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# Analysis of the behaviour of selected $CCK_B$/gastrin receptor antagonists in radioligand binding assays performed in mouse and rat cerebral cortex

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**1** The previously described complex behaviour of the $CCK_B$/gastrin receptor antagonist, L-365,260, in radioligand binding assays could be explained by a variable population of two binding sites. We have investigated whether other $CCK_B$/gastrin receptor ligands (PD134,308, PD140,376, YM022 and JB93182) can distinguish between these sites.

**2** In the mouse cortex assay, Hill slopes were not different from unity and the ligand $pK_I$ values did not differ when either [$^{125}$I]-BH-CCK-8S or [$^3$H]-PD140,376 was used as label as expected for a single site ($G_2$).

**3** In the rat cortex, where previous analysis of replicate ($n=48$) L-365,260 data indicated the presence of two $CCK_B$/gastrin sites ($G_1$ and $G_2$), the competition data for PD134,308, PD140,376, YM022 and JB93182 could be explained by a homogeneous population of $CCK_B$/gastrin sites because the Hill slope estimates were not significantly different from unity. However, the estimated affinity values for JB93182 and YM022 were significantly higher and that for PD134,308 was significantly lower than those obtained in the mouse cortex when the same radioligand was used. In view of our previous data obtained with L-365,260, the rat cortex data were also interpreted using a two-site model. In this analysis, SR27897 expressed ~9 fold, PD134,308 ~13 fold and PD140,376 ~11 fold selectivity for the $G_2$ site. In contrast, JB93182 expressed ~23 fold and YM022 ~4 fold selectivity for the $G_1$ site. If the two-site interpretation of the data is valid then, because of its reverse selectivity to L-365,260, JB93182 has been identified as a compound which if radiolabelled could provide a test of this receptor subdivision.

**Keywords:** $CCK_B$/gastrin receptor; rat cortex; mouse cortex; guinea-pig pancreas; JB93182

**Abbreviations:** CCK-8S, cholecystokinin sulphated octapeptide; BH-CCK8S, Bolton-Hunter CCK8S; HEPES, (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]); EGTA, (ethyleneglycol-bis(β-aminoethylether)N,N,N′,N′-tetraacetic acid); DMF, dimethylformamide

## Introduction

There is a high degree of sequence homology among the cloned $CCK_B$/gastrin receptors from several species (Pisegna *et al.*, 1992). However, the $CCK_B$/gastrin receptor antagonist, L-365,260, expresses species-dependent affinities that were originally attributed to single amino-acid differences within the sixth transmembrane domain of the receptor (Beinborn *et al.*, 1993). More recently, two isoforms of the receptor have been identified which are produced as a consequence of alternate gene splicing (Song *et al.*, 1993). Although agonists are reported not to distinguish between the two receptor isoforms in terms of binding and transduction, L-365,260 has been shown to be 3 fold more potent at competing with $^{125}$I-Bolton-Hunter-labelled-CCK-8S for the human short receptor isoform than for the long receptor isoform (Ito *et al.*, 1994; Wank *et al.*, 1994).

Until recently, there was no evidence from functional bioassays for $CCK_B$/gastrin receptor heterogeneity within a species (Presti & Gardener, 1993). However, we recently reported an analysis of the variation in L-356,260 data obtained in replicate experiments performed over a 3 year period in both radioligand binding (Harper *et al.*, 1996a) and in isolated tissue assays (Roberts *et al.*, 1996a). Overall, the data were not consistent with the pharmacological expression of a single $CCK_B$/gastrin receptor, in both central and peripheral tissues, but they could be accounted for by the variable expression of two $CCK_B$/gastrin receptor subtypes or states which we referred to as gastrin-$G_1$ and -$G_2$. The mouse cortex assay appeared to express a homogeneous population of sites characterized by a $pK_I$ for L-365,260 of 8.41 ($G_2$) whereas the rat cortex assay expressed a variable proportion of two sites characterized by $pK_I$ values of 8.48 ($G_2$) and 7.22 ($G_1$). It was only possible to obtain affinity estimates at the two sites when we simultaneously analysed a larger number of data sets (47/48 individual experiments) because of apparent within-assay variation in the proportion of the two sites and the low subtype selectivity (~18 fold) of L-365,260.

Although the physiological role of the two proposed $CCK_B$/gastrin receptors has not been elucidated, the mRNA coding for the two receptor isoforms, which may be identical to these pharmacologically-defined subtypes, has been shown to vary between tissues (Biagini *et al.*, 1995). Therefore, having made the observation that, under our assay conditions, L-365,260 can distinguish between two $CCK_B$/gastrin receptors we have investigated whether other $CCK_B$/gastrin receptor antagonists could also distinguish between these sites. Accordingly, we have analysed the behaviour of a selection of previously characterized CCK/gastrin receptor ligands in the mouse and rat cortex assays. As in the previous analysis of the behaviour of L-365,260, the data analysed was

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obtained over a number of years as part of a drug discovery programme (e.g. Kalindjian *et al*., 1994). We decided to use the two cortex assays because, if the compounds could distinguish between the two pharmacologically defined subtypes, we expected to obtain simple behaviour in the mouse cortex but variable behaviour in the rat cortex. In addition, we determined the $CCK_A$ receptor-selectivity of the compounds in a guinea-pig pancreas radioligand binding assay. In particular, the $CCK_A$ selective antagonist, SR27897 (Gully *et al*., 1993), was used to confirm that any complexity in cortex data was not a consequence of the compounds interacting with $CCK_A$ receptors in this tissue.

We selected compounds from the large number of $CCK_B$/gastrin receptor ligands that have been described including L-365,260 (Lotti & Chang, 1989), PD134,308 (Hughes *et al*., 1990), L-156,586 (Lam *et al*., 1990), PD136,450 (Horwell *et al*., 1991), PD140,376 (Hunter *et al*., 1993), LY262691 (Howbert *et al*., 1993), YM022 (Nishida *et al*., 1994), L-740,093 (Patel *et al*., 1994), CP-212,454 (Lowe *et al*., 1994), RP72540 (Bertrand *et al*., 1994), RP73870 (Bohme *et al*., 1994; Pendley *et al*., 1995), RPR101367 (Bertrand *et al*., 1995), GV150013 (Corsi *et al*., 1995) and JB93182 (Kalindjian *et al*., 1996). When these compounds were originally described there was no mention of data complexity and no suggestion of $CCK_B$/gastrin receptor heterogeneity. Therefore, the compounds used in this study (PD134,308, PD140,376, YM022, JB93182) were selected on the basis of their structural diversity, in the hope that this approach would increase the likelihood of exposing subtype selectivity, and for the compound-specific reasons detailed below.

YM022 was selected because it is one of the highest affinity $CCK_B$/gastrin receptor antagonists which has been described (Nishida *et al*., 1994). The two 'peptoid' $CCK_B$/gastrin ligands, PD134,308 and PD140,376, were included in the analysis because they appeared to express lower affinities at $CCK_B$/gastrin receptors in the isolated lumen-perfused rat stomach assay (Shankley *et al*., 1997) than at $CCK_B$/gastrin binding sites in the mouse and guinea-pig cortex (Hughes *et al*., 1990; Hunter *et al*., 1993). Moreover, an analysis of the behaviour of L-365,260 in replicate experiments suggested that the rat stomach assay expresses a homogeneous gastrin receptor population with low affinity for L-365,260 (Roberts *et al*., 1996a). Finally, JB93182, was included because data obtained in the lumen-perfused rat stomach assay suggested that this compound had a higher affinity at receptors present in this tissue ($G_1$; Roberts *et al*., 1996b) than for those in the mouse cortex ($G_2$). Therefore, JB93182 might be expected to exhibit complex behaviour in the rat cortex assay and express opposite selectivity to L-365,260 for the proposed two sites.

A preliminary account of this study was presented to the British Pharmacological Society (Harper *et al*., 1996b).

## Methods

### *Preparation of mouse and rat cortex cells*

The preparation of mouse and rat cortex cells, as judged by light microscopy, was based upon the method of Clarke *et al*. (1986). Young adult male mice (Charles River 25–30 g) and male rats (Wistar 250–500 g) were killed by cervical dislocation. The cortex was immediately dissected and placed in ice-cold HEPES-NaOH buffer (pH 7.2 at $21\pm3^{\circ}$C) of the following mM composition: NaCl 130; KCl 4.7; $MgCl_2$ 5; HEPES 10, EGTA 1 plus bacitracin 0.125 g $l^{-1}$. The tissue was weighed and homogenized in 40 ml of ice-cold buffer using a Teflon-in-glass homogenizer. The homogenate was centrifuged at $39,800\times g$ for 20 min at 4°C, the supernatant discarded and the pellet resuspended by homogenization in fresh HEPES-NaOH buffer and recentrifuged. For radioligand binding assays using $^{125}$I-Bolton Hunter labelled cholecystokinin-8S ([$^{125}$I]-BH-CCK-8S), the resulting pellets from rat and mouse cortex were resuspended in HEPES-NaOH buffer to give tissue concentrations of 5 mg ml$^{-1}$ and 2 mg ml$^{-1}$ (original wet weight), respectively. At these tissue concentrations the percentage of added ligand bound in the rat and mouse cerebral cortex assay was $9.2\pm0.7$ and $11.8\pm1.3$% of that added, respectively ($n=3$ to 6; Harper *et al*., 1996a). The non-specific binding in the mouse was $22.8\pm1.9$ and in the rat $49.0\pm1.5$% of the total bound radioligand. Mouse cortex cells were resuspended to give a tissue concentration of 8 mg ml$^{-1}$ (original wet weight) for assays using [$^{3}$H]-PD140,376. At this concentration the percentage of added radioligand bound was $12.3\pm2.4$% and the percentage non-specific binding was $36.0\pm8.1$% ($n=5$).

### *Preparation of guinea-pig pancreatic acini*

Adult male Dunkin Hartley guinea-pigs (200–300 g) were killed by cervical dislocation. The pancreas were removed, dissected from connective tissue and fat and placed in ice-cold HEPES-NaOH buffer. The tissue was weighed and homogenized in 40 ml of ice-cold buffer using a Polytron PT10 ($4\times1$s; setting 10). The homogenate was centrifuged at $39,800\times g$ for 20 min at 4°C and the supernatant discarded. The pellet was resuspended by homogenization in 80 ml of ice-cold HEPES-NaOH buffer, using a Teflon-in-glass homogenizer, and recentrifuged. The final pellet was resuspended in HEPES-NaOH buffer, containing 0.375 $\mu$M PD134,308, to a tissue concentration of 1 mg ml$^{-1}$ (original wet weight) and filtered through 500 $\mu$m pore-size Nytex mesh.

### *Incubation conditions – $CCK_B$/gastrin receptor competition studies*

All CCK/gastrin receptor antagonists were diluted in HEPES-NaOH buffer. Aliquots (50 $\mu$l) of competing ligands at concentrations from 0.10 pM to 100 $\mu$M, were incubated in triplicate with mouse or rat cortex cells (400 $\mu$l) in a final volume of 500 $\mu$l with appropriate buffer containing [$^{125}$I]-BH-CCK-8S (50 $\mu$l; 200 pM) or [$^{3}$H]-PD140,376 (50 $\mu$l; 1.5 nM). Non-specific binding was defined with 1 $\mu$M L-365,260. [$^{125}$I]-BH-CCK-8S has previously been shown not to label $CCK_A$-receptors under these assay conditions (Harper *et al*., 1996a). Assays were incubated for 150 min at $21\pm3^{\circ}$C and then terminated by rapid filtration through pre-soaked Whatman GF/B filters which were washed ($3\times3$ ml) with ice-cold 50 mM Tris-HCl buffer (pH 6.9 at $21\pm3^{\circ}$C). Bound [$^{125}$I]-BH-CCK-8S was determined by counting each filter (1 min) in a LKB Clinnigamma counter. Bound [$^{3}$H]-PD140,376 was determined by liquid scintillation spectroscopy. Filters were transferred into scintillation vials, 5 ml Beckman Ready-Solv HP liquid scintillation cocktail added and after a further 4 h the bound radioactivity determined by counting (5 min) in a Beckman LS6000 liquid scintillation counter.

### *Incubation conditions $CCK_A$ receptor competition studies*

All CCK/gastrin receptor antagonists were diluted in HEPES-NaOH buffer. Aliquots (50 $\mu$l) of concentrations from 1 pM to 100 $\mu$M, were incubated in triplicate with guinea-pig pancreatic acini (400 $\mu$l; containing 0.375 $\mu$M PD134,308) in a final

volume of 500 $\mu$l with appropriate buffer containing [$^{125}$I]-BH-CCK-8S (50 $\mu$l; 200 pM). Non-specific binding was defined with 1 $\mu$M L-364,718. [$^{125}$I]-BH-CCK-8S does not label $CCK_B$/gastrin receptors under these assay conditions because a final assay concentration of 0.3 $\mu$M PD134,308, which would be expected to produce >95% occupancy of $CCK_B$/gastrin receptors, was included in the assay buffer. In addition, high concentrations of L-365,260 (>0.1 $\mu$M) were required to produce significant inhibition of binding (Table 1). After a 150 min incubation at 21±3°C, assays were terminated by rapid filtration through pre-soaked Whatman GF/B filters which were washed (3×3 ml) with ice-cold 50 mM Tris-HCl buffer (pH 6.9 at 21±3°C). Bound [$^{125}$I]-BH-CCK-8S was determined by counting each filter (1 min) in a LKB Clinnigamma counter.

### *Data analysis*

The individual competition curve data were expressed as the percentage of the decrease in specific binding of either [$^3$H]-PD140,376 or [$^{125}$I]-BH-CCK-8S within each experiment. When [$^3$H]-PD140,376 was used as label, dissociation constants ($K_I$) were determined using the Cheng & Prusoff (1973) equation in order to correct for the receptor occupancy by the radioligand,

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

In this equation [L] is the radioligand concentration and $K_D$ is the equilibrium dissociation constant of the radioligand. When ~0.1 nM [$^3$H]-PD140,376 was used as label ($pK_D = 9.89 \pm 0.14$, $n = 4 \pm$ s.e.mean), the correction factor was ~2. In practice, it was not necessary to correct for the radioligand occupancy when ~20 pM [$^{125}$I]-BH-CCK-8S was used as the affinity of CCK-8S at sites in rat and mouse cortex and guinea-pig pancreas was >0.2 nM (i.e. $[L]/K_D < 0.1$).

Competition data were fitted to the following Hill equation, which describes the relationship between the amount of bound ligand (B) and free ligand concentration ([L]), using a derivative-free, non-linear, regression programme (BMDP Statistical Software, Module AR; Dixon, 1992).

$$B = \frac{R.[L]^{n_H}}{IC_{50}^{\,n_H} + [L]^{n_H}} \qquad (2)$$

In the equation, R, the total number of specific binding sites occupied by the radiolabel in the absence of the competing ligand, was fixed in the fitting procedure at a value of 100% because the data were expressed as the percentage of the decrease in specific binding. $n_H$ is the midpoint slope parameter and $IC_{50}$ is the midpoint location parameter which was estimated as $\log_{10} IC_{50}$ on the basis that $IC_{50}$ values are log-normally distributed (Harper *et al*., 1996a).

### *Two-site model*

Data sets were also fitted using the BMDP computer programme to the following two-site model,

$$B = \frac{R_1.[L]}{IC_{501} + [L]} + \frac{R_2.[L]}{IC_{502} + [L]} \qquad (3)$$

This equation describes the binding of a ligand to two independent sites ($R_1$ and $R_2$), governed by the midpoint location parameters $IC_{501}$ and $IC_{502}$, respectively. In practice, the data were expressed as per cent specific binding (i.e. $R_1 + R_2 = 100\%$) so that $R_2$ could be substituted by the term ($100 - R_1$). Again, the $IC_{50}$ values were estimated as base 10 logarithms on the basis that they are expected to be log-normally distributed.

### *Statistical comparison of model parameter estimates*

All data are presented as mean±s.e.mean. Hill equation slope values were tested for differences from unity by Student's *t*-test as were the differences in individual compound $pIC_{50}$ values obtained on the mouse and rat cortex assays. *P* values of <0.05 were considered significant. The goodness-of-fit of the one-site and two-site models were assessed by comparison of the residual variance of the fits to the data using the 'extra sum of squares' principle (for details see De Lean *et al*., 1980).

### *Materials*

$^{125}$I-Bolton Hunter labelled CCK-8S ([$^{125}$I]-BH-CCK-8S) with specific activity of ~2200 Ci $mmol^{-1}$ was obtained from NEN$^{TM}$ Life Science Products, Hounslow, U.K. [$^3$H]-PD140,376 ([[L-3-[(4-aminophenyl)methyl]-*N*-[α-methyl-N-[(tricyclo[3.3.1.1.$^{3,7}$]dec-2-yloxy)carbonyl]-D-tryptophyl]-β-alanine]) with a specific activity of ~50 Ci $mmol^{-1}$ was obtained from Amersham International Plc, Little Chalfont, Buckinghamshire, U.K. L-365,260 (3R-(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-*N*′-3-methylphenyl urea), PD134,308 (CI988) ([R-(R*,R*)]-4-[[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.1.$^{3,7}$]dec-2-yloxy)carbonyl]amino]propyl]amino]-1-phenylethyl]amino]-4-oxobutanoic acid), PD140,376 ((([[L-3-[(4-aminophenyl)-methyl]-*N*-[α-methyl-N-[(tricyclo[3.3.1.1.$^{3,7}$]dec-2-yloxy)carbonyl]-D-tryptophyl]-β-alanine]), SR27897 (1-[[2-(4-(2-chlorophenyl)thiazol-2-yl)aminocarbonyl]indolyl]acetic acid) and YM022 ((R)-1-[2,3-dihydro-1-(2′-methylphenacyl)-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea) were synthesized by James Black Foundation chemists.

HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid), EGTA (ethyleneglycol-bis(β-aminoethylether) N,N,N′,N′-tetraacetic acid), bacitracin and Trizma base® were obtained from the Sigma Chemical Co., Poole, Dorset, U.K.

**Table 1** Analysis of data sets from competition experiments between [$^{125}$I]-BH-CCK-8S and CCK/gastrin receptor ligands for $CCK_A$ binding sites in guinea-pig pancreas

| *Compound* | *$pIC_{50}$±s.e.mean | $n_H$±s.e.mean | *†$pIC_{50}$±s.e.mean | n |
|---|---|---|---|---|
| L-365,260 | 6.50±0.08 | 1.11±0.07 | 6.50±0.08 | 12 |
| JB93182 | 5.29±0.12 | 1.55±0.21 | 5.30±0.12 | 5 |
| PD134,308 | 6.08±0.06 | 1.34±0.21 | 6.07±0.04 | 6 |
| PD140,376 | 6.21±0.09 | 1.16±0.13 | 6.21±0.09 | 4 |
| YM022 | 8.08±0.25 | 1.06±0.10 | 8.03±0.24 | 6 |
| SR27897 | 9.62±0.07 | 1.05±0.22 | 9.63±0.06 | 4 |

The parameter estimates were obtained by fitting the individual replicate competition curve data to the Hill equation (2). *$pIC_{50}$ ~$pK_I$ because $(1+[L]/K_D)$~1.1. †Data analysed with mid-point slope parameter constrained to unity.

All other materials were obtained from Fisons Scientific Apparatus Loughborough, Leics., U.K.

All compounds were dissolved in DMF to give stock concentrations of 10 mM and further dilutions were made in HEPES-NaOH buffer.

# Results

## *Analysis of competition curves in guinea-pig pancreas*

The affinity of compounds at $CCK_A$ binding sites was estimated in the guinea-pig pancreas. This was done to exclude the possibility that any complex data obtained in the cortex $CCK_B$/gastrin receptor assays was due to [$^{125}$I]-BH-CCK-8S also labelling $CCK_A$ binding sites under our assay conditions.

L-365,260, YM022, SR27897, PD134,308, PD140,376 and JB93182 produced concentration-dependent inhibition of the specific binding of [$^{125}$I]-BH-CCK-8S to $CCK_A$ binding sites in guinea-pig pancreas (Table 1). The mean mid-point slope parameter estimates ($n_H$) were not significantly different from unity. Of all the compounds, JB93182 had the lowest, sub-micromolar affinity ($pK_I = 5.29 \pm 0.12$; $n = 5$) for $CCK_A$ receptors.

## *Analysis of competition curves in mouse cortex*

Our previous analysis of the variation in L-365,260 competition curves indicated that the mouse cortex assay expressed a homogenous population of $CCK_B$/gastrin receptors. Therefore, our expectation was that the mean mid-point slope parameter estimates of competition curves for all ligands should not be significantly different from unity. In addition, we did not expect significant variation in the location of the competition curves for each ligand between experiments.

The competition curves for JB93182, L-365,260, YM022, PD134,308, PD140,376 and SR27897 all had mean mid-point slope parameters ($n_H$) which were not significantly different from unity (Table 2). In addition, there was no significant difference between the behaviour of any of the ligands, in terms of mid-point slope parameter and estimated $pK_I$ values ($r = 0.99$, $P < 0.002$), at sites in mouse cortex labelled with either [$^3$H]-PD140,376 or [$^{125}$I]-BH-CCK-8S (Figure 1; Table 2). For instance, the $pK_I$ estimates for JB93182 in the mouse cortex assay, when competing with [$^{125}$I]-BH-CCK-8S and [$^3$H]-PD140,376 for $CCK_B$/gastrin binding sites, were $8.74 \pm 0.15$ ($n = 4$) and $8.88 \pm 0.10$ ($n = 3$), respectively.

## *Analysis of competition curves in rat cortex*

Our previous analysis of the behaviour of L-365,260 suggested that the rat cortex expressed a variable proportion of two $CCK_B$/gastrin sites. However, in the first instance, without prejudice to this previous finding, the data were analysed by fitting the Hill equation (2).

## *Hill equation fitting*

YM022, SR27897, PD134,308, PD140,376 and JB93182 produced concentration-dependent inhibition of the specific binding of [$^{125}$I]-BH-CCK-8S to $CCK_B$/gastrin binding sites in rat cortex (Figure 2). The mean mid-point slope parameter estimates from competition curves for JB93182 (0.82), PD134,308 (0.83) and PD140,376 (0.86) were not significantly different from unity. The mean mid-point slope parameter estimate for L-365,260 (0.85), obtained previously with the exceptionally large data set ($n = 48$), was significantly different from unity (Table 3). The apparent affinity ($pIC_{50}$) values of PD140,376 and SR27897 for $CCK_B$/gastrin binding sites in the rat cortex were not significantly different from those estimated in the mouse cortex. In contrast, the mean apparent affinities expressed by L-365,260 and PD134,308 were both significantly lower, while those expressed by JB93182 and YM022 were significantly higher, than those obtained in the mouse cortex.

## *Two site model fitting*

In view of the previous evidence for the expression of a variable proportion of two $CCK_B$/gastrin binding sites in the

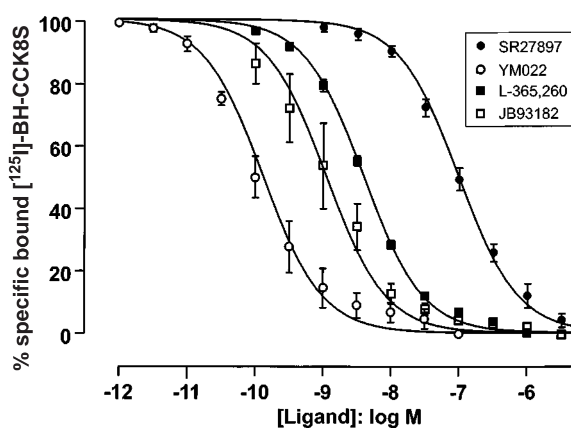


**Figure 1** Competition between [$^{125}$I]-BH-CCK-8S and increasing concentrations of ligands for $CCK_B$/gastrin binding sites in mouse cortex. Data represent the mean ± s.e.mean of 5–47 experiments (see Table 2) where each point was determined in triplicate. The curves shown superimposed on the mean experimental data points were obtained by simulation using equation (2) where the parameters were set at the mean values estimated by fitting each replicate curve to that equation. The parameters used in the simulations are presented in Table 2.

**Table 2** Analysis of multiple data sets from competition experiments between [$^{125}$I]-BH-CCK-8S or [$^3$H]-PD140, 376 and CCK/gastrin receptor ligands in mouse cortex (see text for details)

| | *[$^{125}$I]-BH-CCK-8S* | | | *[$^3$H]-PD140,376* | | | |
|---|---|---|---|---|---|---|---|
| *Ligand* | **$pIC_{50}$* | *$n_H$* | n | *$pIC_{50}$* | *$n_H$* | *$pK_I$* | n |
| L-365,260 | †8.42 ± 0.03 | 1.01 ± 0.02 | 47 | 7.92 ± 0.08 | 0.94 ± 0.06 | 8.17 ± 0.07 | 9 |
| JB93182 | 8.74 ± 0.15 | 0.98 ± 0.11 | 4 | 8.65 ± 0.09 | 0.85 ± 0.08 | 8.88 ± 0.10 | 3 |
| PD134,308 | 8.97 ± 0.10 | 0.90 ± 0.04 | 6 | 8.45 ± 0.14 | 1.14 ± 0.13 | 8.66 ± 0.18 | 5 |
| PD140,376 | 9.23 ± 0.12 | 1.07 ± 0.05 | 6 | 8.80 ± 0.13 | 0.87 ± 0.09 | 9.28 ± 0.13 | 6 |
| YM022 | 9.88 ± 0.12 | 0.91 ± 0.13 | 5 | n.d. | n.d. | n.d. | |
| SR27897 | 7.00 ± 0.07 | 0.91 ± 0.05 | 5 | 6.74 ± 0.12 | 1.24 ± 0.27 | 6.96 ± 0.13 | 5 |

The parameter estimates were obtained by fitting the individual replicate competition curve data to the Hill equation (2). *$pIC_{50} \sim pK_I$ because $(1 + [L]/K_D) \sim 1.1$. †L-235,260 data previously presented (Harper *et al.*, 1996a). n.d. = not determined.

rat cortex, a two-site model (equation 3) was also applied to the data. First, an attempt was made to fit the individual competition curves obtained from replicate experiments for each compound. Given the variation in the proportion of receptors, evident from both the previous analysis of the L-365,260 data and by inspection of the individual curves obtained with the compounds in this study (see Figure 3, for example), the receptor affinity and the receptor ratio was allowed to vary for each replicate curve in the fit.

A satisfactory fit could not be obtained for all the curves. Thus, as observed previously when the L-365,260 data set was analysed, there was not always sufficient information (i.e. biphasicity) within each curve to allow estimation of each of the parameters. Therefore, a second model fit was performed in which all of the replicate curves for each compound were fitted to the model simultaneously. In the model fit the proportion of $R_1$ to $R_2$ was allowed to vary between each replicate curve although single values for $IC_{501}$ and $IC_{502}$ were estimated for each compound. The model fits (equation 3) all converged and estimates of the $pIC_{50}$ of each compound at the gastrin $G_1$ ($pIC_{501}$) and gastrin $G_2$ ($pIC_{502}$) sites were obtained (Table 4). The fit of the data to the two-site model was significantly better than to the Hill equation for each ligand (JB93182, $F_{(53,46)}=10.66$; PD134,308, $F_{(59,52)}=4.40$; PD140,376, $F_{(54,48)}=8.18$; YM022, $F_{(86,76)}=2.27$; SR27897, $F_{(44,39)}=11.46$). The compounds all appeared to express some

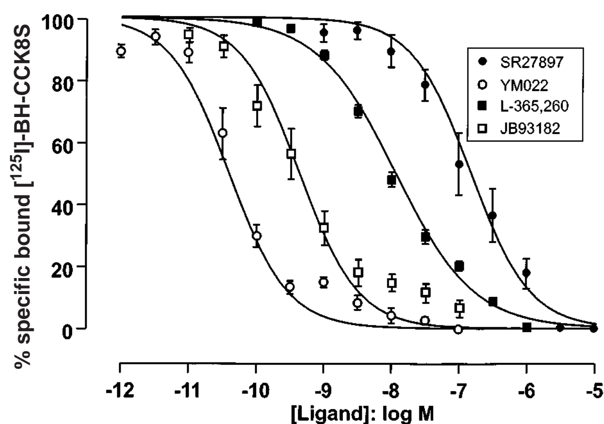


**Figure 2** Competition between [$^{125}$I]-BH-CCK-8S and increasing concentrations of ligands for $CCK_B$/gastrin binding sites in rat cortex. Data represent the mean ± s.e.mean of 5–48 experiments (see Table 3) where each point was determined in triplicate. The curves shown superimposed on the mean experimental data points were obtained by simulation using equation (2) where the parameters were set at the mean values estimated by fitting each replicate curve to that equation. The parameters used in the simulation are presented in Table 3.

**Table 3** Analysis of multiple data sets for competition experiments between [$^{125}$I]-BH-CCK-8S and CCK/gastrin receptor ligands of $CCK_B$/gastrin binding sites in rat cortex (see text for details)

| *Compound* | *$*pIC_{50}$ ± s.e.mean* | *$n_H$ ± s.e.mean* | n |
|---|---|---|---|
| L-365,260 | †7.96 ± 0.05 | **0.85 ± 0.03 | 48 |
| JB93182 | 9.36 ± 0.16 | 0.82 ± 0.10 | 8 |
| PD134,308 | 8.43 ± 0.07 | 0.83 ± 0.09 | 6 |
| PD140,376 | 8.90 ± 0.16 | 0.86 ± 0.11 | 5 |
| YM022 | 10.27 ± 0.07 | 0.99 ± 0.22 | 8 |
| SR27897 | 6.87 ± 0.15 | 1.05 ± 0.20 | 5 |

The parameter estimates were obtained by fitting the individual replicate competition curve data to the Hill equation (2). *$pIC_{50}$ ~$pK_I$ because $(1+[L]/K_D)$ ~1.1. **$n_H$ significantly different from unity. †L-365,260 data previously presented (Harper *et al.*, 1996a).

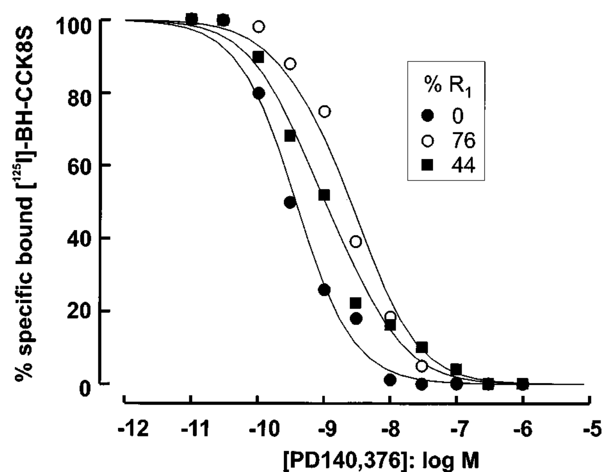


**Figure 3** Representative PD140,376 competition curves obtained in the rat cortex assay using [$^{125}$I]-BH-CCK-8S as the radioligand. The data shown are examples of individual curves obtained in separate experiments ($n=1$ in triplicate). The lines shown superimposed on the individual data sets were obtained using the $R_1$, $R_2$, $pIC_{501}$ and $pIC_{502}$ parameters obtained from the simultaneous two-site model fitting of the individual curves (equation (3) and see Table 4). In the fit, a global $pIC_{501}$ (8.40 ± 0.16) and $pIC_{502}$ (9.44 ± 0.09) were estimated but the percentage of $R_1$ and $R_2$ were allowed to vary for each data set.

**Table 4** Apparent affinities of CCK/gastrin receptor ligands at gastrin-$G_1$ and gastrin-$G_2$ sites in rat cortex (see text for details) using [$^{125}$I]-BH-CCK-8S as radiolabel

| *Compound* | n | *Replicate curve $R_1$ values* (%) | *$G_1$ site $pIC_{501}$* (± s.e.mean) | *$G_2$ site $pIC_{502}$* (± s.e.mean) | *Mouse cortex $pIC_{50}$* (± s.e.mean) |
|---|---|---|---|---|---|
| L-365,260 | 47 | N.A.* | 7.22 ± 0.06 | 8.48 ± 0.06 | 8.42 ± 0.03 |
| JB93182 | 6 | 5, 25, 54, 56, 74, 80 | 9.94 ± 0.16 | 8.57 ± 0.15 | 8.74 ± 0.15 |
| PD134,308 | 6 | 32, 59, 77, 85, 98, 100 | 8.24 ± 0.07 | 9.35 ± 0.21 | 8.79 ± 0.10 |
| PD140,376 | 5 | 0, 44, 57, 68, 76 | 8.40 ± 0.16 | 9.44 ± 0.09 | 9.23 ± 0.12 |
| YM022 | 8 | 0, 3, 17, 45, 48, 65, 70, 100 | 10.54 ± 0.11 | 9.97 ± 0.10 | 9.88 ± 0.12 |
| SR27897 | 5 | 0, 50, 64, 80, 83 | 6.41 ± 0.14 | 7.38 ± 0.08 | 7.00 ± 0.07 |

In the fit to the two site model (equation 3), the $pIC_{50}$ values for the $G_1$ and $G_2$ sites were obtained by applying the model to individual replicate competition curves obtained for each compound in the rat cortex to provide a global estimate with fitting error. The proportion of the two sites was allowed to vary for each replicate ($n$) experiment and third column shows the individual estimates of the percentage of $R_1$ (i.e. $G_1$) sites obtained. The $pIC_{50}$ values for the $G_2$ site in the mouse cortex assay (Table 2) are shown for comparison. These estimates are the mean values of individual curve $pIC_{50}$ estimates. *The data includes examples from the complete range of $R_1$ values from 0 to 100% (see Harper *et al.*, 1996a).

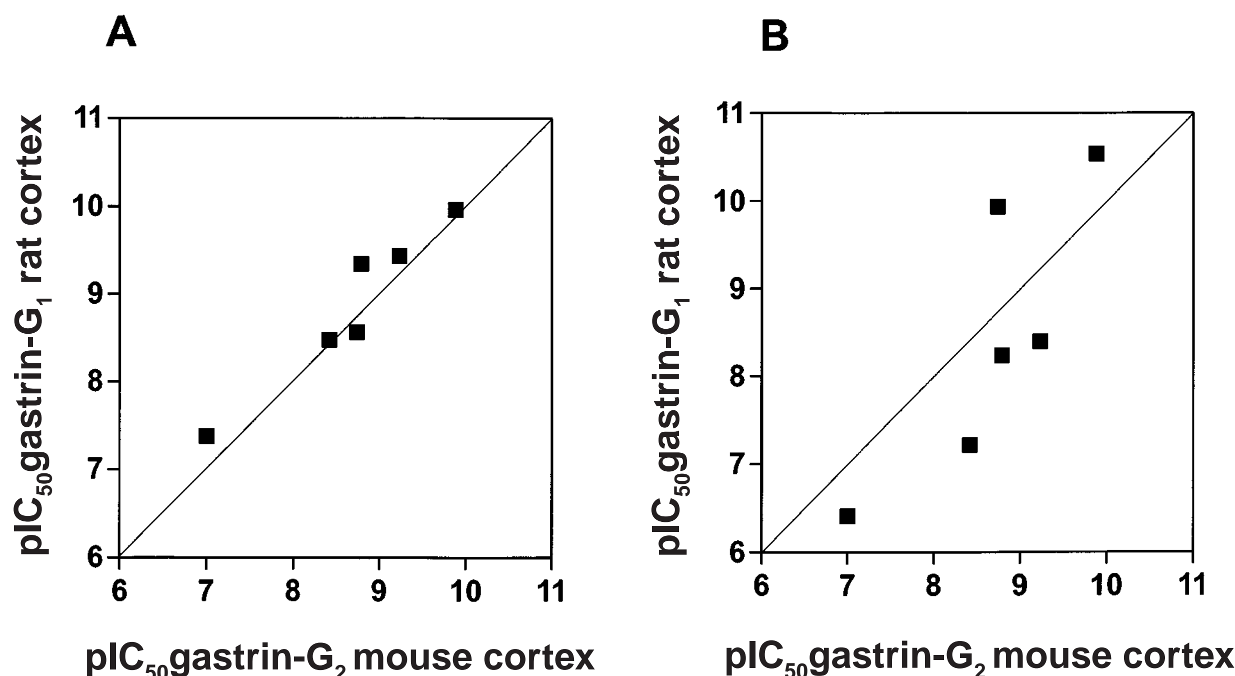


**Figure 4** Comparison of affinity estimates obtained in mouse and rat cortex assays. The line shown superimposed on the data is the line of identity. (A) Comparison of the $G_2$ $pIC_{50}$ values obtained from the mouse cortex data ([$^{125}$I]-BH-CCK-8S as radioligand, Table 2) and the $G_2$ affinity estimate obtained from using the two-site model (equation 3) to analyse the individual replicate curve data obtained in the rat cortex (Table 4). The mouse cortex ($G_2$) $pIC_{50}$ values were indistinguishable from the $G_2$ $pIC_{50}$ values estimated from the two-site model fit of the rat cortex data ($F_{(6,12)}=3.31$). (B) Comparison of the $G_2$ $pIC_{50}$ values obtained from the mouse cortex data ([$^{125}$I]-BH-CCK-8S as label, Table 2) and the $G_1$ estimates obtained using the two-site model (equation 3) to analyse the individual replicate curve data from the rat cortex (Table 4). The mouse $G_2$ $pIC_{50}$ values were significantly different from the rat $G_1$ $pIC_{50}$ values ($F_{(6,12)}=30.91$).

degree of site selectivity (Table 4; JB93182 ~23 fold, PD134,308 ~13 fold, PD140,376 ~11 fold, YM022 ~4 fold and SR27897 ~9 fold).

The mouse cortex ($G_2$) $pIC_{50}$ values were indistinguishable from the corresponding $pIC_{502}$ $G_2$ values estimated from the two-site model fit of the rat cortex data ($F_{(6,12)}=3.31$; Figure 4A) whereas they were significantly different from the rat $G_1$ values ($F_{(6,12)}=30.91$; Figure 4B).

## Discussion

In this study, in an attempt to expose $CCK_B$/gastrin receptor heterogeneity, we investigated the behaviour of a chemically-diverse series of $CCK_B$/gastrin receptor antagonists in the mouse and rat cortex. The low affinities of PD134,308 (6.08), PD140,376 (6.21), L-365,260 (6.50) and JB93182 (5.29) and the high affinity of SR27897 (9.62) in the guinea-pig pancreas assay suggested that any differences between the rat and mouse cortex assays were not due to [$^{125}$I]-BH-CCK-8S labelling differential proportions of $CCK_A$ binding sites in rat and mouse cortex assays. The high Hill slope value associated with an unusually high variance ($1.55 \pm 0.21$; $n=5$) obtained with JB93182 in the $CCK_A$ assay could be due to the high concentrations of the compound which were required to define the competition curve inhibiting the non-specific binding of the radioligand.

In the mouse cortex, the L-365-260 and PD134,308 data were consistent with those previously reported in this tissue when either [$^{125}$I]-BH-CCK-8S (Hughes *et al.*, 1990) or [$^3$H]-pBC264 (Durieux *et al.*, 1992; Bertrand *et al.*, 1994) was used as the radioligand. In addition, the data obtained for all the competing compounds were consistent with our previous observation that this tissue appears to contain a homogeneous population of $CCK_B$/gastrin binding sites. These sites have a high affinity for L-365,260 ($pK_I=8.41 \pm 0.01$, $n=47$; Harper *et al.*, 1996a) and were provisionally termed gastrin $G_2$. In this study, the observation that there was a good correlation between the estimated affinities of the compounds regardless of whether an antagonist ([$^3$H]-PD140,376) or agonist ([$^{125}$I]-BH-CCK-8S) was used to label the sites indicated that any complexity in this, and the previous study, was not a consequence of using an agonist as the radioligand.

In the rat cortex assay, PD134,308, YM022 and L-365,260 had estimated affinity values for $CCK_B$/gastrin binding sites that were similar to those reported previously in rat cortex. For instance, affinity values of 8.20, 10.17 and 7.72 have been reported for PD134,308, YM022 and L-365,260, respectively (Nishida *et al.*, 1994). However, in contrast to our previous study (Harper *et al.*, 1996a), none of these data, at first sight, could be considered to provide compelling evidence for receptor heterogeneity. Thus, the mean Hill slope parameter estimates obtained from the competition curves for PD134,308, PD140,376, YM022, JB93182 and SR27897 were not significantly different from unity (Table 3). However, JB93182 and YM022 expressed significantly higher affinities and PD134,308 expressed significantly lower affinity in the rat cortex assay to that expressed in the mouse cortex assay.

The tissue-dependent affinity of JB93182, YM022 and PD134,308 could be due to these compounds detecting species differences in the amino-acid sequence of the receptor. The species dependence of antagonist affinity has previously been reported for other $CCK_B$/gastrin receptor ligands such as L-365,260 (Lotti & Chang, 1989; Beinborn *et al.*, 1993) and LY247348 (Hunter *et al.*, 1993). However, in view of data obtained for JB93182 and PD134,308 in rat and mouse isolated, lumen-perfused, stomach assays (Roberts *et al.*, 1996b; Shankley *et al.*, 1997), it seems unlikely that speciation

of $CCK_B$/gastrin receptors could account for the differences in antagonist affinity detected in this study. For instance, the behaviour of JB93182 in the isolated, lumen-perfused rat stomach bioassay ($pK_B = 9.90 \pm 0.18$; Shankley *et al*., 1997), in agreement with our previous conclusion (Roberts *et al*., 1996a), could be accounted for by the presence of a homogeneous population of $CCK_B$/gastrin receptors ($G_1$). By contrast, in the mouse stomach, a tissue which was suggested to contain a variable population of gastrin-$G_1$ and gastrin-$G_2$ receptors (Roberts *et al*., 1996a), the slope of the Schild plot for JB93182 was significantly different from unity ($b = 0.60 \pm 0.09$). Moreover, these data could be described by a two-receptor model with $pK_B$ values of $\sim 9.1$ (gastrin-$G_2$) and $\sim 9.9$ (gastrin-$G_1$). Therefore, an alternative explanation for the species differences in the apparent affinity of JB93182, PD134,308 and YM022 is that provided by our original analysis of the complexity in L-365,260 data obtained in rat and mouse cortex assays (Harper *et al*., 1996a) i.e. that the mouse cortex assay expresses one $CCK_B$/gastrin binding site whereas the rat cortex expresses two sites.

In the current study, the finding of Hill slope estimates in the rat cortex which were not significantly different from unity could simply mean that we had insufficient data to expose the two sites. Hill slope parameter estimates from a single-site model fit of a system expressing two sites are dependent on both the affinity of the compound at the two sites and the numbers of each site labelled. Indeed, in the previous study we were only able to obtain compelling evidence that the mean Hill slope of L-365,260 competition curves was significantly different from unity when we analysed an exceptionally large data set (Harper *et al*., 1996a). The difficulty in detecting the two sites appeared to result from the relatively low selectivity of L-365,260 ($\sim 18$ fold) and the between-experiment variability in the ratio of the two sites was an added complicating factor. The large data set allowed the definition of the relationship between the Hill slope value ($n_H$) and the location ($pIC_{50}$) of individual competition curves expected for a system with variable expression of two sites (Harper *et al*., 1996a). Because of this complexity, we only expected to obtain clear evidence for receptor heterogeneity (i.e. Hill slope parameters significantly different from unity) if either the compounds investigated in this study expressed greater receptor selectivity than L-365,260 or if the selectivity of any of the compounds was equivalent to that of L-365,260 and we performed what might be considered to be an unrealistic number of replicate experiments. Therefore, on the basis of the prior knowledge gained from the studies using L-365,260, we considered it justifiable to analyse the data obtained in this study along lines consistent with our original hypothesis, namely, the presence of two $CCK_B$/gastrin binding sites ($G_1$ and $G_2$) in the rat cortex.

A satisfactory fit was obtained when the proportion of $G_1$ and $G_2$ sites was allowed to vary for each replicate competition experiment. This indicated that all of the compounds expressed some degree of selectivity for the two sites and that the proportion of the sites varied between experiments as was found in the original analysis. The finding that the mouse cortex ($G_2$) $pIC_{50}$ values were different from the rat cortex $G_1$ $pIC_{50}$ values (Figure 4B) but indistinguishable from the rat cortex $G_2$ values (Figure 4A) was consistent with our original conclusion that one of the sites is common to both the mouse and rat cortex. Moreover, the new data was also consistent with the previous conclusion that the $G_1$ and $G_2$ sites characterized in the binding studies were equivalent to the sites characterized in the rat ($G_1$) and the mouse ($G_1/G_2$) isolated, lumen-perfused, stomach assays. Thus, the affinity estimates for JB93182 and PD134,308 at the rat cortex $G_2$ and $G_1$ sites (Table 4) were similar to those estimated in the functional assays ($G_2$-receptor: JB93182 $\sim 9.1$, PD134,308 $\sim 9.2$; $G_1$-receptor: JB93182 $\sim 9.9$; PD134,308 $\sim 7.6$; Roberts *et al*., 1996b; Shankley *et al*., 1997).

A reasonable distribution in the variation of the proportion of $R_1$ and $R_2$ sites was present in the replicate experiments for each compound (Table 4) as the two-site model fits converged. However, the fit indicated that within the PD134,308 data set the $R_1$ site was predominant in four of the five replicate experiments. Even in the fifth replicate there were still 32% of the $R_1$ sites present. If the two-site model interpretation is valid, then reliable estimates for $pIC_{501}$ and $pIC_{502}$ are expected only when the data set for each compound includes individual competition curves that lie at either end of the distribution of the $pIC_{50}$ values (i.e. under the conditions where $\sim 100\%$ of either site are present). Thus, for PD134,308 the $G_2$ site $pIC_{50}$ estimate is based on relatively less information and, accordingly, the value was the furthest from that predicted by the mouse cortex assay.

The data obtained in this study are consistent with the hypothesis that there are two, pharmacologically-distinguishable, $CCK_B$/gastrin binding sites in rat cortex (gastrin-$G_1$ and $G_2$). To provide further evidence for the presence of two sites, it is clear that more highly selective ligands are required. However, in the absence of such ligands, one suitable approach for subtype discrimination would be to attempt to label and characterize one of the receptor populations with a receptor-selective radioligand. The current analysis suggests that JB93182 expresses an approximate 23 fold selectivity for the gastrin-$G_1$ site. Therefore, if our interpretation of the rat cortex data is correct it should be possible to label only gastrin-$G_1$ sites in this tissue with a radiolabelled form of JB93182. Therefore, the affinity values obtained for compounds competing with this radioligand should be similar to the gastrin-$G_1$ affinity estimates obtained in this study. Characterization of the binding of [$^3$H]-JB93182 forms the subject of the accompanying report (Harper *et al*., 1999).

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