Characterization of CCK receptors in a novel smooth muscle preparation from the guinea-pig stomach by use of the selective antagonists CI-988, L-365,260 and devazepide

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1 The cholecystokinin receptors mediating motor responses in a novel smooth muscle preparation from the corpus region of the guinea-pig stomach have been characterized by use of five agonist peptides and the antagonists CI-988, L-365,260 and devazepide.

2 Mucosa-denuded strips of circular muscle were contracted in a concentration-dependent manner by the five cholecystokinin (CCK)-related peptides CCK-8S, pentagastrin, gastrin-I, CCK-8US and CCK-4.

3 CI-988 was a powerful antagonist of the response to pentagastrin with an affinity ($pK_B = 9.49$) similar to that obtained in CCK_B receptor binding assays. With CCK-8S as the agonist, CI-988 was approximately 1000 fold less powerful as an antagonist.

4 Devazepide powerfully blocked responses to CCK-8S with an affinity ($pK_B = 9.54$) that was in agreement with reported functional data obtained in pancreatic amylase secretion studies, a system exhibiting CCK_A receptor activity. Devazepide displayed lower affinity against pentagastrin than against CCK-8S.

5 CI-988 blocked responses to pentagastrin in an insurmountable manner in the presence of 3 nM devazepide; a concentration previously shown to block the CCK_A receptor. The nature of the antagonism observed with L-365,260 was unaltered by the presence of devazepide.

6 The guinea-pig stomach corpus smooth muscle preparation contains both subtypes of CCK receptor and will be useful as a pharmacological tool for investigating the functional effects of novel CCK ligands.

Keywords: Cholecystokinin (CCK) receptors; CCK_B antagonists; CI-988; smooth muscle; guinea-pig stomach

Introduction

Cholecystokinin (CCK) and gastrin which share a common COOH-terminal pentapeptide amide sequence (Rehfeld, 1981) interact with at least two receptors that can be distinguished by the relative potencies of sulphated CCK8 (CCK-8S), pentagastrin, gastrin I, unsulphated CCK8 (CCK-8US) and CCK-4 (Jensen, 1989; Knapp *et al.*, 1990). The CCK_A receptor has a high affinity for CCK-8S and a 7000 fold lower affinity for gastrin I (Hughes *et al.*, 1990). This receptor subtype is present on a number of peripheral tissues and cells particularly in the alimentary tract (Zetler, 1984; Innis & Snyder, 1980) but also occurs in some highly localized regions of the brain (Hill *et al.*, 1987) for example in dorsal raphe neurones (Pinnock *et al.*, 1990).

The CCK_B/gastrin receptor is recognised by gastrin, CCK-8S, CCK-8US and CCK-4 with approximately equal affinity (Hughes *et al.*, 1990). Currently the CCK_B and gastrin receptor are pharmacologically indistinguishable (Freidinger, 1989). The CCK_B receptor exists predominantly in the central nervous system (Hughes *et al.*, 1990) while the gastrin receptor is found primarily in the alimentary canal, for example on gastric parietal cells, pancreatic acinar cells and smooth muscle cells (Grider & Makhlof, 1987; Cherner *et al.*, 1988).

CCK receptor heterogeneity has been confirmed following the development of high affinity and selective antagonists. The benzodiazepine ligand devazepide (formerly L-364,718, MK-329) has high affinity for the CCK_A receptor with 150 fold selectivity relative to the CCK_B receptor (Chang & Lotti, 1986). L-365,260, also a benzodiazepine has conversely high affinity for the CCK_B receptor although it does not possess the same degree of selectivity (Lotti & Chang, 1989). Recently developed in our laboratories is a series of dipeptoid ligands with high affinity and high selectivity for the CCK_B/gastrin receptor, the prototype of which is CI-988 (Hughes *et al.*, 1990).

The development of the benzodiazepine antagonists has allowed the characterization of CCK receptors that exist on a variety of smooth muscle preparations with standard organ bath techniques. With few exceptions it has become apparent that the majority of peripheral tissues contain only the CCK_A receptor subtype (Meldrum *et al.*, 1986; Woodruff & Hughes, 1991). Until recently the dearth of a simple and robust smooth muscle functional assay for the CCK_B/gastrin receptor has led a number of groups to examine the ability of CCK analogues to contract isolated, individual smooth muscle cells obtained from the fundic region of the guinea-pig stomach (Bitar & Makhlof, 1982; Collins & Gardner, 1982) or the guinea-pig gallbladder and stomach (Grider & Makhlof, 1990). It has been established that CCK-8S and gastrin I both cause contraction of isolated smooth muscle cells from these regions (Huang *et al.*, 1989). More recently it has been suggested that CCK_B/gastrin receptors mediate the transient contractile response to pentagastrin and CCK-4 in guinea-pig ileum longitudinal muscle myenteric plexus (Lucaites *et al.*, 1991; Patel & Spraggs, 1992).

In studies on isolated cells, conflicting results have been obtained with the antagonists L-365,260 and devazepide with the contractile effect of gastrin in isolated dispersed gastric smooth muscle cells being blocked more effectively by L-365,260 in one study (Huang *et al.*, 1989) but by devazepide in another (Grider & Makhlof, 1990).

In the present study we have employed a previously uncharacterized preparation: the isolated smooth muscle strip from the corpus region of the guinea-pig stomach, in an attempt to shed light on these recent anomalies. A preliminary account of this work has been presented to the British Pharmacological Society (Boyle *et al.*, 1992).

Methods

Tissue preparation

Adult male Dunkin-Hartley guinea-pigs (wt. 330–400 g) with free access to food and water were killed by cervical dislocation and the stomach rapidly removed. The fundus was discarded and the stomach was opened along the greater curvature, pinned out on a petri-dish with the mucosa uppermost. The mucosal and submucosal layers were removed by dissection to reveal the underlying smooth muscle layer. Strips of circular muscle (2 × 25 mm) from the corpus region of the stomach were obtained by cutting inwards, following the striations at the lesser curvature. Muscle strips were mounted in siliconised 3 ml organ baths containing Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 5.9, MgSO₄ 1.2, CaCl₂ 2.5, NaH₂PO₄ 1.2, NaHCO₃ 25.5 and glucose 11.1. The solution was maintained at 37°C and was gassed continuously with a mixture of 95% O₂/5% CO₂. After preliminary experiments the buffer was modified to include 5 µM indomethacin. Isometric contractile responses were measured with Grass FT.03 force-displacement transducers and recorded on Graphtec Linear-corders (Mark VII).

Experimental procedure

Tissues were placed under 1 g tension and allowed to equilibrate for 30 min after which time they were contracted with a submaximal dose of carbamylcholine (10 nM). Using a 12 min dose cycle concentration-response curves were established for a range of agonists that included CCK-8S, CCK-8US, gastrin I, CCK-4 and pentagastrin. Drugs were added in volumes that did not exceed 10 µl. For studies with the antagonists, tissues were exposed to antagonists for 15 min before re-exposure to agonists.

Analysis of results

Contractile responses to exogenously applied agonist were expressed as absolute changes in tension and were transformed to a percentage of the maximal response achieved for that agonist in order to obtain potency values. EC₅₀ values were obtained graphically for individual concentration-response curves using an iterative curve-fitting programme (InPlot) available from GraphPad Software Inc. and are expressed as geometric mean with range. Responses obtained to agonists in the presence of antagonists are expressed as a

percentage of the control maximum response obtained in the same tissue preparation. Agonist concentration-response curves in the absence and presence of increasing concentrations of CCK receptor antagonist were obtained and the method of Arunlakshana & Schild (1959) was used to provide Schild plots of the data, obtain affinity constants for the antagonists, and where applicable confirm the competitive nature of the antagonism. Linear regression for Schild plot analysis was carried out with RS1 curve-fitting software and data are expressed as pA₂ and slope value, with 95% confidence limits in parentheses. Where the slope of the interaction was not significantly different from unity, pK_B values are also supplied.

Compounds

CCK-8S, pentagastrin, CCK-8US, gastrin I and CCK-4 were obtained from Bachem UK Ltd and were dissolved in dimethylsulphoxide (DMSO) at a concentration of 10 mM and subsequently diluted in Krebs-Henseleit solution. Indomethacin, atropine, methysergide, mepyramine and tetrodotoxin were obtained from Sigma Chemical Co. and dissolved in saline. Devazepide and L-365,260 were synthesized by the Medicinal Chemistry Department of Parke-Davis and were dissolved in DMSO at a concentration of 10 mM. CI-988 (4-([2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[tricyclo[3.3.1.1.3⁷] dec-2-yloxy) carbonyl]amino] propyl]amino]-1-phenylethyl] amihno)-4-oxo-[R-(R*,R*)-butanoate *N*-Methyl-D-glucamine), was also synthesized by the Medicinal Chemistry Department at Parke-Davis and was dissolved in Krebs-Henseleit solution.

Results

Effects of agonists

Addition of 5 µM indomethacin to the bathing solution resulted in abolition of the spontaneous contractions of the corpus muscle strip without affecting the potency or efficacy of pentagastrin (Figure 1). Five CCK-related peptides induced concentration-dependent contractions of the stomach preparation. CCK-8S produced a contractile response that was qualitatively similar to that produced by the other agonists but had greater efficacy producing a maximum contraction twice that observed with gastrin, pentagastrin and CCK-4 (Table 1). CCK-8US was of intermediate efficacy producing a maximum contractile response midway between

Figure 1 Representative continuous recording of the dose-dependent contractile action of pentagastrin in the guinea-pig stomach corpus smooth muscle preparation. The top panel of the recording demonstrates the inherent myogenic tone that exists in the tissue. Addition of indomethacin 5 µM removes the spontaneous activity but leaves the responses to pentagastrin unaffected (lower panel).

Table 1 Efficacy and potencies of cholecystokinin (CCK)-related peptides in isolated smooth muscle from the corpus region of the guinea-pig stomach

Agonist	Maximum response (g)	EC ₅₀ (nM)
CCK-8S	8.6 ± 0.54	13.1 (2.1–29.8)
Pentagastrin	4.1 ± 0.22	10.0 (3.2–15.4)
Gastrin 1	3.7 ± 0.35	33.1 (12.5–70.1)
CCK-8US	6.1 ± 0.58	102 (30.3–320)
CCK4	3.9 ± 0.29	214 (60.5–471)

Efficacy values are expressed as mean response in g tension ± s.e.mean for >5 observations. Potency values are expressed as the geometric mean with range in parentheses for >5 observations.

that observed with CCK-8S and the other agonists. In terms only of the respective EC₅₀ values, of the five peptides employed pentagastrin and CCK-8S were the most potent while gastrin I was of intermediate potency relative to the other agonists (Table 1).

Effects of antagonists

The ability of a number of antagonists to influence the contractile responses of the preparation were examined. Atropine, mepyramine and methysergide each at 5 µM caused no significant alterations in the ability of the tissue to contract to either pentagastrin or CCK-8S. Similarly 1 µM tetrodotoxin also was without effect. (Data not shown).

The specific CCK_B receptor antagonist, CI-988, caused a concentration-dependent rightward shift of the concentration-response curve for pentagastrin (Figure 2). Schild plot analysis revealed a pA₂ of 9.49 (95% c.i. 9.29–9.79), a slope of –1.14 (0.48–1.38) and a corresponding pK_B of 9.64 (9.38–9.91). CI-988 was also an antagonist of responses to CCK-8S (Figure 3). The pA₂ value for this interaction was 6.60 (6.13–7.07) with a slope = 0.79 (0.14–1.57). This represents a 1000 fold selectivity for CI-988 when pentagastrin is the contractile agent. L-365,260 also caused a concentration-dependent rightward shift in the concentration-response curves to pentagastrin and CCK-8S (data not shown) with respective pA₂ values of 8.79 (8.49–9.09, slope –0.75 (0.39–1.10)) and 6.86 (6.56–7.16, slope –1.11 (0.55–1.65)) representing a much lower level of specificity than CI-988.

The specific CCK_A receptor antagonist, devazepide, caused a concentration-dependent rightward shift of the concen-

Figure 2 Inhibitory effect of the CCK_B receptor antagonist, CI-988, on control concentration-response curves to pentagastrin (○). Increasing concentrations of CI-988 1 nM (●), 3 nM (Δ) and 10 nM (▲) resulted in a concentration-dependent rightward shift of the pentagastrin concentration-response curve. Data are expressed as percentage of control maximum response to pentagastrin and each point represents the mean ± s.e.mean of not less than 4 observations. **Figure 3** Inhibitory effect of the CCK_B receptor antagonist, CI-988, on control concentration-response curves to CCK-8S (○). Increasing concentrations of CI-988 300 nM (●), 1 µM (Δ) and 3 µM (▲) resulted in a concentration-dependent rightward shift of the CCK-8S concentration-response curve. Data are expressed as percentage of control maximum response to CCK-8S and each point represents the mean (± s.e.mean) of not less than 4 observations.

tration-response curve to CCK-8S (Figure 4) and Schild plot analysis by linear regression revealed a competitive interaction with a pA₂ value of 9.54 (9.95–9.13) a slope of –0.98 (0.48–1.46) and a corresponding pK_B value of 9.51 (9.24–9.77). Devazepide was also a competitive antagonist of responses to pentagastrin with a pA₂ value of 7.55 (7.25–7.85) a slope of –0.81 (0.30–1.26) and a corresponding pK_B value of 7.46 (7.27–7.65).

In order to preclude any interaction of pentagastrin at the CCK_A receptor, the ability of the two CCK_B receptor antagonists CI-988 and L-365,260 to antagonize responses to the pentapeptide were re-examined in the presence of 3 nM devazepide. Under these circumstances L-365,260 produced an almost identical competitive interaction at the CCK_B receptor in comparison to that observed in the absence of devazepide, with a pA₂ of 8.17 (8.00–8.34) a slope of –0.87 (0.58–1.19) and a corresponding pK_B of 8.02 (7.89–8.15). Figure 5 demonstrates that the addition of devazepide to the bathing solution transformed the nature of the antagonism observed with CI-988 against the specific CCK_B receptor agonist pentagastrin. Under these conditions CI-988 produced an insurmountable blockade at the CCK_B receptor.

Figure 4 Inhibitory effect of the CCK_A receptor antagonist, devazepide, on control concentration-response curves to CCK-8S (○). Increasing concentrations of devazepide 1 nM (●), 3 nM (Δ) 10 nM (▲) and 30 nM (□) resulted in a concentration-dependent rightward shift of the CCK-8S concentration-response curve. Data are expressed as percentage of control maximum response to CCK-8S and each point represents the mean (± s.e.mean) of not less than 4 observations.

Figure 5 Insurmountable blockade of the pentagastrin concentration-response curve by increasing concentrations of CI-988 in the presence of 3 nM devazepide. The maximum response of the control (○) concentration-response curve was concentration-dependently: 1 nM (●), 3 nM (△), 10 nM (▲) reduced by CI-988. Data are expressed as percentage of control maximum response to pentagastrin and each point represents the mean ($\pm$ s.e.mean) of not less than 4 observations.

It was apparent, however, that blockade of the CCK_B receptor with 10 nM CI-988 did not affect the nature of the antagonism observed with devazepide against CCK-8S. Under these circumstances devazepide blocked responses to CCK-8S with a pA_2 value of 9.84 (9.45–10.23) a slope of -0.94 (0.48–1.38) and a corresponding pK_B of 9.77 (9.48–10.07).

Discussion

The aim of the present study was to characterize the nature of the contractile effects of a number of CCK-related peptides in a novel smooth muscle preparation obtained from the corpus region of the guinea-pig stomach. CCK_A receptor-mediated contractile effects are readily accessible on a number of tissue preparations from several species, for example guinea-pig gall-bladder and ileum (Zetler, 1984), the pyloric and fundic regions of the rat stomach (Margolis *et al.*, 1989) and guinea-pig trachea (Stretton & Barnes, 1989). Previous attempts to investigate CCK_B receptor-mediated contractions have necessitated the use of isolated smooth muscle cell preparations from guinea-pig stomach (Bitar & Makhoulf, 1982; Grider & Makhoulf, 1990) or in guinea-pig ileum (Patel & Spraggs, 1992) where the response is indirect and apparently due to the release of acetylcholine. The novel preparation investigated in this study provides an accessible and robust means of investigating CCK_A and CCK_B receptor interactions.

Preliminary experiments revealed the ability of the cyclo-oxygenase inhibitor, indomethacin, to inhibit myogenic activity in the preparation without affecting the efficacy or potency of the CCK_B selective agonist pentagastrin or the non-selective agonist CCK-8S. The inability of micromolar concentrations of atropine, mepyramine and methysergide to affect contractions to either agonist suggested that the peptides were not causing release of acetylcholine, histamine or 5-hydroxytryptamine. Similarly the inability of the sodium channel blocker, tetrodotoxin, to influence the contractile response of the preparation suggested that the peptides were producing motor effects via receptors situated post-synaptically on the smooth muscle walls.

The non-selective CCK receptor agonist, CCK-8S, contracted the tissue with the greatest efficacy of the five peptides examined, presumably due to its ability to stimulate receptor-effector coupling at both receptor sites. The intermediate level of efficacy observed with CCK-8US is perhaps due to its ability at higher concentrations to interact with the CCK_A

receptor in the preparation, while the other peptides pentagastrin, gastrin I and CCK-4 retain the ability to interact only with the CCK_B receptor.

The observed efficacy of these peptide agonists coupled with the high potency seen for pentagastrin and gastrin I and the comparatively high potencies observed for CCK-4 and CCK-8US suggest the presence of CCK_B receptors in this smooth muscle preparation that mediate contractile responses. This was confirmed by the observation that two highly selective CCK_B receptor antagonists, CI-988 and L-365,260, both antagonized responses to pentagastrin with affinities that suggested an interaction at the CCK_B receptor. The observed affinity of CI-988 is in close agreement with that observed in radio-ligand binding studies in mouse cortex and in functional electrophysiological studies in the ventromedial nucleus of the hypothalamus (Woodruff *et al.*, 1990) while the affinity observed with L-365,260 is in close agreement with that seen in the guinea-pig ileum where pentagastrin was also used as the agonist, albeit in the presence of devazepide (100 nM) (Patel & Spraggs, 1992).

While the functional affinity of L-365,260, for example in rat hypothalamic brain slices (Kemp *et al.*, 1989) or in guinea-pig dispersed smooth muscle cells (Huang *et al.*, 1989), is lower than its affinity in binding studies; using the guinea-pig isolated stomach preparation as a functional model, the affinity of L-365,260 was maintained in relation to its binding affinity. The 1000 fold lower affinity of CI-988 and the 30 fold lower affinity of L-365,260 obtained against CCK-8S as the agonist as opposed to pentagastrin, suggested that CCK-8S was mediating its contractile response via the CCK_A receptor. This was confirmed with devazepide, the highly selective CCK_A receptor antagonist. Devazepide antagonized responses to CCK-8S both in the presence and absence of CI-988 with equal and high affinity. The pK_B value of 9.5 that was obtained for devazepide is similar to that previously reported for guinea-pig ileum (Chang & Lotti, 1986; Patel & Spraggs, 1992). In the guinea-pig stomach preparation it would seem that the 'non-selective' agonist, CCK-8S, mediates its contractile effects largely through the CCK_A receptor. This is at odds with the higher level of efficacy observed with CCK-8S and suggests that in the presence of CCK_B receptor blockade, the CCK_A receptor assumes total responsibility for contraction of the tissue, possibly due to more efficient receptor effector coupling. The low affinity of devazepide observed against pentagastrin was indicative of an interaction at the CCK_B receptor and is similar to the binding affinity at the CCK_B binding site in guinea-pig cortical membranes (Knapp *et al.*, 1990).

CI-988 and L-365,260 are more powerful antagonists of pentagastrin-induced contractions than devazepide. The data obtained in this study disagree with the observations made in isolated cell preparations wherein the CCK_A selective antagonist, devazepide, was more powerful than L-365,260 at inhibiting the contractile responses to gastrin (Grider & Makhoulf, 1990). However, the data are in agreement with those obtained in a similar isolated cell preparation in which L-365,260 was more powerful than devazepide at blocking the contractile effects of CCK_B-selective agonists (Huang *et al.*, 1989).

In order to preclude an interaction of pentagastrin at the CCK_A receptor, concentration-response curves to this peptide were carried out in the presence of a concentration of devazepide (3 nM) that was ten times its affinity at the CCK_A receptor. Under these circumstances the interaction between L-365,260 and pentagastrin seemed unchanged with the estimated affinity for L-365,260 being similar to that obtained in the absence of devazepide. In contrast, with CI-988 in the presence of devazepide, an insurmountable block at the CCK_B receptor was observed. This effect suggests the possibility that with CI-988, the dipeptoid ligand, but not L-365,260, the benzodiazepine, a previously unrecognised interaction is revealed between the two subtypes of CCK receptor. The selectivity we observed for CI-988 (in the absence of

devazepide) in favour of the blockade of pentagastrin versus CCK-8S suggests that the concentrations of CI-988 that produce an apparently competitive blockade cannot be interacting at the CCK_A receptor to any significant degree. The circumstances of the insurmountable blockade are unique since in the converse experiment where CCK-8S is the agonist and the CCK_B receptor is blocked with CI-988, devazepide retains its competitive action. Indeed no evidence exists to suggest that either compound can influence the binding of the other at either CCK receptor subtype. Therefore the specificity of circumstances under which the effect is observed suggests something other than a simple ligand-

receptor interaction and the possibility of an allosteric interaction between the CCK_A and CCK_B receptors that is revealed under this set of conditions must be considered.

It is possible that such experiments designed to investigate any potential interaction may have to be carried out in this novel preparation if the observed effects are indeed unique to guinea-pig stomach smooth muscle. If radio-ligand binding experiments cannot supply the answer to this interesting observation, then second messenger assays that permit study of receptor-effector coupling may shed light on the reasons for the transformation of the antagonism observed with CI-988 in the presence of devazepide.

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(Received November 11, 1992

Revised March 4, 1993

Accepted March 12, 1993)