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Analysis of variation in L-365,260 competition curves in radioligand binding assays

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1 For several years, we have used the cholecystokinin $(CCK)_B$/gastrin receptor selective antagonist, L-365,260, as a reference compound in a variety of studies in CCK_B/gastrin receptor radioligand binding assays. Here, we have analysed the competition curve data sets obtained between L-365,260 and [^{125}I]-BH-CCK8S in guinea-pig gastric gland and mouse and rat cerebral cortex preparations.

2 Competition curves obtained for L-365,260 in the mouse cortex assay were not different from rectangular hyperbolae (slope = 1.01 ± 0.02) implying the presence of a single population of binding sites ($pK_I = 8.41 \pm 0.01$; data from 47 experiments, slope constrained to unity). However, in the rat cortex and guinea-pig gastric gland assays, the mean slope of the competition curves was significantly less than one and the mean apparent pK_I significantly lower than that obtained in the mouse cortex (slope = 0.85 ± 0.03, 0.90 ± 0.03; apparent $pK_I = 7.98 \pm 0.05$, 8.07 ± 0.05; 48 and 45 experiments, in rat and guinea-pig, respectively). The distribution of the individual pK_I and slope estimates of the competition curves in these two assays was consistent with expectations for the variable expression (in terms of absolute number and proportion) of two binding sites. The two sites were characterized by pK_I values for L-365,260 of 8.50 ± 0.04 and 8.48 ± 0.04 for the high affinity site and 7.32 ± 0.04 and 7.22 ± 0.06 for the low affinity site in guinea-pig and rat, respectively.

3 The affinity estimates for L-365,260, although obtained on different tissues, are consistent with data obtained from the analysis of L-365,260 antagonism of pentagastrin-stimulated responses in mouse and rat stomach (acid secretion) and guinea-pig gastric muscle (isotonic contraction) assays. To this extent, these data suggest the existence of two CCK_B/gastrin receptor subtypes.

Keywords: Cholecystokinin; gastrin; mouse cortex; rat cortex; guinea-pig gastric glands; L-365,260; [^{125}I]-BH-CCK8S

Introduction

The preceding paper (Roberts *et al.*, 1996) describes an analysis of replicate data sets obtained from the interaction between pentagastrin and a selective cholecystokinin $(CCK)_B$/gastrin receptor antagonist, L-365,260, on isolated, gastrointestinal tissue assays. In the isolated, lumen-perfused, immature rat stomach assay, L-365,260 behaved as a simple competitive antagonist acting at a homogeneous population of receptors. A global analysis of the data from five separate, highly-reproducible, experiments obtained over a five year period gave a Schild slope estimate of 1.00 ± 0.10 and a pK_B estimate of 7.54 ± 0.03. In contrast, the replicate data obtained with the same antagonist over the same period in the isolated, lumen-perfused mouse stomach and guinea-pig gastric muscle assays were not consistent with the presence of a single CCK_B/gastrin receptor population. The guinea-pig gastric muscle data were relatively reproducible between experiments but some individual Schild plot slopes and the global analysis slope parameters were significantly less than unity (slope = 0.80 ± 0.07, $pA_2 = 8.56 \pm 0.05$). The data obtained in the mouse stomach were highly variable between experiments and, in addition, significantly more variable than data obtained for the interaction between histamine and the H_2-receptor antagonist, famotidine, on the same assay during the same period. The Schild plot slopes ranged from being extremely flat (0.2) to being not significantly different from unity and the pA_2 values ranged from 7.68 to 8.70. Overall, the results were consistent with model expectations for the variable expression of two CCK_B/gastrin receptors in the mouse stomach and guinea-pig gastric muscle assays.

In this study, we have performed an analysis of L-365,260 competition data obtained over a three year period in three CCK_B/gastrin receptor radioligand binding assays which were routinely used together with isolated tissue assays in a drug discovery programme (Kalindjian *et al.*, 1994). In mouse and rat cerebral cortex and guinea-pig gastric mucosa radioligand binding assays, the non-selective CCK/gastrin receptor agonist [^{125}I]-BH-CCK8S was used as the radiolabel. Ideally, we would have used radioligand binding assays prepared from the same tissues used for the isolated bioassays, but we were unable to obtain a sufficiently high signal-to-noise ratio to permit quantitative analysis when guinea-pig fundus or rat or mouse gastric mucosal tissues were used. An account of an initial analysis of these data was presented to the British Pharmacological Society (Harper *et al.*, 1995).

Methods

Preparation of mouse and rat cerebral cortex cells

The preparation of mouse and rat cortex cells 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 ± 3°C) of the following composition (mM): NaCl 130, KCl 4.7, $MgCl_2$ 5, HEPES 10, EDTA 1 plus 0.125 g l^{-1} bacitracin. The tissue was weighed and homogenized in 40 ml of ice-cold buffer in a Teflon-in-glass homogenizer. The homogenate was centrifuged at 39,800 *g* for 20 min at 4°C, the supernatant discarded and the pellet resuspended by homogenization in fresh HEPES-NaOH buffer and recentrifuged. Rat and mouse cortex cells were resuspended in HEPES-NaOH buffer to give final tissue concentrations of 5 mg ml^{-1} and 2 mg ml^{-1} (original wet weight), respectively. This method of preparation produced predominantly intact cells as judged by light microscopy.

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Preparation of glands from guinea-pig gastric mucosa

The preparation of guinea-pig gastric glands was based on the methods of Soumaron *et al.* (1977), Praissman & Walden (1984) and Chang *et al.* (1986). In brief, male guinea-pigs (Dunkin Hartley 300–500 g) were killed by cervical dislocation and decapitation. Their stomachs were rapidly removed and rinsed with ice-cold gassed (95%O_2;5%CO_2) HEPES-NaOH buffer (pH 7.4 at 21±3°C) of the following composition (mM): NaCl 130, HEPES 15, KCl 4.7, NaH_2PO_4 3, $MgSO_4$ 2, $CaCl_2$ 1, glutamine 4, glucose 5 and containing 0.1% BSA (Buffer A). The gastric mucosa from two guinea-pigs was scraped away from the underlying muscularis mucosa in a glass microscope slide and immediately homogenized in 100 ml ice-cold, gassed, buffer by a Griffith's tube homogenizer. Following a 10 min incubation period at 4°C, the homogenate was centrifuged at 1,900 *g* for 5 min in 10 ml aliquots. The supernatants were discarded and the pellets resuspended in 80 ml ice-cold gassed buffer (containing 0.05% BSA; Buffer B), in a Teflon-in-glass homogenizer, and the homogenate recentrifuged as above. The final pellet was resuspended in a gassed incubation buffer (Buffer C) in a Teflon-in-glass homogenizer to give a final tissue concentration of 4 mg ml^{-1} (original wet weight). Buffer C also contained 1.25 μM each of phosphoramidon, captopril and bestatin, 12.5 μM aprotinin, 1.25 mM dithiothreitol, 0.25 μM Fe (III) nitrate, 5 μM 2-NAP (pK_B at CCK_A receptors = 6.5; Hull *et al.*, 1993), 2% BME [50×] amino acids and 1% MEM [100×] vitamins. These concentrations were reduced by 20% by dilution with the Buffer B in the final incubates. This method of preparation appeared to produce a mixture of gastric glands and cell clumps as judged by light microscopy.

Incubation conditions – competition studies

All CCK/gastrin receptor antagonists were diluted in either Buffer B (gastric mucosa assay) or HEPES-NaOH (cortical assays). Aliquots 50 μl of concentrations from 1 nM to 100 μM, were incubated in triplicate with guinea-pig gastric glands and mouse or rat cortex cells (400 μl) in a final volume of 500 μl with appropriate buffer containing [^{125}I]-BH-CCK8S (20 pM). Non-specific binding was defined with 1 μM L-365,260 or 1 μM PD134,308, another CCK_B/gastrin receptor antagonist, and was shown to be identical with either ligand in each assay. Mouse and rat cortex and guinea-pig gastric gland assays were incubated at 21±3°C for 150, 150 and 100 min, respectively. The 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 (pH 6.9 at 21±3°C). Bound [^{125}I]-BH-CCK8S was determined by counting each filter (1 min) in a LKB Clinnigamma counter. In preliminary experiments (data not shown), reverse-phase h.p.l.c. indicated that the radiolabel and L-365,260 were not degraded to a significant extent during incubation under the conditions of each assay.

Experimental design and protocol

Cells were prepared from the cerebral cortex of at least three rats and mice and from at least two guinea-pigs regardless of the quantity of tissue required. Fully-defined competition curves were obtained by examining a wide range of ligand concentrations at 0.5 log unit intervals. The ability of each concentration of competing ligand to inhibit specific binding was determined in triplicate. All data are presented as mean values±s.e.mean unless indicated.

Data analysis

The individual competition curve data were expressed as the percentage of the decrease in specific binding of [^{125}I]-BH-CCK8S within each experiment. Initially, these data were fitted to the following Hill equation which describes the relationship between the amount of bound ligand (B) and free ligand concentration ([L]) for a single binding site, using a derivative-free, non-linear, regression programme (BMDP Statistical Software, Module AR; Dixon, 1992)

$$B = \frac{R \cdot [L]^{n_H}}{K_I^{n_H} + [L]^{n_H}} \quad (1)$$

In the equation, R is the total number of specific binding sites occupied by the radiolabel in the absence of the competing ligand which was fixed in the fitting procedure at a value of 100%. n_H is the midpoint slope parameter and K_I is the midpoint location parameter which, in practice, was estimated as $\log_{10}K_I$ on the basis that K_I values are log normally-distributed. When the estimated value of n_H was not significantly different from unity, the data were re-fitted with the value of n_H constrained to unity. When the data did not fit the one-site model, that is, the estimate of n_H was significantly different from unity, then the data from each assay were fitted simultaneously to the following equation which describes the binding of a ligand to independent sites (R_1 and R_2), governed by the midpoint location parameters K_{I1} and K_{I2}, respectively.

$$B = \frac{R_1 \cdot [L]}{K_{I1} + [L]} + \frac{R_2 \cdot [L]}{K_{I2} + [L]} \quad (2)$$

In the fit, a single $\log_{10}$ value of K_{I1} and K_{I2} was estimated for all the data. The sum of R_1 and R_2 was constrained to 100% although the proportion of R_1 and R_2 was allowed to vary between each experimental data set.

Materials

[^{125}I]-Bolton Hunter labelled CCK8S ([^{125}I]-BH-CCK8S) with specific activity of 2200 Ci $mmol^{-1}$ was obtained from New England Nuclear Dupont. L-365,260 (3**R**-(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N′-3-methylphenyl urea), and 2-NAP (2-naphthalenesulphonyl-l-aspartyl-(2-phenethyl)amide) were synthesized by James Black Foundation chemists. Captopril, phosphoramidon, bacitracin and bestatin, HEPES, EGTA, Trizma base, bovine serum albumin, (D,L)-dithiothreitol (Cleland's reagent), BME [50×] amino acids, MEM [100×] vitamins and L-glutamine were obtained from the Sigma Chemical Co., Poole, Dorset. Aprotinin (Trasylol) was obtained from Boehringer Mannheim U.K. (Diagnostics and Biochemicals) Ltd, Lewes, East Sussex.

Results

Characterization of mouse and rat cerebral cortex and guinea-pig gastric gland radioligand binding assays

A tissue, concentration-dependent, increase in total binding, non-specific binding and specific binding of [^{125}I]-BH-CCK8S was obtained in cells from the mouse and rat cortex and gastric gland preparations from the guinea-pig (Figure 1). [^{125}I]-BH-CCK8S was used as the radioligand because, in preliminary experiments, the label of choice, [^{125}I]-gastrin (1–17), appeared to be significantly degraded under the assay conditions (data not shown), a previously recognized problem reviewed by Kleveland & Waldum (1991). The relationship between tissue concentration and specific binding was linear up to concentrations of 3, 8 and 9 mg ml^{-1} (original wet weight), in the mouse, rat and guinea-pig preparations, respectively. At tissue concentrations of 2, 5, and 4 mg ml^{-1}, the percentage of added [^{125}I]-BH-CCK8S bound was 9.2±0.7, 11.8±1.3 and 8.4±1.0% (n = 3 to 6), respectively and these concentrations were used in all subsequent experiments. The corresponding non-specific binding was 22.8±1.9, 49±1.5 and 40±1.9% (n = 3 to 6) of the total bound radiolabel.

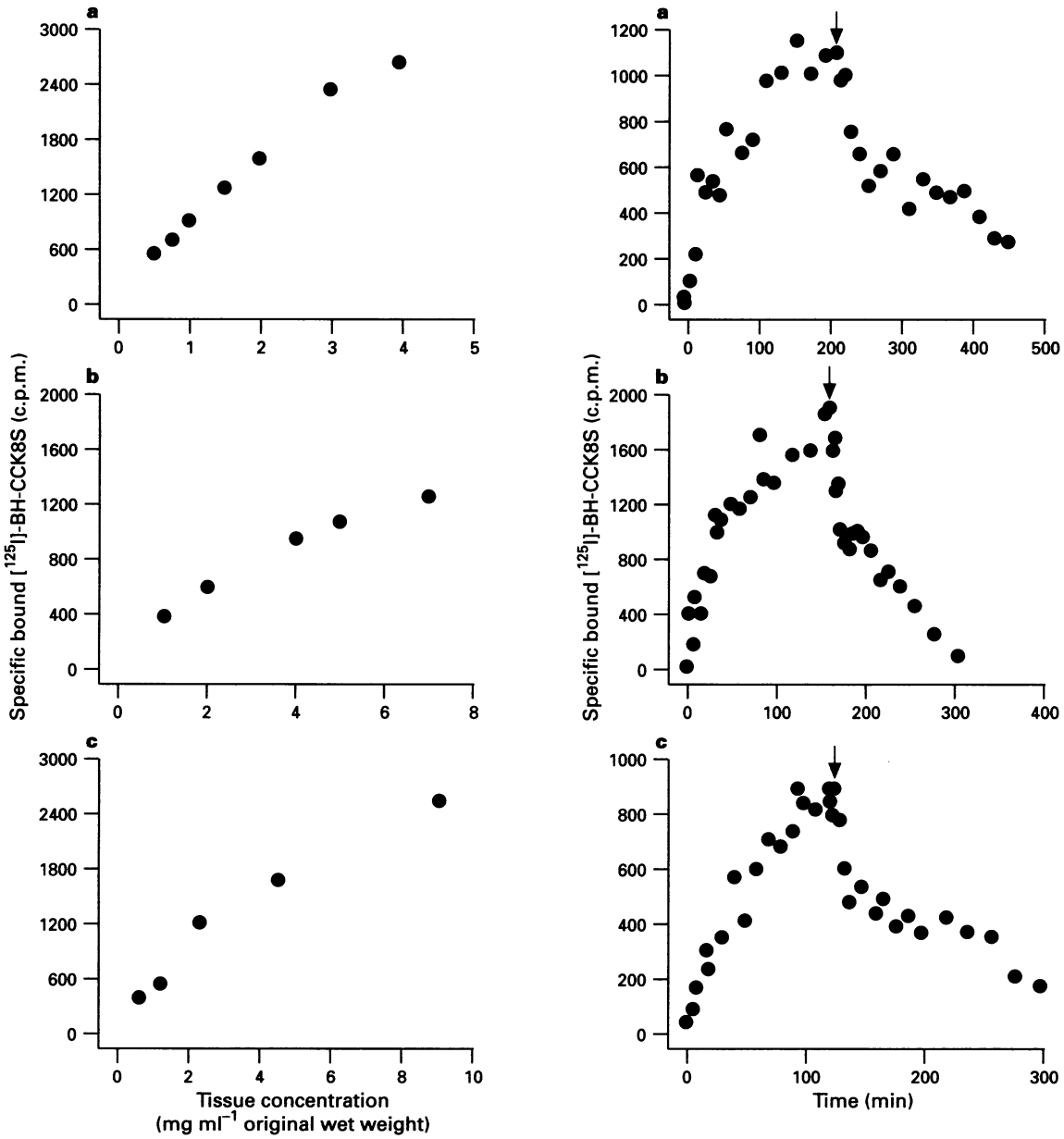

Figure 1 Left hand panels show specific binding of [^{125}I]-BH-CCK8S as a function of increasing concentration of (a) mouse cortex cell, (b) rat cortex cell, and (c) guinea-pig gastric mucosa gland tissue ($n = 3/6$). Aliquots (400 μl) of various concentrations of tissue were incubated in triplicate at 21 ± 3°C with 20 pM [^{125}I]-BH-CCK8S for 150, 150 and 100 min in the mouse, rat and guinea-pig assays, respectively. Total binding and non-specific binding were defined with 50 μl buffer and 50 μl 10 μM L-365,260, respectively (see text for details). Right hand panels show data obtained in association/dissociation experiments in (a) mouse cortex, (b) rat cortex, and (c) guinea-pig gastric mucosa assays (see text for details). Arrows indicate addition of excess (1 μm) unlabelled CCK8S. Data are representative of three replicate experiments in each assay.

The specific binding of [^{125}I]-BH-CCK8S (20 pM) reached equilibrium within 150 min in the mouse and rat cortex and within 80 min in the guinea-pig assay (Figure 1). The total, non-specific and specific binding remained constant for a further 300 min in the mouse and rat assays and 90 min in the guinea-pig assay (data not shown). The reversibility of binding was demonstrated in dissociation experiments in which 20 pM [^{125}I]-BH-CCK8S was incubated until equilibrium was reached and then CCK8S added to give a final assay concentration of 1 μM (Figure 1).

Exclusion of CCK_A binding sites

As judged by the competition curve data obtained with the CCK_A receptor antagonist, 2-NAP, chosen on the basis of its high selectivity (CCK_A receptor, $pK_B \sim 6.5$; CCK_B/gastrin receptor, $pK_B \sim 4.1$ Hull *et al.*, 1993), [^{125}I]-BH-CCK8S did not label a significant number of CCK_A receptor sites in the rat and mouse cortex assays (see Figure 2). However, in the guinea-pig gastric mucosa assay, preliminary experiments indicated that approximately 10–20% of the total specific binding could be displaced by CCK_A receptor selective concentrations (i.e. sub-micromolar) of 2-NAP. Therefore, 4 μM 2-NAP, sufficient to produce greater than 90% occupancy of the CCK_A sites but less than 5% occupancy of CCK_B/gastrin sites, was included routinely in the buffer for this assay.

Analysis of L-365,260 competition curve data

L-365,260 produced a concentration-dependent inhibition of the specific binding of [^{125}I]-BH-CCK8S in all of the competition experiments performed on the three assays (Figure 3). All of the curves obtained in the mouse cortex assay appeared to be monophasic and the mean midpoint slope parameter calculated from 47 replicate experimental data sets was not significantly different from unity ($n_H = 1.01 \pm 0.02$; Table 1).

Therefore, the data were re-fitted to the single-site model with the midpoint slope parameter constrained to unity and, assuming the data was log-normally distributed, a mean pK_I value of 8.41 ± 0.01 was estimated. The large amount of data allowed us to test the validity of the assumption that the data were normally distributed. Tests of normality were performed on the frequency distributions of the individual values expressed as linear and logarithmic quantities according to the methods of Snedecor & Cochran (1967). Only when the K_I values were expressed as pK_I values was there no significant departure from a normal distribution (Figure 4).

When a one-site model with unconstrained slope parameter was fitted to the data from 48 and 45 replicate experiments in the rat cortex and guinea-pig gastric mucosa assays, respectively, it appeared that the pK_I and n_H estimates were more variable than those obtained for the mouse cortex (Figure 5). The mean slope parameter estimates of the competition curves (Table 1, Figure 5) were found to be significantly different from unity (rat, 0.85 ± 0.03, guinea-pig, 0.90 ± 0.03) and the mean midpoint location parameter (referred to as the apparent pK_I) was significantly lower than that obtained in the mouse cortex (rat, 7.96 ± 0.05, guinea-pig, 8.07 ± 0.05; Figure 5). Although the mouse values appeared to be evenly clustered around the mean co-ordinate the individual pK_I values in the rat cortex and guinea-pig mucosa ranged from 8.8 to 7.2 and were not distinguishable from a distribution generated by use of a two-site model (see Figure 5 for details). Examples of individual competition curves selected from the outer limits and the middle of this distribution demonstrated the relationship between n_H and pK_I and are shown in Figure 6b and c together with comparative examples of curves obtained in the mouse cortex assay (Figure 6a). Having established that the data obtained in the rat and guinea-pig assays could not be explained by a one-site model, they were re-fitted to a simple two-site model (equation 2).

Fitting the data to a two-site model

In practice, the two-site model fit did not converge when the individual data sets were used, presumably because there was insufficient biphasicity within any one data set to define two sites. However, it was possible to obtain a fit when all the data from each assay tissue were fitted simultaneously (Table 1). In the fitting procedure a single value of pK_{I1} and pK_{I2} was estimated for all the data. The sum of R_1 and R_2 was constrained to 100% although the proportion of R_1 and R_2 was allowed to vary between individual experimental data sets. The mean value estimated for the percentage of R_2 sites was 47.6 ± 4.3 and 50.8 ± 4.3% in the rat cortex and guinea-pig gastric mucosa, respectively, although there were examples in both data

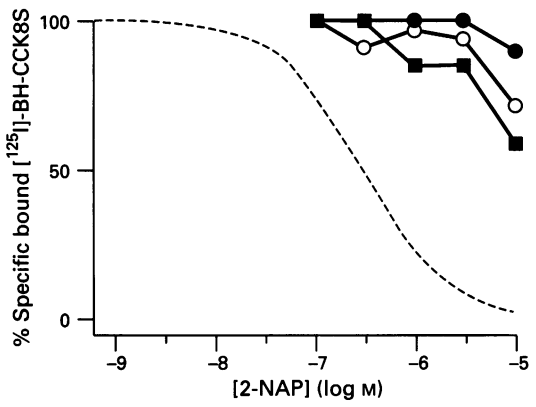

Figure 2 Competition between 2-NAP, a CCK_A receptor antagonist, and the specific binding of [^{125}I]-BH-CCK8S in mouse cortex (●), rat cortex (○) and guinea-pig gastric mucosa (■) assays ($n=3$). Mean competition data are shown in each of the three assays performed under the conditions detailed in the text. The hatched line shows the expected location of a competition curve for 2-NAP on the assumption that the radiolabel only occupies a small fraction of CCK_A receptors (i.e. so that $pIC_{50} \sim pK_I = 6.5$).

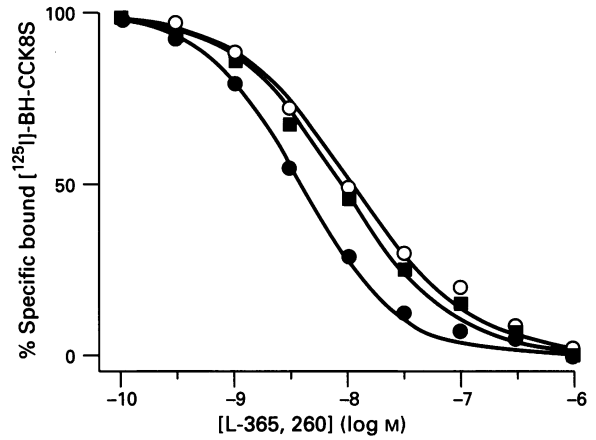

Figure 3 Competition between L-365,260 and the specific binding of [^{125}I]-BH-CCK8S in mouse cortex ($n=47$, ●), rat cortex ($n=48$, ○) and guinea-pig gastric mucosa ($n=45$, ■) assays. The curves shown superimposed on the mean experimental data points were obtained by simulation by use of equation (1) with the parameters set at the mean values estimated by fitting each replicate curve to that equation. The parameters used in the simulations are presented in Table 1.

Table 1 Analysis of multiple data sets from competition experiments between L-365,260 and [^{125}I]-BH-CCK8S at CCK/gastrin sites

	One-site model curve-fitting parameters		
Assay	*No. of experiments*	pK_I	n_H
Mouse cortex	47	8.42 ± 0.03	1.01 ± 0.02
(Mouse cortex$^+$	47	8.41 ± 0.01	constrained)
Rat cortex	48	7.96 ± 0.05	0.85 ± 0.03*
Guinea-pig gastric mucosa	45	8.07 ± 0.05	0.90 ± 0.03*
	Two-site model curve-fitting parameters		
Assay	*No. of experiments*	pK_{II}	pK_{I2}
Rat cortex	48	7.22 ± 0.06	8.48 ± 0.04
Guinea-pig gastric mucosa	45	7.32 ± 0.02	8.50 ± 0.04

Data shown are means ± s.e.mean. For experimental details see text. *Slope significantly different from unity ($P<0.05$). $^+$Mouse cortex data fitted with the slope parameter value constrained to unity.

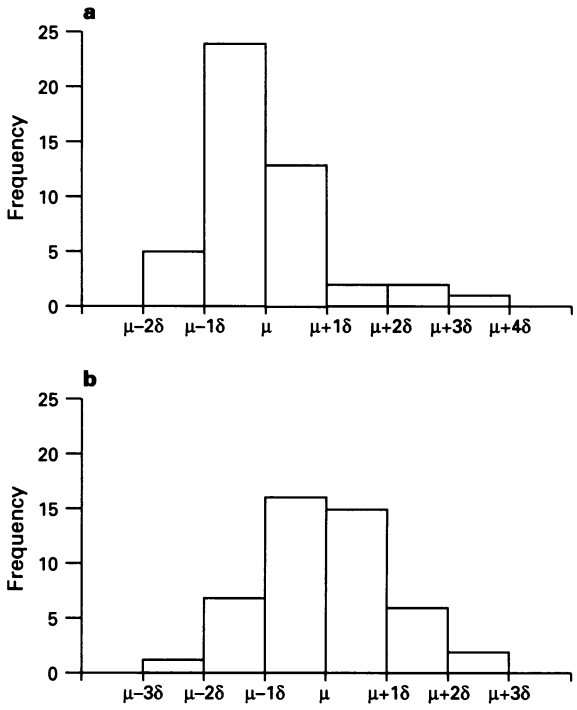

Figure 4 Frequency distributions of individual K_I values estimated for L-365,260 in competition studies with [^{125}I]-BH-CCK8S in mouse cortex ($n=47$). Data were grouped at standard deviation (δ) intervals about the mean (μ). (a) K_I values expressed as a linear quantity. The results of tests for a normal distribution (Snedecor & Cochran, 1967) were as follows: goodness-of-fit to a normal distribution – $\chi^2=5.64$ ($P<0.05$), coefficient of skewness – $\sqrt{b_1}=1.8927$ ($P<0.05$), coefficient of kurtosis – $g_2=4.8567$ ($P<0.05$). Mean K_I value = 4.21 nM ($\equiv pK_I$ value of 8.38), $\sigma^2=1.98$ nM. (b) K_I values expressed as a logarithmic base 10 quantity. Goodness-of-fit to a normal distribution – $\chi^2=1.24$ (not significant, $P>0.05$), coefficient of skewness – $\sqrt{b_1}=0.4272$ (not significant, $P>0.05$), coefficient of kurtosis – $g_2=0.4600$ (not significant, $P>0.05$). Mean pK_I value = 8.41, $\sigma^2=0.18$.

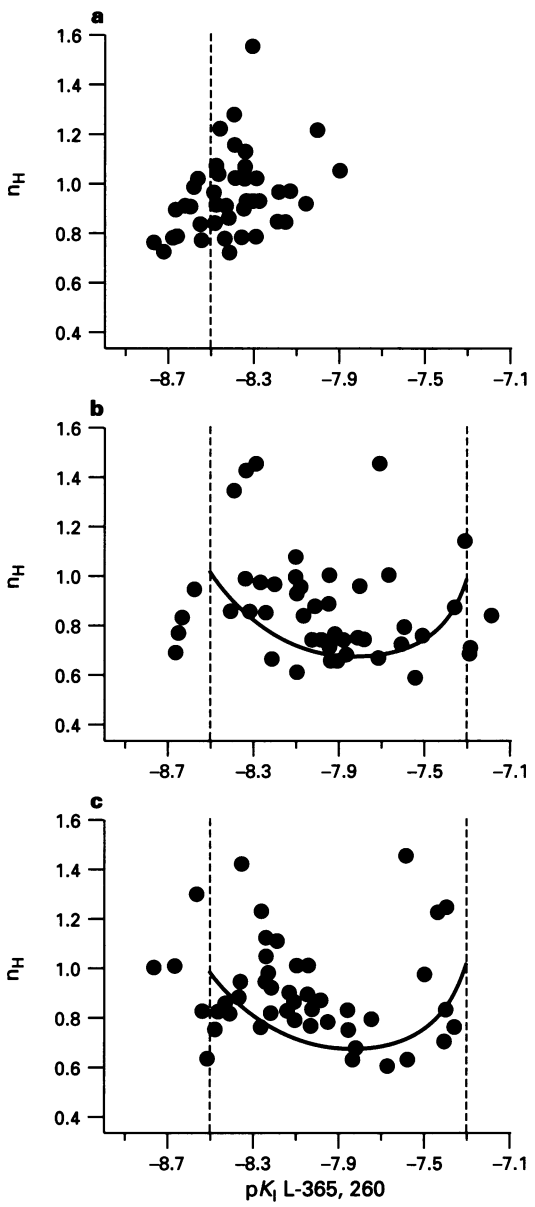

Figure 5 Distribution of individual midpoint slope (n_H) and midpoint location parameter (apparent pK_I-values). The values in (a) mouse cortex, (b) rat cortex and (c) guinea-pig gastric mucosa were estimated by fitting the data from the competition between L-365,260 and the specific binding of [^{125}I]-BH-CCK8S to a one-site model (equation 1). The line superimposed on the rat cortex and guinea-pig gastric mucosa data shows the expected relationship between n_H and pK_I values when competition curves obtained in a two-site system are analysed by a one-site model (equation 1) with unconstrained slope. Competition curves for this simulation were obtained by sequentially altering the proportion of R_1 to R_2 sites from 100% R_1 to 100% R_2 at 5% intervals and by using the two-site model (equation 2) with the L-365,260 pK_I values set at the values obtained from model fitting of the guinea-pig gastric mucosa and rat cortex data ($pK_{I1}=8.49$, $pK_{I2}=7.27$).

sets which were best-fitted by either 100% R_1 or 100% R_2. The pK_I values for the high affinity sites, estimated independently for the rat cortex and guinea-pig gastric mucosa assays, were not significantly different (rat, 8.50 ± 0.04, guinea-pig, 8.48 ± 0.04); similarly the pK_I values for the low affinity sites were not significantly different (rat, 7.32 ± 0.04, guinea-pig 7.22 ± 0.06). Moreover, the estimates made for the high affinity sites were not significantly different from the single site value estimated in the mouse cortex assay. The distribution of the individual pK_I and slope estimates of the competition curves in these two assays were consistent with expectations for the variable expression (in terms of absolute concentrations and proportions between experiments) of two binding sites (Figure 5).

Discussion

L-365,260 has been used as a standard competitor in many CCK_B/gastrin receptor radioligand binding studies (Table 2). Lotti & Chang (1989) first described the species dependency of the affinity of L-365,260 for CCK_B/gastrin receptors; the compound expressed approximately 10 to 20 fold lower affinity in canine brain and gastric mucosa than in guinea-pig mucosa and cerebral cortex tissue from rat, mouse and man. Four years later, Beinborn *et al.* (1993) attributed the differences in the affinity of L-365,260 at CCK_B/gastrin receptors in man, rat and dog to species differences in the amino-acid sequence of the receptor. However, the current consensus view appears to be that there is only one CCK_B/gastrin receptor within each species. This is perhaps remarkable in view of the proliferation over the last decade of recognized subtypes of peptide and non-peptide hormone receptors.

In this study, the competition curves obtained with L-365,260 in the mouse cortex radioligand binding assay were consistent with the model expectations for an interaction at a homogeneous population of receptors ($n_H=1.02\pm0.02$, $pK_I=8.42\pm0.03$, Table 1). Lotti & Chang (1989), Hughes *et al.* (1990) and Durieux *et al.* (1992) obtained K_I values in the same tissue of 5.2 ± 0.2, 2.0 ± 0.3 and 5.2 ± 0.06 nM, respectively, which are equivalent to pK_I values of 8.28, 8.70 and

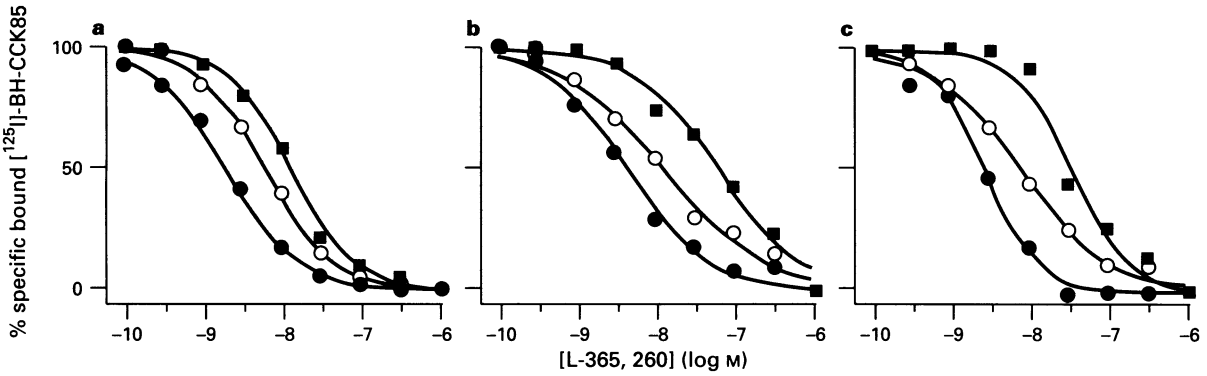

Figure 6 Competition between L-365,260 and the specific binding of [^{125}I]-BH-CCK8S. The data shown are examples of individual curves obtained in separate experiments ($n=1$ in triplicate). The curves shown superimposed on the data points were obtained by fitting each curve to equation (1). (a) Mouse cortex assay. The parameters used were as follows: (●) $pK_I=8.67$, $n_H=0.95$; (○) $pK_I=8.21$, $n_H=1.02$; (■) $pK_I=7.93$, $n_H=1.09$. (b) Rat cortex assay. The parameters used were as follows: (●) $pK_I=8.32$, $n_H=0.85$; (○) $pK_I=7.88$, $n_H=0.67$; (■) $pK_I=7.21$, $n_H=0.83$. (c) Guinea-pig gastric mucosa assay. The parameters used were as follows: (●) $pK_I=8.55$, $n_H=1.31$; (○) $pK_I=8.08$, $n_H=0.79$; (■) $pK_I=7.44$, $n_H=1.23$.

Table 2 Examples of published affinity estimates for L-365,260 in CCK_B gastrin receptor radioligand binding assays

Assay	*Radioligand*	*Temp* (°C)	*Affinity*[1] *(n_H)*	*Reference*
Mouse cortex	[^{125}I]-CCK8S	25	8.69	Lotti & Chang, 1989
Mouse cortex	[^{125}I]-CCK8S	21	8.28	Hughes *et al.*, 1990
Mouse cortex	[^{3}H]-pBC264	25	8.28 (0.88)	Durieux *et al.*, 1992
Mouse cortex	[^{3}H]-pBC264	25	8.28	Bertrand *et al.*, 1994
G-pig cortex	[^{125}I]-CCK8S	25	8.66	Lotti & Chang, 1989
G-pig cortex	[^{3}H]-SNF8702	25	8.79	Knapp *et al.*, 1990
G-pig cortex	[^{125}I]-CCK8S	37	8.39	Lignon *et al.*, 1991
G-pig cortex	[^{3}H]-pBC264	25	8.63 (0.92)	Durieux *et al.*, 1992
G-pig cortex	[^{125}I]-CCK8S	37	7.66	Galas *et al.*, 1992
G-pig cortex	[^{125}I]-CCK8S	22	8.82	Hunter *et al.*, 1993
	[^{3}H]-PD140,376	22	8.74 (1.18)	
G-pig cortex	[^{3}H]-pBC264	25	7.96 (>0.95)	Bertrand *et al.*, 1994
G-pig gastric mucosa	[^{125}I]-G17	25	8.96	Lotti & Chang, 1989
G-pig gastric mucosa	[^{125}I]-leu^{15}G17	25	8.31	Pendley *et al.*, 1993
G-pig gastric mucosa	[^{125}I]-G17	22	7.95	Hunter *et al.*, 1993
	[^{3}H]-PD140,376	22	8.69 (0.81)	
G-pig gastric mucosa	[^{125}I]-leu^{15}G17	25	8.32	Bertrand *et al.*, 1994
Rat cortex	[^{125}I]-CCK8S	25	8.40	Lotti & Chang, 1989
Rat cortex	[^{125}I]-CCK8S	23	8.09	Kuwahara *et al.*, 1992
Rat cortex[3]	[^{125}I]-CCK8S	37	7.7[2]	Wank *et al.*, 1992
Rat cortex	[^{125}I]-CCK8S	–	8.04	Ohtsuka *et al.*, 1992
Rat cortex	[^{3}H]-pBC264	25	7.96 (0.87)	Durieux *et al.*, 1992
Rat cortex	[^{125}I]-CCK8S	25	7.72 (0.72)	Nishida *et al.*, 1994
Rat cortex	[^{3}H]-pBC264	25	7.96	Bertrand *et al.*, 1994
Rat cervical vagus nerve	[^{125}I]-CCK8S	22	7.35	Corp *et al.*, 1993
Rat lateral NTS	[^{125}I]-CCK8S	22	7.66	Corp *et al.*, 1993
Canine cortex	[^{125}I]-CCK8S	25	7.40	Lotti & Chang, 1989
Canine gastric mucosa	[^{125}I]-G17	25	7.64	Lotti & Chang, 1989
Canine parietal cell[3]	[^{125}I]-CCK8S	37	6.89	Kopin *et al.*, 1992
Canine gastric mucosa	[^{125}I]-leu^{15}G17	37	7.17	Pendley *et al.*, 1993
Canine gastrin mucosa	[^{125}I]-CCK8S	37	7.04	Galleyrand *et al.*, 1994
	[^{125}I]-leu^{15}G17		6.85	
Human cortex[3]	[^{125}I]-CCK8S	37	8.00	Pisegna *et al.*, 1992
Human cortex[3]	[^{125}I]-CCK8S	20	8.48	Denyer *et al.*, 1994
Human cortex[3]	[^{125}I]-CCK8S	37	8.42	Lee *et al.*, 1993
Human gastric mucosa[3]	[^{125}I]-CCK8S	37	8.00	Pisegna *et al.*, 1992
Mastomys natalensis	[^{125}I]-CCK8S	24	7.92	Inomoto *et al.*, 1993
Carcinoid tumour	[^{125}I]-G17		7.92	
JURKAT T-cells	[^{125}I]-CCK8S	24	8.48	Lignon *et al.*, 1991 & 1993
AR42J cells	[^{3}H]-PD140,376	22	8.38	Suman-Chauhan *et al.*, 1993
GH3 cells	[^{125}I]-CCK8S	23	8.09	Kuwahara *et al.*, 1992
GH3 cells	[^{125}I]-CCK8S	21	7.60	Smith *et al.*, 1994
PANC-I cells	[^{125}I]-CCK8S	4	8.60	Palmer Smith *et al.*, 1993

[1]Affinity values were originally presented as linear values but here are presented as pK_I or pIC_{50} values. Hill Slopes (n_H) are included where available. [2] Value not presented, this number was estimated by eye from a figure. [3]Issue of origin for receptor DNA expressed in COS-7 cells.

8.28. These values appear to be similar to the mean pK_I value obtained in this study, although the strict numeric comparison of these values is not possible because of the different statistical methods used for generating the values and their errors. In this study, the K_I values were estimated as logarithms because the values in the single site mouse cortex assay were shown to be log-normally-distributed (Figure 3).

In contrast to the mouse cortex, the data obtained from the analysis of replicate experiments in the rat cortex and guinea-pig gastric mucosa radioligand binding assays were not consistent with the presence of a single population of binding sites because the mean slope parameter estimates were significantly less than unity (Figure 4 and Table 1). Although the mean apparent pK_I values obtained from these curves cannot be relied upon for receptor characterization, the values were significantly lower than the value obtained in the mouse cortex.

Before considering the presence of two CCK_B/gastrin binding sites in the rat and guinea-pig assays, we considered whether the large variation in the slope and location of the individual curves in these assays was due to a failure to satisfy the basic criteria for the reliable interpretation of radioligand binding data. One potential problem was that we used cell and gland preparations because we could not obtain adequate specific binding (in terms of % of total and absolute c.p.m.) with membrane preparations. Data from intact cell preparations have been considered to be less reliable than those obtained from membrane preparations (Motulsky *et al.*, 1985). For example, variable rates of intracellular internalization of a single class of radiolabelled receptor, a recognized process for CCK/gastrin receptors (Williams *et al.*, 1988), could produce a sub-population of receptors with less access and hence lower apparent affinity for competing ligands. Another possibility was the depletion or degradation of radioligand and/or competitor, although the current data would require variable and simultaneous loss of radioligand and competitor (see Wells *et al.*, 1980; Goldstein & Barrett, 1987). In practice, it was established that both the radiolabel and competitor were intact throughout the incubation periods used in each of the assays. The most compelling reason for rejecting the idea that inadequate assay conditions could explain the data was provided by the fact that the tissue preparation and assay conditions employed for the rat cortex, although admittedly not the guinea-pig gastric mucosa, were identical to those used for the mouse cortex, with the single exception that the 2 mg ml^{-1} tissue concentration used in the mouse was increased by 2.5 fold (Figure 1). Although the amount of non-specific binding was higher in the rat cortex and guinea-pig gastric mucosa assays, it was calculated to be no more variable in these tissues between replicate experiments than in the mouse cortex. In other words, there was no correlation between the behaviour of L-365,260 and the degree of non-specific binding making it unlikely that the complex behaviour of L-365,260 was due to the high non-specific binding. We have no explanation for the observation that ligand equilibration in the mucosal gland preparation was reached twice as quickly as in the cortical cell preparations.

The relationship between the location and slope of the individual competition curves (Figure 5) and the two-receptor model fits (Table 1) indicated that all of the data obtained on the rat cortex and guinea-pig gastric mucosa assays could be accounted for by a simple two-site model in which the proportion of the sites labelled by [^{125}I]-BH-CCK8S varied between replicate experiments and tissues. The high affinity site in the rat and guinea-pig appeared to be identical to the single site in the mouse as judged by the pK_I values estimated for L-365,260 (Table 1). The possibility that the low affinity site was due to [^{125}I]-BH-CCK8S labelling CCK_A receptors was considered because CCK8S is classified as a non-selective agonist at CCK_A and CCK_B/gastrin receptors (Watson & Girdlestone, 1995). In this respect, it would have been preferable to use a selective CCK_B/gastrin receptor radioligand. However, when the experiments were initiated, [^{125}I]-BH-CCK8S was one of the only readily-available ligands with high specific activity and good batch reproducibility. Our attempts to develop assays with another iodinated ligand, [^{125}I]-gastrin (1–17), were unsuccessful, probably because we were unable to prevent the breakdown of the radioligand despite using recognized enzyme inhibitors, a problem previously discussed by Kleveland & Waldum (1991). The successful development of CCK_B/gastrin receptor assays using [^{125}I]-BH-CCK8S has been demonstrated previously (Praissman & Walden, 1984; Magous *et al.*, 1988; Table 2) and, ultimately, the results obtained with the selective CCK_A receptor antagonist, 2-NAP, demonstrated that significant numbers of CCK_A-sites were not labelled under the assay conditions employed (Figure 2).

When a radioligand with low selectivity for two types of receptor is presumed to be non-selective when it is not, there may be considerable distortion of competition curves and the estimation of the respective affinities of competing ligands (Schutz *et al.*, 1988). Thus, an alternative explanation of the current data could be that [^{125}I]-BH-CCK8S, rather than L-365,260, expresses differential affinity for the two sites. However, if this was the case, the total amount of radioligand bound would have to vary between 5 and 50% of the total amount added to provide the range and degree of displacement of the L-365,260 curves observed between the replicate experiments. In practice, this explanation is unlikely because the % of added ligand bound did not differ significantly between all three assay tissues and within each tissue the total binding of [^{125}I]-BH-CCK8S varied by only $<3\%$ (see results) between replicate experiments. This suggests that the underlying radioligand occupancy did not in fact vary significantly and therefore was not a source of the overall observed variation in the apparent affinity of L-365,260. In view of this the assumption that the IC_{50} values, estimated by fitting the L-365,260 competition curve data to the Hill equation (1), were accurate estimates of pK_I values was not unreasonable (Cheng & Prusoff, 1973) and the degree of variation from this source was probably negligible. In addition, although saturation analyses were not performed, we did on several occasions include CCK8S as a competing ligand in all three assays (data not shown). CCK8S had an apparent affinity of >0.2 nM in all three assays. Assuming that [^{125}I]-BH-CCK8S has the same affinity as CCK8S, then the concentration of [^{125}I]-BH-CCK8S used in the assays (20 pM) would produce at most a K_I error factor of 1.1 so that, for example, a pIC_{50} value of 8.50 would represent a pK_I value of 8.54.

It is not possible to conclude, from the data presented in this study, whether the two sites have any physiological significance. However, as discussed above, CCK8S does not appear to discriminate between the two sites in these particular assays and, similarly, pentagastrin did not appear to discriminate between the sites identified using L-365,260 in three isolated, intact tissue assays (Roberts *et al.*, 1996). In terms of pharmacological significance, the L-365,260 affinity estimates at the two sites are indistinguishable from those estimated in isolated gastric tissue assays prepared from the same three species. The fact that only one of the three intact tissue assays, the guinea-pig gastric muscle assay, was performed at the same temperature (22°C) as the radioligand binding assay does not appear critical to this comparison because L-365,260 has been shown to express the same affinity at rat cortical CCK_B/gastrin receptors labelled with [^{125}I]-BH-CCK8S at incubation temperatures of 37°C and 25°C (see Table 2).

Both glycosylation (Bousso-Mittler *et al.*, 1991) and phosphorylation (Kwatra & Hosey, 1986) have been shown to account for some of the variations in pharmacological profiles attributed to the presence of multiple subtypes of several G-protein-coupled receptor systems. Potential glycosylation sites have been shown to be present in the third intracellular loop of the rat brain sub-cortex CCK_B/gastrin receptor (Wank *et al.*, 1992) and also at amino-acid positions 7, 30 and 36 (Lee *et al.*, 1993) of the human brain CCK_B/gastrin receptor. Similarly, human brain CCK_B/gastrin receptors have the potential to be phosphorylated at sites in the third intracellular loop (Lee *et al.*, 1993) and rat brain CCK_B/gastrin receptors have the po-

tential for protein kinase C phosphorylation at Ser^{82} in the first intracellular loop and also for protein kinase A phosphorylation in the second intracellular loop (Ser^{154}) and cytoplasmic tail (Ser^{442}) (Wank *et al.*, 1992). However, although post-translational modification of a single receptor protein may account for our data it is also possible that the two sites identified in these assays may be related to the multiple CCK_B/gastrin receptor mRNAs shown to be present in rat brain (Jagerschmidt *et al.*, 1994) or indeed to the two human CCK_B/gastrin receptor mRNA isoforms which were identified in the human gastric mucosa (Song *et al.*, 1993). The principle that genes for peptide hormone receptors can give rise to two populations of receptors which can be discriminated by both agonists and antagonists has been established for the mouse bradykinin receptor by McIntyre *et al.* (1993). In addition, when this analysis was being performed, Wank *et al.* (1994) showed that L-365,260 can differentiate between the long and short isoform of the human CCK_B receptor when they are transiently expressed on COS-7 cells.

We do not know why the proportion of the two postulated sites should be so variable between assay replicates. However, variation in receptor density is a well recognized biological variable; for example, Rubin *et al.* (1988) observed previously that there is a four fold circadian change in the concentration of gastrin receptors in the rat fundic mucosa. As yet, we are unable to determine whether the variation is also seasonal because of an uneven distribution of the data throughout the calendar. A mechanism for the change in the proportion of sites might be the differential transcription and/or expression of two isoforms of the CCK_B/gastrin receptor or switching of the post-translational modification of a single receptor protein.

The current analysis gave mean pK_I values of 7.27 and 8.49 for L-365,260 at the two sites, which we refer to as G_1- and G_2-binding sites, respectively. Inspection of the published pK_I data for L-365,260, shown in Table 2, reveals as wide a variation as obtained across the three assays in this study. However, the slopes of the competition curves were not presented in most of these studies and therefore the published data could not be strictly compared to the data obtained in this analysis. In fact, the slope parameters estimated in the current data set were perhaps the most powerful indicators that the complexity and variability of the data were due to the presence of multiple binding sites. As discussed above the published mouse cortex L-365,260 data are consistent with our hypothesis that this tissue predominantly expresses a single G_2-binding site population. We concluded that rat cortex can express both G_1- and G_2-sites in variable proportions. In agreement with this, the L-365,260 affinity values found in other studies are also variable, ranging from 7.7 to 8.4. The quoted affinities of L-365,260 in the other two rat neuronal tissues were both low suggesting the predominant presence of G_1-binding sites. Interestingly, Patel & Spraggs (1992) and Roberts *et al.* (1996) also obtained evidence for a homogeneous population of receptors with a low affinity for L-365,260 in rat gastric secretory assays. Although in this study the guinea-pig gastric mucosa appeared to express both G_1- and G_2-binding sites, only one low affinity value for L-365,260 is presented in Table 2. The low affinity value was obtained when [^{125}I]-gastrin(1–17) was used as the radiolabel (Hunter *et al.*, 1993). In the same study, when [^{3}H]-PD140,376 was used to label receptors in the same tissue it had an affinity of 8.69. If our hypothesis is applicable to this tissue, then [^{3}H]-PD140,376, at the concentration used in the assay (0.2–0.4 nM), predominantly labelled G_2-sites. It may also be suggested that the guinea-pig cortex possesses a variable mixed population of G_1- and G_2-binding sites because there is a large variation in the published L-365,260 affinity values in this tissue (8.82 to 7.66) and additionally because the same authors obtained different affinity values for L-365,260 in this tissue over a period of 12 months (8.39, Lignon *et al.*, 1991; 7.66, Galas *et al.*, 1992). In this study, the two binding sites could only be exposed by analysing large data sets from the rat, mouse and guinea-pig assays. Therefore, in the absence of similar analyses in other tissues and species, which are now underway, we are unable at present to apply the model to other species referred to in Table 2.

In conclusion, the analysis of the variation and complexity of L-365,260 competition curves in radioligand binding assays appears to provide evidence for the existence of two CCK_B/gastrin binding sites. The high affinity sites for L-365,260 were indistinguishable between guinea-pig gastric mucosa and rat and mouse cortical tissues. Thus, the results appear to support the view of Lee *et al.* (1993), namely that the receptors which were referred to as central CCK_B and peripheral gastrin receptors are identical. Clearly, further work with other non-peptide antagonists is required to test the validity of the conclusions. In addition, a similar study with a radiolabelled antagonist would be useful in order to determine whether the two sites represent two receptors or two agonist-induced affinity states of the same receptor. However, if our hypothesis is correct, that the two commercially available antagonist radioligands, [^{3}H]-L-365,260 and [^{3}H]-PD140,376, both have higher affinity at the gastrin-G_2 site, then such a comparative study may not be useful. A high affinity antagonist radioligand is required which is either gastrin-G_1 selective or gastrin-G_1/G_2 non-selective. Ultimately, the development of more highly selective ligands is required for confirmation of the existence of two sites and to elucidate any potential selective physiological or therapeutic roles that they may confer.

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