# Evidence That Cholecystokinin Interacts with Specific Receptors and Regulates Insulin Release in Isolated Rat Islets of Langerhans

EUGEN J. VERSPOHL, HERMANN P. T. AMMON, JOHN A. WILLIAMS, AND IRA D. GOLDFINE

**SUMMARY**

**To determine the nature of the pancreatic islet cell cholecystokinin (CCK) receptor, we studied CCK receptor binding and biologic activity in isolated rat pancreatic islets. Binding of 70 pM $^{125}$I-CCK to collagenase-prepared isolated rat pancreatic islets at 24°C was one-half maximal after 5 min and maximal at 60 min. At 60 min, specific binding was 12% of total radioactivity per 100 μg islet protein; nonspecific binding (in the presence of 1 μM CCK 8) was less than 2% of total radioactivity. Unlabeled CCK 33 inhibited labeled hormone binding one-half maximally at 2 nM; Scatchard analysis showed one binding site ($K_d$, 2.3 ± 0.4 nM; $B_{max}$, 8.1 pmol/mg protein). The agonist selectivity of this binding site was: CCK 8 = CCK 33>desulfated-CCK 8>CCK 4. Two CCK antagonists were studied; N-carbobenzoxy-L-tryptophan was more potent than dibutyryl-cGMP. When the effect of CCK on insulin release from the islets was studied, the order of potency of CCK agonists and antagonists on insulin secretion was the same as the order of their ability to inhibit $^{125}$I-CCK binding. The effect of CCK on insulin secretion was dependent on the glucose concentration in the media. CCK had no effect at 5.6 mM glucose and was fully effective at 11.0 mM glucose. These data, therefore, indicate that: (1) specific binding sites for CCK are present in rat pancreatic beta cells; and (2) CCK acts in concert with glucose to stimulate insulin secretion. DIABETES 1986; 35:38–43.**

The release of insulin from beta cells is under the regulation of metabolic substrates, hormones, and neurotransmitters.[1] It has been known for two decades that glucose administered orally is a more effective stimulant of beta cell insulin release than glucose administered intravenously.[2] Moreover, it has been shown that the intravenous administration of intestinal extracts lowers postprandial blood glucose levels.[3] These observations have led to the concept that an entero-insular axis exists and that intestinal factors are released after a meal ("incretins") that stimulate insulin secretin. The exact nature of these incretins, however, is unknown.[3]

Cholecystokinin (CCK) is a classic gut hormone that is released after food ingestion and regulates pancreatic exocrine function, gallbladder contraction, and bowel motility.[4,5] In addition to its action on gastrointestinal functions, both in vivo and in vitro studies in several species have indicated that CCK stimulates the release of insulin[6–13] and other islet hormones.[6,8,10,14,15] There is also evidence that the CCK tetrapeptide sequence stimulates beta cell growth.[16] Whether CCK acts directly on beta cells, however, remains to be elucidated.

Recently, employing the isolated perfused rat pancreas, we have performed light and electron microscope autoradiographic studies with radioiodinated CCK.[17] In these studies we observed a specific accumulation of this hormone over beta and other islet cells. In addition, CCK was most highly accumulated over beta cells and this accumulation was similar to that of CCK over exocrine acinar cells. Others, employing tritiated CCK, have also detected CCK accumulation over pancreatic islets.[18] These studies raise the possibility, therefore, that islet beta cells, like acinar cells, have receptors for CCK.

In order to better understand the islet cell CCK receptor, in the present study we have utilized isolated rat pancreatic islets. In this preparation, employing radioiodinated CCK, we have detected the presence of CCK receptors by direct binding studies. These studies also indicate that CCK potentiates glucose-induced insulin release. These studies provide additional evidence that CCK may have a role in regulating beta cell function.

From the Cell Biology Laboratory, Mount Zion Hospital and Medical Center, San Francisco, California (E.J.V., J.A.W., I.D.G.); the Departments of Physiology and Medicine, University of California, San Francisco, California; and the Departments of Pharmacology, Institute of Pharmaceutical Sciences, University of Tubingen, West Germany (E.J.V., H.P.T.A.).

Address reprint requests to Ira D. Goldfine, M.D., Cell Biology Laboratory, Mount Zion Hospital and Medical Center, P.O. Box 7921, San Francisco, California 94120.

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## MATERIALS AND METHODS

**Animals.** Wistar rats of either sex weighing between 180 and 250 g were used. They were kept on a standard pellet and tap water diet ad libitum at 22°C with a 12-h light/dark rhythm.

**Chemicals.** Pure natural porcine cholecystokinin (CCK 33) was obtained from Dr. V. Mutt, Karolinska Institute (Stockholm, Sweden). Synthetic CCK 8 and desulfated CCK 8 were gifts from Miguel Ondetti Squibb Institute for Medical Research (Princeton, New Jersey). The following were purchased: synthetic CCK 4 from Research Plus (Denville, New Jersey); $N^2,O^2$-dibutyryl-guanosine 3′,5′-cyclic monophosphoric acid (dibutyryl-cGMP) sodium salt, N-carbobenzoxy-L-tryptophan, pilocarpine hydrochloride, soybean trypsin inhibitor (SBTI), bacitracin, and Hepes from Sigma Chemical Co. (St. Louis, Missouri); bovine serum albumin (BSA) fraction V from Miles Laboratories (Elkhart, Indiana); N-succinimidyl 3-(4-hydroxy, 5$^{125}$I iodophenyl) propionate ($^{125}$I Bolton Hunter Reagent), 2200 Ci/mmol, from New England Nuclear (Boston, Massachusetts); and collagenase (CLS grade) from Worthington Biochemicals Corp. (Freehold, New Jersey). Insulin radioimmunoassay kits were supplied by Isotopendienst West, GmbH, Dreieich, West Germany. Rat insulin was purchased from Novo Research Institute, Copenhagen, Denmark.

**Labeled CCK.** CCK 33 was labeled with $^{125}$I by conjugation with Bolton Hunter reagent [N-succinimidyl 3-(4-hydroxy, 5$^{125}$I iodophenyl) propionate] according to the method of Sankaran et al.[19] with the following two exceptions: (1) the reaction was quenched with 250 μl 0.4 M glycine in 50 mM borate; and (2) the column buffer contained 0.2% BSA instead of gelatin. This $^{125}$I-Bolton Hunter-prepared CCK 33 conjugate ($^{125}$I-CCK) had a specific activity of 250–350 μCi/μg, and is fully biologically active.[19]

**Isolation of rat pancreatic islets.** The isolation procedure of pancreatic islets was the one described by Lacy and Kostianovsky[20] and Kuo et al.[21] with slight modifications as described earlier.[22] Three rats were each pretreated with 0.3 ml of 4% pilocarpine hydrochloride i.p. After 3 h, pancreata were isolated, minced, and washed twice with 20 ml ice-cold Hanks' solution containing 3.7 mM glucose, 1 mg/ml bacitracin, 0.2 mg/ml SBTI, and 0.2% albumin. Pancreas pieces were soaked and shaken in a 37°C water bath in the presence of 650-U collagenase/g tissue suspension. After 15–18 min of incubation the tissue suspension was passed into 10 ml of ice-cold Hanks' solution. Islets were separated by sedimentation and collected as described elsewhere.[20] This method yields 300–500 islets/rat pancreas.

**Binding experiments.** After the islets were isolated, they were washed in ice-cold Hanks' solution using centrifugation. Thirty islets were incubated in 0.15 ml Krebs-Ringer bicarbonate buffer plus 20 mM Hepes (KRBH buffer), pH 7.4, containing 5 mg/ml bovine albumin, 1 mg/ml bacitracin, and 0.2 mg/ml SBTI; 70 pM $^{125}$I-CCK with or without 10 μM unlabeled CCK 8 was then added. The incubation was terminated by cooling the samples, centrifuging at 10,000 rpm for 10 s in a Beckman microcentrifuge, and washing twice with Tris-saline at pH 7.8. All steps were carried out at 4°C. The cut tips of the microfuge tubes were counted in a gamma scintillation counter (Packard Auto-Gamma Scintillation Spectrometer). Degradation of $^{125}$I-CCK was monitored in the supernatant after various time intervals by measuring the solubility of $^{125}$I radioactivity in trichloroacetic acid (10% g/vol).

To establish the apparent receptor affinity (dissociation constant, $K_d$) and the number of specific binding sites ($B_{max}$), competition inhibition of specific $^{125}$I-CCK binding by varying concentrations of unlabeled CCK 33 was analyzed by fitting plots of bound hormone versus free hormone with a nonlinear least-squares computer program that analyzed the data in terms of one nonsaturable and one or two saturable components.[23,24] EC50s of biologic effects were determined after logit-log transformation.[25] For statistical evaluation, multiple comparisons of means were carried out by two-way analysis of variance (F-test).

**Insulin secretion.** To measure insulin secretion, 5 islets were incubated for 60 min at 37°C in the aforementioned Ringers' buffer. Insulin released into the medium by islets was assayed with a radioimmunoassay kit using rat insulin as a standard. Each CCK analogue and antagonist used had been checked for noninterference with the insulin radioimmunoassay. After either counting the cell-associated radioactivity (binding experiments) or after measuring insulin secretion, the protein content of the solubilized pancreatic islets (solubilized with 0.1 N NaOH) was measured using bovine serum albumin as a standard.[26]

## RESULTS

**CCK binding to islets.** The binding of 70 pM $^{125}$I-CCK to isolated rat islets at 24°C was rapid with one-half maximal binding occurring after 5 min and maximal binding occurring

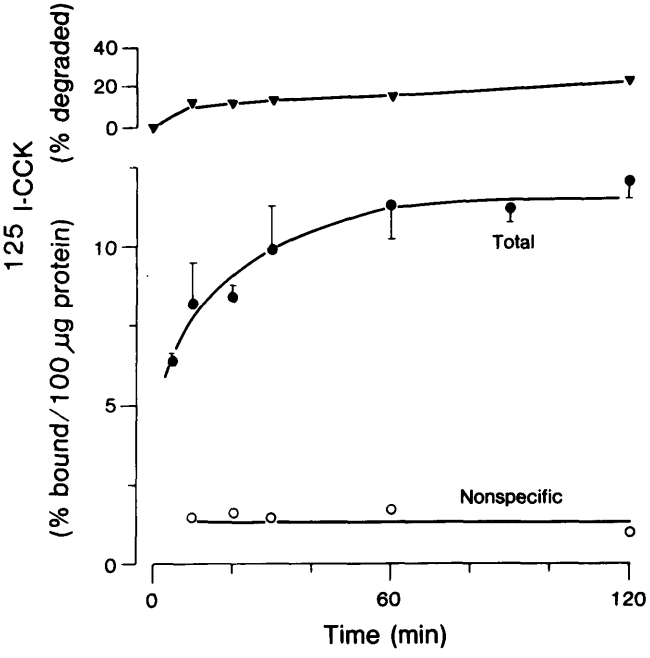


**FIGURE 1. Time course of $^{125}$I-CCK binding by isolated rat islets of Langerhans. Thirty islets were incubated in 0.15 ml buffer for 0–120 min at 24°C with 70 pM $^{125}$I-CCK in the absence (●) and presence (○) of 10 M CCK 8. Degraded $^{125}$I-CCK in the incubation media (upper panel) was measured in samples used for total binding by employing the TCA precipitation method. Results expressed as % bound per 100 μg islet protein (bottom) or as % degraded (top). Each value represents the mean ± SEM of three experiments.**

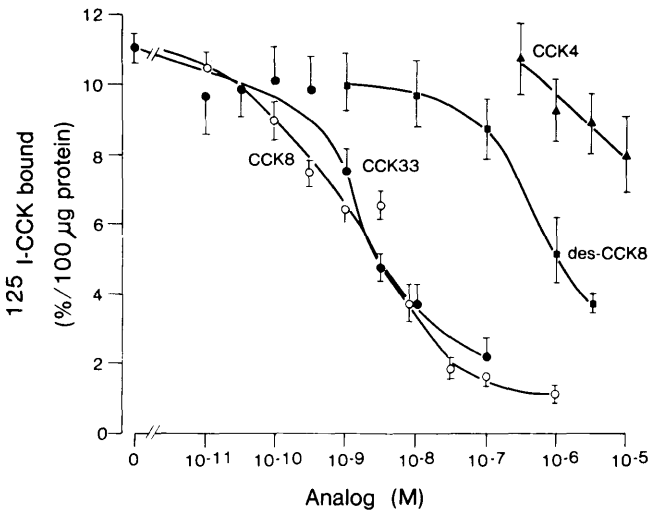


**FIGURE 2. Inhibition of $^{125}$I-CCK binding to isolated rat islets of Langerhans by CCK analogues. Thirty islets were incubated in 0.15 ml KRBH-albumin buffer for 60 min at 24°C with 70 pM $^{125}$I-CCK and increasing concentrations of CCK analogues. Results are expressed as % bound per 100 μg islet protein. Each value represents the mean ± SEM of 3–4 experiments.**

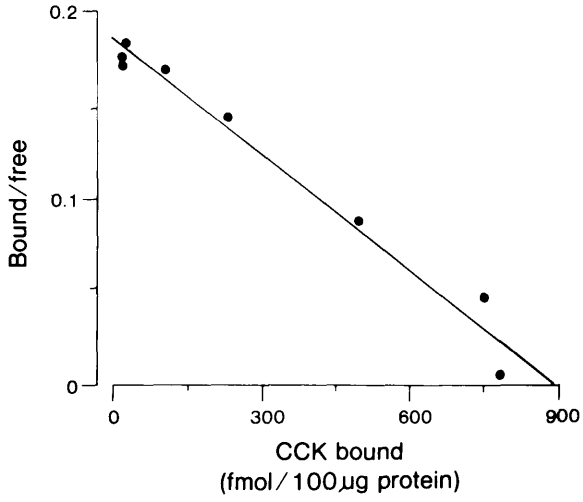


**FIGURE 3. Scatchard analysis of CCK 33 binding to rat pancreatic islets. Data are from one representative experiment similar to that shown in Figure 2.**

at 60 min (Figure 1). At 60 min, maximal binding was 10–12% of total radioactivity per 100 μg islet protein. Nonspecific binding (determined in the presence of 10 μM CCK 8) ranged from 1% to 2% of added radioactivity per 100 μg islet protein. After a 10-min lag, the degradation of tracer was linear for up to 60 min; at this time it was less than 20% of total. Preliminary experiments carried out at 37°C revealed that maximal specific binding occurred within 10 min (3% of added radioactivity) and declined thereafter; CCK degradation at 37°C was higher than at 24°C (<30% after 60 min; data not shown). All subsequent ligand binding studies, therefore, were carried out at 24°C.

The ability of several CCK analogues to inhibit $^{125}$I-CCK binding to rat pancreas islets was examined. Inhibition of specific $^{125}$I-CCK binding by unlabeled CCK 8 and CCK 33 was sigmoidal, and was essentially complete over three orders of magnitude (100 pM–100 nM) (Figure 2). The two curves were not statistically different from each other; the concentrations of both CCK 8 and CCK 33 causing one-half maximal inhibition of $^{125}$I-CCK binding were 1.1–1.4 nM, respectively (Table 1). In contrast, desulfated CCK 8 was 500 times weaker and CCK 4 was 70,000 times weaker than either CCK 8 or CCK 33.

Binding analysis of the competition-inhibition curve generated with unlabeled CCK 33 revealed a single order of binding sites with a $K_d$ of 2.3 ± 0.4 nM (mean ± SEM, N = 4) (Figure 3). The total binding capacity was 8.1 pmol/mg protein.

Two antagonists of CCK binding to its receptor in pancreas and other tissues[27] were tested for their ability to compete for $^{125}$I-CCK binding (Figure 4). Their relative potencies were: N-carbobenzoxy-L-tryptophan>dibutyryl-cGMP (Table 1).

**CCK stimulation of insulin release.** In pancreatic islets, glucose alone stimulated insulin release with a one-half maximal effect occurring at 11.6 ± 0.7 mM (mean ± SEM, N = 3; Figure 5). Addition of 10 nM CCK 8 significantly increased the sensitivity of islets to glucose; a one-half maximal effect of glucose occurred at 9.3 ± 0.6 mM (mean ± SEM, N = 3; $P < 0.01$).* CCK 8, however, did not increase the maximal secretory capacity of the islets. The effect of CCK was dependent on the glucose concentration. No effect of CCK was seen with 5.6 mM glucose, whereas a marked effect was seen at 11.1 mM glucose (Figure 6).

CCK analogues were then studied with 11.1 mM glucose; CCK 8 and CCK 33 were equipotent in increasing insulin release (Figure 7 and Table 1). Desulfated CCK 8 and CCK 4 had reduced potencies that correlated with their reduced receptor binding potencies. The two CCK antagonists, N-carbobenzoxy-L-tryptophan and dibutyryl-cGMP, inhibited CCK-stimulated insulin release with activities similar to those for inhibiting $^{125}$I-CCK binding to its receptor (Table 1 and Figure 8).

TABLE 1
Activity of CCK analogues and antagonists and effects on receptor binding and insulin release

| | Activity (nM) | |
|---|---|---|
| Agonist | Receptor binding | Insulin release |
| CCK 8 | 1.1 | 1.6 |
| CCK 33 | 1.4 | 1.5 |
| Desulfated-CCK 8 | 520 | 370 |
| CCK 4 | >70,000* | 27,000 |
| | Activity (mM) | |
| Antagonist | Receptor binding | Insulin release |
| N-CBZ-L-tryptophan | 1.0 | 1.0 |
| Dibutyryl-cGMP | >1.0* | >1.0* |

Activities were calculated from the concentration required to one-half maximally inhibit $^{125}$I-CCK binding and to one-half maximally regulate insulin release. The above values were calculated from the data in Figures 2, 4, 7, and 8 after logit-log transformation (except those tagged with an asterisk).

*Studies of insulin release were carried out at 37°C because at 24°C, insulin release was not consistent. Since the receptor binding studies and insulin release studies were not carried out at the same temperatures, a precise correlation between receptor occupancy and insulin release could not be obtained.

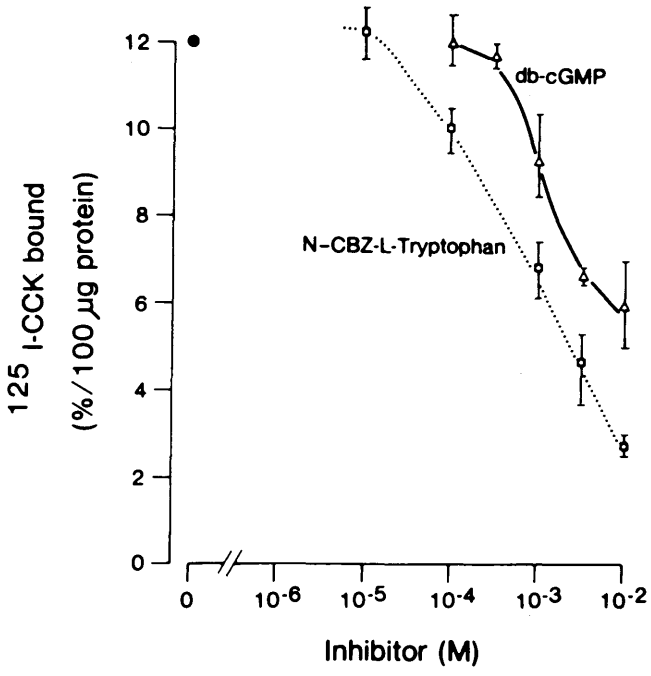


**FIGURE 4. Inhibition by CCK antagonists of $^{125}$I-CCK binding to isolated rat islets of Langerhans. Thirty islets were incubated in 0.15 ml KRBH-albumin buffer at 24°C for 60 min with 70 pM $^{125}$I-CCK and with increasing concentrations of antagonists. Results were expressed as % bound per 100 µg islet protein. Each value represents the mean ± SEM of three experiments.**

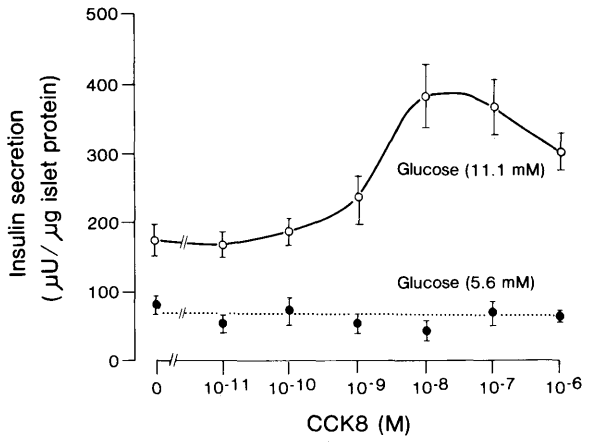


**FIGURE 6. Dependence on glucose concentration of CCK-stimulated insulin release. Five islets were incubated with various CCK 8 concentrations in the presence of either 5.6 or 11.1 mM glucose for 60 min at 37°C. Results are expressed as µU insulin secreted per µg islet protein over 60 min. Each value represents the mean ± SEM of three experiments.**

## DISCUSSION

Recent studies of plasma CCK levels in man and animals indicate that this hormone is promptly released after feeding.[28-32] In addition, plasma CCK levels are elevated after the oral administration of glucose.[31] Earlier in vitro and in vivo studies have indicated that CCK increases insulin secretion in several species including man, dog, and rat.[6-11] While the interpretation of some studies with CCK were complicated by the presence of contaminating intestinal peptides, it has now been clearly demonstrated that both highly purified natural and synthetic forms of CCK have a glucose-dependent beta cell–stimulating activity.[8-11] In the perfused rat pancreas, synthetic CCK increases both insulin release and exocrine pancreatic secretion.[11,12] Moreover, both of these functions are activated at the same concentrations of hormone.[11] In cultured neonatal rat islets, CCK 8 has also been reported to stimulate insulin release.[33] These observations suggest, therefore, that in addition to being a regulator of pancreatic exocrine secretion, CCK may also have effects on the islets of Langerhans.

In the present and previous studies, we find several lines of evidence indicating that specific CCK receptors are present in pancreatic beta cells. First, direct binding studies indicated that islets have specific high-affinity CCK receptors. Second, in EM autoradiographs we localized the majority of these receptors in islets to the beta cell.[17] Third, CCK analogues stimulated insulin release in proportion to their ability to compete for the CCK receptor. These studies therefore suggested a role for CCK in modulating islet cell function.

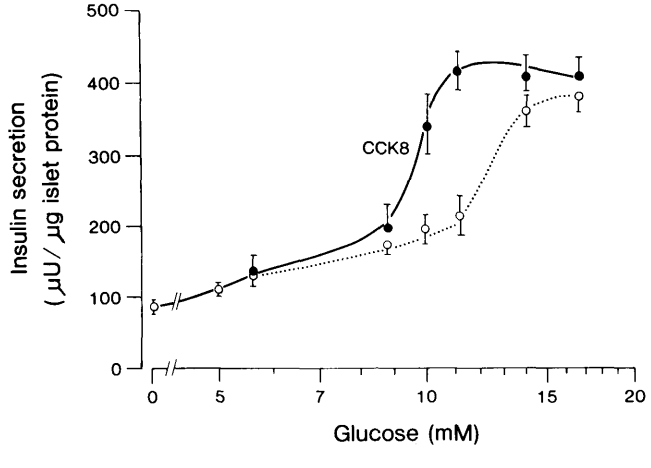


**FIGURE 5. Modulation by CCK of glucose-induced insulin secretion. Five islets were incubated at various glucose concentrations in the absence or presence of 10 nM CCK 8 for 60 min at 37°C. Results are expressed as µU insulin secreted per µg islet protein over 60 min. Each value represents the mean ± SEM of three experiments.**

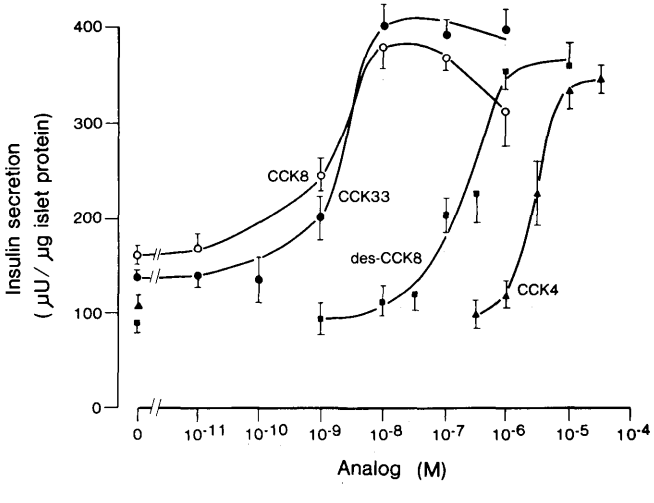


**FIGURE 7. Stimulation of insulin release by CCK analogues. Five islets were incubated in the same buffer with various CCK analogue concentrations for 60 min at 37°C in the presence of 11.1 mM glucose. Each value represents the mean ± SEM of three experiments for each analogue.**

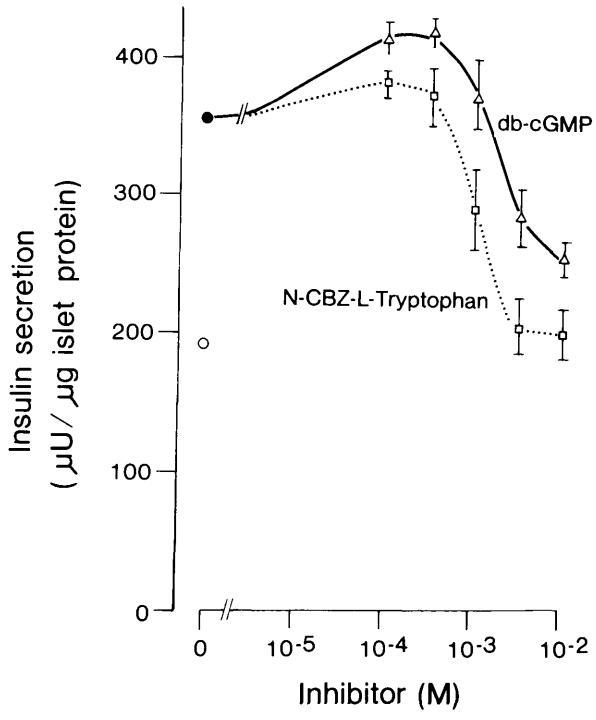


**FIGURE 8. Inhibition of CCK-stimulated insulin release by CCK antagonists. Five islets were incubated with increasing concentrations of antagonists in the presence of 11.1 mM glucose plus 10 nM CCK 8 for 60 min at 37°C. Results are expressed as μU insulin secreted per μg islet protein over 60 min. Each value represents the mean ± SEM of three experiments for each CCK antagonist. Closed circle represents insulin secretion in the absence of both CCK and antagonists.**

Hormonally active CCK has been characterized in gut and plasma. In the small intestine, various forms have been observed.[34–36] These forms include molecules having 58, 39, 33, 22, 8, and 4 amino acids, respectively. All forms include the carboxyl terminal tetrapeptide sequence possessing the intrinsic biologic activity of CCK.[37,38] However, for most high-affinity interactions with gastrointestinal tissues, the sulfated octapeptide sequence is necessary.[5,38] Circulating CCK differs between species. In rat plasma, CCK 8 and a larger sequence (presumably CCK 22) have been detected,[30] whereas in human plasma, CCK 33, an intermediate sequence, and CCK 8 have been detected.[28,31,32,35]

The CCK receptor has been studied most extensively in the pancreatic acinar cell.[5,24,27,38,39] In this cell, CCK 8 is several-fold more potent than CCK 33 in interacting with the CCK receptor, desulfated CCK 8 is 200-fold less potent than CCK 8, and CCK 4 is 10,000–100,000-fold less potent than CCK 8. For interaction with the gallbladder receptor, the potency ratio of these analogues is similar to the pancreas except that CCK 33 and CCK 8 are equipotent.[40] In contrast to the pancreas and the gallbladder, the brain CCK receptor discriminates poorly between all of these CCK analogues.[41,42] Our present studies with pancreatic islets indicate, therefore, that this CCK receptor is similar to the receptors of pancreas and gallbladder since CCK 8 and CCK 33 were equipotent, desulfated CCK 8 was less potent, and CCK 4 had markedly less potency.

It has been suggested that CCK 4 may be an important regulator of islet cell hormone release.[43] In the perfused porcine pancreas, CCK 4, but neither CCK 8 nor CCK 33, stimulated insulin release.[43] In contrast, in the perfused rat pancreas, CCK 4 was 100,000-fold less potent than CCK 8 in stimulating insulin release.[44] In the perfused dog pancreas, CCK 4 was also significantly less potent than CCK 8.[13] In the present studies with isolated islets, CCK 4 was not potent in either stimulating insulin release or in competing for the CCK receptor. One explanation for these data is that considerable species variation may exist in the islet cell response to CCK 4.

Two CCK antagonists were studied. In pancreatic acini and gallbladder, dibutyryl-cyclic GMP is a potent inhibitor of $^{125}$I-CCK binding (with a one-half maximal inhibitory concentration of 50 μM),[27,39] whereas in the brain it is less potent (>1 mM).[45] Since dibutyryl-cGMP had a weak potency (1 mM) in islets, in this respect the islet receptor is more similar to the brain receptor than it is to the pancreatic acinar receptor. Recently it has been reported that modified amino acids such as N-CBZ-tryptophan can inhibit $^{125}$I-CCK binding in pancreatic acini[27] and brain[46] with a similar potency (0.1–1.0 mM). Thus in the case of N-CBZ-tryptophan, the islet receptor is similar to both of these other tissues. In islets, both dibutyryl-cGMP and N-CBZ-tryptophan inhibited $^{125}$I-CCK binding and inhibited CCK-stimulated insulin secretion. These data therefore support the concept that the $^{125}$I-CCK binding sites measured in islets are those involved in CCK action.

Of interest was the observation that the effect of CCK on insulin secretion was observed only at glucose concentrations ranging from 8.8 to 14.0 mM; at 5.6 mM glucose CCK was ineffective. Although this concentration range is narrow, it is the range observed postprandially in various species. In islets, CCK did not increase the maximal amount of insulin that was released; rather, CCK increased the sensitivity of the islets to glucose. In concert with our findings, it has been reported in the perfused rat pancreas that the effect of CCK was dependent on the glucose concentration.[11]

Recent studies indicate that basal circulating CCK levels are in the range of 1 pM and rise to approximately 10 pM after feeding.[30–32,35] In the present studies with islets in vitro, levels of CCK in the range of 1 nM were needed to elicit insulin release. One possibility is that this effect of CCK on islets may not be physiologic. We believe, however, that this possibility is unlikely since in vivo CCK elicits insulin release[6–8] and in the perfused rat pancreas the dose responses for CCK-induced insulin release and CCK-induced amylase release are the same.[11] Another possibility is that islets in vitro, for unknown reasons, are not as sensitive to CCK as islets in vivo. For these reasons further in vivo and in vitro studies of CCK actions on islets are needed to clarify the physiologic role of this hormone on insulin release.

## ACKNOWLEDGMENTS

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