Comparison of Hepatic Elimination of Different Forms of Cholecystokinin in Dogs

Bioassay and Radioimmunoassay Comparisons of Cholecystokinin-8-Sulfate and -33-Sulfate

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Abstract

The influence of hepatic transit on the ability of exogenous cholecystokinin-8-sulfate and -33-sulfate (CCK-8 and CCK-33, respectively) to stimulate gallbladder contraction and exocrine pancreatic secretion, as well as on the peripheral plasma concentration of each agent, was evaluated in five conscious dogs with pancreatic and gallbladder fistulas and complete portacaval transpositions. The gallbladder pressure increments after portal administration of CCK-8 (0.125, 0.25, 0.50, and 1.0 μg/kg per h for 5 min) were diminished by 36, 45, 39, and 25%, respectively, in comparison with those obtained with systemic administration of identical doses of CCK-8 ($P < 0.05$). In a subsequent experiment, the integrated pancreatic juice volume, bicarbonate, and protein secretion were diminished by 22, 32, and 48%, respectively, during a 30-min infusion of CCK-8 (0.10 μg/kg per h) into the portal venous system, in comparison with the results obtained with systemic administration of CCK-8 ($P < 0.05$). In contrast, the gallbladder pressure and pancreatic exocrine secretory responses to portal administration of CCK-33 did not differ significantly ($P > 0.05$) from the results obtained with systemic administration of CCK-33. Radioimmunoassay for CCK-8 in plasma showed that the integrated CCK-8 value during portal administration was significantly lower ($P < 0.05$) than it was during systemic administration. The results for CCK-33, however, did not vary, whether it was given by a systemic or portal route ($P > 0.05$). Thus, the present study demonstrates that CCK-8 is partially inactivated by the liver whereas CCK-33 is not, which suggests that CCK-33 in the circulation may play a significant role in the physiologic regulation of the gallbladder and exocrine pancreas.

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Introduction

Cholecystokinin (CCK)[1] was first isolated from porcine duodenal mucosa and was characterized as a peptide of 33 amino acid residues (1, 2). Immunochemical studies have disclosed five different molecular forms of CCK in the intestinal mucosa, although there may be more (3–6). The biologic potency of these CCK variants, however, is different in the stimulation of gallbladder contraction (7, 8) and pancreatic exocrine secretion (9, 10). The synthesis, release, and catabolism of the individual forms of CCK and their roles as hormones are still unclear.

After CCK is released from the intestine, it must circulate through the liver before it reaches its target organs. The role of the liver in the removal of enteric peptides (such as gastrin fragments smaller than gastrin-9) has been documented (11). Whether the large and small molecular forms of CCK are catabolized in the same manner, however, is unclear. The purpose of this study, therefore, was to evaluate the hepatic removal of CCK-8-sulfate or CCK-33-sulfate (CCK-8 and CCK-33, respectively) from the circulation by means of bioassays and radioimmunoassays (RIAs) for the two different forms of CCK.

Methods

A complete transposition of the portal vein and inferior vena cava was done by the method by Starzl and colleagues (12) on five mongrel dogs, 18–22 kg. During the same operation, a modified Herrera pancreatic fistula (13) and a gastric fistula were prepared. A polyethylene catheter (1.19 mm i.d., 1.70 mm o.d.), with a single side hole (1 mm diam, 1 cm proximal to an occluded end) was placed in the gallbladder through a fundic cystostomy. The patent end of this catheter was brought out from the abdominal cavity through a Thomas-type cannula and then closed and protected with a metal cap. The cystic duct was not ligated (Fig. 1).

The dogs were allowed to recover for 1 mo before the studies were carried out. The portal injection system was operating optimally, since pentagastrin was quantitatively (85%) removed by a single hepatic transit (unpublished findings from our laboratory). CCK-8 (a gift of E. R. Squibb & Sons, Princeton, NJ) or 99% pure porcine CCK-33 (Professor V. Mutt, Karolinska Institute, Stockholm) was infused in 0.15 M NaCl at a constant rate by a pump (Harvard Apparatus Co., Inc., S. Natick, MA). All plastic syringes and tubing for peptide infusions were prewashed with 2% human serum albumin (Buminate 25%; Travenol Laboratories, Inc., Baxter Travenol Laboratories, Glendale, CA).

Experimental design

Experiment 1. Gallbladder pressure study. Dogs were fasted for 18 h but were allowed water ad lib. Gastric and pancreatic fistulas were

1. *Abbreviations used in this paper:* CCK, cholecystokinin; CCK-8 and CCK-33, cholecystokinin-8-sulfate and -33-sulfate, respectively; CU, clinical unit; ICG, indocyanine green; PCT, portacaval-transposed.

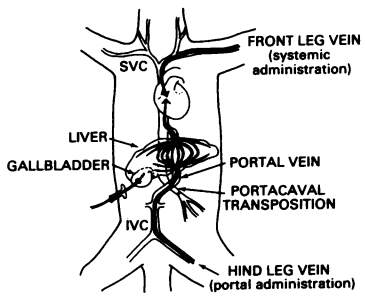

Figure 1. Diagram of operative preparation in dogs. Not shown are the modified Herrera pancreatic fistula (13) and the gastric fistula. IVC and SVC, superior and inferior vena cava, respectively.

open during the studies in order to prevent release of endogenous secretin. A pressure transducer (CD9; Hewlett Packard Co., Palo Alto, CA) was connected to the gallbladder catheter by a saline-filled polyethylene tube; the transducer was secured to the body of the dog with a jacket. The gallbladder was perfused constantly with saline at the rate of 1.0 ml/min by an infusion pump (model 933; Harvard Apparatus Co., Inc.), and gallbladder pressure was recorded by a polygraph (system 7758B; Hewlett-Packard Co.). The rate of increase in pressure of this perfused catheter system after occlusion was 25 cm H_2O/s. Experiments were begun after the gallbladder pressure stabilized at a base line.

Each infusion of CCK-8 or CCK-33 lasted for 5 min, with an interval of at least 15 min between doses. CCK-8 (0.125, 0.25, 0.5, and 1.0 μg/kg per h) or CCK-33 (0.125, 0.25, 0.5, 1.0, and 2.0 μg/kg per h) was given randomly, with the infusion alternating between the systemic venous system (via a frontleg vein) and the portal venous system (via a hindleg vein). The increases in gallbladder pressure, time of onset of gallbladder contractions, and duration of contractions were recorded.

Experiment 2. Exocrine pancreas secretion study. The gastric cannula was open during this study. After an 18-h fast, secretin (0.35 clinical units (CU)/kg per h; Secretin-Kabi; Kabi Group, Inc., Greenwich, CT) was infused via a frontleg vein during all experiments in order to maintain constant pancreatic secretion. After 60 min of secretin infusion, CCK-8 (0.10 μg/kg per h [87 pmol/kg per h]) was infused into a hindleg vein (portal administration) for 30 min. After a 45-min recovery period, CCK-8 (0.10 μg/kg per h) was infused into a frontleg vein (systemic administration) for 30 min.

On another day, an equimolar dose of CCK-33 (0.35 μg/kg per h [89 pmol/kg per h]) was infused in the same manner. Pancreatic juice volume, bicarbonate, and protein secretion were determined by collection of pancreatic juice samples every 15 min. Protein concentrations were measured by the Lowry method (14), with bovine serum albumin used as a standard. Plasma samples were also obtained every 15 min for RIA of CCK-33 and CCK-8 concentrations.

RIAs

Plasma samples were collected every 15 min in chilled glass tubes containing 15 U of heparin (Liquaemin; Organon Diagnostics, West Orange, NJ) and 100 kIU of aprotinin (Novo; Alle-Bagsvaerd, Denmark) per milliliter of blood. Plasma was stored at −20°C until subsequent RIA for CCK-33 or CCK-8. The CCK-33 RIA has been described in detail previously (15, 16). The CCK-33 antiserum does not cross-react with CCK-8 or gastrin.

CCK-8 was measured by a double-antibody procedure. The CCK-8 antibody was generated in New Zealand white rabbits against synthetic CCK-8, which was coupled to bovine serum albumin by the carbodiimide procedure (17). The ID_{50}'s of the CCK-8 antisera for CCK-8, CCK-33, CCK-4-sulfate and gastrin are 0.032, 0.048, 50.0 and 0.105 ng/tube, respectively. In order to measure plasma levels of CCK-8, 2 ml of plasma was applied to a Sep Pak cartridge (C-18; Waters Associates, Millipore Corp., Milford, MA) and eluted by use of 4 ml of 50:50, water/acetronitrile. Samples were then dried under nitrogen gas and reconstituted in assay buffer. This procedure extracts almost all CCK-8 from plasma (90%). Gastrin is poorly (25%) extracted with this procedure.

Assessment of hepatic function

Hepatic function was assessed by the disappearance rate of indocyanine green (ICG) (18, 19). In fasted dogs, ICG (0.5 mg/kg) was given intravenously into the frontleg vein (systemic administration) after a plasma sample was collected. Samples were then collected from a peripheral vein (2, 5, 10, 15, and 20 min after injection of ICG) from an indwelling catheter. Plasma concentrations of ICG were determined spectrophotometrically (805 nm), with the basal plasma sample as the blank. ICG concentrations were graphed against time on semilogarithmic paper, and the ICG disappearance rate constants (K-ICG) were calculated from the half-time ($t_{½}$) according to the formula K-ICG = $(0.693/t_{½})$.

Statistics

Results are expressed as the mean±1 SEM. The *t* test for paired samples was used to analyze data for statistical significance of differences between means. The data depicted in Fig. 2 were analyzed as a three-factor factorial arrangement, with factors defined as treatment (portal and systemic), dose, and dog, which is a random factor. Analyses were done separately for CCK-8 and CCK-33 for each of the three variables (response, onset, and duration). In some cases, the Duncan's multiple range test was used to analyze the data statistically. Differences with a $P < 0.05$ were considered to be significant.

Results

Experiment 1. Gallbladder pressure study. Systemic infusion of graded doses of CCK-33 or CCK-8 resulted in a dose-dependent elevation in gallbladder pressure and in duration of gallbladder contractions (Fig. 2). In addition, infusion of

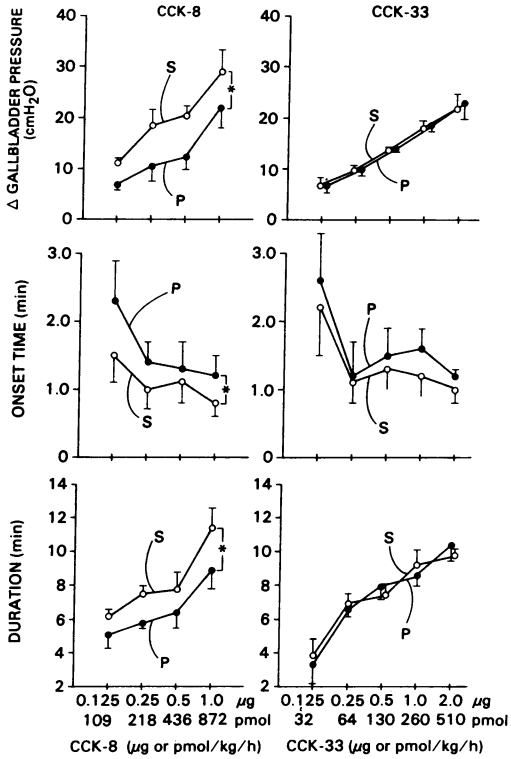

Figure 2. Comparison of gallbladder contraction in five dogs in response to systemic (S) or portal (P) administration of CCK-8 or CCK-33. *$P < 0.05$ in comparison with systemic administration of CCK.

graded doses of CCK-8 or CCK-33 resulted in a dose-dependent decrease in time of onset of gallbladder contraction. Increases in gallbladder pressure and in duration of gallbladder contractions were significantly lower, dose for dose, after portal administration of CCK-8 ($P < 0.05$) than after systemic administration (Fig. 2). The onset time of gallbladder contraction was also significantly longer after portal administration than after systemic administration of CCK-8. The diminutions of increments in gallbladder pressure in response to portal administration of CCK-8 at 0.125, 0.25, 0.5, and 1.0 μg/kg per h were 36, 45, 39, and 25%, respectively, of those achieved with systemic administration ($P < 0.05$).

In contrast, no significant differences were found between portal and systemic administration of CCK-33, in either peak increments of gallbladder pressure, gallbladder contraction onset time, or duration of gallbladder contractions. At the doses tested, on a molar basis, the biologic potencies (gallbladder pressure) of CCK-8 and CCK-33 were identical (Fig. 3).

Experiment 2. Pancreatic secretion study. Pancreatic juice volume, bicarbonate, and protein output attained a steady secretory state within 45 min after the onset of secretin infusion (Fig. 4). Simultaneous administration of CCK-8 or CCK-33 with secretin resulted in prompt elevations in pancreatic juice volume, bicarbonate, and protein output, which remained constant during the 30-min infusion of CCK. After the CCK infusion, pancreatic secretion returned to the steady secretory state within 30 min.

Portal administration of CCK-8 resulted in significantly ($P < 0.05$) lower outputs of pancreatic juice volume, bicarbonate, and protein in comparison with those found with systemic administration (Fig. 4). The integrated responses of pancreatic juice volume, bicarbonate, and protein secretion during portal administration of CCK-8 (1 μg/kg per h) were 78±6, 68±6, and 52±5%, respectively, of those found during systemic administration ($P < 0.05$) (Fig. 5). The protein concentration of pancreatic juice during portal administration of CCK-8 was also significantly lower than that found during systemic administration. The bicarbonate concentration of pancreatic juice during portal administration of CCK-8 was not significantly different from that found during systemic administration (Fig. 4).

No significant differences in the effects of portal and systemic administration of CCK-33 on responses of pancreatic juice volume, bicarbonate, and protein output were found (Fig. 6). Portal and systemic administration of CCK-33 (0.35 μg/kg per h [89 pmol/kg per h]) produced similar integrated responses of pancreatic juice volume, bicarbonate, and protein output (Fig. 5); furthermore, these responses were not different from those achieved with an equimolar dose of CCK-8 given systemically (0.10 μg/kg per h [87 pmol/kg per h]).

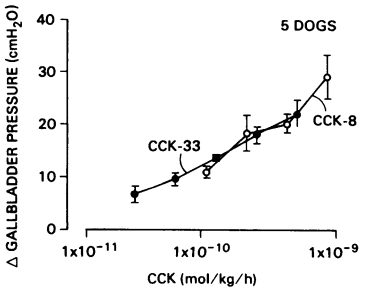

Figure 3. Comparison of systemic administration of CCK-8 and CCK-33 on gallbladder contraction.

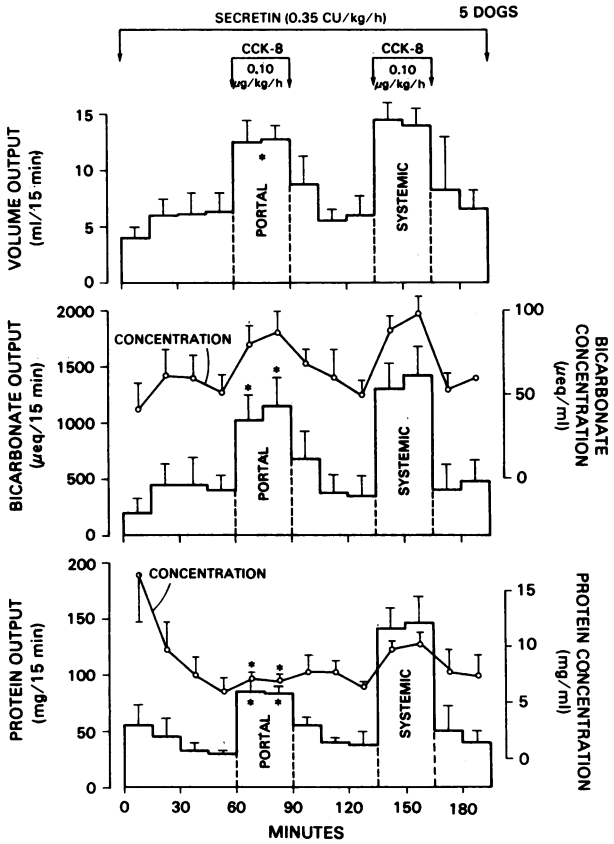

Figure 4. Pancreatic juice volume, bicarbonate, and protein output in response to either portal or systemic administration of CCK-8. *$P < 0.05$ in comparison to systemic administration of CCK-8 at a corresponding time.

Portal and systemic infusion of CCK-8 resulted in significant increases in plasma concentrations of CCK-8 in the peripheral circulation (Fig. 7). When systemic infusion was compared with portal administration, however, the elevation of CCK-8 in the peripheral plasma was significantly higher for the systemic route. The integrated concentration of plasma CCK-8 during portal infusion was significantly lower (113±13 pg·min/ml) than it was during systemic administration (213±30 pg·min/ml) ($P < 0.05$). In contrast to the results with CCK-8, peripheral levels of CCK-33 during portal infusion did not differ significantly from results obtained during systemic administration. Similarly, the integrated CCK-33 levels during portal infusion (2,018±138 pg·min/ml) did not differ signifi-

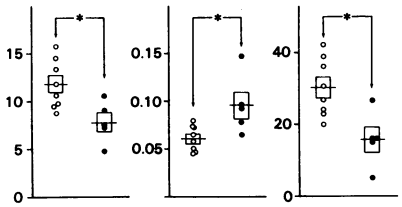

Figure 5. Comparison of pancreatic juice volume, protein, and bicarbonate output in response to systemic or portal administration of CCK-8 or CCK-33. *$P < 0.05$ in comparison with systemic administration of CCK. P/S, ratio of results from portal (P) or systemic (S) administration.

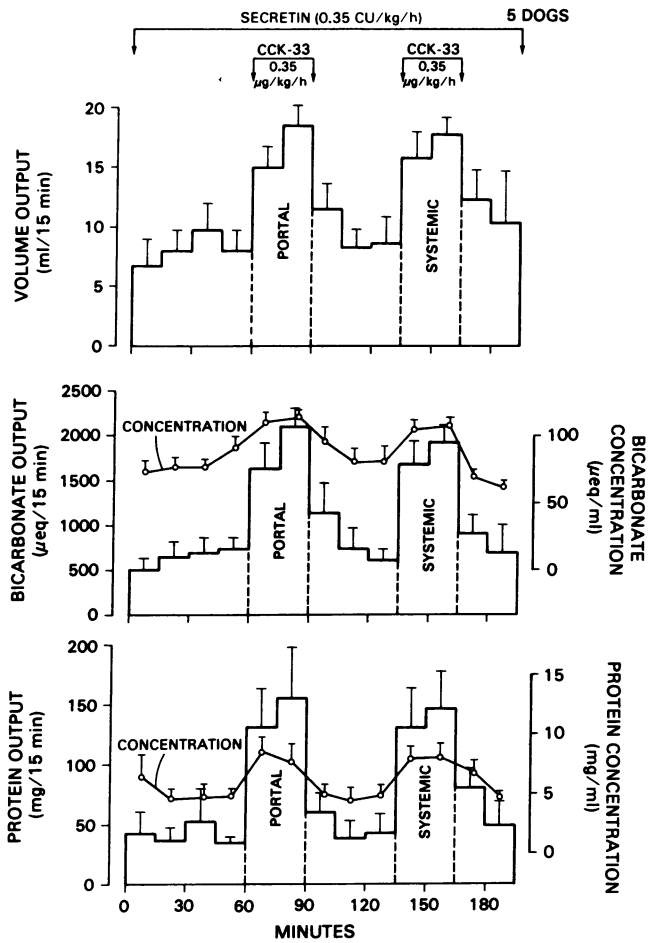

Figure 6. Pancreatic juice volume, bicarbonate, and protein output in response to either portal or systemic administration of CCK-33.

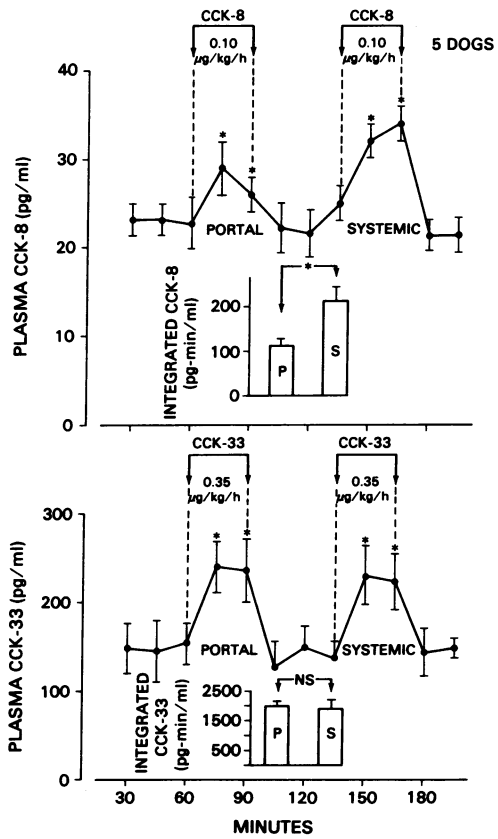

Figure 7. Plasma CCK-8 and CCK-33 levels during portal or systemic infusion of CCK-8 or CCK-33. Integrated CCK-8 and CCK-33 levels are also shown. $^{*}P < 0.05$ in comparison with immediately preceding levels. $^{**}P < 0.05$ in comparison with systemic administration.

cantly in comparison to those found during systemic administration (1,904±311 pg·min/ml) (Fig. 7).

Hepatic function test

Hepatic function tests were performed ~3 mo after portacaval transposition. The dogs did not demonstrate any clinical signs of hepatic dysfunction. The disappearance half-time of ICG was less in the portacaval-transposed (PCT) dogs than in normal control dogs (Fig. 8); the disappearance rate constant of the dye for PCT dogs was significantly higher than that for normal control dogs (Fig. 8), and the percentage plasma ICG retention at 20 min in PCT dogs was significantly lower in comparison with controls.

Discussion

The present study has demonstrated that circulating CCK-8 is partially removed during liver transit, whereas CCK-33 is not affected. These findings suggest that circulating CCK-33 has a major role in the physiologic regulation of the gallbladder and exocrine pancreas, and they extend and confirm earlier reports. Way and colleagues (20), using CCK-33, and Bridgewater and colleagues (21), using an undefined molecular form of CCK, reported that in dogs the pancreatic exocrine response to CCK was not altered by hepatic transit. Two recent studies from Walsh's group, reported in abstract, provide support for our findings. Boettcher and colleagues (22) found that CCK-8, in comparison with CCK-39-sulfate, was preferentially eliminated by hepatic transit, and Eysselein and colleagues (23) reported that the large variants of CCK predominate over small ones in the peripheral plasma, in contrast with what is found in portal plasma. They concluded that small forms of CCK are eliminated preferentially during hepatic transit.

In the present study, the RIA results corroborate those from the biologic assay, which indicate that plasma CCK-8, in comparison with CCK-33, is removed selectively by the liver. Our RIA data for CCK-8 show that the integrated CCK-8 concentrations, during portal infusion of CCK-8, are signifi-

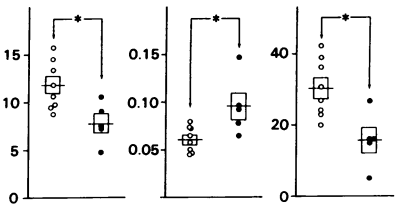

Figure 8. ICG clearance in PCT (○) and normal control (●) dogs. (*Left*) $t_{1/2}$ (*middle*) K-ICG, ICG disappearance rate constant (1/minutes); (*right*) R-ICG, plasma ICG retention (percent at 20 min). $^{*}P < 0.05$, significant difference between PCT and control dogs.

cantly lower than when CCK-8 is given systemically (56% of the systemic value). Similarly, pancreatic protein secretion during portal administration of CCK-8 is 52% of that during systemic administration.

Although we have not specifically measured plasma concentrations of CCK-8 after consumption of food, it is conceivable that the amount of endogenously released CCK-8 is similar to or less than the amount of CCK-8 used in this study (22, 23). The gallbladder pressure study indicates that the rate of hepatic inactivation may depend on the amount of CCK-8 released into the portal system. Therefore, it seems likely that, under normal conditions, >50% of endogenously released CCK-8 is inactivated by a single transit through the liver. In the pancreatic secretory studies, on the other hand, plasma concentrations of CCK-33 were elevated after either portal or systemic administration of CCK-33; more important, these CCK-33 levels are similar to those observed after administration of intraduodenal oleate (24), which suggests that the quantity of exogenous CCK-33 administered in this study is within the physiologic dose.

Multiple molecular forms of CCK have been demonstrated in the mammalian gut and plasma (16, 23, 25–31). Fried and colleagues (16, 24) and Wiener and colleagues (32) have demonstrated, in both dogs and humans, a significant elevation in plasma concentrations of CCK-33 after ingestion of fat. Plasma CCK-33 levels were highly correlated with gallbladder contraction and pancreatic protein secretion. Maton and colleagues (26) demonstrated an equimolar elevation in plasma CCK-8 and CCK-33 concentrations after a fat meal in humans. Chang and Chey (27) showed that ~50% of human plasma CCK detected by RIA after a meat meal is CCK-33. Inoue and colleagues (33) reported that intravenous administration of CCK-33-specific antiserum completely abolished pancreatic protein output in response to bombesin, a stimulant of CCK release in dogs. These findings, taken together, suggest that endogenously released CCK-33 plays a physiologic role in the regulation of the exocrine pancreas and gallbladder contraction.

In contrast, Calam (28), Walsh (25), and Byrnes (29, 30), and their colleagues have contended that CCK-8 is the major form of CCK found in humans after a meal. Rehfeld and colleagues (31) have also reported that CCK-8 is the predominant form released in response to an acidified fat emulsion in perfusion of an isolated preparation of porcine duodenum. Calam, Walsh, and Rehfeld, and their colleagues have suggested that CCK-33 is merely a biosynthetic precursor of smaller variants of CCK and not a major plasma form. It seems, therefore, that considerable controversy remains as to which form of CCK predominates in the circulation.

Note that, at the doses tested, the potency of CCK-33 on exocrine pancreas secretion and on gallbladder contraction was similar (but not identical) on a molar basis to that of CCK-8. In earlier studies, CCK-8 has been reported to be more potent than CCK-33 (7, 8). Recently, however, Lamers and associates (34) demonstrated by bioassay and RIA that CCK-33 and CCK-8 were equipotent on a molar basis. These findings, along with our data, support the concept that CCK-33 plays a major role in the regulation of gallbladder contraction and pancreatic secretion.

Although the present studies do not show desensitization of the gallbladder response to large doses of CCK, previous studies have demonstrated restricted responses of dispersed pancreatic acini and gallbladder smooth muscle to supramaximal doses of CCK (35–37). The disparity between these earlier studies and our data may be due to methodologic differences or to the fact that our study was done in vivo. In addition, our study did not test supramaximal doses of CCK. More important, each dose of CCK was separated by a 15–30 min rest, which might allow the target tissue to respond appropriately without tachyphylaxis.

In dogs prepared with a portacaval transposition, ICG clearance was significantly higher in comparison with that of controls, which indicates that portacaval transposition did not impair hepatic function. In this model, the hepatic uptake might be reduced in comparison with what is achieved with direct infusion into the portal vein, since it is possible that some of the infusate traveling from the hindleg vein might escape to the systemic circulation through collateral pelvic vessels (38). In three dogs with portacaval transposition, we found that the gastric fistula acid output in response to pentagastrin (5 μg/kg per h) given via the hindleg was only 15% of the amount of acid secreted when the pentagastrin was given via the foreleg (unreported findings from our laboratory). This suggests a clearance of 85%, which is similar to the 90% clearance reported after direct portal injection (1, 39).

Although our data do not elucidate the mechanism of hepatic inactivation of CCK, a hepatocyte amidase has been shown to participate in the hepatic inactivation of certain blood-borne peptides such as gastrin (40), vasoactive intestinal peptide (41), insulin (42), and glucagon (43). There is no evidence, however, as to whether this hepatic inactivation is attributable to enzymatic metabolism by the hepatocyte membrane, or to simple elimination into the bile, or to intrahepatic sequestration. According to the study by Strunz and colleagues (11), the liver can inactivate C-terminal fragments of gastrin smaller than gastrin-9. As with gastrin, the smaller variant of CCK, CCK-8, was selectively inactivated by the liver, whereas the larger variant, CCK-33, was unaffected. Thus, the susceptibility of peptides to inactivation by the liver may be related not only to their molecular structure, which could prevent deamination in some cases, but also to the length of the peptide chain.

In conclusion, we have demonstrated the selective hepatic inactivation of CCK-8. Since a larger form of CCK, CCK-33, is not inactivated, our findings suggest that endogenous CCK-33 is not exclusively a biosynthetic precursor of smaller forms of CCK; this is supporting evidence that CCK-33 has its own physiologic role as a circulating hormone.

Acknowledgments

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