Pharmacological Activities of Synthetic Human Cholecystokinin-33 of Which Tyrosine Was Sulfated by Arylsulfotransferase

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The pharmacological activities of synthetic human CCK-33, in which a tyrosine molecule was sulfated by arylsulfotransferase, were investigated in the rat and the guinea-pig. The activities were compared with those of non-sulfated CCK-33 (CCK-33NS), CCK-8 and CCK-4. CCK-33 was about 100 fold more potent than non-sulfated CCK-33(CCK-33NS) but was about 20 fold less potent than CCK-8 in the contraction of the isolated gallbladder of the guinea-pig. In rat pancreatic secretion, intravenous CCK-33 and CCK-8 showed almost the same activity. The potency of each was about 1000 fold more than the individual potency of CCK-33NS, non-sulfated CCK-8 (CCK-8NS) and CCK4. There were no significant differences in gastric acid stimulatory activities among CCK-33, CCK-8, CCK-4, but the activities of CCK-33NS and CCK-8NS were less than those of CCK-33 and CCK-8, respectively. CCK-33 and CCK-8 produced a reduction in the intake of powder chow in doses of 10^{-8} and 3×10^{-8} mol/kg i.p., but CCK-33NS, CCK-8NS and CCK-4 did not. In conclusion, the activities of synthetic human CCK-33 are almost the same as those of CCK-8 on exocrine pancreatic secretion, gastric acid secretion and food intake, but less than CCK-8 on isolated gallbladder contraction.

Keywords CCK-33; CCK-8; non-sulfated CCK; arylsulfotransferase; gallbladder; pancreatic secretion; gastric secretion; food intake

Introduction

Cholecystokinin or C-terminal CCK-8 is used as a stimulant of exocrine and endocrine pancreatic secretion and gallbladder contraction in man.[1] Several investigators have utilized CCK-8 clinically in man as an adjunct to oral cholecystography.[2] It is well established that CCK exists in multiple molecular forms (CCK-33, -39 and -58) in the body. Recently, CCK-58 was isolated from the canine intestine, and the potency in stimulation of pancreatic secretion was compared with that of CCK-8.[3] In addition, synthetic human CCK-33 was evaluated with respect to the biological activity on pancreatic secretion and its potency was compared to that of CCK-8.[4] However, the other biological activities remain undetermined.

Although the sulfation of the tyrosyl residue in the CCK is important for producing biological activities,[5-8] the transfer of the sulfate group to the tyrosine residue is difficult. Arylsulfotransferase (AST) from *Eubacterium* A-44, which is a predominant bacterium of the human intestine, has been reported to catalyze the transfer of the sulfate group from phenolic ester to other phenolic compounds with strict specificity.[9-11] In subsequent studies it was found that the tyrosine of human CCK-33[12] as well as CCK-8[13] was specifically sulfated with AST with high yields.

In order to determine whether CCK-33 could be superior to CCK-8 in a pharmacological aspect, we compared the activities of synthetic human CCK-33, in which a tyrosine molecule was sulfated by AST with those of non-sulfated CCK-33 (CCK-33NS), CCK-8 and CCK-4 in several biological systems: (1) isolated gallbladder contractions in guinea-pig, (2) pancreatic and gastric secretion in rats, (3) food intake in rats.

Materials and Methods

Gallbladder Contraction Male Hartley guinea-pigs, weighing 300—400 g, were killed 24 h after fasting. The gallbladder was removed and rinsed with modified a Locke-Ringer solution (low calcium) containing: NaCl 8.8, $CaCl_2$ 0.04, KCl 0.4, $MgCl_2$ 0.018, Na_2HPO_4 0.08, KH_2PO_4 0.08, glucose 0.5 and $NaHCO_3$ 0.4 (g/l). The gallbladder was cut into a strip of tissue 15—20 mm in length and about 2—3 mm wide. The preparation was mounted in a 15 ml organ bath, containing a low calcium Locke-Ringer solution, with air bubbling and maintained at 32 ± 0.5 °C. The tissue was placed initially under an arresting tension of 0.6—0.7 g and allowed to equilibrate for 30 min. The contraction was measured using a Model IM-300 isometric transducer (FUJIOTEC, Tokyo).

Pancreatic Secretion Male Wistar rats, weighing 270—320 g, 24 h after fasting, were anesthetized with urethane (1.25 g/kg i.p.). The cannula (PE-50) was inserted 3—4 mm into the common bile duct proximal to the duodenum to collect pancreatic juice with bile. Throughout each study bile and pancreatic juice were collected over 15 min periods for 3 h. Since the volume of secretion, estimated by weighting the samples, decreased with time for the first hour, peptide or vehicle was injected into the femoral vein 1 and 2 h after the start of fractionation. Protein content and amylase activity in the juice were used as an index of pancreatic secretion. The protein content was measured by the Lowry method.[14] The amylase activity was measured by modifying Bernfeld's method.[15] That is, 0.1 ml of the sample juice or maltose standards was mixed at 4 °C with 0.4 ml of 0.2 M phosphate buffer (pH 6.9) and 0.5 ml of ice cold starch substrate was added. Immediately after incubation for 10 min at 30 °C, tubes were cooled in ice water. After adding 1.0 ml coloring reagent, it was again incubated for 10 min in boiling water. After cooling to room temperature with ice water, 10 ml of deionized water was added. Amylase activity was determined by absorbance at 540 nm with maltose as a standard.

Gastric Secretion Gastric acid secretion was measured as previously described.[16] Male Wistar rats (200—250 g) were used 24 h after fasting, but allowed free access to water. Animals were anesthetized with urethane (1.25 g/kg i.p.). The trachea was exposed and cannulated. A dual polyethylene gastric cannula[17] was inserted into the gastric lumen after ligation of the pylorus and esophagus. The stomach was perfused with 10 ml saline solution (pH 7.0) through the inlet tube of the gastric cannula and the perfusate was collected from the outlet tube of the cannula. It was titrated for acid content using phenolphthalein as the indicator with 0.01 N NaOH solution. These procedures were repeated at intervals of 20 min for 3 h. The value of a second measurement was determined as the basal secretion. Then the test compound was intravenously administered. Acid stimulatory response returned to basal level 60—80 min after the compound injection, subsequently after 80 min the compound was again administered. The data indicate total acid output during the 60 min after the drug injection.

Food Intake Male Wistar rats, weighing 180—200 g, were housed individually for more than a week before experimentation. The cages provided free access to powdered chow and water in a room with a 12 h

dark/light cycle (lights on at 6:00 a.m.), at 24 °C. Twenty-four hours after fasting, but allowing free access to water, the test compound was administered intraperitoneally to rats at 4:00 p.m., and 30 min later powdered chow was given. Food intake during the first 1 h was recorded by weighting the food at the beginning and the ending of the period. Food was freely given after the measurement. The experiment was done 4 times (Tuesday and Friday) during 2 weeks with the same rats.

Compounds CCK-33NS was synthesized by a solid-phase method and purified by gel chromatography, followed by ion-exchange chromatography, and preparative reverse-phase high performance liquid chromatography (HPLC). The purity and structure of the synthetic CCK-33NS were ascertained by analytical HPLC, amino acid analysis, peptide sequence analysis, and tryptic peptide mapping. Non-sulfated CCK-8 (CCK-8NS) and Boc-CCK-4 (CCK-4) were synthesized by the fragment condensation procedure,[8] and purified by reprecipitation. The purity and structure were ascertained by thin layer chromatography (TLC) and amino acid analysis. CCK-33NS and CCK-8NS were enzymatically sulfated by AST.[13] The purity of all compounds were more than 99%. CCK-33 was dissolved in a 0.1 M CH_3COOH solution containing 1% bovine serum albumin (BSA) and diluted with water at the time of use. CCK-8 and CCK-4 were dissolved in 0.1 M glycine buffer (pH 8.6) and diluted with water at when used.

Statistics Results are expressed as means ± S.E. The significance of results was compared using the Student's unpaired *t*-test. To compare the activities of drugs relative potency they were estimated from a dose-response curve against the logarithm of the dose by the procedure of parallel line assays under a constraint of parallelism. The 95% confidence limits of the ratio was calculated by Fieller's expression.[18] The ratio of dose in the dose-response curve was 3, for example, 10^{-10}, 3×10^{-10} and 10^{-9}.

Results

Gallbladder Contraction Gallbladder strips contracted in the presence of CCK-33, CCK-33NS, CCK-8, CCK-8NS and CCK-4 in a concentration-dependent manner (Fig. 1). The activity of CCK-8 was the most potent of all. The potency ratio of CCK-33NS : CCK-33 was 1 : 97 (60—157, 95% conf. limits), CCK-8NS : CCK-8 was 1 : 1655 (1042—2629, 95% conf. limits), CCK-33 : CCK-8 was 1 : 21 (12—37, 95% conf. limits) and CCK-4 : CCK-8 was 1 : 18360 (9139—36884, 95% conf. limits).

Pancreatic Secretion Maximal increases of pancreatic secretion to all CCKs were observed in the first fraction after the intravenous administration and all responses returned to control levels within 1 h. Table I indicates that the test drugs, CCK-33, CCK-33NS, CCK-8, CCK-8NS and CCK-4 increased amylase and protein output each hour in a dose-dependent manner. In both parameters, CCK-33 and CCK-8 had almost the same activity and the activity was about 1000-fold that of CCK-33NS or CCK-8NS.

Gastric Acid Secretion The basal acid output was 3.7 ± 0.8 μeq/20 min ($n=6$) and was almost constant during 3 h. All CCKs which were tested stimulated gastric acid secretion in a dose-related manner (Table II). There were no significant differences in the activities of CCK-33 and

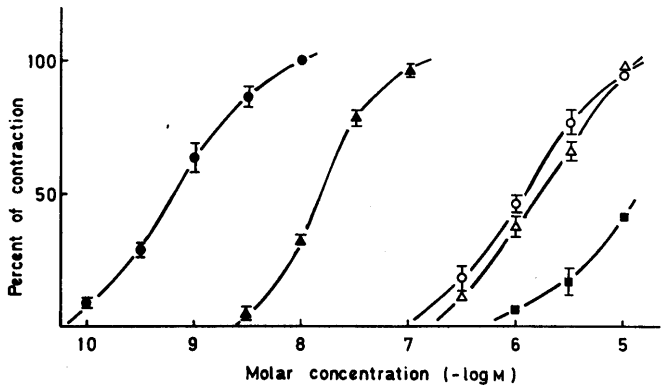

Fig. 1. Relationships between Concentrations (on a log Scale) and Response for Contractions in the Gallbladder of Guinea-Pigs

Responses were normalized to percentage of CCK-8 (10^{-8} M)-induced contraction. —●—, CCK-8; —▲—, CCK-33; —○—, CCK-8NS; —△—, CCK-33NS; —■—, CCK-4. Each point is mean ± S.E. of 3—4 observations.

Table I. Pancreatic Secretion Stimulated by Cholecystokinin

CCK	Dose (mol/kg, i.v.)	n	Amylase output (mM/h)	Protein output (mg/h)
Control		16	5.7 ± 0.7	2.2 ± 0.2
CCK-33	10^{-11}	4	6.7 ± 2.8	4.6 ± 1.2[b]
	10^{-10}	4	21.1 ± 4.4[b]	12.1 ± 2.3[b]
	10^{-9}	4	74.6 ± 12.7[b]	15.9 ± 2.8[b]
CCK-33NS	10^{-9}	8	10.5 ± 2.4[a]	2.9 ± 0.5
	10^{-8}	8	27.4 ± 2.3[b]	6.8 ± 0.4[b]
	10^{-7}	4	36.9 ± 2.7[b]	11.0 ± 2.7[b]
CCK-8	10^{-11}	8	12.5 ± 3.2[b]	5.1 ± 1.3[b]
	10^{-10}	7	24.2 ± 4.0[b]	8.5 ± 1.4[b]
	10^{-9}	3	69.1 ± 11.2[b]	23.8 ± 3.4[b]
CCK-8NS	10^{-9}	4	8.4 ± 1.6	3.2 ± 0.7
	10^{-8}	4	16.3 ± 3.1[b]	5.7 ± 1.4[b]
	10^{-7}	3	30.8 ± 5.6[b]	10.1 ± 0.8[b]
CCK-4	10^{-9}	4	9.8 ± 2.6[a]	4.0 ± 1.0[a]
	10^{-8}	4	13.8 ± 3.8[b]	5.5 ± 1.6[b]
	10^{-7}	4	25.8 ± 4.5[b]	7.0 ± 0.8[b]
	10^{-6}	4	28.3 ± 4.4[b]	8.8 ± 1.1[b]

a) $p<0.05$, b) $p<0.01$ *vs.* control.

Table II. Gastric Acid Secretion Stimulated by Cholecystokinin

CCK	Dose (mol/kg, i.v.)	n	Total acid output (μeq/h)
Control		24	10.3 ± 0.8
CCK-33	10^{-10}	8	11.3 ± 0.6
	10^{-9}	8	23.4 ± 1.4[b]
	2×10^{-9}	8	28.3 ± 3.9[b]
CCK-33NS	2×10^{-9}	6	16.4 ± 0.4[b]
	10^{-8}	6	27.8 ± 2.2[b]
CCK-8	10^{-10}	10	14.1 ± 1.6[a]
	10^{-9}	10	19.4 ± 3.7[b]
	2×10^{-9}	10	39.3 ± 9.0[b]
CCK-8NS	2×10^{-9}	10	13.1 ± 0.8
	10^{-8}	4	30.6 ± 6.8[b]
CCK-4	10^{-10}	10	13.4 ± 2.0
	10^{-9}	10	17.3 ± 1.6[b]
	2×10^{-9}	10	33.2 ± 5.9[b]

a) $p<0.05$. b) $p<0.01$ *vs.* control.

Table III. Inhibitory Effects of Feeding in Fasted Rats

CCK	Dose (mol/kg, i.p.)	n	Food intake (g/h)
Control		28	6.3 ± 0.2
CCK-33	10^{-8}	4	4.1 ± 0.4[a]
	3×10^{-8}	6	3.9 ± 0.6[a]
CCK-33NS	10^{-8}	4	6.7 ± 0.5
	3×10^{-8}	4	6.4 ± 0.4
CCK-8	10^{-8}	6	3.3 ± 0.4[a]
	3×10^{-8}	6	2.1 ± 0.6[a]
CCK-8NS	10^{-8}	3	6.0 ± 0.9
	3×10^{-8}	3	6.8 ± 0.5
CCK-4	10^{-8}	5	7.1 ± 0.3
	3×10^{-8}	5	7.8 ± 0.3

a) $p<0.01$ *vs.* control.

CCK-8. The activities of CCK-33NS and CCK-8NS were less than those of CCK-33 and CCK-8, respectively.

Food Intake The intake weight of the powdered chow during 1 h by a control rat was 6.3 ± 0.2 g ($n=28$). The effects of peptides were evaluated at doses of 10^{-8} and 3×10^{-8} mol/kg i.p. CCK-33 and CCK-8 produced a reduction in the food intake in a dose-related manner, but CCK-33NS, CCK-8NS and CCK-4 did not influence it at these doses. The activity of CCK-8 was slightly more than that of CCK-33 but it was not statistically significant. These results were summarized in Table III.

Discussion

The effects of synthetic human CCK-33 on pancreatic secretion in rats are similar to those reported in dogs with synthetic human CCK-33[4]: there is no significant difference between the effects of synthetic human CCK-33 and those of CCK-8. Studies of Solomon *et al.*[19] and Kontruek *et al.*[20] have clarified that porcine CCK-33 is more potent than CCK-8 in the rat and the dog, respectively, to stimulate pancreatic secretion. The addition of albumin prevented the lose of CCK-33 from solution to a greater degree than that of CCK-8.[19,21] We have confirmed this fact and demonstrated that the addition of BSA in the CCK-33 solution influenced the activity of CCK-33 (Data not shown). Although we have no data on the activity differences between porcine CCK-33 and human CCK-33, differences in the structure of porcine and human CCK-33 may produce a discrepancy between the results of porcine and human CCK-33 in pancreatic secretion. On the other hand, investigators using different species of animals and assay systems have reported that the relative potency of CCK-8 is greater than that of porcine CCK-33.[22-25] However, the activity of intracerebroventricular CCK-8 was less than that of CCK-33.[25]

In the present study, CCK-8 appeared about 20 times more potent than CCK-33 in gallbladder contraction assessed *in vitro*. In pancreatic secretion, the activity of intravenous CCK-33 was almost the same as CCK-8. Even though CCK-8 was more potent than CCK-33 in both the gallbladder and the pancrease, intravenously administered CCK-33 would be metabolized to CCK-8. CCK-33 may be primarily prohormone with CCK-8 being the predominant biologically active form of CCK.[26] In addition, differing sensitivities between the gallbladder and the pancrease to cholecystokinin may contribute to the differences of CCK-33 and CCK-8. The pancreas is more sensitive than the gallbladder to CCK in man.[27] It is difficult to discuss the differences of results between *in vivo* and *in vitro* as further studies are required to make clear these differences.

The presence of sulfated tyrosine as the seventh residue from the C terminus appears to be of crucial importance for the biological activities of CCK. Probably the sulfated residue plays a special role in the interaction between CCK and the receptor site, in producing a stronger binding. It has been reported that sulfation of the CCK-8 is required for binding to the receptor in the pancrease.[28,29] Therefore, the differences in the biological activity between sulfated and nonsulfated CCK-33 as well as CCK-8 on pancreatic secretion and gallbladder contraction, were very pronounced. On the other hand, the differences are smaller on gastric acid secretion. Gastric acid secretion was equally stimulated with sulfated and non-sulfated gastrin.[30] In this study, the potency of CCK-4 in gastric acid secretion was relatively similar to that of CCK-33 or CCK-8. These observations suggest that it requires at least the C-terminal octapeptide sequence containing sulfated tyrosine for CCK-33 or CCK-8 to produce the biological activities in the pancrease and the gallbladder. In the stomach, O-sulfation of tyrosine has no substantial effect on the activity, and a C-terminal tetrapeptide sequence is the active site for producing the action. In addition, present data coupled to the fact that the nonselective CCK/gastrin antagonist, but not the selective CCK antagonist, antagonized the acid response to both pentagastrin and CCK-8,[31] has led to the suggestion that CCK-33 or CCK-8 acts as a potent agonist on gastric receptors, and do not require sulfation.

Cholecystokinin has been demonstrated to inhibit feeding in a variety of species.[32-34] However, most studies of the influence of CCK on food intake have been performed with CCK-8. The present study provides the first demonstration that human CCK-33 as well as CCK-8 reduced food intake. There were no significant differences between CCK-33 and CCK-8, but sulfated CCK-33 and CCK-8 were significantly more potent than non-sulfated CCK-33 and CCK-8, respectively. Schick *et al.*[35] demonstrated that intracerebroventricular CCK-8 was more potent than non-sulfated CCK-8 in the inhibition of feeding in rats. Further studies are required to examine the CNS effect of human CCK-33 on appetite. Initial site of action of intraperitoneal CCK may be peripheral in the present study. Many pharmacological studies have demonstrated that exogenously administered CCK-8 reduces food intake by an initial action in the periphery,[36,37] evoking an abnormal gastrointestinal motor pattern that may induce malaise,[38-40] vomiting[41,42] and inhibition of gastric emptying.[43,44] In conclusion, O-sulfation of tyrosine in human CCK-33 as well as CCK-8 is required to stimulate the gallbladder contraction and the pancreatic secretion and to reduce food intake. The activities of synthetic human CCK-33 are almost the same as those of CCK-8 on exogenous pancreatic secretion, gastric acid secretion and food intake, but not on isolated gallbladder contraction.

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