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Analysis of the variation in the action of L-365,260 at CCK_B/gastrin receptors in rat, guinea-pig and mouse isolated gastric tissue assays

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1 Since L-365,260 was first described as a selective antagonist at cholecystokinin $(CCK)_B$/gastrin receptors, we have used it periodically as a reference compound in isolated tissue assays of guinea-pig gastric muscle and lumen-perfused stomachs from mouse and immature rat. L-365,260 behaved as a surmountable antagonist and produced parallel rightward shifts of pentagastrin concentration-effect curves in each of the replicate experiments. The experiments were performed by several different experimenters in the same laboratories over a five year period.

2 In the isolated, lumen-perfused, immature rat stomach assay, L-365,260 behaved as a simple competitive antagonist (Schild plot slope $= 1.00 \pm 0.10$, $pK_B = 7.54 \pm 0.03$ from a global analysis of the data) acting at a homogeneous population of receptors in five separate, highly-reproducible, experiments. In contrast, the replicate data sets obtained from the interaction in the isolated, lumen-perfused mouse stomach and guinea-pig gastric muscle assays, over the same period, were not consistent with the presence of a single receptor population. The guinea-pig gastric muscle data were relatively reproducible between experiments but some individual Schild plot slopes and the slope estimated from a global analysis of all the data were significantly less than unity (slope $= 0.80 \pm 0.07$, $pA_2 = 8.56 \pm 0.05$ from the global analysis). The data obtained in the mouse stomach were significantly more variable than that obtained in the same assay, during the same period, from the interaction between histamine and the H_2-receptor antagonist, famotidine. The individual Schild plot slopes ranged from being very flat (0.20) to being not significantly different from unity (1.23) and the pA_2 values ranged from 7.68 to 8.70.

3 Overall, the data could be accounted for by assuming the variable expression of two receptor subtypes across the assays. The rat stomach appeared to express a single receptor characterized by a low affinity constant for L-365,260 ($pK_B \sim 7.5$). The guinea-pig gastric muscle and mouse stomach data could be explained by the presence of this receptor and a second one characterized by a high affinity constant for L-365,260 ($pK_B \sim 8.6$). The activity of the two proposed receptor subtypes was consistent between experiments in the guinea-pig and the high affinity receptor appeared to be predominant. In contrast, the mouse stomach data could only be simulated by assuming that the proportion and absolute number of each subtype varied significantly between the replicate experiments.

4 The L-365,260 affinity estimates at the inferred receptor subtypes were indistinguishable from those obtained in a corresponding analysis of the behaviour of L-365,260 in CCK_B/gastrin receptor radioligand binding experiments in guinea-pig gastric gland and mouse and rat cerebral cortex preparations.

Keywords: Cholecystokinin/gastrin/receptor; mouse stomach/immature rat stomach/guinea-pig gastric muscle; L-365,260/pentagastrin

Introduction

Two main classes of cholecystokinin (CCK)/gastrin receptors have been identified: CCK_A and CCK_B/gastrin (see Woodruff & Hughes, 1991). CCK_B/gastrin receptors have been cloned from dog, rat and human tissues (Kopin *et al.*, 1992; Pisegna *et al.*, 1992; Wank *et al.*, 1992; Lee *et al.*, 1993). Species variations in the apparent affinities of the selective CCK_B/gastrin receptor antagonist, L-365,260 have been observed in radioligand binding studies performed on these receptors (Beinborn *et al.*, 1993; see Harper *et al.*, 1996). Site-directed mutagenesis studies have indicated that the differences in affinities may be accounted for by the amino-acid sequence variations in the 6th transmembrane domain of the receptor (Beinborn *et al.*, 1993). These differences between species are not restricted to radioligand binding studies performed on cloned receptors. In fact, the potential for interspecies variation was disclosed in the first study on L-365,260 (Lotti & Chang, 1989), where it was found that it expressed approximately 10 to 20 fold lower affinity at CCK_B/gastrin receptors in canine brain than in guinea-pig stomach and rat, mouse and human brain. Similarly, Patel & Spraggs (1992) showed that L-365,260 was less potent in the rat isolated gastric mucosa than in the guinea-pig isolated ileum, although whether this was due to speciation of receptors or the presence of subtypes could not be discriminated. Although, Makovec *et al.* (1992) proposed that 'the stomach gastrin receptor is not as closely-related to the CCK central receptor as previously hypothesized', this conclusion was based on differences in affinity values found for a range of glutamic acid derived CCK_B/gastrin receptor antagonists determined between gastric mucosa and cerebral cortex from two different species (rabbit and rat for gastric mucosa and cerebral cortex, respectively). Indeed, as far as we are aware, no indications of receptor heterogeneity have been obtained for L-365,260 between or within tissues from a single species (Lotti & Chang, 1989; Roche *et al.*, 1991; Hunter *et al.*, 1993), suggesting that CCK_B/gastrin receptors within a species in both central nervous system and peripheral tissues are similar, if not identical, in terms of pharmacological differentiation. L365,260 is frequently used for the characterization of CCK_B/gastrin receptors and we included it routinely as an internal standard in CCK_B/gastrin receptor radioligand binding assays used in a drug discovery programme (Kalindjian *et al.*, 1994). In addition, the compound was peri-

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odically examined in our standard, CCK_B/gastrin receptor, isolated tissue preparations. It did not always behave as a simple competitive antagonist at a homogeneous receptor population in both types of assays (viz. the isolated tissue and radioligand binding assays). Therefore, it was re-examined in the isolated tissues several more times during the programme. Accordingly, a number of replicate experimental data sets have been obtained which provided an opportunity for investigating between- and within-assay experimental variance. In this paper, we have analyzed replicate data sets from the interaction between pentagastrin and L-365,260 in three isolated gastrointestinal tissue bioassays (isolated, lumen-perfused stomach preparations from mouse and immature rat and guinea-pig, isolated gastric muscle). The analysis includes all the data sets obtained over a five year period by several experimenters within the same laboratories.

We interpret the results in terms of the variable expression of two subtypes of CCK_B/gastrin receptors. An account of an initial analysis of these data has been presented to the British Pharmacological Society (Roberts *et al.*, 1995) and one of the data sets obtained on the guinea-pig gastric muscle assay has been presented previously (Bishop *et al.*, 1995).

Methods

Immature rat and mouse isolated, lumen-perfused, stomach assays

Gastric acid secretion was measured in isolated, lumen-perfused stomachs, prepared essentially as described previously (Black & Shankley, 1985a). Male mice (Charles River CDI, 22–26 g, 18 h fasted, water *ad libitum*) and pre-weaned rat pups (Wistar, 28–52 g, corresponding to an age range of 14 to 21 days) were used. 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 contents of the stomach flushed through with mucosal solution (mM: NaCl 118, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 1.3, glucose 31.6) to remove any remaining food. The stomach was placed into an organ bath containing 40 ml of buffered serosal solution (mM: NaCl 118, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.14, Na_2HPO_4 15.9, $CaCl_2$ 0.65, glucose 31.6). The serosal solution was maintained at 37 ± 1°C and gassed vigorously with 95% O_2 and 5% CO_2. 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 to provide a back pressure to distend the stomach. In the mouse stomach assay, 3-isobutyl-methylxanthine (30 μM) was added to the serosal solution because it appeared that phosphodiesterase inhibition improved the signal-to-noise ratio in preliminary experiments when pentagastrin was used as the agonist. Similarly, for the immature rat stomach preparation the muscarinic cholinoceptor agonist, 5-methylfurmethide (30 nM), was included in the serosal solution as it has been shown to potentiate and amplify pentagastrin-stimulated secretory responses in this species (Welsh, 1992).

The preparations were allowed to stabilize for 90 min before the addition of drugs which were added directly to the serosal solution in the organ bath. Antagonists were allowed to equilibrate for 60 min prior to obtaining a single, cumulative, pentagastrin concentration-effect curve. Because these curves were flat (slope ~0.5) dosing was performed at log unit intervals (0.1 nM to 100 μM). Pentagastrin-stimulated responses were expressed as the change in the pH ($\triangle$pH) of the lumen-perfusate from the basal pH immediately before the first addition of pentagastrin. A continuous record of pH was obtained from chart recorders coupled to a pH electrode amplifier (Fylde Scientific).

Guinea-pig gastric muscle assay

The assay was performed as described by Bishop *et al.* (1995). The stomachs were removed from male guinea-pigs (Dunkin Hartley, 300–500 g). Krebs-Henseleit solution, modified by increasing the K^+ concentration (mM: NaCl 118, KCl 14.7, $CaCl_2$ 2.5, $MgSO_4$ 0.57, KH_2PO_4 1.2, glucose 11, $NaHCO_3$ 25), was injected through a hypodermic needle placed under the serosal and muscle layers of the stomach in the middle region (corpus) between the greater and lesser curvature (avoiding pyloric tissue) to blister the muscle away from the sub-mucosa. One or two strips of muscle (approximately 2.5 cm by 1 cm) were removed from the proximal area of both sides of each stomach and tied with thread to an isotonic transducer under an initial loading tension of 0.35 g. The preparation was incubated in 20 ml of modified Krebs-Henseleit solution at 22 ± 1°C (maintained with the aid of a cooling coil) containing 2.5 mM Ca^{2+} and gassed with 95% O_2 and 5% CO_2. The raising of the concentration of K^+, from 4.7 to 14.7 mM, and the lowering of temperature, from 37°C to 22°C, increased the size of agonist response and improved the signal-to-noise ratio. The CCK_A-receptor selective antagonist, devazepide (10 nM, 100 fold its K_B at CCK_A-receptors in this assay) was included in the modified Krebs-Henseleit solution to block the CCK_A-receptors also coupled to smooth muscle contraction in this assay (Bishop *et al.*, 1995). The preparations were washed every 10 min for 30 min after which the antagonist or vehicle was incubated for 60 min before a single, cumulative, pentagastrin concentration-effect curve was obtained. Tissue length was continuously recorded with an isotonic transducer and responses were expressed as changes in mm chart record. The total amplification of the recording system was such that 200 mm of trace was equal to 1 mm of tissue length in all experiments.

Eight preparations were used simultaneously in each assay and randomised block designs were used throughout for the allocation of experimental treatments to each organ bath.

Analysis

Individual agonist concentration-effect curves were fitted to the Hill equation,

$$E = \frac{\alpha[A]^{n_H}}{[A]_{50}^{n_H} + [A]^{n_H}}, \quad (1)$$

to provide estimates of midpoint slope (n_H), midpoint location ($\log[A]_{50}$) and upper asymptote (α) as described previously (Black & Shankley, 1985). The effect of drug treatment on these parameters was assessed by one-way analysis of variance (ANOVA) and the Bonferroni modified t test for multiple comparisons (Wallenstein *et al.*, 1980). P values of less than 0.05 were considered to be significant.

Analysis of competitive antagonism: one receptor model When the minimum criteria for competitive antagonism were satisfied, that is the antagonist produced parallel, rightward shift of the agonist-concentration-effect curves with no change in upper asymptote, data were analyzed according to the methods described by Black *et al.* (1985a). pK_B values were estimated by fitting the individual midpoint location values, obtained in the absence ($\log[A]_{50}$) and presence of antagonist ($\log[A]_{50B}$), to the following derivation of Schild equation,

$$\log[A]_{50B} = \log[A]_{50} + \log(1 + [B]^b/10^{\log K_B}). \quad (2)$$

If the Schild plot slope parameter (b) was not significantly different from unity, it was constrained to a value of unity and the data re-fitted to provide a pK_B estimate. When the estimated value of b was significantly different from unity, a pA_2 value was calculated from the data obtained with the lowest concentration of antagonist which produced a significant dose-

ratio with the Schild equation (dose-ratio = $\log[A]_{50B}/\log[A]_{50} = \log[B] + pA_2$). To provide a graphical display of the results, individual dose-ratios were calculated from the mean control $\log[A]_{50}$ values within each experiment and these data expressed as mean values in Schild plot space.

Compounds

Boc-pentagastrin (Cambridge Research Biochemicals Ltd, UK) was dissolved in dimethylformamide (DMF) to a concentration of 40 mM, serially diluted to 4 mM in DMF and subsequently in water down to 0.4 μM. L-365,260 (3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N'-(3-methylphenyl)urea) (synthesized by chemists at the James Black Foundation) was initially diluted in DMF to 4 mM and all subsequent dilutions were also made in DMF. Devazepide (3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-1H-indole-2-carboxamide) (synthesized by JBF chemists) was diluted in DMF to 2 mM. 3-Isobutyl-1-methyl-xanthine (Sigma Chemical Co. Ltd., UK) was diluted in DMF to 40 mM. 5-Methyl furmethide (a gift from Wellcome Research Laboratories, Beckenham, UK) was dissolved in water to 40 mM and subsequently diluted to 40 μM. Molar stock solutions of histamine dihydrochloride (Sigma) were neutralised by addition of sodium hydroxide (Black *et al.*, 1981). Famotidine (Sigma) was dissolved in 0.1 N HCl (40 mM) and diluted in water.

Results

Analysis of the interaction between L-365,260 and pentagastrin in immature rat and mouse stomach and guinea-pig gastric muscle assays

Several replicate data sets were obtained in the immature rat stomach, mouse stomach and guinea-pig gastric muscle assays. Each data set consisted of families of pentagastrin concentration-effect curves obtained in the absence and presence of several concentrations of L-365,260. Due to the current analysis being performed retrospectively, the experimental designs were not uniform with respect to either the number of antagonist treatment groups or the absolute antagonist concentrations employed within each experiment (see Table 1). The replicate data sets were compared in terms of the parameters of the one-receptor competitive antagonism model by using the $\log[A]_{50}$ value estimated for each pentagastrin concentration-effect curve as the basic unit for analysis. Thus, the Schild plot slope (b) and pK_B parameters were estimated by fitting the $\log[A]_{50}$ values directly to equation (2). In addition, it was possible to perform a global analysis of the data by simultaneously fitting the $\log[A]_{50}$ values obtained across all the replicate experiments in one tissue. In this way, single parameter values were obtained with standard errors which represented both the within and between replicate data set experimental variance of each assay (Table 2).

The particular form of the Gaddum-Schild equation (2) was selected on the basis that the $\log[A]_{50}$ values were expected to be normally-distributed. The large amount of data available allowed the validity of this assumption to be tested. Tests of normality were performed on the frequency distributions of the individual control curve $\log[A]_{50}$ values expressed as linear or logarithmic quantities according to the methods of Snedecor & Cochran (1967). No significant departure from a normal distribution was found when they were expressed as logarithmic values. In contrast, a significant deviation was found when the linear values were analyzed (Table 3).

In the first instance, the replicate data sets obtained in the assays were analyzed individually by use of the one-receptor competitive antagonism model. In all the replicate experiments performed in the three assays, L-365,260 produced parallel, rightward shift of the pentagastrin concentration-effect curves with no change in upper asymptote. In addition L-365,260, at all the concentrations employed (range 3 nM − 3 μM, see Table 1), had no significant effect on the baseline activity of the assays (data not shown). The results obtained by fitting the individual

Table 1 Analysis of the interaction between L-365,260 and pentagastrin in replicate experiments in mouse stomach, immature rat stomach and guinea-pig gastric muscle assays and between histamine and famotidine in the mouse stomach assay by use of the one receptor competitive antagonism model

Individual fits replicate no. (month)	n	*Control p[A]$_{50}$*	*[L-365,260](−log M)*	*Schild slope parameter* (b)	*pK$_B$ (pA$_2$)*
Immature rat stomach					
1. (12)	33	8.07±0.09	7.3, 6.9, 6.5, 6.1	1.07±0.16	7.61±0.12
2. (05)	28	8.13±0.08	7.5, 7.0, 6.5, 6.0	0.88±0.10	7.80±0.12
3. (06)	25	7.39±0.13	7.5, 7.0, 6.5, 6.0	1.11±0.15	7.41±0.12
4. (02)	24	7.56±0.20	7.0, 6.5, 6.0, 5.5	0.76±0.15	7.51±0.19
5. (07)	43	7.30±0.09	7.0, 6.5, 6.0, 5.5	0.62±0.15*	(7.71±0.11)
Mouse stomach					
1. (03)	28	8.27±0.07	7.5, 7.0, 6.5	1.23±0.23	8.47±0.16
2. (02)	47	8.11±0.24	8.3, 7.7, 7.3, 6.9, 6.5, 6.1	0.89±0.12	7.88±0.14
3. (07)	30	7.92±0.08	7.5, 7.0, 6.5, 6.0	0.20±0.19*	(8.31±0.20)
4. (09)	41	8.08±0.16	8.3, 7.7, 7.3, 6.9	0.31±0.22*	(8.67±0.27)
5. (11)	32	8.38±0.25	7.5, 7.0, 6.5, 6.0	0.20±0.15*	(8.70±0.34)
6. (02)	43	7.99±0.20	8.0, 7.5, 7.0, 6.5, 6.0	1.17±0.16	7.68±0.16
Guinea-pig gastric muscle					
1. (10)	29	7.52±0.11	8.5, 8.0, 7.5	0.80±0.14	8.38±0.10
2. (03)	24	7.82±0.15	8.2, 7.7, 7.2	0.68±0.12*	(8.96±0.19)
3. (12)	28	7.55±0.16	8.0, 7.5, 7.0	0.86±0.15	8.39±0.11
4. (03)	55	7.68±0.12	8.5, 8.1, 7.7, 7.3, 7.0, 6.5	0.77±0.06*	(8.78±0.16)
Mouse stomach histamine/famotidine					
1. (06)	34	5.21±0.08	7.5, 7.0, 6.5, 6.0, 5.5	0.98±0.04	7.50±0.05
3. (06)	36	5.61±0.18	7.5, 7.0, 6.5, 6.0	0.82±0.11	7.51±0.15
2. (09)	38	5.24±0.06	7.5, 7.0, 6.5, 6.0	0.91±0.12	7.69±0.13

The data are listed in chronological order. When the Schild plot slope parameter was not significantly different from unity, the slope was constrained to unity and the data re-fitted to equation (2) and a pK_B value estimated (see Methods for details). Data are shown expressed as mean±s.e.

*$P<0.05$ significantly different from unity.

$\log[A]_{50}$ values to the one receptor competitive antagonism model (equation 2) are shown in Table 1. Typical examples of the agonist concentration-effect curve data obtained in each assay are shown in Figure 1. They were chosen for having parameter estimates close to the mean values estimated in the global analysis of the data (Table 2). Individual Schild plots constructed from all of the replicate data sets are shown in Figure 2.

Immature rat stomach assay

In the rat stomach assay, L-365,260 behaved as a simple competitive antagonist at a homogeneous receptor population because the Schild plot slope parameter (b) was not significantly different from unity in four out of the five experiments (Table 1). However, there was a significant difference in control curve locations between the replicate experiments as judged by ANOVA so that equation (2) could not be used directly to estimate a global pK_B value. Therefore, individual $\log[A]_{50B}$ values were adjusted within each replicate experiment to account for this variation and to allow a common $\log[A]_{50}$ control value to be estimated in the global analysis. This was achieved by normalising the individual $\log[A]_{50}$ values by taking the ratio between the overall mean control value and the mean control value of each replicate and multiplying each individual value by this factor. In the global model fit, the value of the Schild plot slope parameter was not significantly different from unity ($b = 1.00 \pm 0.10$; d.f. = 150) and a pK_B value of 7.54 ± 0.03 (d.f. = 150; Table 2) was estimated in the second fit made with b fixed at unity.

Guinea-pig gastric muscle assay

In this assay, b was significantly different from unity in two out of the four experiments (Table 1). The between replicate variance was less than that obtained in the mouse assay (see below) and there were no significant differences in the control curve locations between experiments. The value of the Schild plot slope parameter ($b = 0.80 \pm 0.07$; d.f. = 133, Table 2) estimated in the global model fit was significantly different from unity ($P < 0.05$) and, therefore, a pK_B value was not estimated. However, within the two experiments in which the values of b were not significantly different from unity, pK_B values (8.39 ± 0.11, 8.38 ± 0.10) were estimated from the constrained model fits and from the other two experiments pA_2 values (8.96 ± 0.19, 8.78 ± 0.16) were estimated. These values were similar to the high affinity values estimated in the mouse assay. Overall, the pA_2 value calculated from the lowest significant dose-ratios was 8.56 ± 0.05. The replicate Schild plots obtained in the guinea-pig assay (Figure 2c) appeared to have an inflexion in them as judged by the fact that, in each case, the dose-ratios obtained at concentrations of L-365,260 between 10 and 30 nM were lower than expected by linear, unit slope, extrapolation from the dose-ratios obtained at lower concentrations. Linear regression of the individual log(dose-ratio − 1) values obtained in all the experiments at concentrations less than 10 nM gave a slope parameter value (1.18 ± 0.30) which was not

Table 2 Global analysis of the interaction between L-365,260 and pentagastrin from replicate experiments in mouse stomach, immature rat stomach and guinea-pig muscle assays and histamine and famotidine in the mouse stomach assay by use of the one receptor competitive antagonism model

Global model fits	n	*Control* $p[A]_{50}$	*[L-365,260]* (−log M)	*Schild slope parameter* (b)	pK_B
Rat stomach	153	7.66 ± 0.06	7.5–5.5	1.00 ± 0.10	7.54 ± 0.03
Mouse stomach	221	8.03 ± 0.08	8.3–6.0	0.71 ± 0.09*	N/A
Guinea-pig gastric muscle	136	7.57 ± 0.06	8.5–6.5	0.80 ± 0.07*	N/A
			[Famotidine] (−log M)		
Mouse stomach Histamine/famotidine	108	5.41 ± 0.09	7.5–5.5	0.90 ± 0.09	7.66 ± 0.11

When the Schild plot slope parameter was not significantly different from the unity, slope was constrained to unity and the data refitted to equation (2) and a pK_B value estimated (see Methods for details). Data are shown expressed as mean ± s.e.
*$P < 0.05$ significantly different from unity.

Table 3 Analysis of the distribution of pentagastrin control curve $[A]_{50}$ values, expressed as linear and logarithmic values, estimated in isolated, lumen-perfused, immature rat ($n = 29$) and mouse ($n = 43$) stomach and guinea-pig, isolated gastric muscle ($n = 29$) assays

(a) Linear values

	Rat stomach	*Mouse stomach*	*Guinea-pig gastric muscle*
Goodness-of-fit to a normal distribution χ^2	50.06 ($P < 0.05$)	36.61 ($P < 0.05$)	162.25 ($P < 0.05$)
Mean $[A]_{50}$ (nM)	34.6	13.3	34.0
σ (nM)	31.1	18.1	31.6

(b) Logarithmic base 10 values

	Rat stomach	*Mouse stomach*	*Guinea-pig gastric muscle*
Goodness-of-fit to a normal distribution χ^2	8.50 (NS)	2.35 (NS)	3.75 (NS)
Coefficient of skewness ($\sqrt{b_1}$)	−0.31 (NS)	−2.36 (NS)	−0.25 (NS)
Coefficient of kurtosis[1] (g_2)	0.35 (NS)	0.11 (NS)	0.81 (NS)
Mean $p[A]_{50}$ value	7.66	8.12	7.62
σ	0.45	0.46	0.37

The results of tests for a normal distribution (Snedecor & Cochran, 1967) are shown: NS-not significant $P > 0.05$.
[1]Calculated according to Geary (1936) for tests on data sets where $n < 40$.

significantly different from unity and an apparent pK_B value of 8.86. Moreover, although there was insufficient data obtained to perform a formal test, the log (dose-ratio − 1) values obtained with concentrations above 30 nM appeared to revert back to unit slope as though the plots were biphasic (Figure 2c).

Mouse stomach assay

In this assay, the value of b was significantly different from unity in three out of six experiments (Table 1). The between replicate experimental variability was greater than that obtained in the immature rat stomach and guinea-pig gastric muscle assays (see below). It was not necessary to normalise the $\log[A]_{50}$ values before performing the global model fit to the one-receptor model because there were no significant differences in the control curve location between the experiments. The global estimate of the Schild plot slope parameter ($b = 0.71 \pm 0.09$; d.f. = 218, Table 2) was significantly different from unity ($P < 0.05$) and, therefore, the estimation of a meaningful pK_B value was precluded. However, constrained model fits were made of the data obtained in the three replicate experiments, in which b was not significantly different from unity (Table 1). Two of the pK_B estimates (7.88 ± 0.14, 7.68 ± 0.16) were similar to the global value estimated in the rat assay (7.54 ± 0.03) although the other value was significantly higher (8.47 ± 0.16). The pA_2 values estimated from the lowest significant dose-ratios in the other three experiments (8.31 ± 0.20, 8.70 ± 0.34, 8.67 ± 0.27) were consistently higher than the global value estimated from the rat data.

Analysis of the behaviour of L-365,260 between assays

To facilitate the comparison of the behaviour of L-365,260 between assays the data were normalised with respect to antagonist concentration. This was achieved using a rearranged Schild equation ($pA_2 = \log(([A]_{50B}/[A]_{50}) - 1) + \log[B]$) to convert the

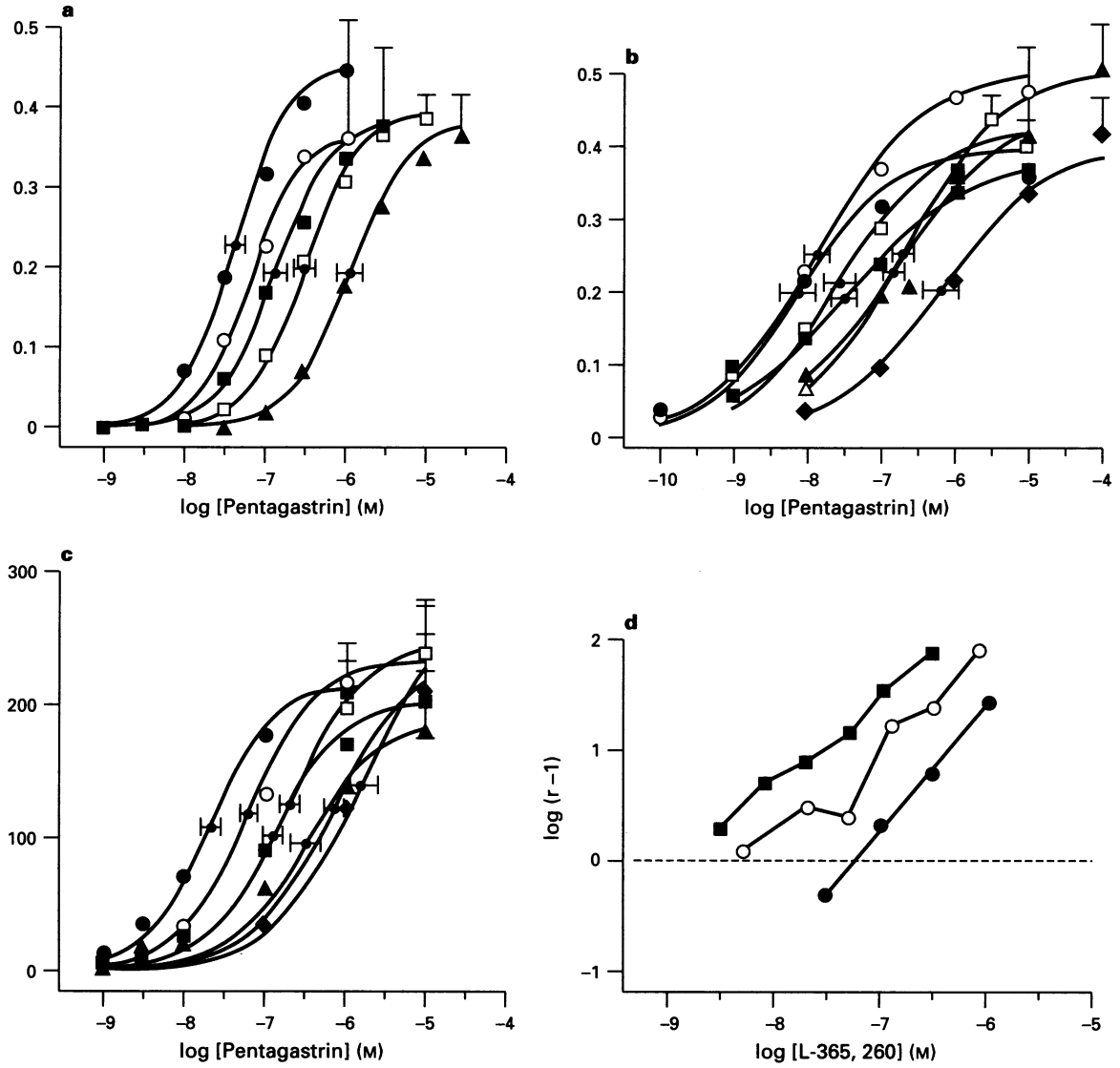

Figure 1 Pentagastrin concentration-effect curves obtained in the absence and presence of L-365,260. The curves shown superimposed on the mean data points were simulated by use of the Hill equation (1) with the mean parameters obtained by the curve fitting procedure. Ordinate scales for (a) and (b) ΔpH and for (c), mm chart record (where 200 mm = 1 mm tissue length). (a) Isolated, lumen-perfused, immature rat stomach assay. Curves were obtained in the absence (●) and presence of 30 (○), 100 (■), 300 (□) and 1000 (▲) nM L-365,260 ($n = 5/7 \pm$ s.e.mean). (b) Isolated, lumen-perfused, mouse stomach assay. Curves were obtained in the absence (●) and presence of 5 (○), 20 (■), 50 (□), 125 (▲), 300 (△) and 800 (◆) nM L-365,260 ($n = 5/7 \pm$ s.e.mean). (c) Guinea-pig isolated gastric muscle assay. Curves were obtained in the absence (●) and presence of 3 (○), 8 (■), 20 (□), 50 (▲), 100 (△) and 300 (◆) nM L-365,260 ($n = 5/7 \pm$ s.e.mean). (d) Schild plots corresponding to the data shown in panels (a), (b) and (c) and the experiment replicate numbers 3, 2 and 4 in Table 1 in the rat (●), mouse (○) and guinea-pig (■) assays, respectively. The line shown superimposed on the rat stomach data was simulated with unit slope and $pK_B = 7.41$.

individual $\log[A]_{50B}$ values into pA_2 values calculated from the mean control $\log[A]_{50}$ value within each experiment (Figure 3). The families of pA_2 values for L-365,260 between the three assays were significantly different ($F = 81.1$; total d.f. = 394). Subsequent Bonferroni modified t tests indicated that the pA_2 values were significantly different between each assay (t_{calc}: rat/mouse = 5.4, rat/guinea-pig = 96.5, mouse/guinea-pig = 8.4; total d.f. = 394). F-tests performed on the pA_2 values indicated that the mouse data were significantly more variable than the rat ($F = 2.05$, d.f. = 122, 164) or guinea-pig ($F = 2.33$; d.f. = 106, 164) and that the variability of the rat and guinea-pig assay was indistinguishable ($F = 1.13$; d.f. = 122, 106). An attempt was made to determine if the high variability of the mouse assay was restricted to the interaction between L-365,260 and pentagastrin or if it was a general feature of this assay. An identical analysis was performed on the mouse stomach pA_2 data from the interaction between histamine and the selective histamine H_2-receptor antagonist, famotidine. In the three replicate experiments, the Schild slope parameters were not significantly different from unity (Table 1, Figure 2d) and the corresponding

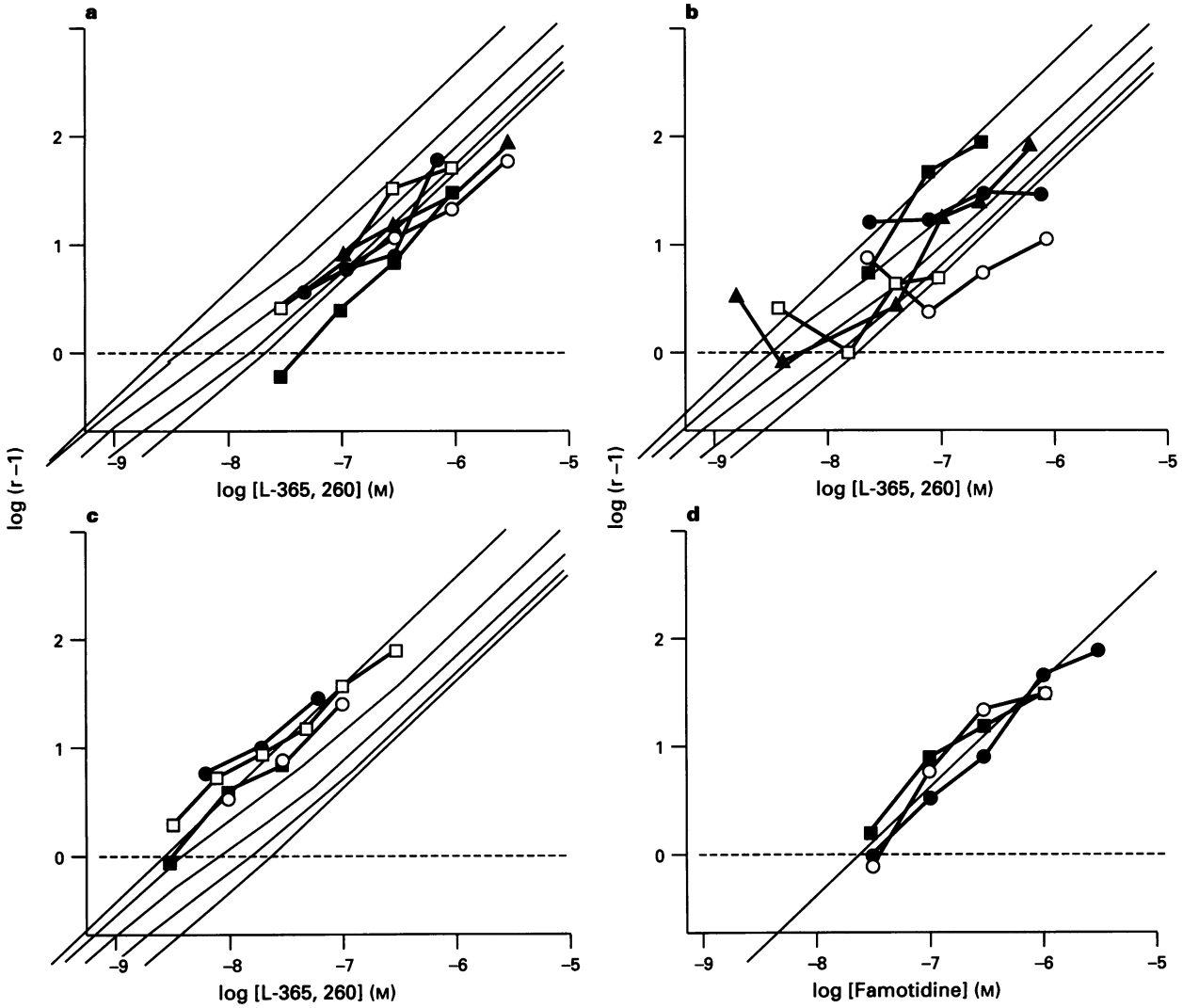

Figure 2 Schild plots from the analysis of the interaction between L-365,260 and pentagastrin in (a) immature rat stomach, (b) mouse stomach and (c) guinea-pig gastric muscle assays and (d) from the interaction between histamine and a histamine H_2-receptor antagonist, famotidine, in the mouse stomach assay. The individual Schild plots correspond to the replicate data sets obtained on each assay presented in Table 1. The line shown superimposed on the data in panel (d) was simulated with unit slope by use of the global pK_B value 7.66 (see text for details). The lines shown superimposed on the L-365,260 data were simulated by use of equation (7) of the Appendix in which a two receptor, addition of stimuli, model of agonism in which the agonist is capable of producing maximum tissue effect by activation of either receptor is described. In the simulations, which are the same in each panel, the affinity of L-365,260 for the low (pK_{B1}) and high (pK_{B2}) affinity receptors were fixed at the global pK_B estimated in the immature rat stomach assay (7.54) and the mean pA_2 in the guinea-pig gastric smooth muscle assay (8.56), respectively. It was assumed that the maximum amount of stimulus which can be produced by activation of each receptor was identical so that $\alpha_1 = \alpha_2$, and, by a simple cancelling of the common factor, α, between numerator and denominator, under these conditions equation (7) simplifies to,

$$\text{dose} - \text{ratio} = \frac{[A]_{50B}}{[A]_{50}} = 1 + \frac{(K_2 \cdot K_{B2} + K_1 \cdot K_{B1})[B] + (K_1 + K_2)[B]^2}{K_{B1} \cdot K_{B2}(K_1 + K_2) + (K_2 K_{B1} + K_1 \cdot K_{B2})[B]},$$

and the parameters which govern the operational selectivity of the agonist are K_1 and K_2. The two extreme, linear, unit slope, Schild plots were simulated assuming that only one of the two receptors was activated. The five biphasic Schild plots were simulated by fixing the value of K_2 at 1.0 and setting the values of K_1 at 10, 3, 1, 0.3, 0 for each plot from left to right. This transition has the effect of making pentagastrin more potent (at ~0.5 log unit intervals) at the receptor characterized by the low pK_B value for L-365,260.

pK_B values were indistinguishable. The global Schild slope parameter was not significantly different from unity ($b = 0.90 \pm 0.09$) and a global pK_B value of 7.66 ± 0.11 was estimated. When the famotidine data were expressed as pA_2 values, it was found to be significantly less variable than that obtained with L-365,260 ($F = 2.26$, d.f. = 107, 164) in the same assay.

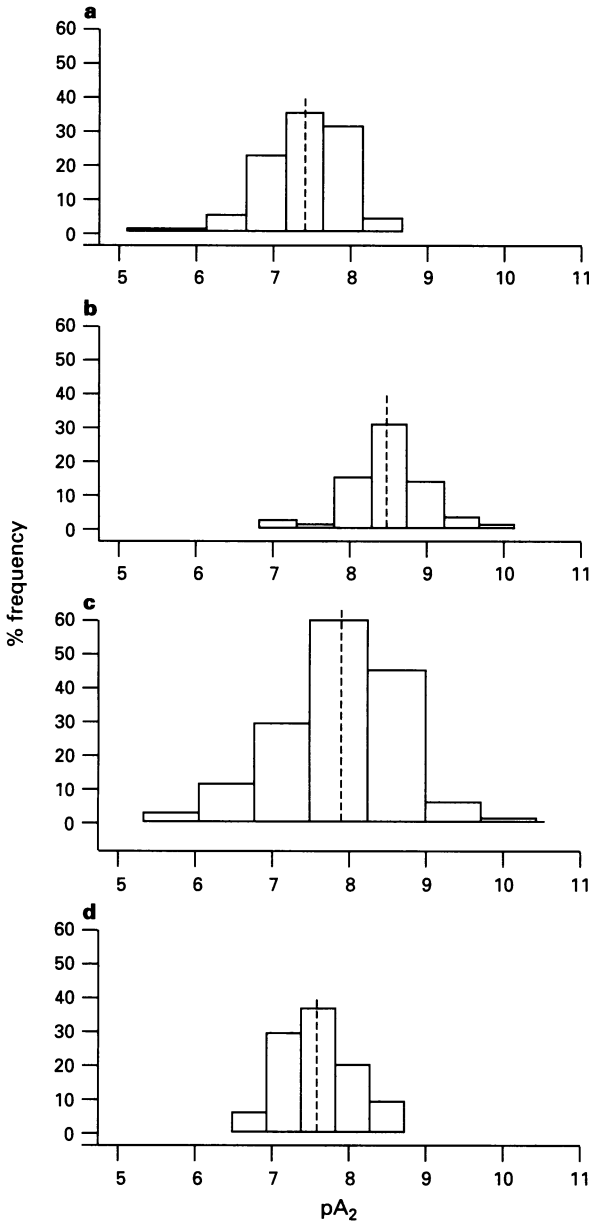

Figure 3 Histograms showing the frequency distribution of individual pA_2 values estimated in replicate analyses of the interaction between L-365,260 and pentagastrin in (a) immature rat stomach, (b) guinea-pig gastric muscle and (c) mouse stomach assays and (d) from the interaction between histamine and a histamine H_2-receptor antagonist, famotidine, in the mouse stomach assay. The data obtained in each assay were grouped at intervals equal to one standard deviation (i.e. bar width = 1 s.d.) and the mean pA_2 values are indicated by the hatched lines. The descriptive statistics of the pA_2 data were as follows:

Assay:	*Rat stomach*	*Mouse stomach*	*Guinea pig gastric muscle*	*Mouse stomach (histamine data)*
n	123	165	107	86
Mean	7.54	7.93	8.56	7.61
s.d.	0.51	0.73	0.48	0.44
s.e. mean	0.05	0.06	0.05	0.05
Variance	0.26	0.53	0.23	0.20
Range	2.86	4.59	2.90	1.99
(min–max)	(5.75–8.61)	(5.61–10.20)	(6.92–9.82)	(6.61–8.60)

Discussion

The current analysis indicates that L-365,260 behaves as a surmountable antagonist in the immature rat stomach, mouse stomach and guinea-pig gastric smooth muscle assays but that its behaviour is only compatible, consistently, with a competitive mechanism of action at a single population of receptors in the immature rat stomach assay. In spite of this, the rat assay appeared to be the most variable in terms of the pentagastrin control curve location which was significantly different between replicate experiments (range 7.39 to 8.13). These differences may be due to the known variability in gastrin receptor density. For example, Rubin *et al.* (1988) showed that rats, both fed and fasted, exhibited a 4-fold circadian variation in fundic mucosal gastrin receptor concentration expressed in terms of number of binding sites/mg^{-1} protein. According to the operational model of agonism (Black & Leff, 1983), a 4-fold receptor density change would produce a 0.6 log unit shift of a high efficacy agonist concentration-effect curve which would account for most of the variation observed. Another source of variation in receptor density may be associated with the stage of development of the rats used in this study (28–52g equivalent to 14 to 21 days post-natal). Takeuchi *et al.* (1981) could only detect gastric mucosal gastrin receptors in a radioligand binding assay from the time of weaning which occurs between day 20 and 25 in the rats used in the current study. Evidently, in our study, there was sufficient gastrin receptor density for the stimulation of gastric acid secretion by pentagastrin in our mainly pre-weaned rats although it maybe that the gastrin receptor density is changing rapidly during this period of their development. Stomachs from immature animals were used because it has been shown that only thin-walled stomachs will respond reliably to secretogogues in the lumen-perfused, whole stomach assay system (Welsh, 1992). Overall, the data obtained in the immature rat stomach were entirely congruent with those obtained previously from the same interaction in a rat isolated gastric mucosa sheet assay by Patel & Spraggs (1992) who estimated a pA_2 value of 7.6.

The interaction between pentagastrin and L-365,260 in an assay of guinea-pig gastric muscle has been examined previously (Boyle *et al.*, 1993), in the absence and presence of the selective CCK_A-receptor antagonist, devazepide (3 nM). However, these studies were performed under isometric conditions at 37°C rather than isotonic conditions at 22°C adopted in the current assay; pA_2 values of 8.79 (95% c.l. 8.49–9.09) and 8.17 (95% c.l. 8.00–8.34) were obtained in the absence and presence of devazepide, respectively. In agreement with the current data, the corresponding Schild plots were both flat (0.75, 0.87, respectively), although the 95% c.l. were relatively large (0.39–1.10 and 0.58–1.19, respectively) so that an interaction at a single receptor could not be excluded in this study. In our study, performed in the presence of devazepide, the guinea-pig gastric muscle assay was the least variable. The control curve locations spanned less than 0.3 of a log unit. However, all of the Schild plot slope values were less than unity, although in only two of the four cases was the deviation significant, and the range of the slope values was narrower than in the other assays (Table 1). In addition, the slope estimated in the global analysis of the data was also significantly less than unity. Evidently, the guinea-pig gastric muscle data did not reliably fit the one-receptor model.

The mouse stomach data were the most variable in terms of the effects of L-365,260 regardless of whether the data were expressed in terms of the competitive antagonism model (Table 1, Figure 2b) or, without prejudice to mechanism of action, as individual pA_2 values (Figure 3). The finding was contrary to our expectations which were based on our previous experience of the assay. The compilation and analysis of the data from the interaction between histamine and the histamine H_2-receptor

antagonist, famotidine, confirmed that the between-replicate variance was not a general feature of oxyntic cell secretion in this assay. Furthermore, the replicate pentagastrin control curve locations were not especially variable. In fact, this variation was less than in the rat assay and no greater than in the guinea-pig gastric muscle assay (Table 1). Therefore, it appears that the variation has to be attributed to an action of L-365,260. This variation was evident in terms of the Schild plots which varied in slope and location (Figure 2b) and the individual pA_2 values ranged from low values which were similar to the pK_B expressed in the rat assay to high values which were similar to the pA_2 values estimated in the guinea-pig assay.

Previously, we have found that antagonist pK_B values can be significantly underestimated in the mouse stomach assay and the degree of underestimation was shown to be related to lipophilicity of several histamine H_2-receptor and muscarinic cholinoceptor antagonists (Shankley *et al.*, 1988). We speculated that the underestimation was due to the loss of lipophilic compounds into the acid secretion causing a net lowering of the concentration in the region of the oxyntic cell receptors. Subsequently, Welsh *et al.* (1992) found in a comparative study that the degree of pK_B underestimation in the rat stomach was significantly less than in the mouse stomach. L-365,260 is a lipophilic compound ($logP_{oct/Krebs} = 3.9$; Laurence Wright personal communication) whose activity would under-read by approximately four log units if the same loss model applied. However, the mean pA_2 value obtained in the mouse assay (7.93 ± 0.06) was significantly higher than the pK_B estimated in the rat assay (7.54 ± 0.05). In addition, four out of six individual affinity estimates made in the mouse assay were not significantly different from the mean pA_2 value obtained in the guinea-pig gastric muscle assay where no underestimation would be anticipated. Therefore, irrespective of the explanation for the complex behaviour of L-365,260 between and within the three assays, it would appear that its affinity is not underestimated to a significant extent in the mouse stomach assay. We considered the possibility that this might be due to pentagastrin acting indirectly in the mouse stomach by the release of histamine (Black *et al.*, 1985b) and that the loss of antagonist might be less in the region of the histamine-releasing cells than in the region of the oxyntic cells. However, we have previously performed an analysis which suggested that the pK_B value for atropine ($logP_{oct/H_2O} = 1.82$) was underestimated to the same extent in both cell types in the mouse stomach assay at muscarinic cholinoceptors on both cell types (Black & Shankley, 1985).

Another potential source of variation was the batch purity and solubility of L-365,260. In practice several different batches of L-365,260 were used in the study. However, each synthesis was strictly quality-controlled and purity was assessed by nuclear magnetic resonance (n.m.r.) and optical rotation. It was initially necessary to dissolve L-365,260 in DMF. However, the reproducibility of the rat stomach data, where the highest concentrations of the compound were used, and the mouse cortex radioligand binding assay data (Harper *et al.*, 1996) suggests that its limited aqueous solubility was not responsible for the data complexity.

Overall, the analysis of the mouse stomach and guinea-pig gastric muscle replicate data indicated that L-365,260 did not behave as a simple competitive antagonist at a homogeneous receptor population and appeared to expose between-replicate experimental variation. Several features of the data led us to consider whether the variable expression of two receptor subtypes could explain these results. First, all of the mouse Schild plot data lay within the space delineated by the considerably less variable affinity estimates made in the rat stomach and guinea-pig gastric muscle assays (Figure 2, Table 1). Second, two of three Schild plots with slopes which were not significantly different from unity had corresponding affinity values (7.68 and 8.47) which were similar to the rat and guinea-pig mean values respectively. In addition, as judged by inspection of Figure 2c, there was a suggestion of biphasicity in the replicate Schild plots obtained in the guinea-pig gastric muscle assay. Furthermore, a parallel analysis of the behaviour of L-365,260 in guinea-pig gastric gland and mouse and rat cerebral cortex CCK_B/gastrin receptor radioligand binding assays also produced results which were consistent with the expression of two receptor subtypes (Harper *et al.*, 1996).

Biphasic agonist concentration-effect curves can be a distinguishing feature of two receptor systems (see Kenakin, 1992). However, no biphasicity was detected in any of the pentagastrin concentration-effect curves obtained in the absence or presence of L-365,260 in the guinea-pig gastric muscle or mouse stomach assays. The simplest two-receptor model which can account for monophasic agonist curves, under conditions where both receptors are activated by an agonist, is in the form of that originally described by Furchgott (1981) and Lemoine & Kaumann (1983). In both models, activation of the receptor produces two intracellular stimuli which add algebraically. Pharmacological effect is given by a saturable function of the sum of these stimuli. In the model used by us (see Appendix), monophasic agonist concentration-effect curves are obtained when the parameters are set so that agonist activation of either receptor generates sufficient stimulus to produce maximum effect (see Appendix). According to the model, it is possible to obtain biphasic Schild plots with a selective antagonist. These plots have two asymptotes with unit slope and the lower asymptote is always equal to the higher pK_B value for the antagonist (see Appendix for details). Therefore, if this model is applicable to the current data, the mean pA_2 value calculated from the lowest significant dose-ratios obtained in the guinea-pig gastric muscle assay (8.56 ± 0.05) will approximate to the affinity of L-365,260 for one of the two inferred receptors. The upper asymptote is governed by the relative selectivity of the agonist and antagonist in the system and is less than the lower pK_B value for the antagonist.

In this study, the aim was to explore the conservative hypothesis that the data obtained across the three isolated tissue assays could be accounted for by the presence of two receptors defined in terms of their affinity for L-365,260. If the model was applicable and the low dose-ratios in the guinea-pig assay provided an estimate of the affinity of L-365,260 for one receptor, then the rat stomach data, because it was consistent with the one receptor model, provided the affinity of L-365,260 ($pK_B = 7.54 \pm 0.03$) for the other receptor. Using these affinity values, Schild plots were simulated by use of the two receptor model (Figure 2) and equation (7) presented in the Appendix. In the simulations, the different Schild plots were obtained by simply varying the operational selectivity of the agonist in terms of the potency of pentagastrin at the receptor characterized by the low affinity for L-365,260. In the model, the operational selectivity subsumes the affinity and efficacy (intrinsic and tissue-dependent) of the agonist. It is evident that a set of parameters could be obtained which could account for the guinea-pig gastric muscle data (Figure 2c). The guinea-pig data were best simulated when the parameters were set so that pentagastrin was 3-fold more potent at the receptor for which L-365,260 expresses high affinity. In mechanistic terms, this model description could be attributable to the presence of a 3-fold higher concentration of the high affinity receptors.

The mouse stomach data could also be simulated by the two-receptor model (Figure 2b). However, in this simulation, it was necessary to vary the operational selectivity of pentagastrin for the two receptor subtypes to account for the between-replicate experimental variation. If a difference in the level of expression of the two receptor subtypes is the source of the between-replicate experimental variation in the behaviour of L-365,260, then the mouse stomach may be a useful tissue for studying the regulation of receptor expression. The between-replicate variation in this tissue was so great that, if the individual experiments had been analyzed in isolation, on three out of six occasions it would have been concluded that the data were consistent with receptor homogeneity. However, on two out of three of these occasions, the receptor would have been

regarded as being indistinguishable from that in the rat, whereas on the third occasion, it would be regarded as being indistinguishable from that expressed predominantly in the guinea-pig gastric muscle assay. Although we have insufficient data to relate this variation to a particular source, there does not appear to be a relationship between the calendar month during which the experiments were performed and any parameters describing the data (Table 1).

The experiments represented here were not conducted according to a global experimental design with an entailed statistical approach. This means that our retrospective analysis of collections of experiments suffers from a lack of optimal constraints. We are aware of the dangers of both data selection and excessive analysis. To the first danger, all available data have been included irrespective of the known internal variations such as the experience of the investigators. To the second danger, we simply claim that we have been confronted with a degree of variation among the data sets that, in our opinion, could not be ignored. We chose to consider receptor heterogeneity as a possible source of this variation not only because of the systematic nature of the variation but also because we obtained further evidence of receptor heterogeneity in CCK_B/gastrin receptor radioligand binding assays performed over the same time period (Harper *et al.*, 1996).

In conclusion, the data obtained are compatible with the existence of two subtypes of gastrin receptors whose pharmacological expression varies significantly between the three assays and within replicate experiments in the mouse stomach assay. The rat stomach appeared to express a single receptor characterized by a low affinity constant for L-365,260 ($pK_B \sim 7.5$). The guinea-pig gastric muscle and mouse stomach data could be explained by the presence of this receptor and a second one characterized by a high affinity constant for L-365,260 ($pK_B \sim 8.6$). The activity of the two receptors was fairly consistent between experiments in the guinea-pig and the high affinity receptor appeared to be predominant. In contrast, the mouse stomach data could only be simulated by assuming that the proportion and absolute number of each receptor varied significantly between the replicate experiments. Clearly, further studies, with different agonists and antagonists, are required to substantiate these conclusions. It has been shown that different amino-acid residues in the CCK_B/gastrin receptor are involved in the binding of the gastrin-related peptides and benzodiazepine-derived antagonists, like L-365,260 (Beinborn *et al.*, 1993). In view of this, it will be of particular interest to see whether other chemical classes of antagonists can expose evidence of receptor heterogeneity between and within different tissues and species.

The L-365,260 affinity estimates at the inferred receptor subtypes are indistinguishable from those we obtained in a similar analysis of the behaviour of L-365,260 in CCK_B/gastrin receptor radioligand binding experiments in guinea-pig gastric gland and mouse and rat cerebral cortex preparations (Harper *et al.*, 1996). The relationship between our data, the results obtained by other groups who have quantified the activity of L-365,260 in a number of different bioassays and the molecular biological characterization of CCK_B/gastrin receptors are considered in the accompanying paper (Harper *et al.*, 1996).

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Appendix

Simulation of Schild plots for non-selective antagonists by use of a two receptor, addition of stimuli, model of agonism

A two-receptor, addition of stimuli, model of agonism was first developed by Furchgott (1981) and by Lemoine & Kaumann (1983). Kenakin (1992) further explored the behaviour of the model. The analysis of the data in the accompanying paper was performed by use of the following algebraic formulation of a two-receptor model. The details of this model were kindly provided by Paul Leff, Astra Research, Loughborough, U.K. In the model, two intracellular stimuli (S_1 and S_2) are produced as a rectangular hyperbolic function of the agonist concentration ([A]) as follows,

$$[S_1] = \frac{\alpha_1[A]}{K_1 + [A]}, \quad (1); \qquad [S_2] = \frac{\alpha_2 \cdot [A]}{K_2 + [A]}, \quad (2)$$

where α_1 and α_2 are the maximum concentrations of S_1 and S_2 which can be produced and K_1 and K_2 are the midpoint location parameters of the functions. These functions subsume an explicit model of agonism which would include parameters relating to the affinity and efficacy of the agonist at each receptor. Therefore, in terms of the production of stimulus, K_1 and K_2 can be considered as the agonist $[A]_{50}$ values at each receptor. Pharmacological effect, E is assumed to be given by a rectangular hyperbolic function of the sum (S_T) of S_1 and S_2,

$$E = \frac{E_M \cdot S_T}{K_S + S_T}, \quad (3)$$

where E_M is maximum effect and K_S the midpoint location of the E/$[S_T]$ curve. Substitution of equations (1) and (2) into (3) gives E as a quadratic function of [A],

$$E = \frac{E_M((\alpha_1 \cdot K_2 + \alpha_2 \cdot K_1)[A] + (\alpha_1 + \alpha_2)[A]^2)}{K_S \cdot K_1 \cdot K_2 + (K_S(K_1 + K_2) + \alpha_1 \cdot K_2 + \alpha_2 \cdot K_1)[A] + (K_S + \alpha_1 + \alpha_2)[A]^2}, \quad (4)$$

When activation of either receptor can produce maximum effect, that is when the condition $\alpha_1, \alpha_2 >> K_S$ applies, then equation (4) reduces to,

$$E = \frac{E_M \cdot [A]}{K_S/(\alpha_1/K_1 + \alpha_2/K_2) + [A]}. \quad (5)$$

Under these conditions, variation in K_1 and K_2 values are always accompanied by parallel displacements of the log agonist concentration-effect curves. The midpoint location of the overall agonist concentration-effect curve, $[A]_{50}$, is given by,

$$[A]_{50} = K_S/(\alpha_1/K_1 + \alpha_2/K_2). \quad (6)$$

If only one or other receptor was present then $[A]_{50}$ would be given by $K_1/(\alpha_1/K_S)$ and $K_2/(\alpha_2/K_S)$, respectively. With both receptors present, the ratio of these two terms defines the operational selectivity of the agonist, that is, $(\alpha_1/K_1)/(\alpha_2/K_2)$. The effects of a competitive antagonist (B) on the $[A]_{50}$ value are obtained in the usual way by multiplying the location parameters, K_1 and K_2, in equation (6) by factors $(1 + [B]/K_{B1})$ and $(1 + [B]/K_{B2})$, respectively. The corresponding Schild equation is given by,

$$\frac{[A]_{50B}}{[A]_{50}} = 1 + \frac{(\alpha_1 \cdot K_2 \cdot K_{B2} + \alpha_2 \cdot K_1 \cdot K_{B1})[B] + (\alpha_1 \cdot K_2 + \alpha_2 \cdot K_1)[B]^2}{K_{B1} \cdot K_{B2}(\alpha_1 \cdot K_2 + \alpha_2 \cdot K_1) + (\alpha_1 \cdot K_2 \cdot K_{B1} + \alpha_2 \cdot K_1 \cdot K_{B2})[B]}. \quad (7)$$

This equation has the general features shown in the Schild plots presented in the accompanying paper (Figure 2). Whether a particular system displays an inflexion producing a biphasic Schild plot with unit slope straight line asymptotes depends on the selectivities of the agonist and antagonist involved. When agonist and antagonist have opposite selectivities and/or when the antagonist is completely non-selective, then unit-slope linear Schild plots are predicted. Under all conditions, the intersection of the lower asymptote with the log [B] axis corresponds to the log K_B value for the antagonist at the receptor for which the agonist is selective. In contrast, the extrapolation of the upper asymptote to the log [B] axis does not give the other log K_B value but actually underestimates the value making the antagonist appear more potent. This is because the low dose-ratio information reflects competition only at the receptor through which the agonist is more potent. Higher dose-ratio information reflects competition of the antagonists at both receptors. Mathematically this result can be proven as follows. Equation (7) can be written in Schild plot form,

$$\log(r - 1) = \frac{\log(a.[B] + b.[B]^2)}{c + d.[B]}, \quad (8)$$

in which,

r = dose-ratio
$a = \alpha_1 \cdot K_2 \cdot K_{B2} + \alpha_2 \cdot K_1 \cdot K_{B1}$
$b = \alpha_1 \cdot K_2 + \alpha_2 \cdot K_1$
$c = K_{B1} \cdot K_{B2}(\alpha_1 \cdot K_2 + \alpha_2 \cdot K_1)$
$d = \alpha_1 \cdot K_2 \cdot K_{B1} + \alpha_2 \cdot K_1 \cdot K_{B2}$

At low values of [B], equation (8) approximates to the equation of the lower asymptote, $\log(r-1) = \log[B] + \log(a/c)$, and at high values of [B] to the equation of the upper asymptote, $\log(r-1) = \log(b/d)$. The intercepts of the two asymptote lines are defined by the condition when $\log(r-1)$ equals zero. The lower asymptote is given by,

$$-\log[B] = pK_{B1} + log\,(1 + \frac{K_{B1} \cdot \alpha_2 \cdot K_1)}{K_{B2} \cdot \alpha_1 \cdot K_2}$$

When both the agonist and antagonist are selective for R_1, so that $K_{B1} < K_{B2}$ and $\alpha_1.K_2 > \alpha_2 K_1$, then this equation simplifies to

$-\log[B] = pK_{B1}$ because the term on the right hand side of the equation becomes log (1) which is zero. The upper asymptote is defined as follows,

$$-\log[B] = pK_{B2} + \log(1 + \frac{K_{B1} \cdot \alpha_2 \cdot K_1)}{K_{B2} \cdot \alpha_1 \cdot K_2}$$

In this case, the conditions $K_{B1} < K_{B2}$ and $\alpha_1.K_2 > \alpha_2.K_1$ mean that the term on the right hand side of the equation is not insignificant but makes the intercept a larger quantity than pK_{B2}. Therefore, the intercept overestimates the affinity constant K_{B2}.

References

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