AN ENTERIC SIGNAL REGULATES PUTATIVE GASTROINTESTINAL
PRESYPATHETIC VASOMOTOR NEURONS IN RATS

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ABSTRACT

Ingestion of a meal results in gastrointestinal hyperaemia and is associated with the systemic and paracrine release of a number of peptide hormones, including cholecystokinin (CCK), and 5-hydroxytryptamine (5-HT). Systemic administration of CCK-octapeptide inhibits a subset of presympathetic neurons of the rostroventrolateral medulla (RVLM) that may be responsible for driving the sympathetic vasomotor tone to the gastrointestinal viscera. The aim of this study was to determine whether endogenous release of CCK and/or 5-HT also inhibits CCK-sensitive RVLM neurons. The effects of intraduodenal administration of secretagogues sodium oleate (SO)/soybean trypsin inhibitor (SBTI) on circulating levels of CCK and 5-HT were examined. In separate experiments, the discharge rates of barosensitive, medullospinal, CCK-sensitive RVLM presympathetic vasomotor neurons were recorded following rapid intraduodenal infusion of SO/SBTI or water. Alternatively, animals were pre-treated with CCK₁ receptor antagonists devazepide or lorglumide or the 5-HT₃ antagonist MDL-72222, before SO/SBTI administration. Secretagogue infusion significantly increased the level of circulating CCK, but not 5-HT. SO/SBTI produced a significant decrease (58%) in the neuronal firing rate of CCK-sensitive RVLM neurons when compared to water (5%). CCK₁ receptor antagonists did not reverse SO/SBTI-induced neuronal inhibition (58%) whereas the 5-HT₃ antagonist significantly attenuated the effect (22%). This study demonstrates a functional relationship between a subset of RVLM presympathetic vasomotor neurons and meal-related signals arising from the gastrointestinal tract. It is likely that endogenously-released 5-HT acts in a paracrine fashion on gastrointestinal 5-HT₃ receptors to initiate the reflex inhibition of these neurons, resulting in gastrointestinal vasodilatation by withdrawal of sympathetic tone.
KEYWORDS

rostroventrolateral medulla; cholecystokinin; devazepide; MDL-72222; 5-hydroxytryptamine
INTRODUCTION

Gastrointestinal hyperaemia is a physiological consequence of food consumption. Gastrointestinal hormones such as CCK have been implicated in gastrointestinal vasodilatation associated with postprandial increase in splanchnic blood flow. The involvement of the sympathetic vasomotor system in this response is poorly understood. We have recently suggested the possibility that food-related signals may produce gastrointestinal vasodilatation via a reflex withdrawal of sympathetic vasomotor outflow (39, 45). In healthy individuals the sympathetic nervous system compensates for postprandial splanchnic blood pooling by activation of sympathetic vasoconstrictor drive to skeletal muscle resistance vessels in response to baroreceptor unloading. In patients with cardiovascular disease associated with diabetic neuropathy or autonomic dysfunction, poor baroreflex compensation for blood pooling in the splanchnic bed (9) may result in serious and even life-threatening consequences including angina pectoris and stroke (17, 31).

Postprandial release of CCK in the process of food digestion has been well documented and leads to gall-bladder contraction, satiety (35), pancreatic exocrine secretion, increased gastric motility and gastrointestinal hyperaemia (27). In rats, foods rich in protein (21, 29) and fats (20) are the most potent stimulants of CCK release (19). Fatty acids such as sodium oleate (SO) appear to function by direct stimulation of CCK-containing enteroendocrine cells of the gastrointestinal mucosa (3). Proteins entering the gut postprandially bind to trypsin to form an inactive complex, facilitating CCK release by removing the inhibitory influence of the protease on CCK-releasing peptides (16). Similarly, soybean trypsin inhibitor (SBTI) acts to prevent the digestion of trypsin-sensitive peptides by inhibiting trypsin activity (50), making it a potent stimulus for CCK-release in rats (29).
It has been suggested that CCK and 5-HT act synergistically to activate vagal afferents (24). Early *in vivo* studies supported a role for 5-HT in the vasodilator responses elicited by CCK, secretin or mucosal irritation (12). Osmotic stimuli and the products of carbohydrate digestion stimulate the release of 5-HT from enterochromaffin cells lining the gastrointestinal mucosa (53).

Premotor sympathoexcitatory (presympathetic) vasomotor neurons in the rostroventrolateral medulla (RVLM) are thought to have a pivotal role in blood pressure regulation (5, 41). Intravenous administration of CCK sulphated octapeptide selectively inhibits splanchnic but not lumbar sympathetic nerve activity (39) and is also selective for a subset of barosensitive neurons in the rostroventrolateral medulla (RVLM) (39, 40). Postprandially-released CCK may act in a paracrine fashion on subdiaphragmatic vagal afferent fibres (38) lying within the wall of the gastrointestinal mucosa, to indirectly inhibit RVLM presympathetic neurons. We hypothesized that these CCK-sensitive RVLM neurons may control gastrointestinal vascular resistance, and demonstrated that the response to exogenous CCK was dependent on 5-HT$_3$ receptors and central NMDA receptors (38, 47). However, the physiological significance of these findings was unclear since it was not known whether endogenous release of CCK could mediate this effect via similar receptor interactions.

The aim of this study was to investigate whether the physiological release of endogenous CCK and 5-HT, stimulated by intraduodenal secretagogue administration, could also inhibit the activity of CCK-sensitive RVLM neurons, and to determine whether this involved CCK$_1$ and/or 5-HT$_3$ receptors.
MATERIALS AND METHODS

Animals All experiments were performed using male Sprague-Dawley rats (300-450 g, n=34) obtained from the Animal Resources Centre, Perth, Western Australia. This study was approved by the Animal Ethics Committee of Austin Health (Heidelberg, Victoria, Australia) and complied with principles outlined in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Materials CCK-octapeptide (CCK-8, sulfated form) was purchased from the American Peptide Company, Sunnyvale, CA, U.S.A. Phenylbiguanide (PBG) was purchased from Aldrich Chemical, Milwaukee, WI; lorglumide (sodium salt), SO (cis-9-octadecanoic acid sodium salt) and SBTI were purchased from Sigma-Aldrich, St. Louis, MO, U.S.A.; MDL-72222 was purchased from Tocris, Ellisville, MO, U.S.A.; devazepide (L-364,718) was purchased from Merck Research Laboratories, Rahway, NJ, U.S.A.

Animal preparation Arterial and venous cannulation of male Sprague-Dawley rats for the measurement of arterial blood pressure and for intravenous drug administration respectively, were carried out as described previously (39, 40). Briefly, rats were anaesthetised with halothane, tracheostomised and ventilated with 100% O₂ containing 1.3-1.5% halothane (50-60 breaths/min; 1 ml/kg). This was followed by cannulation of the left jugular vein and carotid artery for intravenous drug administration and measurement of arterial blood pressure, respectively. Adequacy of anesthetic depth was verified by absence of a response to toe-pincho r corneal probing. For duodenal administration of either SO/SBTI or water, a cannula (single lumen polyethylene catheter: internal diameter 0.58 mm, external diameter 0.96 mm; Critchley Electrical Products P/L, Silverwater, NSW, Australia) was inserted into the duodenum in the aboral direction 0.5 cm distal to the pyloric sphincter and secured to the abdominal wall with a silk suture.
Enteric modulation of presympathetic vasomotor neurons was performed using glass microelectrodes (2 mm OD) filled with 2% Pontamine sky blue in 0.5M sodium acetate. An intracellular amplifier in bridge mode (Intra 767, World Precision Instruments, Sarasota, FL, U.S.A.) was used for recordings and a window discriminator (Fintronics, Orange, CT, U.S.A.) for signal amplification (400 Hz to 4 kHz bandpass); an oscilloscope and audio amplifier were used to monitor extracellularly-recorded signals. The procedures used for location, identification and extracellular single unit recording of RVLM neurons have been described previously (39, 40). Prior to neuronal recording, an inflatable occlusive cuff was placed on the abdominal aorta for precisely controlled elevation of arterial blood pressure for baroreceptor stimulation; a bipolar electrode was placed onto the dorsolateral funiculus of the spinal cord for antidromic activation of RVLM neurons; another bipolar electrode was placed on the mandibular branch of the facial nerve, stimulation of which created a field potential used to identify the location of RVLM neurons. After completion of all surgery neuromuscular blockade was induced by administration of pancuronium (1-2 mg/kg, i.v.).

The dose of CCK used to determine RVLM neuronal sensitivity to CCK was based on previous studies in our laboratory (39). Only barosensitive, medullospinal RVLM neurons with positive collision tests were used in this study. These were then tested for responsiveness to systemic administration of the 5-HT₃ agonist PBG (von-Bezold-Jarisch reflex; 5-10 µg/kg i.v.) and CCK-8 (4 µg/kg, i.v.). The doses of CCK-8 and PBG used in this study were submaximal, as determined from previous studies (39, 46, 48). Only neurons that were inhibited by CCK were selected for this study, with the exception of those in the CCK-insensitive control group.

**Effect of SO/ SBTI or water on neuronal firing rate** A single neuron was studied in each experiment. Neuronal discharge was then monitored for up to 1 hour following the rapid
(over 30 seconds) intra-duodenal infusion of 1-2 ml of SO (dissolved in warm tap water, 100 mM final concentration) / SBTI (0.8% stock solution in distilled water, 0.2% final concentration). Two control groups were selected in this study. One group consisted of barosensitive, spinally-projecting RVLM neurons that were not CCK-sensitive, and these were tested for response to intraduodenal infusion of SO/SBTI. In other control animals, the neuronal discharge of CCK-sensitive neurons was monitored following 1-2 mls of tap water administered in place of SO/SBTI. In both groups, neuronal discharge was monitored for one hour subsequent to duodenal infusion. As a standard procedure for all neuronal activity evaluated in this study, the percentage decrease in discharge rate was calculated by dividing the difference between the mean basal firing rate and mean firing rate during the period of sustained maximal inhibition (counted over a standard period of 70 seconds) by the mean basal firing rate, and expressing this as a percentage.

**Effect of CCK₁ receptor antagonism on SO/SBTI inhibition of RVLM neurons** To determine whether postprandial release of CCK contributed to the inhibition of RVLM neuronal activity, a group of animals was pre-treated with either of the CCK₁ receptor antagonists devazepide (5 animals; 0.5-1.0 mg/kg, i.v.) or lorglumide (1 animal; 10 mg/kg, i.v.). Each antagonist was administered in separate experiments after location of a CCK-sensitive, RVLM presympathetic neuron. Following administration of the antagonist and return of arterial pressure and/or neuronal firing rates to baseline levels (approximately 20 minutes), the absence of a response to intravenously administered CCK was verified and 1ml SO/SBTI infused intraduodenally as described above. The neuronal discharge rate was then monitored for up to one hour and periods of inhibition were analysed and compared to mean baseline levels.

**Effect of 5-HT₃ receptor antagonism on SO/SBTI inhibition of RVLM neurons** To determine whether postprandial release of 5-HT contributed to the inhibition of RVLM
neuronal activity, a group of animals was pre-treated with the 5-HT$_3$ receptor antagonist MDL-72222 (0.5 mg/kg, i.v.) following the same procedure as described above for the CCK$_1$ receptor antagonists.

**Circulating CCK and 5-HT levels following intra-duodenal infusion of SO/SBTI** In addition to the general procedures described above, arterial blood collections were made from animals directly preceding, and for one hour following the infusion of SO/SBTI into the proximal duodenum. In the case of circulating CCK levels, blood was collected at 20, 40 and 60 minutes following secretagogue-infusion. In a separate set of experiments, blood was collected for the determination of circulating 5-HT levels at 15, 30, 45 and 60 minutes post-infusion because a smaller volume was required for assay. These experiments were not performed in conjunction with neuronal recording experiments since removal of blood was likely to compromise the conditions required for stable extracellular recording of RVLM presympathetic neurons.

**Radioimmunoassay of plasma CCK.** Blood (900 µl) was collected into eppendorf tubes containing 100 µl heparin (1000 U/ml). After collection, the blood samples were immediately placed on ice and centrifuged. Plasma was stored at −20°C until the time of assay. Plasma CCK concentration was measured in ethanol-extracted plasma as described previously (52). Briefly, 800 µl of absolute ethanol was added to 400 µl of plasma and the mixture centrifuged for removal of coagulated protein. The ethanol extracts were then divided into 2 x 400 µl aliquots, evaporated to dryness and stored at −20°C until time of assay. Antiserum 92128 which has negligible cross-reactivity with gastrins was used in the assay. $^{125}$I-Bolton-Hunter-CCK-8 (Amersham International, Piscataway, NJ, USA) was used as a tracer. Charcoal stripped, ovine extracted, plasma tubes were used for the CCK-8 non-sulphated standard curve. The ID$_{50}$ was 3.5 fmol/ml and the intra- and inter-assay coefficients of variation were 7 and < 14%, respectively.
**Determination of whole blood 5-HT** After collection of blood (500 µl) into eppendorf tubes, platelet disruption was achieved by addition of 0.5 ml ascorbic acid (9 mg/ml in distilled water) followed immediately by 0.2 ml perchloric acid (4M). Tubes were vortexed for 20 seconds then centrifuged at 12,000g for 10 minutes. Supernatants were stored at -20°C and assayed within 3 days of collection. Biochemical assays were performed using a reverse-phase high pressure liquid chromatography machine fitted with a 15 cm Spherisorb 5 µ C18 column (Waters Australia, Rydalmere, NSW, Australia). A Shimadzu LC10 injector system (Shimadzu Scientific Instruments Oceania Pty Ltd, Mount Waverly, Victoria, Australia) was used for delivery of samples. Elution of the compound was monitored by a fluorescence detector (Kratos model FS970; Shimadzu Scientific Instruments Oceania Pty Ltd, Mount Waverly, Victoria, Australia; excitation wavelength 200 nm, emission Corning 7-60 filter). The mobile phase contained 0.1M potassium dihydrogen phosphate, 0.4M perchloric acid and 0.1% sodium heptanesulphate in 10% acetonitrile (pH 4.0) and was run through the column at 2 ml/min. The detection limit was 1 ng/ml.

**Data analysis and statistics** Arterial blood pressure, heart rate, extracellular action potentials and stimulation pulses were recorded using a Cambridge Electronic Design data acquisition system (CED, Cambridge, UK) and Spike2 software. Data are expressed as mean ± s.e. mean. Statistical analyses were performed using a one-way ANOVA followed by a Tukey-Kramer test using GraphPad Instat version 3.06 (Graph Pad Software, San Diego CA, USA).
RESULTS

**Electrophysiological characterization of RVLM presympathetic neurons and sensitivity to CCK** All units examined in this study were spontaneously active and were silenced or considerably slowed by elevation of arterial blood pressure (AP; Fig. 1A). In addition, all were transiently inhibited by administration of the 5-HT3 receptor agonist PBG (Fig. 1A). Apart from the CCK-insensitive controls, all neurons selected for this study were clearly inhibited by systemic administration of CCK-8 administration (Fig. 1A) and were spinally-projecting (collision test positive; Fig. 1B).

**Effect of water on RVLM neuronal firing rate** The fluctuations in neuronal firing rates (FR) observed following infusion of 1-2 ml tap water into the duodenum ranged from 0 to 13% below baseline, with a mean decrease of 5 ± 2% (baseline FR 22 ± 3 spikes/s; mean conduction velocity (CV) 4.4 ± 0.6 m/s; n=6). These small fluctuations in firing rate were short-lived and all cells showing any sign of inhibition recovered during the course of the recording. Periods of inhibitory activity were analysed and compared to baseline levels. These changes were not associated with any significant increases in AP and were usually accompanied by a decrease in AP (mean -7 ± 5 mmHg; n=4) and heart rate (HR; mean -8 ± 5 bpm; n=4). Similar blood pressure and heart rate fluctuations were observed in the two animals with no change in FR.

**Effect of SO/SBTI on RVLM neuronal firing rate** Infusion of SO/SBTI into the proximal duodenum produced between 29-100% inhibition in RVLM neuronal spike discharge, with a mean decrease in FR of 58 ± 7% (mean conduction velocity 3.3 ± 0.5 m/s, baseline firing rates 11 ± 2 spikes/s; n=10). Neuronal discharge rate was monitored for up to 1 hour following infusion of SO/SBTI into the duodenum, with maximal inhibition of neuronal discharge occurring between 22-50 minutes post-infusion (mean 31 ± 3 min).
Patterns in firing rates emerged whereby periods of sustained inhibition (greater than 10 minutes) were gradually followed by periods of partial recovery. In two cases, cell firing was monitored for only 30 and 45 minutes because of instability and loss of recording. The inhibitory phase of these cells was nevertheless monitored for a period of 10 and 25 minutes respectively, and was sustained. In all other cases, once the cell showed signs of sustained inhibition, the firing rate did not return to pre-SO/SBTI levels during the course of the recording. Inhibition was invariably accompanied by a fall in arterial pressure and heart rate with an average decrease of \(-22 \pm 4\) mmHg and \(-19 \pm 4\) bpm (n=10) respectively, confirming that the inhibition was not arterial pressure-dependent. Arterial pressure and heart rate changes were not significantly different from the tap water control experiments \((P>0.05)\).

Figure 2 depicts the effect of SO/SBTI on the neuronal discharge of an RVLM presympathetic vasomotor neuron. Twenty minutes after infusion of SO/SBTI the firing rate was reduced by 70% (Fig. 2B). At forty minutes post-infusion partial recovery of neuronal activity was evident (Fig. 2C). Note that the blood pressure during maximal inhibition fell by 45 mmHg and the heart rate by 11 beats/min. The inhibition of RVLM neuronal firing rate produced by infusion of SO/SBTI into the duodenum was significantly different from infusion of water alone (Fig. 3; \(P<0.001\)).

**CCK\(_1\) receptor antagonism** Pre-treatment with devazepide or lorglumide before intraduodenal infusion of SO/SBTI did not attenuate the inhibitory effect of SO/SBTI on neuronal firing rate. Maximal inhibition (with respect to baseline levels) ranged from 26 - 95% (mean 58 \pm 12\%); conduction velocity 3.5 \pm 0.3\ m/s, baseline firing rates 7 \pm 1\ spikes/s; n=6) and occurred between 15 and 54 minutes (mean 41 \pm 6\ min) post-SO/SBTI. The inhibition observed in the group pre-treated with CCK\(_1\) receptor antagonists was not significantly different to that observed in the SO/SBTI-infused group without pretreatment \((P>0.05)\), but was significantly different to that observed in the water control group \((P<0.001;\ Fig. 3)\).
Following SO/SBTI infusion, sustained inhibition of neuronal firing rate was observed, with firing rate returning to baseline levels in only one case. Inhibition was invariably accompanied by a fall in arterial pressure and heart rate with an average decrease of \(-27 \pm 5\) mmHg \((P>0.05\) compared to control) and \(-40 \pm 13\) bpm \((P<0.05\) compared to control) \((n=6)\) respectively, again confirming that the inhibition was not pressure-dependent.

### 5-HT\(_3\) receptor antagonism

Pre-treatment with MDL-72222 before intra-duodenal infusion of SO/SBTI significantly attenuated the inhibitory effect of SO/SBTI on neuronal firing rate \((P<0.05)\). Maximal inhibition (with respect to baseline levels) ranged from 0 – 41 % (mean 22 ± 7 %; conduction velocity 3.5 ± 0.4 m/s, baseline firing rates 14 ± 2 spikes/s; \(n=6\)) and occurred between 15 and 48 minutes (mean 29 ± 6 min) post-SO/SBTI. The inhibitory effect of SO/SBTI in the group pre-treated with the 5-HT\(_3\) receptor antagonist was not significantly different to that of the water control group \((P>0.05)\) but was significantly different to the inhibition observed in the SO/SBTI treated animals, with \((P<0.05)\) or without \((P<0.01)\) CCK\(_1\) receptor antagonist pretreatment (Fig. 3). The periods of suppressed firing rate were not sustained and cell firing recovered within the one-hour recording period. Periods of inhibitory activity were analysed and compared to baseline levels. These changes were invariably accompanied by a decrease in blood pressure (mean -32 ± 17 mmHg; \(n=5\) not including the experiment in which there was zero % decrease in firing rate; \(P>0.05\) compared to control) and heart rate (mean -29 ± 9 bpm; \(n=5\)), confirming that the inhibition was not pressure-dependent.

### Effect of SO/SBTI on non-CCK-sensitive controls

Following intraduodenal administration of SO/SBTI, the magnitude of the inhibition observed in CCK-insensitive neurons was comparable to that observed in control and MDL-72222 pre-treatment experiments \((P > 0.05\) in both cases), with fluctuations in neuronal firing rate ranging from 0 to 22% (mean neuronal inhibition 11 ± 4%; baseline firing rate 15 ± 7 spikes/s; mean
conduction velocity $1.3 \pm 0.4$ m/s; $n=5$ see Figure 3 for summary). Inhibitory periods were not associated with any significant increases in arterial pressure but rather with a decrease in blood pressure (mean $-18 \pm 6$ mmHg; $n=4$ excluding the experiment in which there was zero % decrease in firing rate; $P>0.05$ compared to control) and heart rate (mean $-21 \pm 17$ bpm; $n=4$). In all cases the inhibition was transient and was followed by full recovery to pre-infusion discharge rates.

**Effect of SO/SBTI on circulating levels of CCK** Infusion of SO/SBTI into the duodenum invariably produced an elevation in circulating levels of CCK-8. Baseline levels of $8.0 \pm 1.9$ pM rose to maximal plasma concentrations of $20.1 \pm 2.9$ pM ($P<0.05$; $n=5$) at 20 minutes post-infusion, amounting to an average 2.5 fold increase in circulating levels of CCK-8 (Fig. 4). Individual peak increases in CCK levels varied from 1.5 to 7 fold and occurred between 20-60 minutes post intra-duodenal infusion of SO/SBTI.

**Effect of SO/SBTI on whole blood serotonin** The concentration of serotonin in arterial blood was not significantly altered following SO/SBTI administration (Fig. 5). Baseline levels of $208.4 \pm 54.5$ ng/ml rose to a maximum of $244.0 \pm 60.8$ ng/ml ($P>0.05$; $n=6$) at 15 minutes post-infusion and returned to baseline level ($205.2 \pm 35.4$ ng/ml) at 60 minutes post-infusion.
DISCUSSION

We have previously demonstrated that systemic administration of exogenous CCK inhibits the firing rate of a sub-population of RVLM presympathetic neurons (termed CCK-sensitive neurons)(39, 40). On the basis of these observations, we predicted that simulation of postprandial conditions by infusion of secretagogues into the duodenum would also inhibit CCK-sensitive RVLM presympathetic vasomotor neurons, and this hypothesis was tested in the present study. Before duodenal secretagogue infusion, the sensitivity of RVLM neurons to CCK was tested using intravenous administration of the peptide. In accordance with our previous findings, we found that CCK-sensitive neurons had axonal conduction velocities (mean 3.6 ± 1.4 m/s) consistent with lightly-myelinated axons (40). Intraduodenal secretagogue administration inhibited the firing rate of CCK-sensitive RVLM presympathetic neurons, but not that of RVLM neurons that were CCK-insensitive. Intraduodenal administration of water in place of SO/SBTI had no significant effects on the firing rate of RVLM presympathetic vasomotor neurons. Surprisingly, the antagonist studies demonstrated the inhibitory effects of secretagogue administration on CCK-sensitive RVLM neurons to be independent of CCK₁ receptor activation but instead contingent on 5-HT₃ receptor activation.

Apart from CCK, several other gastrointestinal hormones are released during digestion including somatostatin, leptin, gastrin, secretin, peptide YY, enteroglucagon, neurotensin (13, 43) and serotonin, (32). Although the ability of CCK to augment gastrointestinal blood flow has been demonstrated (4, 10, 15, 39), other gastrointestinal hormones have also been implicated in postprandial gastrointestinal hyperaemia (4, 36). SO/SBTI may trigger the release of several hormones that may act on vagal afferents to inhibit RVLM presympathetic neuronal discharge. A possible candidate is serotonin, given that it been shown to activate cardiopulmonary and gastrointestinal vagal afferents via a 5-
HT₃ receptor-dependent mechanism (2, 8). In support of this proposal, all RVLM neurons in this study, including those that were CCK-sensitive, were also sensitive to systemic administration of the selective 5-HT₃ receptor agonist PBG.

5-HT may play a gastrointestinal vasodilator role in response to cholecystokinin (12). Our laboratory has previously demonstrated that pre-treatment with the selective 5-HT₃ receptor antagonist MDL-72222 attenuates the inhibitory effect of exogenously-administered CCK on CCK-sensitive RVLM presympathetic neurons (38). It was hypothesised that the actions of CCK may have been partly dependent on 5-HT₃ receptor activation, i.e. CCK may release 5-HT which acts on 5-HT₃ receptors to inhibit RVLM neuronal activity. In examining the effects of CCK on feeding, many have suggested that the actions of CCK are partially dependent on 5-HT₃ receptor activation (1, 6, 14). Furthermore, primary vagal afferent neurons are potently stimulated by endogenous 5-HT and CCK release (26, 53), and a subpopulation of nodose ganglion neurons possess both high affinity CCK₁ receptors and 5-HT₃ receptors (24).

The present study demonstrated that pre-treatment with the 5-HT₃ receptor antagonist MDL-72222 significantly attenuated the inhibitory effect of SO/SBTI on RVLM neuronal firing, whereas pre-treatment with the CCK₁ receptor antagonist devazepide had no effect. These results suggested that the secretagogue-induced inhibition of RVLM neurons observed was not CCK₁ receptor mediated but instead dependent on 5-HT₃ receptor activation. It is conceivable that SO/SBTI triggers an integrated response involving the release of CCK and other hormones such as 5-HT that result in vagal afferent activation and reflex inhibition of RVLM presympathetic neuronal discharge. However, since MDL-72222 readily crosses the blood brain barrier, the possibility that it acts centrally cannot be discounted.

Our study corroborated the findings of previous investigations showing that infusion of SO or SBTI into the duodenum produces an increase in the levels of circulating CCK (42,
50), although the basal circulating CCK concentrations observed in the present study were approximately two-fold higher than those reported elsewhere. Others reported peak levels following SBTI (28, 30) or SO (19) to be below 11 pM compared with 20 pM observed in our laboratory. However, basal levels reported in other studies were lower than those observed in the present study (below 1 pM compared to our 8 pM) as were the increases following secretagogue infusion (up to 16 fold compared to our 2.5 fold). These discrepancies may be due to several differences in the protocols employed by others including the use of fasted, conscious animals. In support of these claims Li and colleagues (20), who also performed studies on anesthetised animals and examined the effects of intraduodenal SO administration on endogenous CCK release, obtained basal and post-secretagogue CCK levels similar to ours (20).

RVLM presympathetic vasomotor neurons examined in this study that were inhibited by intravenous administration of CCK-8 were referred to as CCK-sensitive. The inhibitory effects of intravenously administered CCK-8 on the discharge of CCK-sensitive RVLM presympathetic neurons is blocked by the CCK₁ receptor antagonist devazepide (38 and present study). We have proposed that CCK-sensitive RVLM presympathetic neurons may drive the sympathetic vasomotor outflow to the gastrointestinal vasculature and may therefore be involved in postprandial gastrointestinal hyperaemia. Duodenal infusion of SO/SBTI reduced the discharge rate of CCK-sensitive, but not that of CCK-insensitive RVLM presympathetic vasomotor neurons. This was a secretagogue-dependent mechanism since infusion of water alone into the duodenum had no significant effect on neuronal discharge, and was independent of MAP elevation since no change or a modest decrease in MAP was noted during the periods of maximal neuronal inhibition.

CCK₁ receptor antagonists did not reverse the inhibitory effects of SO/SBTI on RVLM presympathetic neuronal discharge, suggesting that this inhibition was not mediated by
activation of the CCK<sub>1</sub> receptor. The dose of devazepide chosen in the present study blocked the inhibitory actions of exogenously administered CCK-8 on splanchnic sympathetic nerve activity and CCK-sensitive RVLM neurons (39 and present study). Furthermore, many investigators have demonstrated that similar doses of devazepide are capable of reversing the effects of endogenously-released CCK on pancreatic enzyme secretion (23, 33, 34), vagal afferent nerve discharge (7, 11, 18), gastric emptying (51) and food intake (37), suggesting that the dose chosen in our study is likely to have been adequate.

It is possible that despite an elevation in circulating levels of CCK, its concentration may have been insufficient for activation of subdiaphragmatic vagal afferents. As a result, the inhibitory effects of SO/SBTI on RVLM neuronal discharge were not blocked by devazepide. This prompted the conclusion that secretagogue-induced inhibition of CCK-sensitive RVLM neurons is not dependent on CCK<sub>1</sub> receptor activation. Instead, the concomitant release of 5-HT from enterochromaffin cells following secretagogue infusion appears to be of greater importance.

Rat chow stimulates pancreatic protein secretion via a CCK-independent mechanism involving serotonin-release from enterochromaffin cells (EC) (22). The normal rat diet consists of 4-5% fat (42) and includes the high-protein soybean meal (Barastoc G.R.2 rat and mouse feed content; Ridley Agriproducts Pty Ltd, Pakenham, Victoria); SO is a digestion product of fat (49). The CCK-secretagogues SO and SBTI used in our study are possible substitutes for the fat and protein content of rat chow respectively, and may have stimulated the release of serotonin from EC cells without elevating its circulating levels. Li and colleagues demonstrated that luminal non-CCK-mediated pancreatic secretion was eliminated following administration of the 5-HT synthesis inhibitor p-chlorophenylalanine (22). In another study (25), they also showed that maltose and hypertonic saline increased the intraluminal concentrations of 5-HT without affecting its circulating levels, and that the
monoamine acts in a paracrine fashion to induce pancreatic secretion by stimulating 5-HT$_3$ receptors on gastrointestinal vagal afferents. Similarly, the release of 5-HT from enterochromaffin cells in response to SO/SBTI in the present study, may have stimulated 5-HT$_3$ receptors located on vagal sensory fibres in a paracrine fashion to produce a reflex decrease in sympathetic vasomotor outflow. This is supported by the results demonstrating an attenuation of the SO/SBTI-induced inhibition of RVLM neuronal discharge rate following 5-HT$_3$ receptor blockade.

The absence of a significant increase in arterial 5-HT concentration following SO/SBTI infusion into the duodenum is most probably due to its rapid and complete clearance as it passes through the pulmonary circulation (44). This also supports the notion that the inhibitory effects of serotonin on RVLM neuronal discharge are likely to be mediated by luminal release and paracrine activation of 5-HT$_3$ receptors on gastrointestinal vagal afferents, and not by activation of cardiopulmonary 5-HT$_3$ vagal afferents and the von Bezold-Jarisch reflex. Furthermore, others have demonstrated that nodose neuronal responses to digestion products and luminal osmolarity, are dependent on the endogenous release of 5-HT from enterochromaffin cells lining the gastrointestinal mucosa (53). These responses were reported to occur within 5 minutes of continuous infusion of various test solutions. Although we have reported the maximal effects of secretagogue infusion on CCK-sensitive RVLM sympathetic neurons to occur (on average) at 31 minutes post-infusion, neurons demonstrated signs of inhibition as early as 5 minutes (mean 10 ± 1; n = 10) post-infusion. The reflex effects of intraduodenal nutrient infusion on RVLM neurons in our experiments may be slightly delayed when compared to other preparations for several reasons including administration of pancuronium, the position of gut due to positioning rat in stereotaxic frame and bolus versus continuous infusion of nutrients into gut.
Our results demonstrate that a subset of RVLM presympathetic neurons are sensitive to systemic administration of both CCK-8 and PBG as illustrated in Figure 6. These neurons may drive presympathetic vasomotor outflow to the gastrointestinal vasculature to produce gastrointestinal vasodilatation by an indirect intramedullary pathway similar to the von Bezold-Jarisch reflex. This reflex possibly functions via a trisynaptic medullary pathway whereby neurons in the nucleus of the solitary tract which receive excitatory vagal afferent inputs, activate inhibitory GABAergic caudal ventrolateral medullary (CVLM) neurons. These in turn, inhibit RVLM presympathetic neurons (45), resulting in the reflex withdrawal of sympathetic vasomotor tone and subsequent vasodilatation (Fig. 6). We have found that inhibition of CVLM neurons by local microinjection of the GABA\textsubscript{A} receptor agonist muscimol abolishes baroreflex-mediated sympathoinhibition as well as CCK-induced inhibition of splanchnic nerve discharge (Sartor and Verberne, unpublished observations).

Postprandial stimuli inhibited CCK-sensitive RVLM presympathetic neurons but not CCK-insensitive neurons. Devazepide failed to reverse the secretagogue-induced inhibition of CCK-sensitive RVLM presympathetic neurons, suggesting that the inhibition was independent of CCK\textsubscript{1} receptor activation. This implies that these neurons are not inhibited by the postprandial release of CCK but by some other hormone released in response to SO/SBTI. Since these neurons are also sensitive to exogenous administration of PBG, it seems plausible that serotonin released postprandially may act in a paracrine fashion (25, 53) on subdiaphragmatic vagal afferent neurons to produce gastrointestinal vasodilatation (Fig 6).

The observation that only a subset of RVLM presympathetic neurons is CCK-sensitive may be used to distinguish them from other RVLM presympathetic vasomotor neurons in order to elucidate their role in gastrointestinal circulatory control. This is the first study to describe a relationship between a group of physiologically identified pre-sympathetic vasomotor neurons of the RVLM and an enteric signal. Although these neurons are CCK-
sensitive, their physiological role in gastrointestinal postprandial hyperaemia seems to be independent of CCK₁ receptor activation. Instead, their contribution to gastrointestinal vascular perfusion may depend on activation of 5-HT₃ receptors probably located on CCK-sensitive subdiaphragmatic vagal afferents to elicit a novel gut-brain reflex. Further examination of these neurons and their role in gastrointestinal perfusion may lead to improved drug therapies for the treatment of postprandial gastrointestinal hyperaemia which can be symptomatic in susceptible individuals with compromised reflex sympathetic vasomotor control.
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REFERENCES


FIGURE LEGENDS

Figure 1. Physiological properties of a CCK-sensitive RVLM presympathetic vasomotor neuron. A: Neuronal discharge rate was reduced by aortic occlusion (AOc), administration of PBG (10 µg/kg, i.v.) and CCK-8 (4 µg/kg, i.v.). The neuron characterised in this example had a firing rate of 22 spikes/s and a spinal conduction velocity of 6.2 m/s (constant latency spike of 4.5ms). From top to bottom, traces represent i) firing rate (FR; spikes/sec); ii) heart rate (HR; bpm); iii) spike amplitude (mV) and iv) arterial pressure (AP; mmHg). B: Collision test for the cell depicted in panel A. Arrow represents constant latency antidromic spike (traces 1, 4 and 5 from bottom). Collision of the antidromic spike (asterisks) occurred when application of spinal stimulation was within the critical interval.

Figure 2. The effect of SO/SBTI on the discharge rate of an RVLM presympathetic vasomotor neuron. A: pre-infusion recording; B: 20 minutes post-infusion demonstrating 70% inhibition of cell firing rate and C: at 40 minutes post-infusion showing partial recovery of neuronal discharge.

Figure 3. The inhibitory effects of intraduodenal administration of SO/SBTI on RVLM neuronal firing rate with or without antagonist pre-treatment; comparison with water and non-CCK-sensitive controls. Duodenal infusion of SO/SBTI alone ( ) or with CCK₁ receptor antagonist pre-treatment (devazepide or lorglumide; ) resulted in a decrease in RVLM neuronal firing rate significantly different (***P<0.001) from the water control ( ). The effect of SO/SBTI on RVLM neuronal firing rate was significantly attenuated after 5-HT₃ receptor antagonist pretreatment (MDL-72222; ) when compared with SO/SBTI alone (**P<0.01) or with CCK₁ receptor antagonist pretreatment (*P<0.05). The effect of SO/SBTI
on non-CCK-sensitive neurons (**) was not significantly different to the water control or MDL-72222 pretreatment experiments, but was significantly different to the experiments in which SO/SBTI was given alone (**P<0.01) or with CCK₁ receptor antagonist pretreatment (**P<0.01).

Figure 4. The concentration of CCK-8S (pM) found in arterial blood just preceding and at 20, 40 and 60 minutes following the infusion of SO/SBTI into the duodenum. At 20 minutes post-infusion, the circulating CCK concentration of is significantly different from basal levels (P<0.05).

Figure 5. The concentration of 5-HT (ng/ml) found in arterial blood just preceding and at t= 15, 30, 45 and 60 minutes following the infusion of SO/SBTI into the duodenum.

Figure 6. A possible mechanism of action of endogenously-released CCK, 5-HT and/or other enteric signals following SO/SBTI infusion into the duodenum. Enteric signals including CCK and 5-HT may act in a paracrine fashion to innervate a subpopulation of subdiaphragmatic vagal afferent nerves within the mucosal wall. Activation of vagal afferents possessing both high affinity CCK₁ receptors and 5-HT₃ receptors may trigger a vagal depressor reflex. Stimulation of chemosensitive vagal afferents activate medullary NTS neurons which in turn may project to inhibitory GABAergic CVLM neurons to inhibit CCK-sensitive and PBG-sensitive RVLM sympathetic neurons with efferent projections to the gastrointestinal vasculature. The result would be withdrawal of sympathetic vasomotor tone and gastrointestinal vasodilatation.
Figure 1.

A

FR (sp/s)

AOc  PBG  CCK-8

HR (bpm)

Unit (mV)

AP (mmHg)

5 s

B

1mV  5 ms

*  *
Figure 2.

A  FR (spikes/s)  25
HR (bpm)  450
Units (mV)  2
AP (mmHg)  100

B  FR (spikes/s)  25
HR (bpm)  450
Units (mV)  2
AP (mmHg)  100

C  FR (spikes/s)  25
HR (bpm)  450
Units (mV)  2
AP (mmHg)  100

5 s
Figure 3.

SO/SBTI  Water control  CCK\textsubscript{\textalpha} antagonist pretreatment  5-HT\textsubscript{\textbeta} antagonist pretreatment  Non CCK-sensitive control

(n=10)  (n=6)  (n=6)  (n=6)  (n=5)

% Reduction in neuronal firing rate

* \( p < 0.05 \)
** \( p < 0.01 \)
*** \( p < 0.001 \)
Figure 4.
Figure 5.
Figure 6.