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Review Article

Discovery of a Cholecystokinin-Releasing Peptide: Biochemical Characterization and Physiological Implications

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Abstract

Recent studies indicate that the secretion of CCK is mediated by a trypsin sensitive peptide secreted by the proximal small intestine that has been designated "CCK-releasing factor" (CCK-RF). This CCK-RF was found to be identical to the porcine diazepam binding inhibitor by peptide sequencing and mass spectrometry analysis. This peptide is present in abundance in the epithelial cells in the duodenal mucosa. Its release into the lumen is mediated by intestinal submucosal cholinergic neurons. Functionally, this peptide appears to mediate feedback regulation of pancreatic secretion and CCK release in response to peptone and lipid stimulation. It fulfills all the criteria as a physiological regulator of CCK secretion. This represents the first chemical characterization of a luminally secreted enteric peptide functioning as an intraluminal regulator of intestinal hormone release.

Key Words: diazepam binding inhibitor, cholecystokinin, pancreatic secretion, enteric peptide, feedback regulation

Physiological studies in animals and humans show that cholecystokinin (CCK) plays an important role in mediating postprandial pancreatic enzyme secretion. Recently we investigated the mechanisms responsible for feedback modulation of CCK release by intraluminal proteases. This has led to the discovery and characterization of a trypsin-sensitive CCK-releasing peptide which is secreted into the proximal intestine. In this communication, we review the biochemistry and physiology of this novel peptide which appears to play a critical role in the secretion of CCK.

Feedback Regulation of Cholecystokinin Release

Experimental evidence in animals suggests that the presence of pancreatic enzymes in the duodenum exhibit a feedback control of pancreatic exocrine secretion. Raw soybean, or an isolated soybean trypsin inhibitor (SBTI) markedly stimulates the pancreas in rats (1-3). Green and Lyman (4) found that in rats with bile pancreatic duct fistulas, removing pancreaticobiliary juice (PBJ) from the intestine results in a large increase in pancreatic enzyme secretion. Return of PBJ or infusion of the PBJ components trypsin or chymotrypsin suppresses pancreatic enzyme secretion. Therefore, feedback inhibition of pancreatic enzyme secretion in the rat is mediated by proteolytic enzymes in the intestinal lumen.

Similar feedback control systems have been shown in chickens (5), pigs (6) and humans (7, 8). In man, intraduodenal trypsin perfusion inhibits phenylalanine-, oleic acid- and meal-stimulated chymotrypsin and lipase outputs (7). This inhibitory effect is protease specific since suppression is not observed with intraduodenal perfusion of lipase or amylase. A similar phenomenon was observed in chronic pancreatitis patients (9). In contrast, Dlugosz (10) and Hotz (11) found no effect on pancreatic

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Table 1. Amino Acid Analysis of CCK-Releasing Peptide

Amino acid	nmole	ratio	integral
Asx	0.68	10.1	10
Thr	0.28	4.1	4
Ser	0.38	5.6	6
Glx	0.87	12.8	13
Pro	0.15	2.2	2
Gly	0.59	8.8	9
Ala	0.60	8.9	9
Cys	0.00	0.0	0
Val	0.23	3.4	3
Met	0.02	0.4	0
Ile	0.32	4.7	5
Leu	0.42	6.3	6
Tyr	0.22	3.2	3
Phe	0.14	2.0	2
Lys	0.88	13.1	13
His	0.11	1.6	2
Arg	0.11	1.7	2
Total	6.00		89

Amino acid composition of the purified porcine CCK-RP analyzed with a Hitachi amino acid analyzer (model L8500) after hydrolysis of the samples in 6M HCl containing 1% phenol at 110°C in an evacuated tube.

enzyme secretion with intraduodenally infused aprotinin, a trypsin inhibitor. Similar findings were reported using the new trypsin inhibitor, FOY-305 (12). However, neither compound strongly inhibits human chymotrypsin. On the other hand, Liener (13) showed that Bowman-Birk soybean trypsin inhibitor, an inhibitor of chymotrypsin and elastase, markedly stimulates pancreatic enzyme secretion in humans, suggesting that not only trypsin, but also other proteases such as chymotrypsin and elastase, need to be removed to evoke stimulation of pancreatic enzyme secretion in humans.

In subsequent studies, diversion of bile pancreatic juice and duodenal infusion of SBTI in the rat were shown to increase plasma CCK levels and pancreatic enzyme secretion (14). On the other hand duodenal infusion of trypsin abolishes the increase in plasma CCK. Intravenous infusion of proglumide or L364, 718, a specific CCK antagonist, abolishes the increase in pancreatic enzyme secretion following bile pancreatic juice diversion (14, 15), indicating that feedback regulation of pancreatic secretion by trypsin is mediated by CCK release. The increases in plasma CCK levels and pancreatic secretion after diversion of pancreatic juice appear to be mediated by a trypsin-sensitive substance secreted by the proximal intestine that has been designated "CCK-releasing peptide" (CCK-RP) (16, 17). When trypsin is present, this peptide is inactivated. This newly discovered CCK-RP may act as a mediator of pancreatic enzyme secretion in response to dietary protein intake in rats. Dietary protein in the intestine competes for the trypsin (16, 17, 18) that would otherwise inactivate CCK-RP. The resulting increase of CCK-RP in the lumen enhances CCK release and stimulates pancreatic enzyme secretion.

CCK-RP is not the only peptide which stimulates CCK release. Fushiki (19) recently isolated a new peptide from rat pancreatic juice and named it pancreatic monitor peptide. This peptide stimulates CCK release and enzyme secretion when administered into the rat proximal intestine by an atropine-insensitive mechanism (20, 21). Pancreatic monitor peptide is found exclusively in zymogen granules of pancreatic acinar cells (22) and is acid stable, heat resistant and has a molecular weight of approximately 6500 daltons. It is important to note that the pancreatic monitor peptide present in the pancreatic juice does not explain the feedback mechanism originally reported by Green and Lyman (4), who demonstrated that diversion of pancreatic juice causes an increase in amylase secretion, while our CCK-RP can. In addition, we showed that CCK-RP but not the pancreatic monitor peptide mediates protein-stimulated CCK release in rats.

Extraction, Purification and Sequencing of CCK-Releasing Peptide (Diazepam Binding Inhibitor) from Porcine Intestinal Mucosa

To characterize CCK-RP we extracted and purified this peptide from porcine intestinal mucosa by HPLC. In each purification step, HPLC fractions were assayed for stimulation of pancreatic enzyme secretion and CCK release using anesthetized rats with bile pancreatic and intestinal fistulas. In the final HPLC purification step we obtained a pure peptide rich in Asx, Glx and Lys by amino acid analysis (Table 1). The intact peptide was subjected to amino acid sequence analysis, but we failed to obtain a specific sequence suggesting that the peptide has a blocked N-terminus. After tryptic digestion of the peptide and separation by HPLC, we determined the sequences of eight fragments which were identical to the corresponding fragments of porcine diazepam binding inhibitor (DBI) (Fig. 1), originally isolated from rat brain (23). Analysis by mass spectrometer revealed a molecular weight of 9810 which corresponds to the molecular weight of porcine intestinal DBI_{1-86}, indicating that we have purified and sequenced a CCK-releasing peptide from the porcine small intestine which is identical to porcine DBI_{1-86} (24).

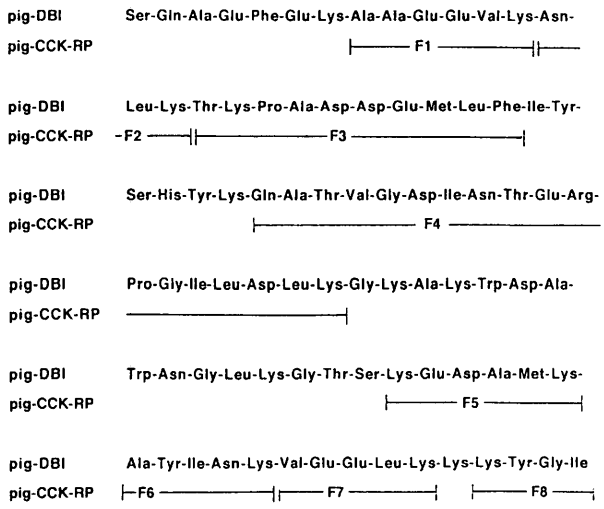

Fig. 1. Comparison of the amino acid sequence of porcine DBI_{1-86} and fragments of porcine CCK-RP following tryptic digestion.

Biochemistry and Physiology of Diazepam Binding Inhibitor (DBI)

DBI is a 9-KD polypeptide first isolated in 1983 from rat brain (23). Using oligonucleotides corresponding to a partial amino acid sequence of this peptide, cDNAs for DBI were obtained from rat, human, bovine and mouse brain cDNA libraries (25-28). The deduced amino acid sequences of DBI from various species are highly homologous in the charged COOH-terminal region but show differences in the NH_2-terminal region. DBI and its two major processing products DBI_{33-50} (octadecaneuropeptide) and DBI_{17-50} (triakontatetraneuropeptide) are unevenly distributed in the brain with the highest concentrations being present in the hypothalamus, amygdala and cerebellum (29). DBI is also present in peripheral tissues (30, 31, 32) including the adrenal cortex, Leydig cells of the testis and liver. Furthermore, significant amounts of DBI-like immunoreactivity have been detected in the gut and the highest concentrations were found in the duodenum and antrum (33). In the intestine, DBI-like immunoreactivity (DBI-LI) was localized only to the epithelial cell layer in both enterocytes and goblet cells (33). Myenteric neuronal cells were devoid of DBI-LI (33). In situ hybridization using a 35S-labeled cRNA probe showed that DBI mRNA is located in the mucosa (33), clearly indicating that, in the rat, intestinal epithelial cells synthesize DBI-like material; however the physiological function(s) of DBI in the gastrointestinal tract remains unknown.

DBI and its natural processing products have a distribution pattern that corresponds to that of benzodiazepine (BZD) recognition sites. In the brain, there are two types of BZD recognition sites, each thought to be associated with a specific protein, including the "central" and "peripheral" BZD binding sites. The "central" BZD recognition site is prevalent in neurons and is associated with $GABA_A$ receptors, which are ligand-gated Cl- channels (34-37). Studies of recombinant $GABA_A$ receptors expressed in a kidney tumor cell line after transfection with a combination of α, β, γ subunit cDNAs have shown that there are at least two different allosteric modulatory sites for BZDs in the extracellular domain of the $GABA_A$ receptor. One site requires the presence of specific α (38) and γ subunits and is the binding site for the anxiolytic BZDs (diazepam), the axiogenic β-carboline carboxylate esters (BCS) (β-carboline-3-carboxylic acid ethyl ester methyl amide) and the BZD antagonist flumazenil (34-37). A second site is stimulated by 4' chlorodiazepam, a convulsant BZD ligand (39) which down regulates $GABA_A$ receptors (40) in a manner that is dependent on the presence of receptor γ subunits but is resistant to flumazenil inhibition (36).

The "peripheral" BZD recognition site (39) is prevalent in glia (39, 41) and is preferentially associated with a protein in the outer mitochondrial membrane (42, 42) that regulates the access of cholesterol to the inner mitochondrial membrane, where a specific type of cytochrome P450 catalyzes the conversion of cholesterol into pregnenolone (44, 45). In addition to the brain, the "peripheral" BZD recognition site is also abundant in peripheral steroidogenic cells (adrenal gland and testis) (46), epithelial cells in the kidney and intestine (33) and hepatocytes. More recent studies suggest that these "peripheral" mitochondrial BZD recognition sites are also present on the plasma membrane of interstitial Leydig cells (47) and intestinal epithelial cells (33). Currently it is unknown which BZD binding sites (central vs peripheral) mediate the ability of DBI to release CCK.

DBI has multiple biological actions, some of which are mediated by BZD binding sites and some of which are unrelated to BZD binding sites. In animal models, DBI induces anxiety and proconflict responses (48). In primary cultures of mouse spinal cord neurons, DBI reduces γ-aminobutyric acid (GABA)-evoked chloride channel opening (49). These activities are related to the actions of DBI on the "central" BZD binding site. In addition DBI stimulates mitochondrial steroid biosynthesis in adrenocortical, Leydig and glial cells by a "peripheral" BZD binding site-dependent mechanism (29, 46). More recently DBI has been shown to be a paracrine/autocrine stimulator of Leydig cell proliferation acting via a plasma membrane "peripheral" BZD binding site independent of mitochondrial function (47). Some reported DBI actions are unrelated to known BZD binding sites.

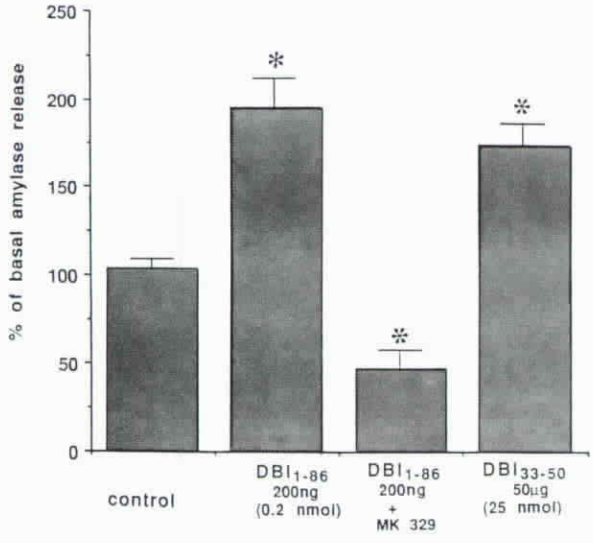

Fig. 2. Effect of intraduodenal DBI_{1-86} and its fragment DBI_{33-50} on amylase output in anesthetized and atropinized rats. Atropine (150 µg/kg/hr) was infused and bile-pancreatic juice diverted throughout the experiment. After a basal period, DBI_{1-86} or DBI_{33-50} dissolved in 0.05M sodium bicarbonate was infused intraduodenally over 15 minutes. To evaluate whether the CCK antagonist MK-329 can block the effect of DBI, MK-329 was dissolved in 1:1 dimethylsulfoxide (DMSO): polysorbate 80 and diluted with saline to a final concentration of 1%. Values are means±SW of seven experiments. *p<0.05. (From Herzig K.H., I. Schön, K. Tatemoto, Y. Ohe, Y. Li, U.R. Fölsch, C. Owyang. Proc Natl Acad Sci (USA) 93: 7927, 1996).

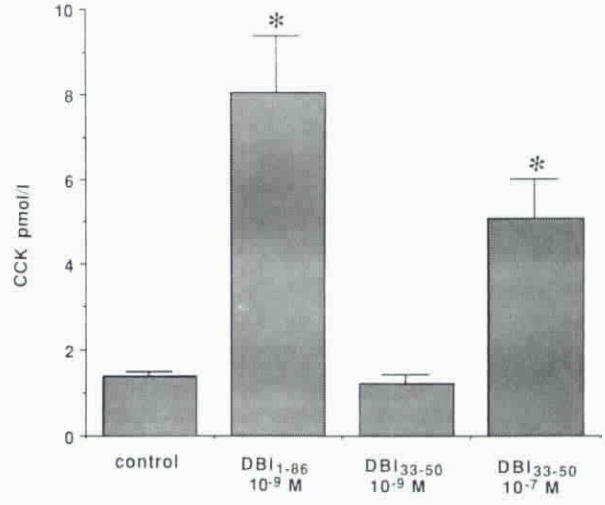

Fig. 3. Comparison of DBI_{1-86} and DBI_{33-50} on CCK release from dispersed intestinal mucosa cells. Values are means ± SE of at least four experiments. *p<0.05. (From Herzig K.H., I Schön, K. Tatemoto, Y. Ohe, Y. Li, U.R. Fölsch, C. Owyang. Proc Natl Acad Sci (USA) 93: 7927, 1996).

Porcine and rat DBI markedly decreases the late phase of glucose-induced insulin release from perfused rat pancreas (50, 51) independently of known BZD binding sites. In addition to the above described actions, our recent studies indicate that DBI is capable of releasing CCK both *in vivo* and *in vitro* and these data are presented in the following section.

Demonstration That DBI_{1-86} and DBI_{33-50} (Octodecaneuropeptide) Release CCK In Vivo and In Vitro

Porcine DBI was synthesized using an automatic peptide synthesizer on P-alkoxybenzyl alcohol resin with a FMOC protection strategy (24). Using our in vivo anesthetized rat model, intraduodenal perfusion of synthetic porcine DBI_{1-86} (200 ng) over 15 min stimulated amylase output by 222±16% (Fig. 2), which was abolished by the CCK antagonist MK-329 (1 mg/kg) (Fig. 2) suggesting that DBI-stimulated amylase release is mediated by CCK. Furthermore we showed that stimulation of amylase secretion by DBI was accompanied by an increase in plasma CCK levels from 0.8±0.1 pM to 9±2.2 pM. Similarly, we also showed that intraduodenal perfusion of DBI_{33-50} (ODN) (50 µg) stimulated amylase secretion (Fig. 2) and CCK release. Pretreatment of DBI_{1-86} or DBI_{33-50} with trypsin but not lipase completely abolished the stimulating properties of these peptides. In separate studies DBI_{1-86} and its fragments had no effects on amylase secretion from isolated acini.

We performed *in vitro* studies to demonstrate that DBI stimulates CCK secretion (24) using a perfusion system of dispersed rat intestinal mucosa cells as described by Bouras et al (52). KC1, the calcium ionophore, A23187 and GRP stimulated CCK release. In this system, DBI_{1-86} (10^{-9}–10^{-13}M) dose-dependently stimulated CCK release with a maximal effect at 10^{-9}M (Fig. 3). Similarly DBI_{33-50} (10^{-7}M) also released CCK although it was less potent than DBI_{1-86}. The release of CCK was unaffected by tetrodotoxin indicating that DBI directly stimulates CCK release without neural involvement.

In separate studies, we examined the effects of DBI_{33-50} on CCK released from STC-1 cells (53). The STC-1 cell line, kindly provided by Dr. Douglas Hanahan (UCSF), was derived from an intestinal endocrine tumor in mice that carried transgenes for the rat insulin promoter linked to the simian virus 40 large T antigen and the polyoma virus small T antigen (54, 55). Similar to the results obtained from the perfusion system of dispersed rat intestinal mucosa cells, we showed that DBI_{33-50} (10^{-8}–10^{-5}M) stimulated CCK release from STC-1 cells in a dose-dependent manner. At 10^{-7}, DBI_{33-50} increased CCK release from 0.9 pM to 5.9±0.5 pM. We also showed that DBI_{33-50} (10^{-8}–10^{-6}M) elicited Ca^{2+} oscillations in STC-1 cells in a dose-dependent manner (Fig. 4) (53). This action was abolished by Ca^{2+} free medium.

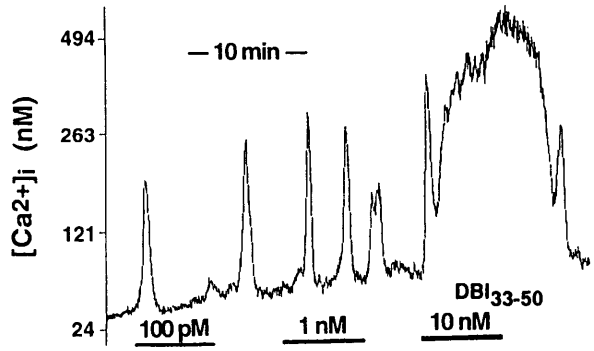

Fig. 4. Ca^{2+} oscillations evoked by DBI_{33-50} in fura 2-loaded individual STC-1 cells. DBI_{33-50} elicited Ca^{2+} oscillations at concentrations ranging from 0.1 to 1,000 nM. Data are representative of 54 determinations. $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration. (From Yoshida H., Y. Tsunoda, C. Owyang. Am J. Physiol 276: G694, 1999).

L-type Ca^{2+} channel blockers nifedipine and diltiazem (3-10 μM) markedly attenuated DBI-stimulated Ca^{2+} oscillations. In other cell types L-type Ca^{2+} channels are activated by cAMP-protein kinase A. DBI_{33-50} failed to stimulate cAMP formation in STC-1 cells. Similarly, DBI_{33-50} had no effect on *myo*-inositol 1, 4, 5-trisphosphate concentration ($[IP_3]$). In addition, inhibitors of phospholipase C (U-73122), phospholipase A_2 (ONO-RS-082), and protein tyrosine kinase (genistein) did not alter the Ca^{2+} oscillations elicited by DBI_{33-50}. It appears that DBI_{33-50} acts directly on STC-1 cells to elicit Ca^{2+} oscillations via the voltage-dependent L-type Ca^{2+} channels, resulting in the secretion of CCK. Mediation of this action is by intracellular mechanisms independent of the traditional signal transduction pathways, including phospholipase C, phospholipase A_2, protein tyrosine kinase, and cAMP systems.

Evidence that Diazepam Binding Inhibitor is a Physiological Regulator of CCK Secretion

At least three peptides release CCK when given intraduodenally. These include monitor peptide in pancreatic juice (54), luminal CCK-releasing factor (LCRF) from intestinal secretions (55), and DBI from porcine and rat intestinal mucosa (24). We hypothesized that DBI fulfills the criteria to be the CCK-releasing peptide responsible for feedback regulation of pancreatic secretion and postprandial CCK release. To test this hypothesis, we need to demonstrate that DBI is localized to the duodenal mucosa, that is secreted intraduodenally in parallel with CCK secretion into the circulation, and intraduodenal administration of DBI antisera that will abolish CCK release stimulated by protein and lipid.

We performed immunohisto-chemical staining studies and showed that DBI is abundant in duodenal villus enterocytes distinct from CCK-containing crypt cells (56). Using an anesthestized rat model equipped with bile-pancreatic and intestinal cannula, we demonstrated that bile pancreatic juice diversion (BPJ) for 2 hr caused a 2-fold increase in pancreatic protein output and an increase in plasma CCK from 0.7±0.4 to 9.2±1.8 pM, accompanied by a 2-2.5 fold increase in luminal CCK-RP (56). At 5 hr after BPJ diversion, pancreatic secretion, plasma CCK and luminal CCK-RP returned to basal levels. Intraduodenal DBI antisera administration (titer 1:40,000), but not preimmune rabbit serum, abolished pancreatic secretion and increased CCK levels following BPJ diversion (56). To demonstrate that DBI causes the postprandial rise in plasma CCK, we showed that, at 5 hr following BPJ diversion, intraduodenal 5% peptone or 10% oleic acid caused 62±10 and 78±6% increases in pancreatic secretion, respectively, and net increases of 6±1 and 7.4±0.8 pM of plasma CCK over basal, respectively, accompanied by a 2-fold increase in luminal DBI immunoreactivity. Intraduodenal anti-DBI antiserum (titer 1:40,000) abolished increases in pancreatic secretion and plasma CCK in response to peptone and oleic acid. Intraduodenal bile infusion inhibited the stimulatory effects of peptone and oleic acid. Therefore our studies have conclusively showed that trypsin-sensitive DBI stimulates CCK release and pancreatic secretion. It is present in the duodenal mucosa and is released into the proximal intestinal lumen in sufficient quantities to account for the effects of physiological stimuli. Immunoneutralization studies indicate that DBI antisera abolishes CCK release stimulated by peptone and oleic acid. Thus DBI meets all criteria to be the CCK-RP responsible for mediating feedback regulation of pancreatic secretion and postprandial CCK release.

Enteric Neural Circuitry Responsible for Luminal Secretion of DBI

We next investigated the mechanisms by which peptone in the testine stimulates the secretion of the DBI which mediates CCK secretion, and examined the enteric neural circuitry responsible for secretion (57). We used a "donor-recipient" rat intestinal perfusion model to quantify the DBI secreted in response to nutrient stimulation. Infusion of concentrated intestinal perfusate collected from donor rat perfused with 5% peptone caused a 62±10% increase in protein secretion and an elevation of plasma CCK levels to 6.9±1.8 pM in the recipient rat. The stimulatory effect of the intestinal washings was abolished when the donor rats were pretreated with

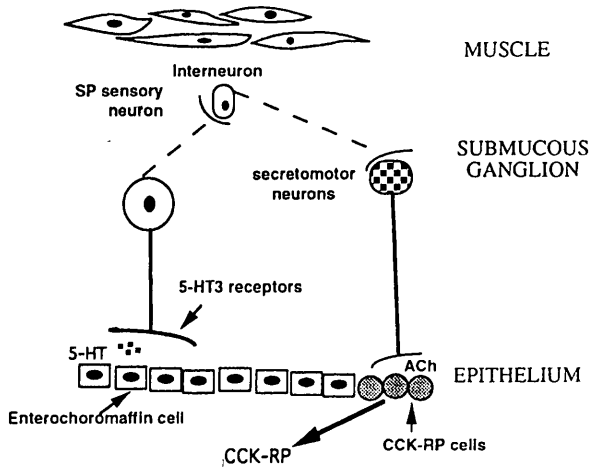

Fig. 5. Proposed enteric circuitry controlling CCK-RP secretion. Peptone in the lumen stimulates the release of 5HT from enterochromaffin cells which activates 5HT receptors on sensory substance P. neurons in the submucous plexus. Signals are then transmitted to cholinergic interneurons and to epithelial CCK-RP containing cells via cholinergic secretomotor neurons. (From Li Y., C. Owyang. J. Clin Invest 97: 1463, 1996).

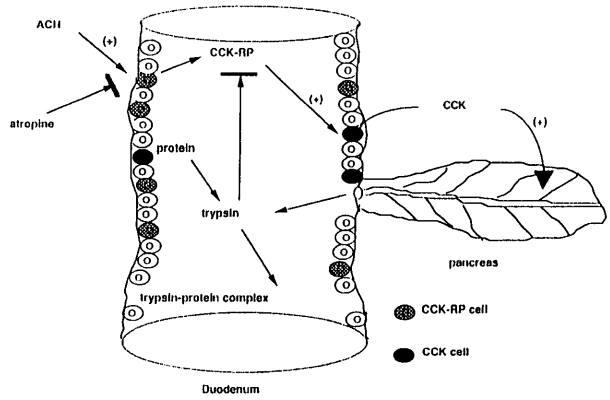

Fig. 6. A schematic representation of the postulated mechanism by which CCK-RP stimulates the secretion of CCK postprandially. CCK-releasing peptide (CCK-RP) is being secreted into the proximal small intestine and inactivated by trypsin. Postprandially, when food enters the duodenum, protein binds to trypsin and prevents CCK-RP from being inactivated. CCK-RP stimulates CCK cells in the duodenum to release CCK into the bloodstream. CCK in turn stimulates pancreatic enzyme secretion. (From Herzig K.H., I. Schön, K. Tatemoto, Y. Ohe, Y. Li, U.R. Fölsch, C. Owyang. Proc Natl Acad Sci (USA) 93: 7927, 1996).

atropine or hexamethonium but not with guanethidine or vagotomy. Mucosal application of lidocaine but not serosal application of benzalkonium chloride which ablates the myenteric neurons in the donor rats also abolished the stimulatory action of the intestinal washings. Furthermore, treatment of the donor rats with a $5HT_3$ antagonist and a substance P antagonist also prevented the secretion of DBI. These observations suggest that peptone in the duodenum stimulates serotonin release which activates the sensory substance P neurons in the submucous plexus. Signals are then transmitted to cholinergic interneurons and to epithelial DBI containing cells via cholinergic secretomotor neurons (Fig. 5). This enteric neural circuitry which is responsible for the secretion of DBI may in turn play an important role in the postprandial release of CCK (57).

Conclusion

Until recently, postprandial CCK release was believed to be mediated by the direct action of nutrients on CCK-containing cells in the intestine. The discovery of DBI as a CCK-releasing peptide provides new insight into the physiologic regulation of CCK secretion. The characterization of this trypsin-sensitive, luminally secreted enteric peptide functioning as an intraluminal regulator of CCK release also provides a mechanism to explain feedback regulation of pancreatic secretion by trypsin (Fig. 6).

The existence of a feedback regulatory pathway in humans may have important clinical implication. It is conceivable that in patients with chronic pancreatitis, decreased pancreatic enzyme secretion may result in elevated plasma CCK levels, reflecting a failure in the feedback modulation of CCK release. This may cause hyperstimulation of the pancreas and produce pain. Effective enzyme replacement therapy may reduce pancreatic stimulation, decrease intraductal pressure, and diminish pain. Large doses of pancreatic extract have relieved pain in some patients with chronic pancreatitis.

Acknowledgements

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