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RECEPTOR BINDING OF CHOLECYSTOKININ ANALOGUES IN ISOLATED RAT PANCREATIC ACINI

Ryuichiro Doi, Ryo Hosotani, Kazutomo Inoue,
Nobutaka Fujii*, Haruaki Yajima*,
Phillip L.Rayford** and Takayoshi Tobe

First Department of Surgery, Faculty of Medicine,
Kyoto University, Kyoto 606, Japan

*Faculty of Pharmaceutical Sciences,
Kyoto University, Kyoto 606, Japan

**Department of Physiology and Biophysics,
University of Arkansas for Medical Sciences,
Little Rock, Arkansas 72205

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SUMMARY: The receptor binding of CCK analogues was determined in terms of the inhibition of [125I]CCK binding in isolated rat pancreatic acini. The inhibition curve produced by CCK-8 showed the same feature as that produced by synthetic human CCK-33. The relative potency values of CCK analogues to half-maximally inhibit specific CCK binding were calculated; CCK-8 was equal to human CCK-33, 3-fold stronger than natural porcine CCK-33 and 39, and 700-fold stronger than the unsulphated form of synthetic human CCK-33. Our data suggest that CCK-33, one of the longer molecular forms of CCK, is as important as CCK-8 in the mechanism of physiological actions of CCK.

Cholecystokinin (CCK) is the principal gastrointestinal peptide known to stimulate pancreatic enzyme secretion. Biologic actions of CCK on pancreatic acinar cells have been well studied in isolated acini from guinea pig [1,2] and rat pancreas [3]. In addition, the presence of CCK receptor on pancreatic acini has been demonstrated by binding studies with labeled CCK [4]. Studies on the binding affinity of CCK analogues have demonstrated that longer molecular forms of CCK such as porcine CCK-33 have less potent affinity to the receptor of rat pancreatic acini than CCK-8 [4]. Although biologic activities of longer molecular forms of CCK have been reported to be less potent than CCK-8 [2,5,6], recent studies which re-evaluated the relative potencies of CCK analogues have demonstrated that CCK-8 is not more potent than CCK-33 [7,8] in vivo.

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Recently, the gene structure of human CCK has been elucidated [9] and subsequently total synthesis of human CCK-33 has been successfully accomplished [10]. In previous studies [10,11], we have investigated the biological activity of synthetic human CCK-33 and have demonstrated that the relative potency of human CCK-33 was equal to CCK-8 in stimulating pancreatic protein output in in vivo dogs and rats. Absence of impure substances which may influence the interpretation of bioassay is a great advantage of totally synthesitzed peptides over natural peptides. In the current study, we report re-evaluation of relative potencies of CCK analogues in the inhibition of specific CCK binding to the receptor of isolated rat pancreatic acini, by use of synthetic human CCK-33, CCK-8 and other natural CCK analogues.

MATERIALS AND METHODS

Materials

The following substances were purchased: soybean trypsin inhibitor (type I-S), bovine serum albumin (BSA, fraction V) from Sigma Chemical Co. (St.Louis, Missouri); purified collagenase (type CLSPA) from Worthington Biochemicals (Freehold, New Jersey) ;minimal Eagle's medium amino acid supplement (MEM) from Gibco (Grand Island, New York); CCK-8 from Peptide Institute (Osaka, Japan); [125I]CCK-8 labeled with Bolton and Hunter reagent ([125I]CCK, 1950 Ci/mmol) from Amersham (Tokyo, Japan).

Synthetic human CCK-33 (hCCK-33) and unsulphated form of human CCK-33 (desCCK-33) were kindly provided by Professor H. Yajima (Faculty of Pharmaceutical Sciences of Kyoto University, Kyoto, Japan) [10,11]; porcine CCK-33 (pCCK-33) and CCK-39 (pCCK-39) from Professor V. Mutt (Karolinska Institute, Stockholm, Sweden).

Cell preparation

Isolated pancreatic acini were prepared by enzymatic digestion according to the method of Williams et al.[3]; the details of the procedure have been described previously [12,13]. In brief, male Sprague-Dawly rats fasted overnight were lightly anesthetized with diethylether and sacrificed. About 1.0 g of pancreas was obtained and injected by means of a 28-gauge needle with 5 ml of collagenase solution (67 U/ml) in Krebs-Henseleit-bicarbonate buffer (KHB, pH 7.4) containing 0.1 mM Ca2+, 11.1 mM glucose, 2 mg/ml BSA, 0.1 mg/ml soybean trypsin inhibitor and MEM, which was equilibrated with 95 % O_2 and 5 % CO_2. Injected pancreatic tissue was incubated at 37 °C with shaking 120 c/min in 50 ml polycarbonate Erlenmyer flask. After 10 min, the dissociation medium was replaced with 5 ml fresh medium and the pancreatic tissue was further incubated for approximately 30 min. The pancreatic tissue was then mechanically dissociated by forceful pipetting through plastic pipettes with decreasing orifices, filtered through 150 μm mesh nylon cloth and purified by a gradient centrifugation with KHB containing 4 % BSA at 50xg for 4 min. Acinar cells were washed twice with the same buffer and once with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered Ringer solution (HR buffer, pH 7.4) containing 10 mM of HEPES, 1.28 mM Ca2+, 11.1 mM glucose, MEM, 0.1 mg/ml soybean trypsin inhibitor and 5 mg/ml BSA, which was equilibrated with 100 % O_2.

Receptor binding study

CCK binding to its receptors was determined by the modified method of Sankaran et al.[4]. Pancreatic acinar cells were resuspended in fresh HR buffer at an acinar protein concentration of approximately 0.3 mg/ml. The protein concentration was determined by the method of Bradford [14]. Two-milliliter aliquots were distributed in 10-ml polystyrole vials, [125I]CCK (10 pM) plus various concentrations of unlabeled CCK analougues were added to the suspension, and incubation was carried out for 60 min at 37 °C with shaking (60 c/min). After incubation, one ml of the cell suspension was taken from each vial, centrifuged at 300xg for 2 min at 4 °C. The pellets thus obtained were washed twice with 0.9 % NaCl at 4 °C and the radioactivity associated with the washed acinar pellet was counted for 5 min with the gamma scintillation counter (Aloka, Japan). Total radioactivity in the incubation medium from each vial was also determined. Nonspecific binding was determined by incubating acinar cells with labeled CCK in the presence of an excess of CCK-8 (500 nM). Each incubation was performed in duplicate vials and experiments were replicated in six or seven times. Results are expressed as the means±SEM. Slopes of dose-response curves were determined by regression analysis using log-logit transformation.

RESULTS

Total [125I]CCK binding to isolated rat pancreatic acini at 37 °C was rapid, being half maximal at 15 min and maximal at 30-90 min, of which the characteristics were identical with those reported by Sankaran et al.[4].

Effects of unlabeled CCK-8 and hCCK-33 on specific CCK binding to isolated rat pancreatic acini were studied over the concentration range from 5pM to 100nM (Fig. 1). The inhibition curve produced by CCK-8 showed the same feature as that produced by hCCK-33. Both unlabeled CCKs progressively inhibited the

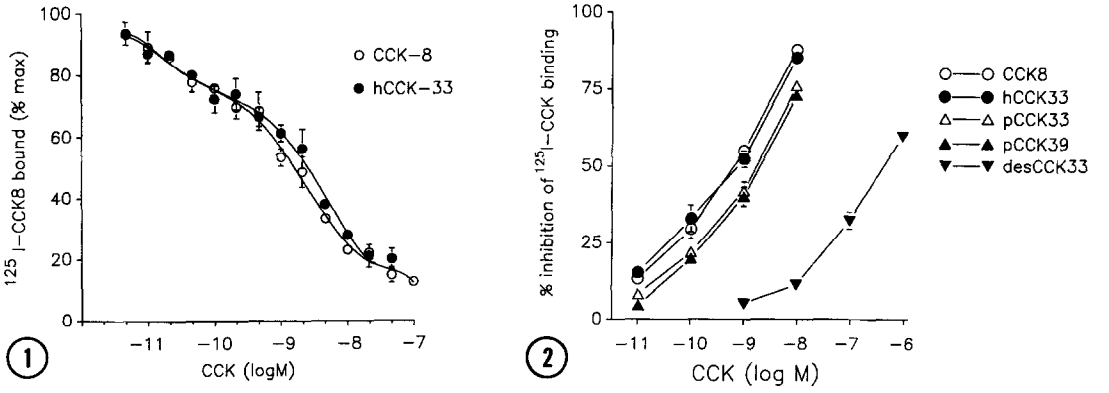

Figure 1. Effect of CCK-8 and hCCK-33 on specific CCK binding to acini. Isolated rat pancreatic acini was incubated with [125I]CCK (10pM) plus various concentrations of unlabeled CCK. Each value is the mean±SEM of three experiments.

Figure 2. Inhibition of [125I]CCK binding to isolated rat pancreatic acini, expressed as percent of maximum, is plotted against the various concentration of CCK analogues. Each value is the mean±SEM of four experiments.

Table 1
Potency values of CCK analogues in the inhibition of specific CCK binding to isolated rat pancreatic acini

Analogue	Inhibition of receptor binding
hCCK-33	1.024
CCK-8	1.000
pCCK-33	0.436
pCCK-39	0.358
desCCK-33	0.001

CCK-8 was assigned a value of 1.0.

specific CCK binding, and the competitive inhibition produced was biphasic. One phase of inhibition occurred over the range of 5-100 pM, and the second phase occurred over the range of 0.1-33 nM.

Potencies of CCK and its analogues in the inhibition of [125I]CCK binding were compared (Fig. 2). The relative potency of hCCK-33 was equal to CCK-8 in the inhibition of specific CCK binding, pCCK-33 and 39 were less potent than CCK-8, and desCCK-33 was much less potent than other CCK analogues. The relative potency values of CCK analogues to half-maximally inhibit specific CCK binding were calculated (Table 1). CCK-8 was equal to hCCK-33, 3-fold stronger than pCCK-33 and 39, and 700-fold stronger than desCCK-33 in the inhibition of specific CCK binding.

DISCUSSION

In the current study we examined the relative potencies of CCK analogues in the inhibition of specific CCK binding to the receptor of isolated rat pancreatic acini, and first demonstrated that hCCK-33 was exactly equipotent to CCK-8. The purity of hCCK-33 used in the current study was ascertained by analytical HPLC and amino acid analysis after acid hydrolysis, and the presence of sulphated tyrosine residue in position 27 was confirmed by the leucin-aminopeptide digestion technique [10]. In a previous report [11], we have shown that on a molar basis the relative potency of hCCK-33 was equal to that of CCK-8 in stimulating pancreatic protein output in dogs. Recently,

Solomon et al.[7] and Konturek et al.[8] have tested the biological activities of CCK analogues in several bioassay systems and have shown that both CCK-33 and CCK-8 were important mediators of actions of CCK on the pancreas. The current study strongly suggests that CCK-33 is as important as CCK-8 in the mechanism of physiological actions of CCK.

The sulphated tyrosine residue was demonstrated to have an important role in the receptor binding of hCCK-33; hCCK-33 was about 750 times more potent than desCCK-33 on a molar basis in the inhibition of specific CCK binding. Similar results have been reported with a carboxyl-terminal heptapeptide of CCK (CCK-7) in isolated acini from guinea pig, mouse and rat pancreas [16]. It has also been demonstrated that sulphate ester was necessary for optimal biologic activity of CCK-7 [16] and CCK-8 [2] in stimulating amylase release in isolated pancreatic acini. We have previously reported sulphated tyrosine residue of hCCK-33 was substantially required to stimulate protein output in dog pancreas [15]. Our present data and previous reports strongly suggest sulphated tyrosine residue plays an important role in the receptor binding and subsequent biological actions of longer molecular forms of CCK in the pancreas.

Several structural analogues of CCK have been available for evaluation of the biological effects; pCCK-33, a 33-amino acid peptide, which was the first of the analogues to be isolated [17]; pCCK-39, also called CCK variant, a 39-amino acid peptide [18]; and CCK-8, the carboxyl-terminal octapeptide of pCCK-33 and 39, first isolated as a cleavage product of pCCK-33 and then synthesized. Since the isolation of CCK-8, there have been numerous reports of the relative potencies of CCK analogues. In terms of pancreatic secretion, the relative potency of CCK-8 has been reported to be stronger than those of pCCK-33 and 39 in guinea pig [2] and rat pancreatic acini [4], and in in vivo rats [5] and dogs [6]. It has been reported that pCCK-33 and 39 were less potent than CCK-8 in the inhibition of specific CCK binding in rat pancreatic acini [4]. In the current study, we also demonstrated the similar results but differences in potencies between hCCK-33 and pCCK-33, 39. We demonstrated the

importance of the sulphate ester on tyrosine residue for optimal biologic activity of CCK-33. Difference in the amino acid sequences of the peptides, impure substance or unsulphated tyrosine residue in the natural peptide may affect the relative potency of CCK-33. Interplay between receptor occupation, mobilization of cellular calcium, and amylase release has recently been studied using CCK-JMV-180, a synthetic CCK analogue [19]. Relationship between molecular form of CCK and such intracellular mechanisms should be clarified in the further studies.

We demonstrated that synthetic hCCK-33 was equipotent to CCK-8 in the inhibition of specific CCK binding to the receptor of isolated rat pancreatic acini and the sulphated tyrosine residue may have an important role in the receptor binding of hCCK-33. Our current data strongly suggest that CCK-33, one of longer molecular forms of CCK, is as important as CCK-8 in the mechanism of physiological actions of CCK.

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