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Pharmacological analysis of the CCK_B/gastrin receptors mediating pentagastrin-stimulated gastric acid secretion in the isolated stomach of the immature rat

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1 The CCK_B/gastrin receptors mediating pentagastrin stimulation of gastric acid secretion by histamine release and by direct stimulation of oxyntic cells have been characterized in the immature rat isolated stomach assay. This was achieved by estimating antagonist affinity values for competitive antagonists from three distinct chemical classes (L-365,260, PD134,308 and JB93190) in the absence and presence of a high concentration of the histamine H_2-receptor antagonist, famotidine (30 μM).

2 Pentagastrin produced concentration-dependent stimulation of gastric acid secretion in the absence and presence of famotidine. Famotidine depressed the maximum secretory response to pentagastrin although the degree of depression varied between experimental replicates (25-60%). This variation was attributed to the histamine-release mediated component of acid secretion, as judged by the consistency of the maximum responses obtained in the presence, but not absence, of famotidine.

3 All three CCK_B/gastrin receptor antagonists behaved as surmountable antagonists in the absence and presence of famotidine. JB93190 ($pK_B \sim 9.1$, ~ 8.9, in the absence and presence of famotidine, respectively) was approximately 30 fold more potent than either L-365,260 ($pK_B \sim 7.4$, ~ 7.1) or PD134,308 ($pK_B \sim 7.6$, ~ 7.4).

4 It was assumed that the famotidine treatment converted pentagastrin-stimulated acid secretion from a combination of an indirect action due to the release of histamine and a direct action on the oxyntic cell to solely a direct action on the oxyntic cell. A simple mathematical model of this two-receptor system was developed. The direct and indirect components were assumed to sum to produce the total response to pentagastrin obtained in the absence of famotidine. It was found that this model could account quantitatively for the behaviour of the three antagonists without invoking a difference in antagonist affinity for the CCK_B/gastrin receptors mediating the direct and indirect actions of pentagastrin. However, a conclusion of receptor homogeneity has to be qualified because the model was also used to generate simulations which indicated that the analysis could only detect antagonist affinity differences of greater than one log-unit between enterochromaffin-like (ECL) and oxyntic cell CCK_B/gastrin receptor populations.

Keywords: Pentagastrin; histamine; CCK_B/gastrin receptors; gastric acid secretion

Introduction

The peptide hormone, gastrin, is a member of the cholecystokinin (CCK) family of peptides with which it shares a common carboxyl-terminal pentapeptide sequence (Rehfeld, 1981). The receptors mediating the actions of these peptides have been classified as CCK_A and CCK_B/gastrin (see Woodruff & Hughes, 1991). CCK_B/gastrin receptors have been cloned from rat (Wank *et al.*, 1992), *Mastomys* (Nakata *et al.*, 1992), rabbit (Blandizzi *et al.*, 1994), dog (Kopin *et al.*, 1992) and human (Pisegna *et al.*, 1992; Lee *et al.*, 1993; Ito *et al.*, 1993) tissues. These cloned receptors show a high degree of homology. However, interspecies differences in the apparent affinity of the CCK_B/gastrin receptor antagonist, L-365,260, have been obtained in radioligand binding assays performed on both native tissues (Lotti & Chang, 1989; Harper *et al.*, 1995) and cell line receptor expression systems (Beinborn *et al.*, 1993). This is consistent with functional, intact tissue, studies showing differences in the potency of L-365,260 in rat and guinea-pig assays (Patel & Spraggs, 1992; Roberts *et al.*, 1995). It has been suggested that these differences may be due, at least in part, to single amino acid variations in the region of the receptor relating to the 6th transmembrane domain (Beinborn *et al.*, 1993). Until recently there was no evidence for functional differences in CCK_B/gastrin receptors within a single species (Presti & Gardner, 1993). However, a recent study analysing the antagonism of L-365,260 in *in vitro* assays from rat, mouse and guinea-pig concluded that, in the mouse and guinea-pig, its behaviour was inconsistent with that of a simple competitive antagonist at a pharmacologically-defined, single receptor site (Roberts *et al.*, 1995). A similar conclusion was reached from an analysis of the behaviour of L-365,260 in radioligand binding assays performed on rat and mouse cerebral cortex and guinea-pig gastric mucosa tissue (Harper *et al.*, 1995).

To date, only one CCK_B/gastrin receptor gene has been identified within an individual species. However, following cloning of the *Mastomys* ECL carcinoid tumour CCK_B/gastrin receptor, stringent hybridization techniques revealed CCK_B/gastrin receptor mRNA of different transcript sizes in brain and stomach tissues (Nakata *et al.*, 1992). Subsequently, the human CCK_B/gastrin receptor gene has been shown to be alternatively spliced to yield two different mRNAs (Song *et al.*, 1993); a long (452 amino acids) and a short isoform (447 amino acids). The long isoform differs by possessing the additional

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pentapeptide sequence Gly-Gly-Ala-Gly-Pro in the region relating to the putative third intracellular loop of the receptor. This region is thought to be important for G-protein coupling and hence signal transduction (Savarese & Fraser, 1992). RNase-protection assays have determined that a single isoform is preferentially expressed in the human brain, stomach and pancreas, and subsequent S1 nuclease mapping has shown this to correspond to the short isoform (Ito *et al.*, 1994). The long isoform has been found to be conserved in canine, rat and *Mastomys* CCK_B/gastrin receptors (Ito *et al.*, 1993; Lee *et al.*, 1993; Pisegna *et al.*, 1992). Separate expression of the two human CCK_B/gastrin receptor isoforms in mouse fibroblasts showed the same characteristics in their agonist binding and signal transduction pathways (Ito *et al*, 1994). However, L-365,260 has been shown to be 3 fold more potent at inhibiting [^{125}I]-Bolton–Hunter(BH)-CCK-8 binding to the short isoform compared to the long isoform (Wank *et al.*, 1994).

In the rat, pentagastrin has been shown to stimulate gastric acid secretion both indirectly, via histamine release, and directly (Sandvik *et al.*, 1987; Welsh *et al.*, 1992a), by interacting with CCK_B/gastrin receptors situated on enterochromaffin-like (ECL) and oxyntic cells, respectively. It was the aim of this study to determine whether the CCK_B/gastrin receptors on oxyntic and ECL cells in the rat stomach could be distinguished pharmacologically. To achieve this, CCK_B/gastrin receptor antagonists from three distinct chemical classes were used: L-365,260 (Lotti & Chang, 1989), a 1,4-benzodiazepine, PD134,308 (Hughes *et al.*, 1990), a dipeptoid and JB93190 (Kalindjian *et al.*, 1996), a benzimidazole. These antagonist studies were performed in the absence and presence of famotidine, a selective histamine H_2-receptor antagonist, in order to convert the pentagastrin-stimulated acid secretory mechanism from a combination of direct and indirect action to a direct action at the oxyntic cell.

A preliminary account of these data was presented to the British Pharmacological Society (Hills *et al.*, 1996).

Methods

Immature rat isolated, lumen-perfused, stomach assays

Gastric acid secretion was measured in isolated, lumen-perfused stomachs, prepared essentially as described (Welsh *et al.*, 1993). Pre-weaned rat pups (Wistar, 28–42 g, corresponding to an age range of 14–21 days) were killed by cervical dislocation. 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 and the contents of the stomach flushed through the mucosal solution (mM: NaCl 118, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 1.3, glucose 31.6) to remove any remaining food. A second cannula was ligated tightly into the incision. 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°C and gassed vigorously with 95% O_2 and 5% CO_2. The stomachs were perfused from the fundic to the pyloric cannulae with warmed mucosal solution gassed with 100% O_2 at a rate of 1 ml min^{-1} and the perfusate passed over a pH-electrode system adjusted for height to provide 12 cmH_2O intragastric pressure. The selective acetylcholine M-receptor agonist, 5-methylfurmethide (30 nM), was included in the serosal solution as it has been shown (Welsh, 1992) to potentiate (i.e. leftward shift) and amplify (increase the maximum response) pentagastrin concentration-effect curves in this species. When used, the histamine H_2-receptor antagonist famotidine (30 μM: 1000 fold the K_B at histamine H_2-receptors, Welsh *et al.*, 1992b) was present in the serosal solution throughout the experiment.

Six preparations were used simultaneously and experimental treatments were allocated to each organ bath according to randomized block designs. The effect of each CCK_B/gastrin receptor antagonist on pentagastrin concentration-effect (E/[A]) curves, obtained in the absence and presence of 30 μM famotidine, were investigated within individual experiments which were performed consecutively over a four to five week period. The effect of the same concentration of famotidine on histamine E/[A] curves was determined in a separate experiment. Agonist-stimulated responses were expressed as the change in the pH (ΔpH) of the lumen perfusate from the basal pH immediately before the first addition of pentagastrin. This response metameter was chosen in preference to [H^+] as a previous analysis has shown that pH values obtained under basal and secretogogue-stimulated conditions in isolated stomach assays are normally-distributed (Shankley, 1985; see Appendix). The preparations were allowed to stabilize for 60 min before the drug addition made directly to the serosal solution. Antagonists were allowed to equilibrate for 60 min before a single cumulative, pentagastrin or histamine E/[A] curve was obtained. Pentagastrin was dosed at either one or half-log unit intervals (1 nM to 100 μM). When histamine was the agonist, half-log unit dose intervals (0.1 μM to 300 μM) were used.

Data 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 (expressed as log[A]$_{50}$) and upper asymptote (α) as described previously (Black & Shankley, 1985). For analysis and display purposes the individual parameter estimates for each treatment group were expressed as mean $\pm$ s.e.mean and single curves simulated and shown superimposed upon the mean experimental data. The effect of drug treatments on these parameters was assessed by one-way analysis of variance (ANOVA). P values of less than 0.05 were considered to be significant.

Analysis of competitive antagonism

When the minimum criteria for competitive antagonism were satisfied, that is the antagonist produced parallel, rightward shift of agonist E/[A] curves with no change in upper asymptote, data were analysed 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 equation,

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

where [B] is the antagonist concentration, K_B is the antagonist equilibrium dissociation constant, b is the Schild plot slope parameter. If b was not significantly different from unity, it was constrained to a value of unity and the data refitted to provide a pK_B estimate. The substitution of $10^{\log K_B}$ for K_B in equation (2) was based on the assumption that the K_B values were log-normally distributed. In one analysis (JB93190 in the absence of famotidine), the agonist E/[A] curves were significantly steeper in the presence of the antagonist and so the criteria for competitive antagonism were not strictly met. Therefore, the pK_B estimate obtained was referred to as an apparent pK_B (pK_B') as recommended by Jenkinson (1991).

The goodness-of-fit of the two-receptor model simulations (see results) to the experimental data was assessed using a chi-squared (χ^2) analysis where χ^2 was calculated by comparing the

mean observed data points with the expected values obtained by model simulation from the following equation:

$$\chi^2 = \sum \frac{(\text{observed} - \text{expected})^2}{\text{m}(\text{expected}^2) + \text{c}} \quad (3)$$

The denominator of this equation is a correction factor applied to account for the approximately linear relationship which was found between the observed variance (σ^2) and the square of the observed effect values for the data from individual experiments (see Welsh *et al.*, 1995). The values of m (slope) and c (intercept) were estimated by linear regression analysis on these data.

Drugs

Pentagastrin (Bachem, California) 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 (3**R**(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N'*-(3-methylphenyl)urea; a gift from Merck Sharp & Dohme Research Labs.), PD134,308 (4-{[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-v[[(tricyclo[3,3,1,$1^{3,7}$]dec-2-yloxy)carbonyl]amino]propyl]-amino]-1-phenylethyl]amino}-4-oxo-[**R**-(**R***,**R***)]-butanoate; a gift from Parke Davis Ltd.) and JB93190 (5-[[[(1**S**)-[[(3,5-dicarboxyphenyl)amino]-carbonyl]-2-phenylethyl]amino]-carbonyl]-6-[[(1-adamantyl methyl)amino]carbonyl]-benzimidazole; James Black Foundation) were initially dissolved (to 4 mM) and subsequently diluted in DMF. 5-Methylfurmethide iodide (a gift from Wellcome Research Labs.) was dissolved (to 3 mM) and subsequently diluted to 30 μM in water. Famotidine (Sigma) was dissolved in dilute HCl (~0.01 N) to 40 mM).

Pentagastrin was prepared each day. L-365,260, PD134,308, JB93190, 5-methylfurmethide and famotidine were prepared at the beginning of individual experiments and kept frozen for the duration of that experiment. The total volume of drug added to each 40 ml bath in any one experiment did not exceed 1 ml.

Results

Pentagastrin and histamine control concentration-effect curves in the immature rat stomach

Pentagastrin and histamine produced concentration-dependent increases in gastric acid secretion (Figure 1). Pentagastrin and histamine control E/[A] curve data were fitted to the Hill equation (1) and the mean parameter values estimated are shown in Table 1. The mid-point location ($p[A]_{50}$) and the midpoint slope (n_H) parameter values of the three replicate control pentagastrin E/[A] curves were consistent between individual antagonist experiments although the upper asymp-

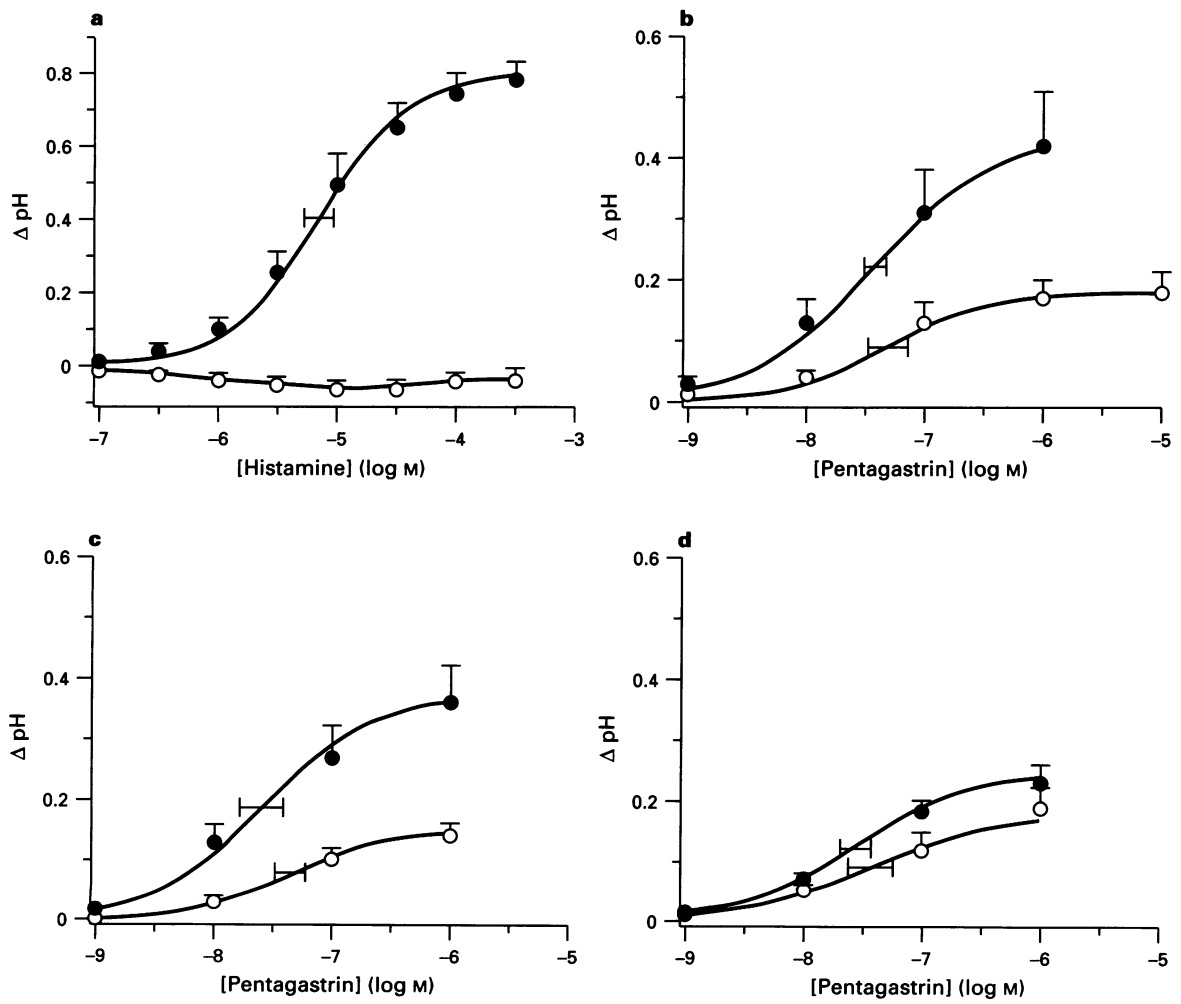

Figure 1 Histamine (a, $n=7$) and pentagastrin (b–d) E/[A] curves obtained in the absence (●) and presence (○) of famotidine (30 μM) in the immature rat isolated, lumen-perfused, stomach assay. The pentagastrin control E/[A] curves are shown from three experiments performed to quantify the activity of the CCK_B/gastrin receptor antagonists, L-365,260 (b, $n=7$), PD134,308 (c, $n=6$) and JB93190 (d, $n=8$). The lines superimposed on the mean experimental data were simulated by use of the Hill equation (1) with the parameter values set at the mean values obtained by curve fitting shown in Table 1. Error bars represent s.e.mean.

Table 1 Hill equation parameters obtained by fitting individual concentration-effect curve data from cumulative dosing of immature rat isolated, lumen-perfused, stomach preparations with histamine or pentagastrin in the absence and presence of the histamine H_2-receptor antagonist, famotidine (30 μM)

		Control				*Famotidine-treated*		
Agonist	*d.f.*	α (ΔpH)	$p[A]_{50}$	n_H	*d.f.*	α (ΔpH)	$p[A]_{50}$	n_H
Histamine	6	0.80±0.05	5.15±0.13	1.17±0.13	8	−0.04±0.03	–	–
Pentagastrin								
(L-365,260)	6	0.44±0.09	7.41±0.09	0.86±0.04	5	0.18±0.03	7.30±0.17	1.01±0.11
(PD134,308)	5	0.37±0.06	7.60±0.18	0.94±0.07	5	0.15±0.03	7.36±0.13	1.08±0.09
(JB93190)	7	0.24±0.03	7.57±0.13	0.88±0.05	5	0.18±0.03	7.44±0.19	0.78±0.09

The values are given for the pentagastrin control curves obtained in each of the three CCK_B/gastrin receptor antagonist experiments. $p[A]_{50}$ and n_H values are not given for histamine in the presence of famotidine because no significant response was obtained with up to 0.3 mM histamine. d.f. refers to the degrees of freedom (=no. of replicates curves−1). Data shown are means±s.e.mean.

Table 2 Competitive antagonism model parameters estimated by use of data from the interaction between pentagastrin and three CCK_B/gastrin receptor antagonists obtained in the absence and presence of the histamine H_2-receptor antagonist famotidine (30 μM)

		Control				*Famotidine treated*		
Antagonist	ΔpH *basal*	pK_B	*b*	*d.f.*	ΔpH *basal*	pK_B	*b*	*d.f.*
L-365,260	0.02±0.04	7.40±0.10	0.81±0.10	30	−0.03±0.01	7.10±0.12	0.84±0.10	35
PD134,308	0.02±0.05	7.61±0.11	0.92±0.10	28	−0.03±0.02	7.36±0.10	1.20±0.11	23
JB93190	0.08±0.12	9.08±0.10*	1.22±0.15	24	−0.02±0.01	8.94±0.15	1.32±0.24	24

d.f. refers to the degrees of freedom (=no. of values −2) in the model fit to equation (2) of log $[A]_{50}$ values obtained in the absence and presence of antagonist. The columns show the effect of the highest concentration of the antagonist on basal acid secretion (ΔpH basal±s.e.mean) and, from an analysis of all the data, the negative logarithm of the antagonist equilibrium dissociation constant (pK_B±s.e.) and the Schild plot slope parameter (b±s.e.). *Apparent pK_B (for details of the analysis see text).

totes (α) of the curves varied considerably. In each of the three antagonist experiments, pentagastrin was ~240 fold more potent than histamine but maximum secretory responses were significantly lower.

Effect of histamine H_2-receptor blockade on histamine and pentagastrin concentration-effect curves

When famotidine (30 μM; ~1000 fold K_B), previously shown to behave as a competitive, surmountable antagonist of histamine in this assay (Welsh *et al.*, 1992b), was included in the serosal bathing solution throughout the experiment, basal pH values in control experiments were significantly higher (basal pH: absence = 4.43±0.06, n = 20; presence = 4.69±0.04, n = 17; $P<0.05$). This concentration of famotidine abolished the secretory action of exogenously-administered histamine (0.1–300 μM; Figure 1a). Therefore, it was assumed that any pentagastrin response obtained in the presence of famotidine was due to a direct action on oxyntic cells, that is, it was independent of an action of endogenous histamine on oxyntic cell histamine H_2-receptors.

In two of the three antagonist experiments (Figure 1b–d), famotidine significantly depressed the pentagastrin E/[A] curve maxima ($P<0.05$, d.f. = 10 and 11; Table 1). In the third experiment the mean maximum response value was lower in the presence of famotidine but this reduction was not significant as tested. Similarly, the mean log $[A]_{50}$ values of the pentagastrin E/[A] curves were all increased in the presence of famotidine but these rightward shifts were not significant as tested. The degree of depression of the curve maxima, when expressed as a percentage of the corresponding control curve maxima, varied between 25 and 60%. However, the maximum ΔpH (Table 1) obtained in the presence of famotidine was less variable between experiments (0.15–0.18 pH units) compared to pentagastrin alone (0.24–0.44 pH units).

Antagonist studies

The three CCK_B/gastrin receptor antagonists, as well as their corresponding vehicle controls, had no significant effect on basal acid secretion either in the absence or presence of famotidine (Table 2). In the absence of famotidine, L-365,260 (0.1–3 μM; Figure 2a) and PD134,308 (0.1–3 μM) satisfied the basic criteria for competitive antagonism. That is, they produced parallel, concentration-dependent, rightward shifts of the pentagastrin E/[A] curves with no significant effect on the curve upper asymptotes. Subsequent analysis gave Schild slope parameter estimates which were not significantly different from unity (Table 2). In contrast, in the absence of famotidine, JB93190 (1 nM–30 nM) also shifted pentagastrin E/[A] curves to the right without affecting the maximum response but produced a significant increase in the slope of the E/[A] curves ([JB93190], n_H values: control, 0.88±0.05; 1 nM, 0.86±0.06; 3 nM, 0.83±0.08; 10 nM, 1.10±0.07; 30 nM, 1.09±0.10). Although one of the criteria for competitive antagonism was not satisfied, the log $[A]_{50}$ data obtained in the absence and presence of JB93190 were fitted to the derivation of the Schild equation (2). The Schild plot slope parameter estimated was not significantly different from unity and a second model fit, performed with this parameter constrained to unity, converged to yield a pK_B' value of 9.08±0.10 (d.f. = 34). This value was similar to the affinity estimate (9.01±0.17) which was calculated from the lowest significant dose-ratio obtained in the presence of 1 nM JB93190, a concentration which did not have a significant effect on the slope of the pentagastrin E/[A] curve.

In the presence of famotidine (L-365,260 data shown in Figure 2b), the basic criteria for competitive antagonism were all satisfied and the pK_B values estimated for each antagonist are presented in Table 2.

Development and application of a two receptor model

The gastric acid secretory action of pentagastrin in the immature rat stomach assay comprises a direct and indirect component (see Introduction). In addition to a direct activation of CCK_B/gastrin receptors on oxyntic cells, pentagastrin acts at CCK_B/gastrin receptors situated on ECL cells to release histamine which stimulates acid secretion by activation of oxyntic cell histamine H_2-receptors. Based on the data shown in Figure 1a, it was assumed that the high concentration of the histamine H_2-receptor antagonist, famotidine (30 μM), abolished the histamine-mediated (indirect) component to expose

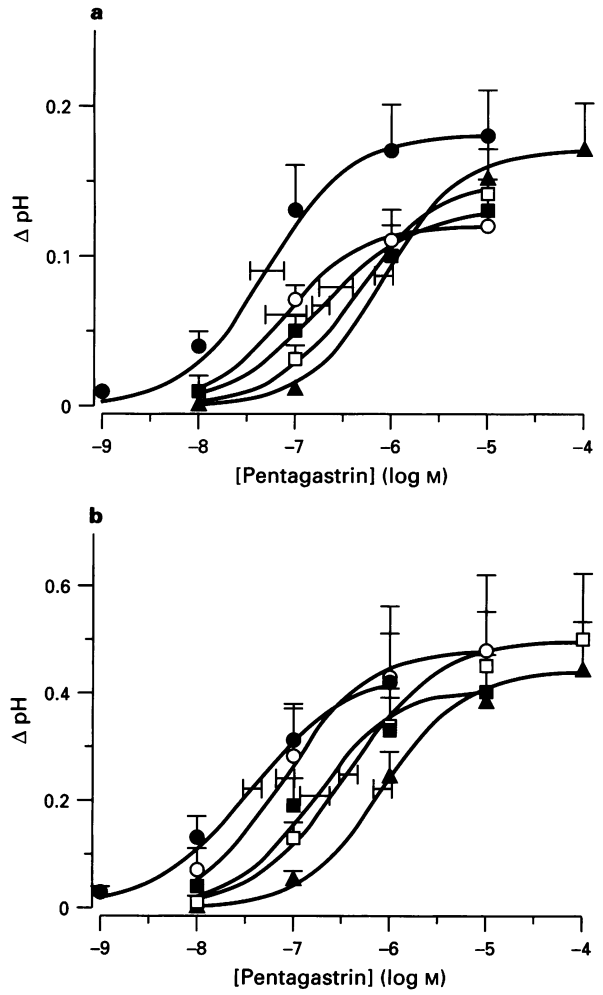

Figure 2 Immature rat isolated, lumen-perfused, stomach assay. Pentagastrin E/[A] curves obtained in the absence (●) and presence of L-365,260 (μM; ○, 0.03; ■, 0.1; □, 0.3; ▲, 1.0) both in the presence (a) and absence (b) of famotidine (30 μM). The lines superimposed on the mean experimental data ($n = 6/8$) were simulated by use of the Hill equation (1) with the parameter values set at the mean values obtained by curve fitting. Error bars represent s.e.mean.

the direct action of pentagastrin at the oxyntic cell. Therefore, the antagonist pK_B estimates obtained in the presence of famotidine could be assumed to characterize the oxyntic cell CCK_B/gastrin receptors.

The problem was how to characterize the ECL cell receptor population from the data obtained in the absence of famotidine under which condition pentagastrin appears to act at both ECL and oxyntic cells. The approach taken was to build a mathematical model which included the minimum necessary features to describe the system. For simplicity it was assumed that the total secretory response to pentagastrin was given by the algebraic sum of its two cellular actions. Thus, the histamine-mediated component could be calculated as the difference between the responses to pentagastrin obtained in the absence and presence of famotidine. In practice, the difference between the fitted mean pentagastrin E/[A] curves obtained in the absence and presence of famotidine was measured, arbitrarily, at 0.5 log unit concentration intervals and the data generated, representing the mean histamine-mediated pentagastrin E/[A] curve, were fitted to the Hill equation (1). Each of the CCK_B/gastrin receptor antagonist experimental data sets were treated in this way. With this information it was possible to simulate quantitative expectations for the effect on the total pentagastrin response of competitive antagonists expressing selectivity for either the ECL or oxyntic cell CCK_B/gastrin receptors (Figure 3). Simulations were obtained with the following equation:

$$E_{total} = E_{direct} + E_{indirect} \quad (4)$$

where,

$$E_{direct} = \frac{\alpha_1[A]^n}{\left([A]_{50_1}\left(1+\frac{[B]}{K_{B_1}}\right)\right)^n + [A]^n}, \text{ and}$$

$$E_{indirect} = \frac{\alpha_2[A]^m}{\left([A]_{50_2}\left(1+\frac{[B]}{K_{B_2}}\right)\right)^m + [A]^m}$$

E is pharmacological effect, [A] is agonist concentration, α_1 and α_2, $[A]_{50_1}$ and $[A]_{50_2}$, n and m, are the upper asymptotes, mid-point locations and mid-point slopes of the Hill equations used to describe the acid secretory actions of pentagastrin at oxyntic and ECL cells, respectively. [B] is the antagonist concentration, and K_{B_1} and K_{B_2} are the antagonist equilibrium dissociation constants for the oxyntic and ECL cell CCK_B/gastrin receptor populations, respectively. To facilitate the simulation process, mean experimental E/[A] curve data were normalized to their respective upper asymptotes on the basis that none of the antagonist treatments produced a significant effect on the maximum secretory responses to pentagastrin.

In order to determine if the experimental data were indicative of receptor heterogeneity, the hypothesis that CCK_B/gastrin receptors on oxyntic and ECL cells were identical was tested. Simulations for the three antagonist data sets were obtained by assuming that K_{B_1} and K_{B_2} in equation (4) were both equal to the K_B values estimated, by Schild analysis, from the data obtained in the presence of famotidine (Table 2). Chi squared analysis indicated that there were no significant differences ($P > 0.05$) between the observed and expected values for the model simulations of the L-365,260 ($\chi^2 = 7.95$, d.f. = 24) and JB93190 ($\chi^2 = 16.08$, d.f. = 20) data obtained. However, there was a significant difference between observed and expected values for the data obtained with PD134,308 ($\chi^2 = 78.2$, d.f = 20). An example of the model simulations is shown in Figure 4.

Discussion

The aim of this study was to determine if ECL and oxyntic cell CCK_B/gastrin receptors could be distinguished pharmacologically with three competitive antagonists. A high concentration of the selective histamine H_2-receptor antagonist, famotidine, was used to convert the total secretory response to pentagastrin, a combination of direct (oxyntic) and indirect (ECL) actions, to a response assumed to be mediated solely by activation of oxyntic cell CCK_B/gastrin receptors. The concentration of famotidine (30 μM) employed was sufficient to abolish the response to 300 μM exogenously-administered histamine (Figure 1a). If pentagastrin was capable of elevating endogenous levels of histamine to higher concentrations than this in the region of the oxyntic cell histamine H_2-receptor then pentagastrin would be expected to produce the same maximum response as achieved with exogenous histamine. In practice, pentagastrin was a partial agonist in the system with respect to exogenous histamine. Therefore, the assumption that 30 μM famotidine abolished the histamine-mediated component of pentagastrin-stimulated acid secretion appears to be reasonable. The acetylcholine (ACh) M-receptor agonist, 5-methylfurmethide, was included in the assay in an attempt to improve the signal-to-noise ratio. It is possible that 5-methylfurmethide expresses a differential effect on ECL and oxyntic cells although this would not be expected to undermine the analysis which is based on null methods.

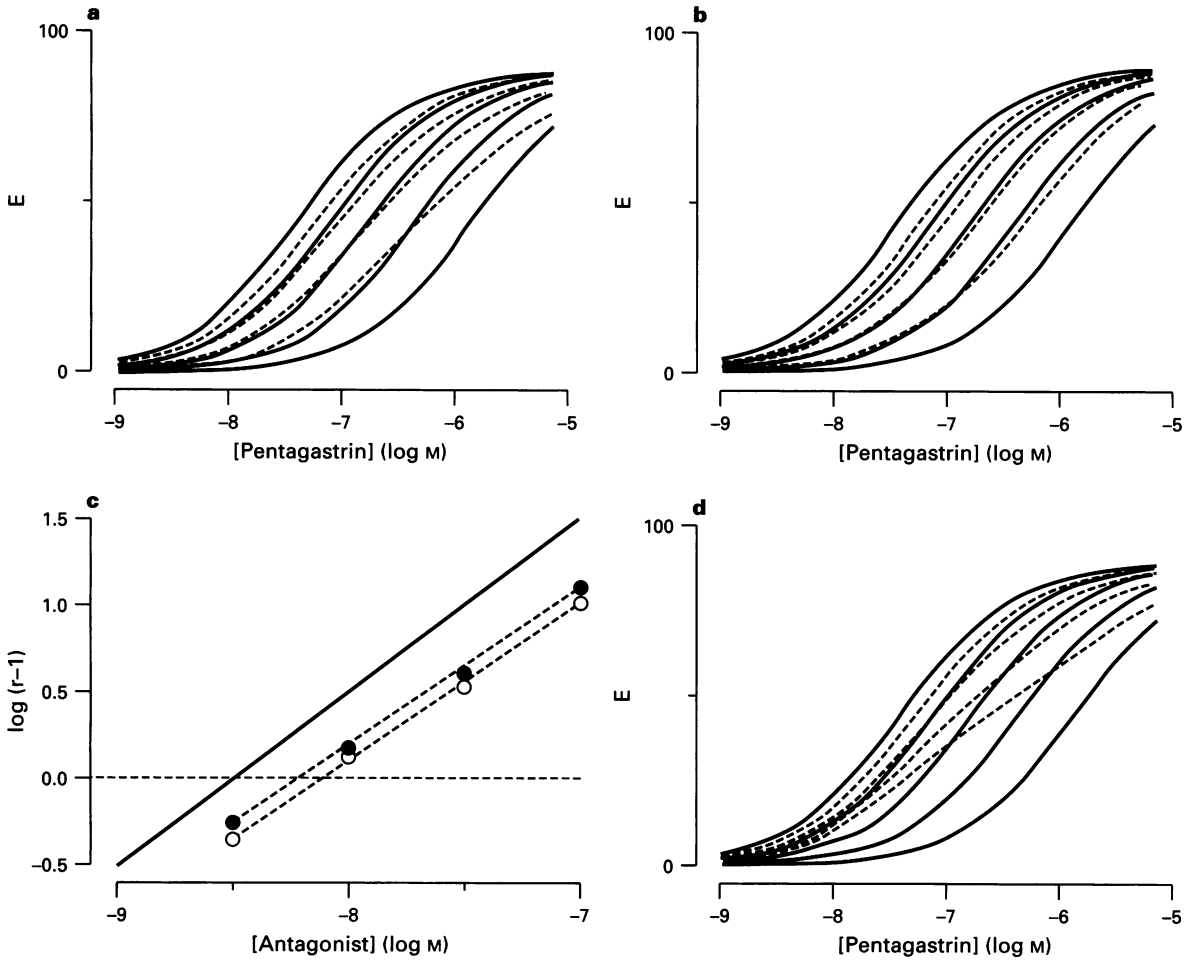

Figure 3 Two receptor model simulations of the stimulation of gastric acid secretion by pentagastrin in the immature rat isolated stomach. (a) and (b) Two receptor model simulations (equation 4) of the theoretical effect of a competitive antagonist, in the absence of famotidine, on a pentagastrin E/[A] curve. The solid lines show the parallel rightward shift with no change in upper asymptote expected for an antagonist which does not discriminate between the ECL and oxyntic cell CCK_B/gastrin receptor (i.e. $K_{B_1} = K_{B_2}$). The hatched lines show the E/[A] curves expected for an antagonist expressing 10 fold higher affinity for (a) the ECL cell ($K_{B_1} = 10 \cdot K_{B_2}$) or (b) the oxyntic cell (i.e. $K_{B_1} = 0.1 \cdot K_{B_2}$). (c) The Schild plots corresponding to (a, ●) and (b, ○). The solid line shows the expected Schild plot, with unit slope, for a non-selective antagonist (i.e. $K_{B1} = K_{B_2} = 3$ nM). Other parameter values in equation 4 were set as follows: $\alpha_1 = 41\%$, $\alpha_2 = 49\%$, $[A]_{50_1} = 32$ nM, $[A]_{50_2} = 49$ nM, $n = 1.01$, $m = 0.78$ and $[B] = 0, 3, 10, 30, 100$ nM. (d) The two receptor model simulation obtained from the same parameter values used in (a) and (b) but with the antagonist expressing 3 log units of selectivity for the ECL cell ($K_{B_1} = 1000.K_{B_2}$).

Previously, we found that histamine H_2-receptor blockade effectively abolished the acid secretory response to pentagastrin in isolated preparations of mouse and guinea-pig gastric mucosa (Black *et al.*, 1985b; Shankley *et al*, 1992) precluding the pharmacological characterization of any oxyntic cell CCK_B/gastrin receptors in these types of assay in these species. In this study, with the immature rat stomach assay, the effects of famotidine in the three replicate experiments were qualitatively consistent with previous experiments in which the histamine H_2-receptor antagonist tiotidine was used (Shankley *et al.*, 1992 Welsh *et al.*, 1992a). In the presence of H_2-receptor blockade, pentagastrin produced a significant, concentration-dependent, stimulation of acid secretion presumably by activation of oxyntic cell CCK_B/gastrin receptors. The only difference in experimental protocol in the current experiments was inclusion of the ACh M-receptor agonist, 5-methylfurmethide, to potentiate the pentagastrin response (see Methods). This could account for the fact that in the current experiments histamine H_2-receptor blockade reduced the basal acid secretion, a result not found in our previous studies. Previously, we have shown that 5-methylfurmethide-stimulated acid secretion is inhibited by histamine H_2-receptor blockade in the immature rat stomach assay (Welsh *et al.*, 1995). The 0.26 pH unit decrease in basal secretion is numerically equivalent to 30% of the maximum response which was obtained by addition of exogenous histamine in the absence of famotidine (0.80 pH, Table 1). According to the Schild equation, this level of basal histamine would not be expected to alter significantly the effectiveness of famotidine (30 μM $\cong$ 1000 fold K_B) when blocking the subsequent action of exogenous or pentagastrin-released histamine. However, the significant effect of famotidine on basal acid secretion did raise another potential problem with the analysis. The comparison of data expressed as changes from basal values (i.e. ΔpH) which are different is potentially misleading. However, in this instance, we believe the analysis is still valid because the direct, oxyntic cell, and histamine-release mediated effects of pentagastrin appeared to be additive. Therefore, it seems reasonable to suppose that the basal component of histamine-mediated secretion which was exposed by treatment with famotidine is also additive with respect to the agonist-stimulated secretion.

Notwithstanding the inclusion of 5-methylfurmethide in the serosal bathing solution, the pentagastrin E/[A] curve $p[A]_{50}$ values were similar to those previously obtained in the immature rat stomach assay (Welsh, 1992), and with those obtained in a rat gastric mucosal sheet assay (Patel & Spraggs, 1992). The intrinsic activity of pentagastrin was consistent between replicate experiments when pentagastrin

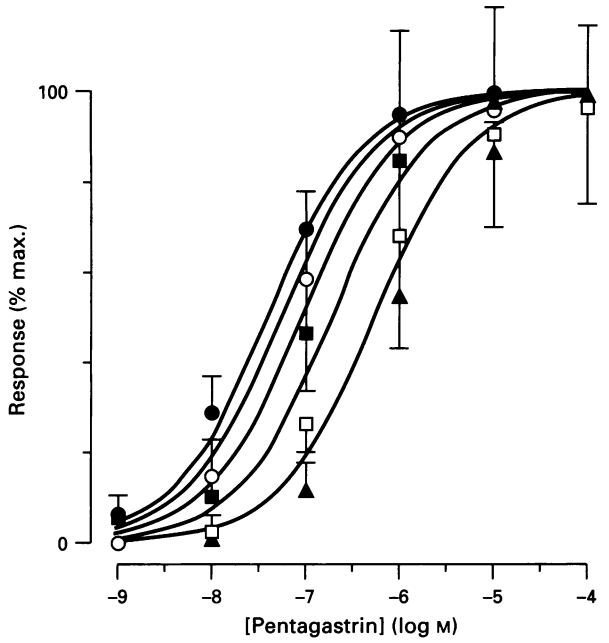

Figure 4 Two-receptor model simulations of the interaction between pentagastrin and L-365,260, in the absence of famotidine, in the immature rat isolated, lumen-perfused, stomach assay. Pentagastrin E/[A] curves were obtained in the absence (●) and presence of L-365,260 (μM; ○, 0.03; ■, 0.1; □, 0.3; ▲, 1.0). The lines superimposed on the mean experimental data ($n=6/8$) were simulated assuming no difference in the affinity of L-365,260 for the oxyntic and ECL cell CCK_B/gastrin receptors from equation (4) with the parameter values set as follows: $K_{B_1}=K_{B_2}=79.3$ nM ($=pK_B$ estimate of 7.10 made in the presence of famotidine), $\alpha_1=41\%$, $\alpha_2=49\%$, $[A]_{50_1}=32$ nM, $[A]_{50_2}=49$ nM, n = 1.01, m = 0.78 and [B] = 0, 0.03, 0.1, 0.3, 1.0 μM. For details of the analysis see text. Error bars represent s.e.mean.

was acting as a direct stimulant of oxyntic cells in the presence of famotidine (Table 1). In contrast, in the absence of famotidine the variation in intrinsic activity was far greater, suggesting that it was due to variation in the maximum amount of histamine which could be released by pentagastrin. The ECL cell capacity to release histamine is determined, in part at least, by histidine decarboxylase, an inducible enzyme whose activity has previously been recognised as a biological variable (Dimaline *et al.*, 1993). In an attempt to reduce the variation between experiments which we had observed previously, we chose to study each antagonist in the absence and presence of famotidine within a single experiment by use of a randomized block design. In view of the relatively large between-experiment variation in the maximum responses to pentagastrin obtained in the absence of famotidine, it would have been better to have performed an experiment in which the design allowed all three antagonists to be studied simultaneously. In practice, the individual experiments were performed consecutively over a period of about four to five weeks.

The antagonists were chosen from three chemical classes on the basis that such diversity would increase the probability of exposing any receptor heterogeneity. In practice, all three compounds behaved as surmountable competitive antagonists in the presence of famotidine (Table 2) and therefore provided no evidence for oxyntic cell CCK_B/gastrin receptor heterogeneity. The pK_B values estimated for L-365,260 and PD134,308 (Table 2) were consistent with those obtained from previous studies on rat gastric tissues (e.g. Patel & Spraggs, 1992; Prinz *et al.*, 1994; Roberts *et al.*, 1995). In the absence of famotidine, the pK_B values estimated for the three antagonists did not differ significantly from the corresponding values obtained in the presence of famotidine, the largest difference was only 0.3 log units (Table 2).

In the presence of famotidine, it was reasonable to assume that only a single population of CCK_B/gastrin receptors was involved, at least in terms of cellular localization, so that the pK_B values estimated could be considered to be reliable for the characterization of the oxyntic cell receptors. In contrast, in the absence of famotidine, it was evident that pentagastrin was acting on at least two cellular populations of CCK_B/gastrin receptors. The question was whether the finding that the pK_B values estimated in the absence and presence of famotidine were not significantly different provided sufficient evidence for the conclusion that the ECL and oxyntic cell receptors are pharmacologically indistinguishable. In practice, this question was addressed by developing a mathematical model of the system to generate experimental expectations.

For the application of the model (equation 3) to the experimental data, the antagonist affinity values obtained in the presence of famotidine by Schild analysis were chosen because under these conditions we expected only a single cellular (i.e. oxyntic cell) population of CCK_B/gastrin receptors to be involved. The analysis revealed that an excellent goodness-of-fit could be obtained for the L-365,260 and JB3190 data by assuming that the antagonists expressed the same affinity at both the ECL and oxyntic cell CCK_B/gastrin receptors. This was not the case for the PD134,308 data where the χ^2 analysis indicated a significant difference between observed and predicted values. However, inspection of the model simulation of the PD134308 data revealed that the deviation of the model from the data was in the form of a small (~0.15 log unit), parallel shift of the pentagastrin E/[A] curves obtained in the presence of the various concentrations of PD134,308. It was as though the pK_B value estimated for PD134,308 in the presence of famotidine (7.36 ± 0.10) was marginally too low for both the ECL and oxyntic cell receptors. The χ^2 test treated the pK_B estimate as though it was error free. Therefore, a second analysis was performed with the PD134,308 pK_B value fixed at a value (7.50) which lies within the 95% confidence intervals of the original estimate, and a good overall fit was obtained ($\chi^2=14.25$, d.f. = 20).

Evidently, the hypothesis that the ECL and oxyntic cell CCK_B/gastrin receptors are homogenous could not be rejected. However, the question remained as to what difference in antagonist affinity for the two cellular populations could have been detected in the analysis. Clearly, a large difference (e.g. $K_{B_1}=1000.K_{B_2}$) would have been readily detectable (Figure 3d). In the presence of increasing concentrations of such a selective antagonist, the pentagastrin E/[A] curves would become progressively biphasic. By progressively decreasing the antagonist selectivity in the model, it was found that a one log unit difference in pK_B values was the minimum that could be detected in this particular assay system. Such a difference would produce about 0.6 log unit underestimation of the pK_B for the antagonist in the absence of famotidine (Figure 3c) which could just be discriminated given the size of the variances associated with the pK_B values actually estimated in the current study (see Table 2). Similarly, this degree of antagonist selectivity although not producing overt biphasicity (Figure 3a and b) is predicted to produce significant changes (decreases) in the apparent slope of the pentagastrin E/[A] curves as judged by fitting to the Hill equation (1).

Previously, we found differences of approximately 1–1.5 log units in the apparent affinity of L-365,260 for CCK_B/gastrin receptors both between and within assays (Harper *et al.*, 1995; Roberts *et al.*, 1995). Wank *et al.* (1994) showed that a 0.5 log unit difference in the affinity of the same compound for the two isoforms of the human CCK_B/gastrin receptor. Therefore, although we found no evidence for receptor heterogeneity, we must conclude that the assay lacks the necessary discriminatory power to be certain of detecting the difference in antagonist affinity that we might have expected for L-365,260 from previous studies. Similarly, if PD134,308 and JB93190 discriminate between oxyntic and ECL cell CCK_B/gastrin receptors then we conclude that they express less than one log unit selectivity for either cellular receptor population.

Appendix

The choice of response metameter in rodent isolated, lumen-perfused, stomach assays

Before our modification of the mouse stomach assay (Black & Shankley, 1985) which allowed fully-defined agonist concentration-effect curves to be obtained in each preparation by cumulative dosing, the choice of pH or $[H^+]$ as a response metameter in isolated gastric acid secretory assays would have appeared arbitrary. Gastric acid secretion had been expressed both as $[H^+]$ (Bunce & Parsons, 1976; Szelenyi & Vergin, 1980; Angus & Black, 1982) and pH (Angus *et al.*, 1980). As part of the development of the mouse stomach assay we analysed the effect of metameter selection on basal and stimulated gastric acid secretion (Shankley, 1985). Standard parametric statistical analyses, routinely employed by pharmacologists, for the comparison of data within and between experimental treatment groups assume that bioassay measurements are normally-distributed (see Gaddum, 1945; Burns, 1950). Therefore,

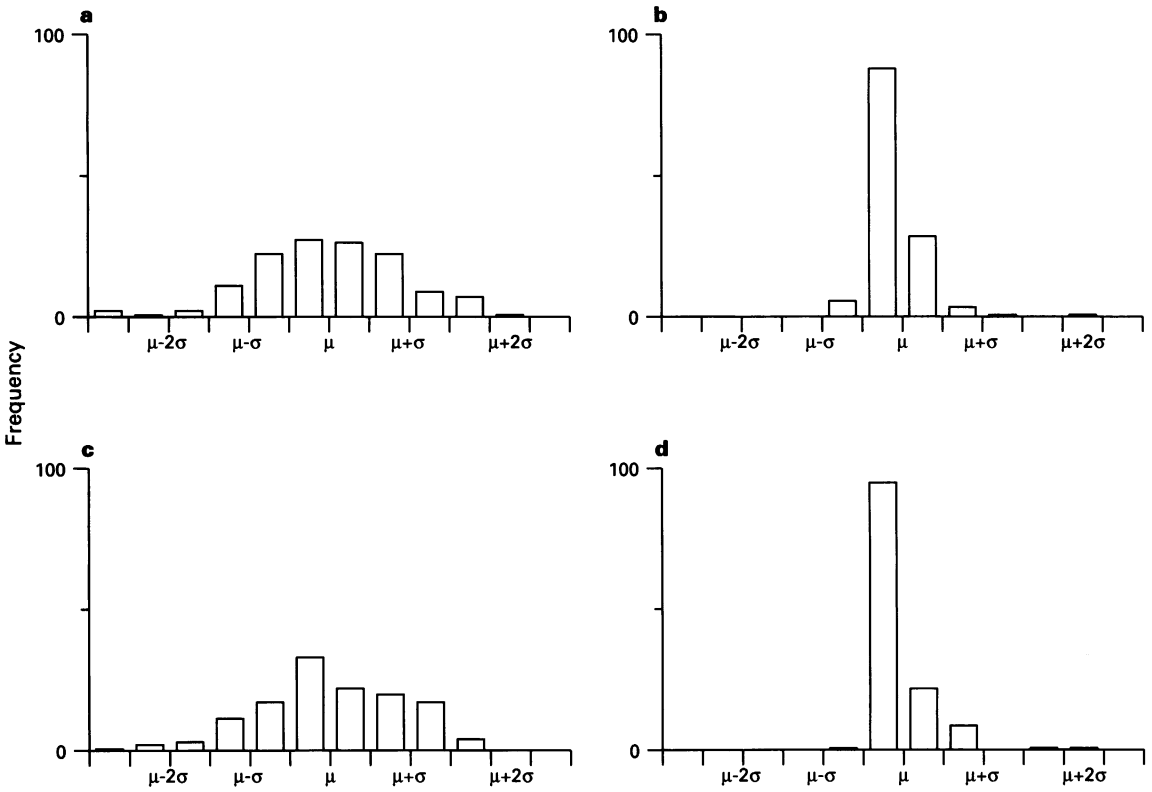

Figure 5 Frequency distributions of basal (a, b) and maximal pentagastrin-stimulated (c, d) gastric acid secretion expressed as pH (a, c) and $[H^+]$ (b, d) in the immature rat isolated, lumen-perfused stomach assay.

Table 3 Tests of normality for frequency distributions of basal/histamine-stimulated gastric acid secretion in the mouse stomach assay and basal/pentagastrin-stimulated secretion in the immature rat stomach assay

Mouse stomach		
Test	*Response metameter*	
Basal secretion	*pH*	*$[H^+]$(M)*
(I) $\chi 2$ (goodness-of-fit)	3.48 (d.f. = 13)	64.8 (d.f. = 13)*
(II) $\sqrt{b_1}$ (coefficient of skewness)	−0.061 (s.d. = 0.153)	1.782 (s.d. = 0.153)*
(III) g_2 (coefficient of kurtosis)	−0.095 (s.d. = 0.306)	4.373 (s.d. = 0.306)*
Histamine-stimulated secretion	*pH*	*$[H^+]$(M)*
(I) $\chi 2$ (goodness-of-fit)	5.34 (d.f. = 13)	30.8 (d.f. = 13)*
(II) $\sqrt{b_1}$ (coefficient of skewness)	0.239 (s.d. = 0.181)	1.189 (s.d. = 0.181)*
(III) g_2 (coefficient of kurtosis)	0.173 (s.d. = 0.362)	4.496 (s.d. = 0.362)*
Rat stomach		
Basal secretion	*pH*	*$[H^+]$(M)*
(I) $\chi 2$ (goodness-of-fit)	9.07 (d.f. = 9)	216 (d.f. = 9)*
(II) $\sqrt{B_1}$ (coefficient of skewness)	−0.484 (s.d. = 0.213)	7.029 (s.d. = 0.213)*
(III) g_2 (coefficient of kurtosis)	1.594 (s.d. = 0.426)*	59.149 (s.d. = 0.426)*
Pentagastrin-stimulated secretion)	*pH*	*$[H^+]$(M)*
(I) $\chi 2$ (goodness-of-fit)	10.2 (d.f. = 9)	260 (d.f. = 9)*
(II) $\sqrt{b_1}$ (coefficient of skewness)	−0.639 (s.d. = 0.213)	8.201 (s.d. = 0.213)*
(III) g_2 (coefficient of kurtosis)	1.894 (s.d. = 0.426)*	77.806 (s.d. = 0.426)*

For details of methods used see text. *Significant at $P<0.05$ level (see Snedecor & Cochran, 1989 for details of analysis and significance testing).

Table 4 Hill equation (1) fitting parameters for histamine and pentagastrin concentration-effect curve data obtained in the mouse stomach assay

Response metameter	*Agonist*	n	$p[A_{50}]$	α	n_H
$\Delta[H^+](\mu M)$	Histamine	6	4.93±0.05	154.0±4.5	0.99±0.07
ΔpH	Pentagastrin	5	7.97±0.18	74.8±4.3	0.69±0.04
	Histamine	6	5.17±0.05	0.51±0.02	0.92±0.05
	Pentagastrin	5	8.17±0.13	0.36±0.04	0.67±0.05

Data shown are means±s.e.mean. For details of methods used see text (Methods).

the frequency distributions of basal and maximal histamine-stimulated acid secretion data, expressed as pH and $[H^+]$ were determined. Tests of normality were performed on these distributions according to the methods of Snedecor & Cochran (1989). The analysis has now been extended to include basal and maximal pentagastrin-stimulated gastric acid secretion data from the isolated, lumen-perfused, immature rat stomach assay. The frequency distributions of the rat data are presented in Figure 5 and the results of the tests of normality on both assays are presented in Table 3.

No significant departure from normality was found in the distribution of basal and histamine-stimulated (mouse) or pentagastrin-stimulated (rat) gastric acid secretion when responses were expressed as pH as judged by the χ^2 analysis (Table 3). However, there was a significant deviation from normality in the distribution of basal and histamine-stimulated secretion when responses were expressed as $[H^+]$. In addition, analysis of the mouse data indicated that only the distribution of the $[H^+]$ values exhibited significant skew and kurtosis (Table 3). The rat pH data distribution did exhibit moderate kurtosis and skewness but this was trivial compared with the degree calculated for the distribution of $[H^+]$ values.

The choice of pH or $[H^+]$ as response metameter does not affect the analysis of competitive antagonism which is based on null methods. However, the choice of metameter can change the shape, location and relative maxima of agonist concentration-effect curves. In practice, due to the relatively small range of pH values covered in an agonist concentration-effect curve in the gastric assays (~0.15 to 0.7 pH in this study), the effect of expressing the response as $[H^+]$ rather than pH on the location and midpoint slope parameters obtained by fitting the data to the Hill equation is found to be negligible (Table 4). However, the mouse stomach assay data (Table 4) does illustrate how the comparison of agonist intrinsic activity values may be confounded. Thus, pentagastrin may be considered to produce either 48.5±2.7% or 70.5±6.8% of the maximum response to histamine depending on whether pH or $[H^+]$ is chosen.

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