding at the CCK_2 receptor is consistent with ternary complex formation between agonist, receptor and G-protein (Harper *et al*., 1996b), according to the model proposed by Kent *et al*. (1980). However, it is notable in this study that pentagastrin and CCK-8S competition curves had n_H values that were not significantly different from unity and additionally, that pK_I values were comparable to pEC_{50} values obtained in the rat stomach assay (for example 37°C pEC_{50} pentagastrin = 7.33; CCK-8S = 6.74; pK_I pentagastrin = 6.70, CCK-8S = 6.87), suggesting limited formation of ternary complex. Therefore, it is possible that CCK_2 receptor agonists, as defined by the rat stomach, were not thermodynamically distinguished from antagonists in the rat cortex because the formation of ternary complex was restricted due to limited availability of G-protein.

In consideration of the possibility that the lack of high-affinity agonist binding was the reason we did not find thermodynamic discrimination of agonists and antagonists, an obvious idea would be to repeat these studies under conditions where CCK-8S and pentagastrin express pK_I values greater than pEC_{50} values obtained in the rat stomach. It is possible that this could be achieved by using a different buffer for the preparation of the rat cerebral cortex membranes. However, in light of studies that have shown that the CCK_2 receptors can couple to multiple G-proteins resulting in activation of phospholipase C (PLC), phospholipase A_2 and also adenylate cyclase (Detjen *et al*., 1997; Pommier *et al*., 1999; Wu *et al*., 1999; Mazzocchi *et al*., 2004), there is an added complexity of studies using different tissues to determine pK_I and intrinsic activity measurements. This is because the G-proteins recruited by receptor states in the rat cerebral cortex for ternary complex formation may differ from those activated in the rat stomach (see Kenakin, 2003). This possibility is supported by studies which have shown that histamine-stimulated acid secretion in the rat stomach resulting from activation of CCK_2 receptors on enterochromaffin cells (Sandvik and Waldum, 1991; Shankley *et al*., 1992; Prinz *et al*. 1993; Lindström *et al*., 1997; Lindström *et al*., 1999) is a consequence of the activation of Gq, leading to PLC activation and production of inositol trisphosphates (IP_3) with subsequent mobilization of intracellular calcium (Prinz *et al*., 1993; Prinz *et al*., 1994; Seva *et al*., 1994; Kinoshita *et al*., 1998), whereas in CNS cells, the CCK_2 receptor has been shown to activate Gq, leading to intracellular calcium mobilization (Kuwahara *et al*., 1993; Kaufman *et al*., 1994; Smith and Freedman, 1996; Muller *et al*., 1997), but it has also been shown to activate Gs, leading to an increase in cAMP (Kombian *et al*., 2006). Therefore, it is feasible that even if the thermodynamic studies were repeated under conditions where CCK-8S and pentagastrin expressed pK_I values greater than pEC_{50} values obtained in the rat stomach, agonists and antagonists would still not be thermodynamically discriminated. Consequently, it would seem most sensible to repeat these thermodynamic studies at the CCK_2 receptor in a system where it is possible to measure both ligand affinity (pK_I) and intrinsic activity (α). This could be achieved by performing radioligand-binding studies and second messenger assays in a cell line endogenously expressing the CCK_2 receptor or in cells expressing the recombinant CCK_2 receptor.

It is uncertain whether we would have found thermodynamic discrimination of agonists and antagonists at the CCK_2 receptor if we had been able to use more direct methods of measuring ΔS° and ΔH°, such as isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) (see Perozzo *et al*., 2004; Bruylants *et al*., 2005). However, these techniques generally require higher receptor densities than are usually present in biological tissues. In addition, many of the thermodynamic studies of GPCRs that have used ITC or DSC have used only an N-terminal fragment of the GPCR (for example Grauschopf *et al*., 2000; Rajagopalan *et al*., 2005; Fernando *et al*., 2006). We wished to measure the thermodynamic parameters resulting from binding to the intact CCK_2 receptor so that the magnitude of ΔS° and ΔH° would not only result from the binding interactions between ligand and receptor but also from changes in receptor conformation or from G-protein coupling resulting from the binding of ligands with intrinsic efficacy.

Care clearly must be taken not to over interpret the changes in entropy ($\Delta S^{\circ\prime}$) and enthalpy ($\Delta H^{\circ\prime}$) associated with ligand binding when they have been measured utilizing the approach used in this study; this is because only $\Delta G^{\circ\prime}$ is measured experimentally, whereas $\Delta S^{\circ\prime}$ and $\Delta H^{\circ\prime}$ are estimated using the van't Hoff plots. However, despite this caveat and there being no apparent thermodynamic distinction between peptides, peptoids, benzodiazepines or indole-based ligands (Table 4), there do appear to be some interesting features of the thermodynamic parameters of ligands of similar structure. For instance, the *S* enantiomer of L-365,260 bound with lower affinity to CCK_2 receptors than the *R* enantiomer (Table 2). However, the balance of the thermodynamic parameters for this *S*-L-365,260 show a greater increase in entropy ($\Delta S^{\circ\prime}$) and smaller decrease in enthalpy ($\Delta H^{\circ\prime}$) upon binding than that resulting from the binding of *R*-L-365,260 (Table 4). The changes associated with the binding of *S*-L-365,260 relative to that of *R*-L-365,260 may be because the chirality of these benzodiazepine ligands is important in determining the precise nature of their interactions with the receptor. Thus, the greater decrease in $\Delta H^{\circ\prime}$, associated with the binding of *R*-L-365,260, could be a consequence of this ligand-forming hydrogen bonds and electrostatic interactions with the receptor which are absent or not formed as strongly when *S*-L-365,260 binds. The partly compensating large increase in $\Delta S^{\circ\prime}$ associated with

the binding of S-L-365,260 is likely to result from a stronger hydrophobic interaction of this ligand, the consequence of which is to strip more water molecules from the receptor and hence increase the entropy of the overall binding process.

The higher affinity of YM022 and *R*-L-740,093, relative to *R*-L-365,260 (Table 2), appears to result from an increase in $\Delta S^{\circ\prime}$ relative to that underlying the binding of *R*-L-365 260 (Table 4). These thermodynamic changes are consistent with YM022 and *R*-L-740,093 accessing additional hydrophobic binding sites. This would evidently be through the 5-phenyltolylcarbonylmethyl group at the N_1 position of benzodiazepine ring in YM022 (see Figure 1) and through the azabicyclo[3.2.2]nonan-3-yl group at the five position of the benzodiazepine ring in *R*-L-740,093 (see Figure 1).

The linear relationship between mean $\Delta H^{\circ\prime}$ and $T\Delta S^{\circ\prime}$ (Figure 9a) has been reported for numerous GPCRs and ligand gated ion channels, such as β-adrenoceptors, adenosine A_1 or A_{2A}, dopamine D_2, 5-beta hydroxytryptamine receptor 1 (5-HT_{1A}), glycine, γ-aminobutyric acid ($GABA_A$) and nicotinic receptors (see Borea *et al*., 1998). This relationship indicates that enthalpy–entropy compensation may exist for this 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

This study is the first to investigate thermodynamics of ligand binding at CCK_2 receptors. The data suggest that the binding of agonists and antagonists at CCK_2 receptors labelled with [^{3}H]JB93182, in rat cerebral cortex, cannot be thermodynamically discriminated. Therefore, measurement of the thermodynamic parameters underlying binding at the CCK_2 receptor cannot be used to predict whether a ligand possesses intrinsic efficacy at this receptor.

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Conflict of interest

This work was funded by Johnson and Johnson. There is no conflict of interest.

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