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Protection induced by cholecystokinin-8 (CCK-8) in ethanol-induced gastric lesions is mediated via vagal capsaicin-sensitive fibres and CCK_A receptors

[1]S. Evangelista & C.A. Maggi

Pharmacology Department, Menarini Pharmaceuticals, via Sette Santi 3, 50131 Firenze, Italy

1 We have investigated the effect of intravenous injection of cholecystokinin-8 (CCK-8) and other peptides on gastric lesion formation in response to an intragastric perfusion with 25% ethanol in rats anaesthetized with urethane.

2 Intravenous injection of CCK-8 (50–100 nmol kg^{-1}), but not bombesin (1–100 nmol kg^{-1}), calcitonin gene-related peptide (1–50 nmol kg^{-1}), neurokinin A (1 μmol kg^{-1}) or substance P (100 nmol kg^{-1}), induced protection against gastric haemorrhagic lesions produced by ethanol.

3 The CCK_A-antagonist L-364,718 (2.45 μmol kg^{-1}, i.v.) increased the lesion index induced by ethanol and reversed the protective effect of CCK-8 (50 nmol kg^{-1}, i.v.). The CCK_B-antagonist L-365,260 (5 μmol kg^{-1}, i.v.) and a lower dose of L-364,718 (0.25 μmol kg^{-1}, i.v.) were ineffective.

4 The gastric protective effects afforded by CCK-8 (50 nmol kg^{-1}, i.v.) were not observed in vagotomized-rats and were reduced by capsaicin pretreatment. In capsaicin-pretreated rats there was a worsening of gastric lesions induced by ethanol-perfusion as compared to those observed in vehicle-pretreated rats.

5 These results demonstrate that the mucosal protective effect of CCK-8 involves, at least in part, the activation of CCK_A-receptors and is mediated by vagal capsaicin-sensitive fibres.

Introduction

Cholecystokinin-8 (CCK-8), the active fragment of CCK-33, is a peptide present in the gastrointestinal tract (Ekblad *et al.*, 1985) where it exerts an important role as physiological mediator of functions such as motility and emptying of the stomach (Raybould & Taché, 1988) and pancreatic secretion (Williams, 1982). These effects have been reported to be mediated through peripheral CCK_A receptors and blocked by the specific receptor antagonist L-364,718 (Evans *et al.*, 1986).

Recently, the inhibitory effect of CCK-8 on gastric motility and emptying has been shown to be partly mediated through vagal capsaicin-sensitive afferents (Raybould & Taché, 1988). Capsaicin is a selective neurotoxin that, when administered systemically to neonatal rats, induces a lifelong chemical sensory denervation (Holzer, 1988). Capsaicin-sensitive afferents are also involved in a local defence mechanism(s) against gastroduodenal ulcers (Holzer *et al.*, 1990a). Thus newborn rats treated with capsaicin are more prone than controls to develop gastric lesions in response to a variety of ulcerogenic stimuli (Evangelista *et al.*, 1988). The observation that the subcutaneous administration of CCK-8 reduced gastric lesions induced by ethanol (Evangelista *et al.*, 1987) prompted us to determine the possible involvement of capsaicin-sensitive afferents in the protective effects induced by CCK-8 and the influence of vagotomy on the same phenomenon. For comparison, similar experiments were carried out with other peptides contained in capsaicin-sensitive fibres such as bombesin, calcitonin gene-related peptide, neurokinin A and substance P (Holzer, 1988).

In view of the recent development of CCK_A- and CCK_B-receptor antagonists, namely L-364,718 and L-365,260 (Evans *et al.*, 1986; Bock *et al.*, 1989), these drugs were used to determine if the protective effects of CCK-8 on ethanol-induced gastric lesions might involve CCK_A- or CCK_B-receptors.

[1] Author for correspondence.

Methods

Experimental animals and procedures

Male albino rats, Sprague-Dawley Nossan strain, weighing 280–310 g, were housed at constant room temperature (21 ± 1°C), relative humidity (60%) and with 12 h light-dark cycle (light on 06 h 00 min). The animals were deprived of food for 20 h before the experiments but allowed free access to tap water.

Surgical preparation and induction of gastric lesions

The rats were anaesthetized by a subcutaneous injection of urethane (1.5 g kg^{-1}). The body temperature was maintained at 36–37°C by means of a heating lamp and tracheostomy was performed and the trachea cannulated to ensure a patent airway.

The gastric lumen was continuously perfused by a technique described previously (Holzer & Lippe, 1988). Briefly, a soft catheter (i.d. 0.8 mm) was inserted into the stomach through an incision in the cervical esophagus and held in place by a ligature. This catheter was connected to a peristaltic pump, and saline at pH 7 and 37°C was perfused through the gastric lumen at a rate of 0.7–0.8 ml min^{-1}. Gastric outflow was collected by means of a cannula (i.d. 3 mm) inserted into the stomach via an incision in the duodenum and held in place by two ligatures around the duodenum. At the beginning of the experiment the stomach was flushed with 50 ml of bodywarm saline to remove any solid contents. After a 60 min period, peptides under study were injected i.v. and 5 min later the gastric lumen was perfused with 25% ethanol.

In some experiments the vagi were cut bilaterally at cervical level, when the oesophagus was intubated (about 60 min before ethanol challenge), and the animals were artificially ventilated by means of a ventilator (Basile, Varese, Italy) for small rodents (60 strokes min^{-1}, 0.8 ml 100 g^{-1} body wt). At the end of the experiments (after 30 min of ethanol perfusion)

the animals were killed, their stomachs excised and examined for the presence of lesions by an observer unaware of the treatments. Each individual lesion (red streaks) was measured along its greatest length with the aid of a binocular microscope (magnification $\times 10$) to determine the lesion index (Holzer & Lippe, 1988). Lesions smaller than 1 mm were assigned a rating of 1, lesions measuring 1–2 mm were assigned a rating of 2 and lesions measuring more than 2 mm were given a rating according to their length in mm. The overall total was designated the 'lesion index'.

In order to assess the mechanisms of the antilesion effect of CCK-8, L-364,718 (0.25–2.45 μmol kg^{-1}, i.v.) or L-365,260 (5 μmol kg^{-1}, i.v.), at doses reported to antagonize specific *in vivo* receptor-mediated CCK-8 effects (Lotti *et al.*, 1987; Lotti & Chang, 1989), were administered 5 min before CCK-8.

Sensory denervation

On their second day of life, rats received capsaicin 50 mg kg^{-1}, by subcutaneous injection. This treatment is known to cause a permanent degeneration of unmyelinated afferent neurones (Holzer, 1988). Control animals received an equal volume of vehicle (10% ethanol, 10% Tween 80 and 80% saline v/v/v). All injections were performed under ether anaesthesia. The rats were then grown to adulthood and used for experiments 2–3 months after this treatment.

Drugs

L-364,718 (3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3yl)-1H-indole-2-carboxamide) and L-365,260 ((3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3yl)-N^1-(3-methyl-phenyl)mea) (Merck Sharp & Dohme) were dissolved in 1:1 dimethylsuphoxide (DMSO): Tween 80. The solution was sonicated and then diluted with saline to a final concentration of 8% DMSO and Tween 80. Controls were treated with the same vehicle. Peptides were purchased from Peninsula and dissolved in 0.9% saline. All drugs were administered i.v. in a volume of 1 ml kg^{-1} except for capsaicin (Sigma) which was given in 2 ml kg^{-1} by the subcutaneous route.

Statistics

All data related to the lesion index are expressed in the figures as mean ± s.e. and analyzed by means of the analysis of variance followed by Dunnett's test to determine differences among means.

Results

Effect of cholecystokinin-8 and other peptides

In the first series of experiments we investigated the effect of some peptides on the formation of haemorrhagic lesions in the glandular mucosa of the stomach produced by the intraluminal perfusion of 25% ethanol. The development of lesions was reduced by CCK-8 (Figure 1), but not by bombesin (1–100 nmol kg^{-1}), calcitonin gene-related peptide (1–50 nmol kg^{-1}), neurokinin A (1 μmol kg^{-1}) or substance P (100 nmol kg^{-1}), injected 5 min before the ethanol-perfusion ($n = 6$ for each peptide).

As shown in Figure 1 the protective effect induced by 50 nmol kg^{-1} i.v. of CCK-8 was not further enhanced by increasing the dose of CCK-8. The possibility was investigated that the effect of CCK-8 on ethanol-induced gastric lesions might have involved systemic changes in the cardiovascular system. Intravenous injection of 50 nmol kg^{-1} of CCK-8 produced a rapid and negligible increase in blood pressure (5 ± 0.7 mmHg at 5 min after CCK-8 injection; $n = 4$).

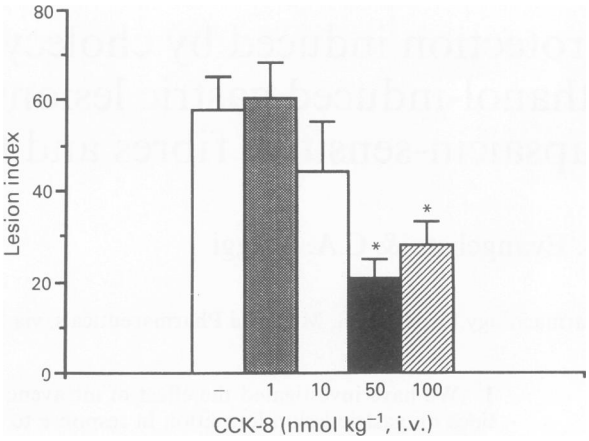

Figure 1 Effect of cholecystokinin-8 (CCK-8) (1–100 nmol kg^{-1}, i.v.) on gastric lesion formation caused by the intragastric perfusion of 25% ethanol for 30 min. CCK-8 was administered 5 min before the start of ethanol perfusion. Columns show mean values with s.e. indicated by vertical bars, $n = 8$; * $P < 0.05$ as compared to controls.

Effect of CCK receptor antagonists

Pretreatment with the CCK_A-receptor antagonist L-364,718 (2.45 μmol kg^{-1}, i.v.; see Lotti *et al.*, 1987) prevented the antiulcer effect of CCK-8 (Table 1) while a lower dose of L-364,718 (0.25 μmol kg^{-1}, i.v.) or the CCK_B-receptor antagonist L-365,260 (5 μmol kg^{-1}, i.v.) were without effect (Table 1). In the animals pretreated with L-364,718 (2.45 μmol kg^{-1}, i.v.). CCK-8 at a dose of 50 nmol kg^{-1} i.v. was unable to give any protective effect against the lesions induced by ethanol (Table 1).

It should be noted that pretreatment with L-364,718, at the dose that blocked the effect of exogenous CCK-8, aggravated the ethanol-induced gastric lesions, while pretreatments with L-364,718 at 0.25 μmol kg^{-1} i.v. or L-365,260 (5 μmol kg^{-1}, i.v.) were ineffective (Table 1).

Effect of vagotomy and capsaicin pretreatment

Protection against gastric lesions induced by CCK-8 (50 nmol kg^{-1}, i.v.) in vehicle-pretreated rats (63% reduction of lesion index as compared to controls) was reduced in capsaicin pretreated- and totally abolished in vagotomized rats (Figure 2). Treatment with CCK-8 (50 nmol kg^{-1}, i.v.) or vehicle in vagotomized rats produced almost similar values in lesion index following the intragastric perfusion with ethanol (Figure 2).

Table 1 Effect of L-365,260 or L-364,718 on cholecystokinin-8 (CCK-8) protection in gastric lesion formation caused by the intragastric perfusion of 25% ethanol

Treatments and dose	*Lesion index*
Vehicle + saline	58 ± 7
Vehicle + CCK-8 50 nmol kg^{-1}, i.v.	24 ± 4*
L-365,260 5 μmol kg^{-1}, i.v. + saline	65 ± 10
L-365,260 5 μmol kg^{-1}, i.v. + CCK-8 50 nmol kg^{-1}, i.v.	26 ± 8*§
L-364,718 0.25 μmol kg^{-1}, i.v. + saline	71 ± 12
L-364,718 0.25 μmol kg^{-1} i.v. + CCK-8 50 nmol kg^{-1} i.v.	32 ± 7*§
L-364,718 2.45 μmol kg^{-1}, i.v. + saline	99 ± 15**
L-364,718 2.45 μmol kg^{-1}, i.v. + CCK-8 50 nmol kg^{-1}, i.v.	79 ± 10*

Values are expressed as mean ± s.e., $n = 8$–10. * $P < 0.05$ and ** $P < 0.01$ as compared to vehicle + saline group; § $P < 0.05$ as compared to respective antagonist (L-365,260 or L-364,718) + saline group.
L-365,260 or L-364,718 and CCK-8 were injected 10 and 5 min respectively before the start of ethanol perfusion.

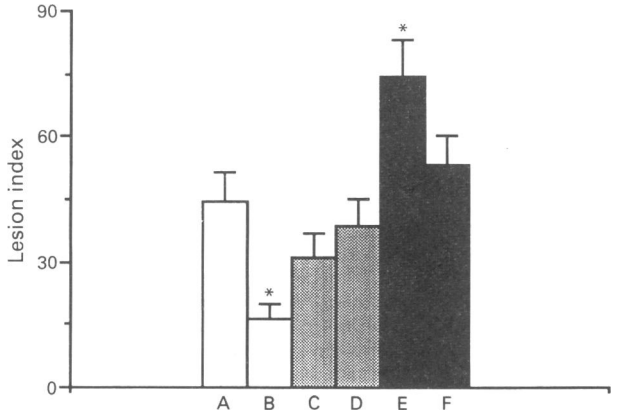

Figure 2 Gastric lesion formation caused by the intragastric perfusion of 25% ethanol for 30 min in controls (open columns), vagotomized (stippled columns) or capsaicin pretreated rats (solid columns). Groups refer to the following treatments carried out 5 min before the start of an ethanol perfusion: A, C and E = vehicle; B, D and F = CCK-8 50 nmol kg^{-1}, i.v. Columns show mean values with s.e. indicated by vertical bars, $n = 12$. * $P < 0.05$ and compared to group A (controls).

In rats pretreated with capsaicin, CCK-8 (50 nmol kg^{-1}, i.v.) produced only a 29% reduction of lesion index and was not significantly different as compared to capsaicin-pretreated vehicle treated rats (Figure 2). Capsaicin-pretreatment aggravated the lesion index induced by 25% ethanol as compared to vehicle-pretreated rats, while the effect of ethanol in vagotomized animals was not significantly different from that of sham-operated animals (Figure 2). Neither vagotomy nor capsaicin affected the lesion index in the absence of ethanol perfusion. When we increased the lesion index (by intragastric perfusion of 50% ethanol), to obtain a value similar to that found in capsaicin-pretreated rats (see Figure 2), CCK-8 (50 nmol kg^{-1}, i.v.) produced a significant reduction in lesion index (36 ± 8, $n = 4$) with a 59% reduction in this value as compared to controls (90 ± 5, $n = 4$). Therefore the reduction in the protective effect of CCK-8 observed in rats pretreated with capsaicin or the CCK_A-receptor antagonist is probably not ascribable to the aggravation of lesions produced by the pretreatments.

Discussion

The present results show that intravenous injection of CCK-8 affords protection against ethanol-induced gastric lesions in rats. This effect of CCK-8 is not shared by intravenous administration of other gut peptides such as bombesin, calcitonin gene-related peptide, neurokinin A or substance P, although some of the latter peptides have been shown to be effective after subcutaneous administration (Evangelista *et al.*, 1987; 1989) but the enzymatic breakdown strongly influences the effects of peptides when given intravenously. On the other hand the intravenous route for peptides is likely to be more suitable to obtain a specific effect as compared to the subcutaneous administration.

The protective effect of peptides given systemically could not be attributed to any systemic haemodynamic effect, since CCK-8 was devoid of significant effects on blood pressure up to a dose of 50 nmol kg^{-1} i.v.

The protective effect of CCK-8 is dependent on the integrity of the vagus nerve and, at least in part, mediated by capsaicin-sensitive afferents. Neurochemical studies have shown that sensory neurones supplying the stomach are of dual origin (vagal and spinal; Green & Dockray, 1988). Our results show that the protective properties of CCK seem to be restricted to the stimulation of sensory neurones of vagal origin.

Capsaicin-sensitive nerves appear to control the susceptibility of the gastric mucosa to ulcerogenic stimuli since their ablation aggravates (Evangelista *et al.*, 1988; Esplugues & Whittle, 1990; present results) and stimulation reduces (Holzer & Lippe, 1988) lesion formation induced by ethanol. A local release of sensory neuropeptides has been shown to play a pivotal role in these protective mechanisms (Holzer *et al.*, 1990b; Whittle *et al.*, 1990) through the regulation of local microvascular protective response to challenge (Holzer *et al.*, 1990a). In light of the above we cannot exclude the possibility that the protective effect of CCK-8 against mucosal damage might involve local release of sensory neuropeptides and increase in gastric blood flow. On the other hand, the complete failure of CCK-8 to afford ulcer protection within a short time from vagotomy, strongly suggests the involvement of reflex mechanisms. Indeed, it appears unlikely that the sensory neuropeptide content in vagal primary afferents in the stomach might have been depleted within 60 min from nerve section. It should be noted that acute vagotomy was more effective than capsaicin pretreatment in preventing the protective effect of CCK-8. Although the exact mechanism underlying this difference cannot be immediately understood on the basis of the present findings, a hypothesis to be considered is that CCK-8 stimulates certain vagal afferents which were unaffected by capsaicin pretreatment.

The recent development of potent and highly selective CCK receptor antagonists has made possible detailed studies on the physiological functions of CCK. Thus, peripheral CCK_A-receptors are involved in pancreatic secretion, stomach and gallbladder motility and anorexia (Evans *et al.*, 1986) and CCK_A binding sites have been specifically demonstrated on the vagus nerve and shown to be transported towards the periphery (Moran *et al.*, 1987). Receptors for CCK are well known to be present in the stomach (Sutliff *et al.*, 1987) and they have been considered to be mainly involved in the gastric motility effects of CCK. This study shows that CCK_A-receptors might also have a role in regulating the susceptibility of the gastric mucosa to injury. In fact the CCK_A-receptor-antagonist (L-364,718) prevented the protective effect of CCK-8 while the CCK_B-receptor antagonist (L-365,260) was ineffective. This effect was obtained with a dose of the CCK_A-receptor antagonist which has been shown to block selectively other *in vivo* peripheral effects mediated by CCK_A-receptors (Lotti *et al.*, 1987). Conversely L-365,260, which binds with high affinity to gastrin and central CCK_B-receptors (Lotti & Chang, 1989), did not affect the protective response to CCK-8 at the dose which selectively inhibits gastrin-stimulated gastric acid secretion (Lotti & Chang, 1989). Furthermore, administration of the CCK_A-receptor antagonist alone aggravated ethanol-induced lesions, implicating a possible role of endogenous CCK in the regulation of gastric mucosal integrity in response to injury.

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