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Distinct Requirements for Activation at CCK-A and CCK-B/Gastrin Receptors: Studies with a C-Terminal Hydrazide Analogue of Cholecystokinin Tetrapeptide (30–33)

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SUMMARY

We describe here the properties of *tert*-butyloxycarbonyl-Trp-Leu-Asp-Phe-$NHNH_2$ (A-57696), a C-terminal hydrazide analogue of *tert*-butyloxycarbonyl-CCK_4 (Boc-Trp-Met-Asp-Phe-NH_2), at four cholecystokinin (CCK) receptor-bearing tissues, the guinea pig pancreas and gall bladder (Type A), guinea pig cortex (Type B), and NCI-H345 cells, a human small cell lung cancer cell line that expresses CCK-B/gastrin receptors. Using ^{125}I-Bolton-Hunter-cholecystokinin octapeptide (26–33) (^{125}I-Bolton-Hunter-CCK_8) as the radioligand, A-57696 was found to be selective for cortical CCK-B receptors (IC_{50} = 25 nM), compared with pancreatic CCK-A receptors (IC_{50} = 15 μM). A-57696 behaved as a competitive antagonist in reversing CCK_8-stimulated pancreatic amylase secretion and phosphoinositide breakdown. By Schild analysis, its K_d was determined to be 4.7 and 6.8 μM in amylase and phosphoinositide assays, respectively. A-57696 (100 μM) did not elicit gall bladder contraction, and it inhibited contractions induced by CCK_8. The K_d of A-57696 at gall bladder CCK-A receptors was 19 μM. In contrast, A-57696 behaved as a partial agonist (80% of maximal CCK_8 response) in stimulating calcium mobilization at CCK-B/gastrin receptors on NCI-H345 cells. A-57696 and CCK_8 inhibited each other in calcium mobilization experiments utilizing the fluorescent dye Indo-1. Stimulatory actions of CCK_8 and A-57696 were reversed by the CCK-B-selective (*R*)-L-365,260 (100 nM), whereas at the same concentration, the CCK-A-selective (*S*)-L-365,260 was ineffective. Binding studies using ^{125}I-Bolton-Hunter-CCK_8 and ^{125}I-gastrin indicated that binding sites labeled by these two ligands displayed similar affinities for CCK_8, desulfated CCK_8, gastrin, A-57696, and both enantiomers of L-365,260. A-57696 represents a new class of CCK-A peptide antagonist at guinea pig pancreas and gall bladder. Its contrasting functional activities at guinea pig CCK-A and CCK-B/gastrin receptors in a human tumor cell demonstrate that, in addition to the previously described differences in binding specificity for selective agonists and antagonists, CCK-A receptors and CCK-B/gastrin receptors have different requirements for activation.

Receptors for the brain-gut peptide CCK have been classified into CCK-A (alimentary) and CCK-B (brain) types, with the pancreas and cerebral cortex as examples of tissues that express the respective types (1, 2). The CCK-A receptors have high affinity for CCK_8 and low affinity for CCK_4 (Trp-Met-Asp-Phe-NH_2) and desulfated CCK_8. In contrast, CCK-B receptors show high affinity for these different fragments of CCK_8. At present, several classes of CCK-A antagonists have been identified and they fall into the following catagories: 1) fragments of CCK_8, 2) derivatives of glutaramic acids and other amino acids, 3) benzodiazepine analogs, 4) derivatives of cyclic nucleotides, and 5) derivatives of substance P_{4-11} (3–7). Most recently, Merck scientists reported a benzodiazepine-based CCK-B-selective antagonist, L-365,260 (8, 9). Here we report studies on the hydrazide derivative of Boc-[Leu^2]-CCK_4, Boc-Trp-Leu-Asp-Phe-$NHNH_2$ (A-57696) and demonstrate that this peptide behaves as an antagonist at the CCK-A receptors in the guinea pig pancreas and gall bladder. In contrast, A-57696 exhibits partial agonist activity (~80% of CCK_8) in stimulating calcium mobilization in NCI-H345 cells, which express CCK-B/gastrin receptors (see Refs. 10 and 11 and below). The contrasting agonist/antagonist properties of A-57696 at guinea pig CCK-A and CCK-B/gastrin receptors provide the first evidence that CCK-A and CCK-B/gastrin receptors have distinct requirements for activation.

Materials and Methods

CCK_8, phosphoramidon, and bestatin were purchased from Peptides International (Louisville, KY). Heat-inactivated fetal calf serum,

ABBREVIATIONS: CCK, cholecystokinin; CCK_8, cholecystokinin octapeptide (26-33); CCK_4, cholecystokinin tetrapeptide (30–33); PI, phosphoinositide; BSA, bovine serum albumin; BH, Bolton-Hunter; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)piperazine ethanesulfonic acid; indo-1/AM, indo-1 acetoxymethyl ester; Boc, *tert*-butyloxycarbonyl; MEM, minimum essential medium; CCK_8(DS), desulfated cholecystokinin octapeptide (26–33).

EGTA, HEPES, BSA, and aprotinin were from Sigma Chemical Co. (St. Louis, MO). ^{125}I-B-H-CCK$_8$, ^{125}I-gastrin (specific activity, 2200 Ci/mmol), and [^{3}H]myo-inositol were from New England Nuclear (Boston, MA). Collagenase (CLSPA) was from Worthington Biochemical Corp. (Freehold, NJ). Indo-1/AM was purchased from Molecular Probes (Eugene, OR). Male guinea pigs (225–300 g) were from Sasco (Omaha, NE). MEM amino acids and vitamins were from KC Biologicals (Lenexa, KS). L-364,718 was obtained from Mr. W. Henckler, Chemical Data Department, Merck & Co., Inc. (Rahway, NJ). Boc-Trp-Leu-Asp-Phe-NHNH$_2$ (A-57696) was synthesized at our company.

Amylase assay. Guinea pig acini were prepared as described (12). Isolated acini were resuspended in 100 volumes of Krebs-Ringer HEPES buffer containing 0.1% BSA, 1% MEM amino acids, 1% MEM vitamins, 0.01 mg/ml aprotinin, pH 7.4 (buffer A), and were revitalized for 30 min at 37° with changes of fresh buffer every 15 min. Into 1.5-ml microcentrifuge tubes were added 300 μl of CCK$_8$, A-57696, or buffer, and 200 μl of acini (1 ml of acini/100 ml of buffer) suspension. Final volume of the assay was 500 μl. The incubation medium was buffer A plus 3 μM phosphoramidon and 100 μM bestatin as protease inhibitors. Tubes were incubated for 30 min at 37° under 100% O_2 and amylase activity in the medium and cell pellet were separately determined by ABBOTT-VP bichromatic analyzer as described (13). The maximal response to CCK$_8$ (0.3 nM) varied 3- to 8-fold after corrections for amylase activity in the medium at the beginning of drug incubation.

PI breakdown assay. Guinea pig acini were isolated and resuspended in 2 ml of buffer A. Twenty microcuries of [^{3}H]inositol were added and cells were incubated for 1 hr at 37° under 100% O_2. [^{3}H] Inositol was removed by centrifugation and acini were resuspended in 50 volumes of buffer A plus 10 mM LiCl, phosphoramidon, and bestatin. Into 12 × 75 mm glass tubes were added 100 μl of CCK$_8$, A-57696, or buffer and 200 μl of acini. The final volume of the assay was 300 μl. After incubation at 37° for 30 min, the reaction was terminated with 1 ml of chloroform/methanol (2:1) solution and inositol phosphate fractions were collected as described (13). For this study, inositol phosphates, inositol bisphosphates, and inositol trisphosphates were collected together by elution with 1 M ammonium formate from AG 1-X 8 columns. The maximal stimulation by CCK$_8$ (100 nM) varied 20- to 40-fold over control.

Gall bladder contraction. Guinea pig gall bladder was excised and mounted in a 10-ml chamber filled with Krebs-Ringer buffer, at 37°. Isometric contractions were recorded with a Grass polygraph. Baseline tension was set at 0.3 g and tensions generated by CCK (100 nM) varied between 2 and 4 g. The EC$_{50}$ of CCK$_8$ was 5 nM.

Binding assays in guinea pig cortex and pancreas. Guinea pig pancreatic and cortical membranes were isolated as described for the cortex (14). The final suspension for the pancreatic membranes and cortical membranes were 60 and 15 ml/g of wet weight, respectively. The buffer was 20 mM HEPES, 1 mM EGTA, 118 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, pH 7.4, containing 0.05% BSA, 3 μM phosphoramidon, and 100 μM bestatin. Assays were performed in Skatron polystyrene tubes. Twenty-five microliters of ^{125}I-BH-CCK$_8$ (30,000 cpm; ~30 pM), 25 μl of various concentrations of A-57696, and 200 μl of membrane suspension were mixed and pancreatic tissues were incubated for 30 min at 37°. Cortical membranes were incubated for 150 min at 30°. The incubation was terminated by filtration with a Skatron Cell Harvester on SS32 (Schleicher and Schuell) glass microfiber filter mats. The nonspecific binding, defined as binding of ^{125}I-BH-CCK$_8$ in the presence of 1 μM CCK$_8$, was 5–15% in the pancreatic membranes and 10–25% in the cortical membranes.

Binding studies in NCI-H345 cell membranes. Membranes were prepared as described for the cortex (14). The final suspension for the cell membranes was 40 ml/g. The incubation buffer was the same as the buffer used in the cortical and pancreatic membranes. Fifty microliters of radioligand (40,000 cpm for ^{125}I-BH-CCK$_8$ and 80,000 cpm for ^{125}I-gastrin), 200 μl of membrane, and 50 μl of competing peptides were mixed and incubated for 120 min at room temperature. The nonspecific binding of ^{125}I-gastrin was defined as binding in the presence of 1 μM gastrin, and for ^{125}I-BH-CCK$_8$ 1 μM CCK$_8$ was used. Nonspecific binding represented 40 and 30% for BH-CCK$_8$ and gastrin, respectively.

Measurement of intracellular calcium in NCI-H345 cells. Culture conditions were as described (10), except that the medium was modified to RPMI 1640 with 2.5% fetal bovine serum (heat inactivated), 5 μg/liter sodium selenite, 5 mg/liter human transferrin, 5 mg/liter insulin, penicillin (100 units/ml) and streptomycin (100 μg/liter). Cells (~200,000/ml) were loaded with 1 μM indo-1/AM for 1 hr, washed, and resuspended in Dulbecco's phosphate-buffered saline, pH 7.4, plus 0.1% BSA and 0.1% glucose. Intracellular Ca^{2+} levels were monitored with a SLM 8000C spectrofluorimeter with settings of 350 nm excitation and 405 and 480 nm emissions. Calibrations of $[Ca^{2+}]_i$ were done as described (15):

$$[Ca]_i = K_d(R - R_{min})/(R_{max} - R)$$

where R_{min} and R_{max} were the ratios (480/405) obtained in the presence of excess EGTA (10 mM) and digitonin (50 μM), respectively. K_d is assumed to be 240 nM, and R is the ratio in the presence and the absence of CCK. Basal calcium levels were 147 ± 3 (three experiments) and the maximal levels of calcium stimulated with 1 μM CCK$_8$ were 357 ± 30 (three experiments).

Results

Table 1 lists the IC$_{50}$ values of CCK$_8$, A-57696, L-364,718, CR-1409 (4, 5), and the *R*- and *S*-enantiomers of L-365,260 (8) in inhibiting ^{125}I-BH-CCK$_8$ binding to cortical and pancreatic membranes. Both L-364,718, a benzodiazepine derivative, and CR-1409, a proglumide derivative, were over 100-fold more potent at pancreatic CCK-A receptors. In contrast, A-57696 was ~300–500 times more potent at cortical CCK-B receptors. In agreement with published data (8, 9), (*R*)-L-365,260 showed selectivity for CCK-B receptors (IC$_{50}$ ratio, ~190), whereas the *S*-enantiomer was selective for CCK-A receptors (IC$_{50}$ ratio, ~0.06).

Figs. 1 and 2 show that A-57696 behaved as a competitive antagonist of CCK-A receptors in guinea pig pancreas. At 100 μM, this peptide did not stimulate amylase secretion or enhance PI hydrolysis. In amylase secretion assays (Fig. 1), increasing concentrations of A-57696 shifted the dose-response curves for CCK$_8$ to the right in a parallel manner. The K_d of A-57696, derived from the Schild plot, was 4.7 ± 0.77 μM (four experiments). Similarly, in the PI breakdown study (Fig. 2), A-57696 acted as a competitive inhibitor of CCK$_8$. Its K_d value, determined from the Schild plot, was 6.8 ± 0.5 μM (four experiments). Because the slope of the Schild transformation from

TABLE 1

IC$_{50}$ values of various CCK-A- and CCK-B-selective antagonists in inhibiting ^{125}I-BH-CCK$_8$ binding in guinea pig cortical and pancreatic membranes

The IC$_{50}$ values of these compounds in the cortex and pancreas were determined using ^{125}I-BH-CCK$_8$ as the radioligand, as described in Materials and Methods. IC$_{50}$ values were determined from the Hill analysis and were the means of duplicate samples repeated at least twice.

Compound	IC$_{50}$		IC$_{50}$ ratio (pancreas/cortex)
	Cortex	Pancreas	
	nM		
CCK$_8$	8	0.4	0.05
A-57696	27	14,000	520
L-364,718	120	0.92	0.008
CR-1409	10,000	110	0.01
(*R*)-L-365,260	15	2,800	190
(*S*)-L-365,260	480	28	0.06

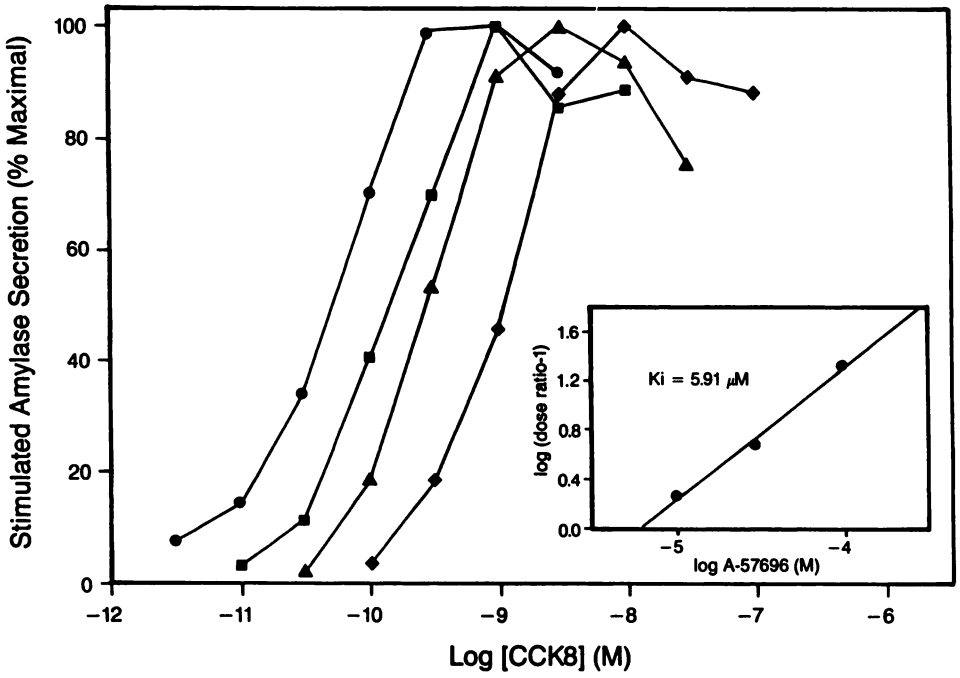

Fig. 1. Effect of A-57696 on the dose-response curves of CCK_8-stimulated amylase secretion in pancreatic acinar cells. Acini were incubated with the indicated concentrations of CCK_8 in the absence (●) and presence of increasing concentrations of A-57696 [10 μM (■), 30 μM (▲), and 100 μM (♦)]. After 30 min, the amylase activity in the medium and cell pellet were determined separately, as described in Materials and Methods. Results are expressed as a percentage of the response to 0.3 nM CCK_8 in the absence of A-57696. *Inset*, Schild plot of Fig. 1. The average slope ± SE from four experiments was 0.97 ± 0.05 and the K_d was 4.7 ± 0.77 μM.

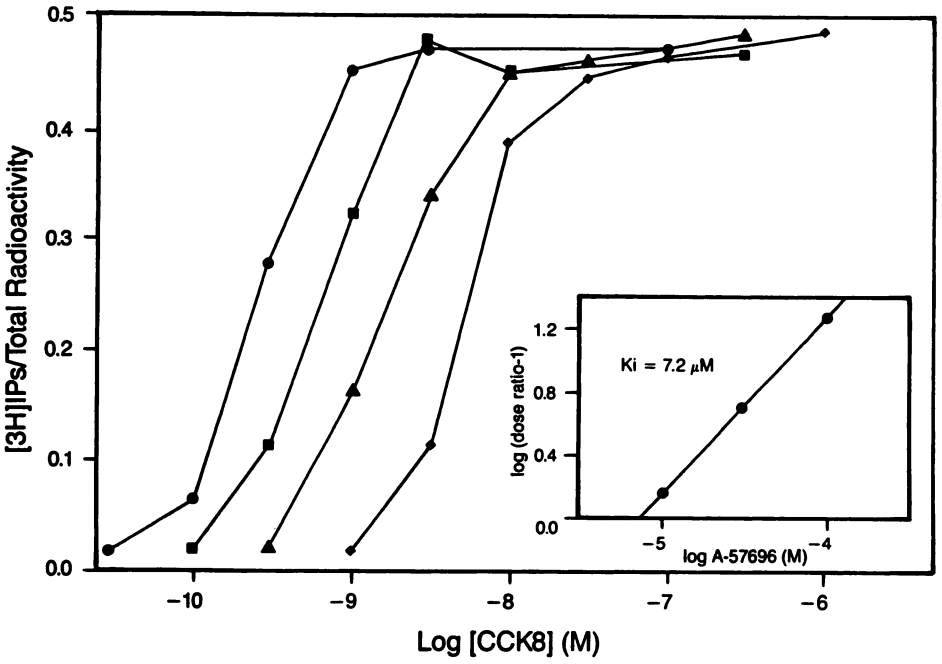

Fig. 2. Effect of A-57696 on the dose-response curves of CCK_8-stimulated PI hydrolysis. Acini were incubated with the indicated concentrations of CCK_8 in the absence (●) and presence of increasing concentrations of A-57696 [10 μM (■), 30 μM (▲), and 100 μM (♦)]. After 30 min, incubations were terminated by chloroform/methanol, as described in Materials and Methods. Total radioactivity is the total amount of radioactivity in the aqueous phase that was applied to the Bio-Rad AG-1X8 column. [^{3}H]IPs represent the amount of radioactivity eluted with 1 M ammonium formate/0.1 M formic acid. *Inset*, Schild plot of Fig. 2. The slope of the plot from four different experiments was 1.03 ± 0.06 and the K_d value was 6.8 ± 0.5 μM.

both assays was close to unity (see legends to Figs. 1 and 2), A-57696 may be regarded as a competitive antagonist of CCK-A receptors. In other studies, two similar CCK_4 analogs, Boc-Trp-Met-Asp-Phe-$NHNH_2$ and Boc-Trp-Leu-Asp-Phe-NH_2, were shown to possess ~20–35% of the CCK_8 activity in the amylase release assay.

In the guinea pig gall bladder, A-57696 also behaved as a CCK antagonist. At 10 μM, A-57696 reversed the response to CCK_8 (5 nM) by 17–28% (three experiments); at 30 μM, it inhibited the contractions induced by CCK_8 (5 nM) by 33–57% and, at 100 μM, A-57696 inhibited the response to CCK_8 (5 nM) by 77–95%. Using the Cheng and Prusoff equation, the K_d of A-57696 was determined to be 19 ± 4.2 μM (three experiments).

Fig. 3 shows the dose-response curves for CCK_8, A-57696, and Boc-CCK_4 in the calcium assays in NCI-H345 cells. In contrast to its antagonistic properties at guinea pig CCK-A receptors in the pancreas and gall bladder, A-57696 was a partial agonist, possessing ~80% of the excitatory response of CCK_8. The potency of A-57696 (EC_{50}, ~30 nM) agreed well with its binding affinity for the cortical CCK-B receptor.

Fig. 4 shows the stereoselective inhibition of L-365,260 on stimulatory responses induced by A-57696 (Fig. 4, *left*) and CCK_8 (Fig. 4, *right*) in the calcium assays. At 100 nM, the CCK-B-selective (*R*)-L-365,260 antagonized the stimulatory responses to CCK_8 (30 nM) and A-57696 (100 nM). In contrast, 100 nM concentrations of the CCK-A-selective (*S*)-L-365,260 failed to inhibit the responses to CCK_8 and slightly inhibited the responses to A-57696.

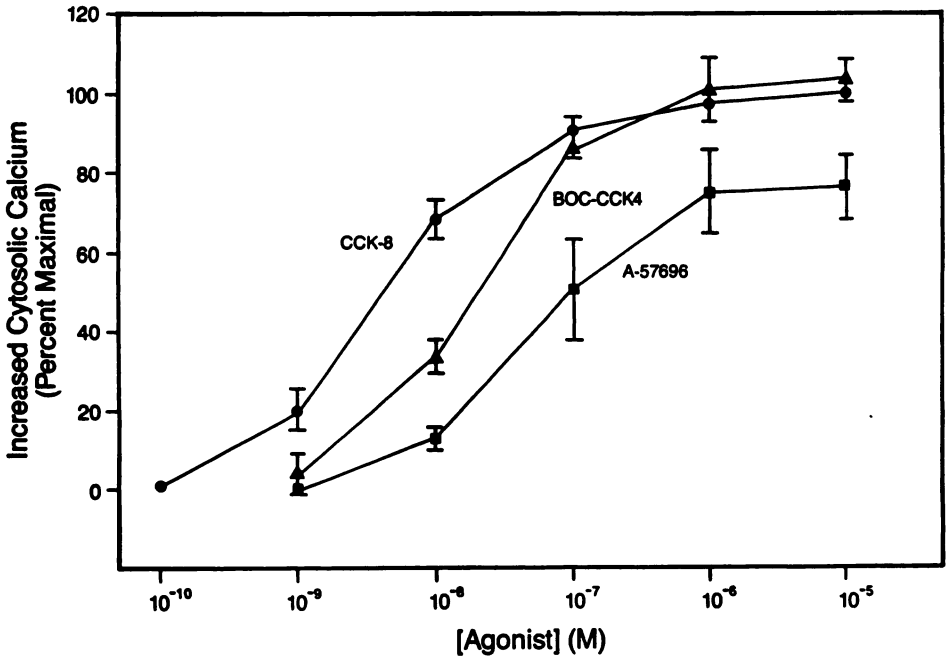

Fig. 3. Dose-response curves for CCK_8, Boc-CCK_4, and A-57696 in calcium mobilization in NCI-H345 cells. Cells were loaded with 1 μM indo-1/AM for 1 hr and washed and indo-1 fluorescence was measured, as described in Materials and Methods. For each concentration of peptide, the maximum elevation of $[Ca]_i$ was determined and expressed as a percentage of the response to 1 μM CCK_8, which was determined with the same group of cells. The results shown were the average ± standard error from three experiments.

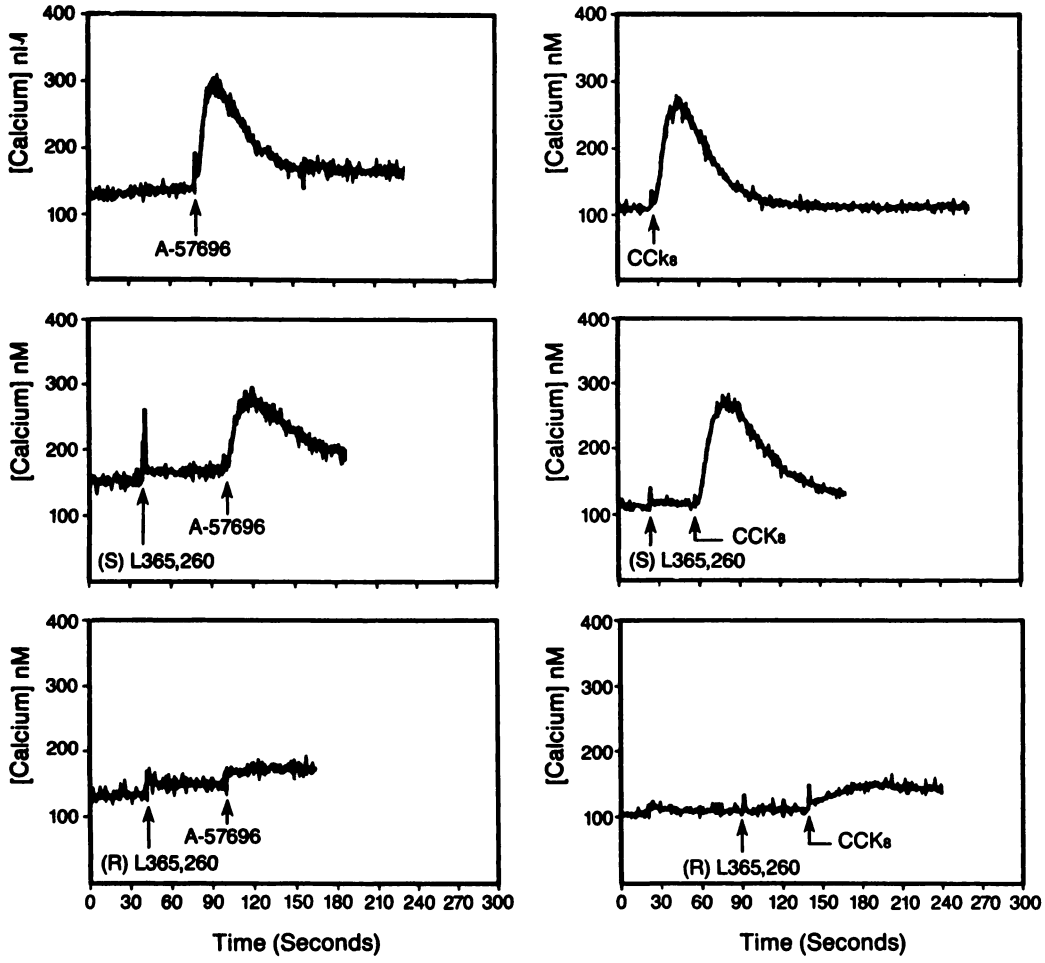

Fig. 4. Effects of enantiomers of L-365,260 (100 nM) on calcium mobilization induced by 100 nM A-57696 (*left*) and 30 nM CCK_8 (*right*). Results shown were taken from one experiment, which was repeated at least twice.

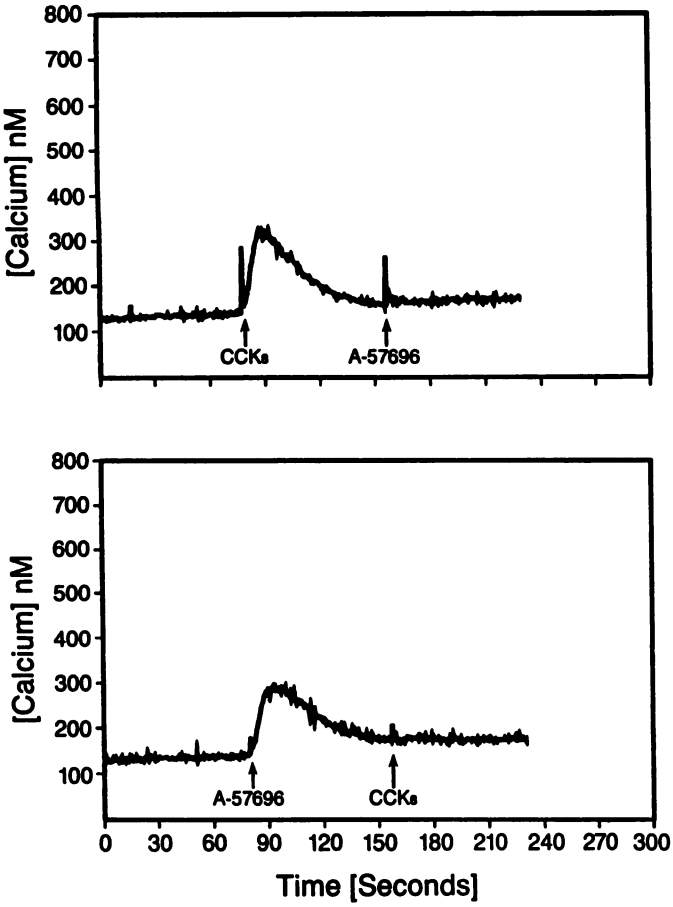

Fig. 5. Effects of prior additions of CCK_8 (30 nM) (*top*) and A-57696 (100 nM) (*bottom*) on the calcium-mobilizing responses of cells to subsequent additions of A-57696 (100 nM) and CCK_8 (30 nM). Results were taken from one experiment, which was repeated at least twice.

Fig. 5 (*top*) shows that prior activation of intracellular calcium pools by 30 nM CCK_8 prevented the response to subsequent addition of 100 nM A-57696 in NCI-H345 cells. Similarly, prior addition of 100 nM A-57696 prevented the response to 30 nM CCK_8 (Fig. 5, *bottom*). These observations, in conjunction with the stereospecific inhibition by the *R*-enantiomer of L-365,260 (Fig. 4), indicate that both peptides activate the same CCK-B/gastrin receptors and release the same internal pool of calcium. In other series of experiments, it was determined that prior treatment with a high concentration of bombesin (1 μM) inhibited the response to CCK_8 (1 μM) and A-57696 (10 μM) by 50 and 75%, respectively. Prior additions of CCK_8 (1 μM) and A-57696 (10 μM) inhibited the response of cells to bombesin (1 μM) by 20 and 30%, respectively.

Because of the similarities between gastrin and CCK-B receptors (8), the binding specificity of ^{125}I-BH-CCK_8 and ^{125}I-gastrin was investigated in NCI-H345 cell membranes. As shown in Table 2, the potencies of CCK_8, CCK_8(DS), gastrin, Boc-CCK_4, A-57696, and the *R*- and *S*-enantiomers of L-365,260 in displacing the binding of these two radioligands were similar, indicating that both radioligands bind to similar receptor sites. Thus, the receptor sites on NCI-H345 are referred to as CCK-B/gastrin receptors.

Discussion

We describe here the binding affinity and efficacy of a hydrazide derivative of CCK_4 at CCK-A and CCK-B/gastrin receptors. A-57696 is a relatively weak antagonist at CCK-A receptors in the guinea pig pancreas and gall bladder, but it exhibits high affinity for cortical CCK-B receptors (IC_{50} = 27 nM) and is a relatively potent agonist (EC_{50} ~100 nM) at CCK-B/gastrin receptors in NCI-H345 cells. The binding of A-57696 is highly selective for the CCK-B receptor, compared with the CCK-A receptor (Table 1). In guinea pig pancreas, it does not exhibit agonist activity at 100 μM and it inhibits the responses of CCK_8 in both the amylase and PI assays. Schild analyses from both assays show slopes close to unity, indicative of competitive antagonism. A-57696 is also a CCK-A antagonist in guinea pig gall bladder. In contrast, A-57696 possesses partial agonist (~80%) activity by stimulating intracellular calcium levels in NCI-H345 cells (11), a variant of NCI-H209 cells whose binding characteristics have been described (10). As demonstrated in the present report, the binding properties of CCK receptors on NCI-H345 is of the CCK-B/gastrin type (Table 2), where CCK_8, gastrin, CCK_8(DS), Boc-CCK_4, A-57696, and (*R*)-L-365,260 have affinities in the nanomolar range. In addition, (*R*)-L-365,260, a CCK-B-selective antagonist, inhibits the calcium-mobilizing responses of CCK_8 and A-57696, whereas (*S*)-L-365,260, a CCK-A-selective antagonist, was ineffective. The contrasting functional properties of A-57696 at guinea pig CCK-A and CCK-B/gastrin receptors on human small cell lung cancer cell lines demonstrate that, in addition to having differential binding specificity, CCK-A and CCK-B/gastrin receptors have different requirements for activation. Specifically, replacement of the C-terminal amide with hydrazide and replacement of the Met by Leu in Boc-CCK_4 decreased the ability of Boc-CCK_4 to activate guinea pig CCK-A receptors, but the ability of the ligand to bind and activate the CCK-B/gastrin receptors is maintained.

At present, the existence of both A and B types of CCK receptors in the central nervous system has been demonstrated (1, 16), but their functional significance in brain has not been clearly defined. In particular, there has been a lack of measurable CCK-B receptor-coupled responses in brain. The characterization of CCK-B/gastrin receptors in small cell lung carcinoma cell lines (10, 11) has created an avenue to explore the functions of CCK-B/gastrin receptors. In the present study, we have demonstrated that CCK-B/gastrin receptors mediate Ca^{2+} mobilization in NCI-H345 cells. It remains to be determined whether A-57696 will behave as an agonist in the cortical CCK-B receptor systems such as the excitatory responses reported for CCK_8 in a subpopulation of cortical neurons (17, 18).

Finally, it is of interest to note that CCK-B and gastrin

TABLE 2

Affinities of CCK_8-related peptides and (*R*)- and (*S*)-L-365,260 for ^{125}I-BH-CCK_8 and ^{125}I-gastrin binding sites in NCI-H345 cells

The IC_{50} values of these compounds were determined as described in Materials and Methods. The results were the means of duplicate samples repeated at least twice.

Compound	IC_{50}	
	^{125}I-BH-CCK_8	^{125}I-Gastrin
	nM	
CCK_8	0.31	1.1
CCK_8 (DS)	8.5	25
Gastrin	3.6	5.2
Boc-CCK_4	5.0	3.4
A-57696	9.5	14
(*R*)-L-365,260	12	12
(*S*)-L-365,260	200	150

receptors are quite similar. Using ^{125}I-gastrin and ^{125}I-BH-CCK$_8$ as radioligands, many CCK tetrapeptide analogs were shown to exhibit identical affinities for CCK-B sites labeled by both radioligands in guinea pig cortex.[1] In other studies, it has been shown that many CCK antagonists have similar affinities for gastric glands and cortical CCK-B receptors (8, 9). The similarity between gastric gland and CCK-B/gastrin receptors on NCI-H345 cells has also been observed.[2] In the present study (Table 2), we also demonstrated that binding sites labeled by ^{125}I-BH-CCK$_8$ and ^{125}I-gastrin are similar in NCI-H345 cells. Recently, species differences in the functional properties of a CCK fragment at pancreatic CCK-A receptors have been described, raising the possibility of CCK-A receptor heterogeneity (19). The expression (20–22) and cloning of CCK receptors will help to resolve the similarities and differences among the multiple classes of CCK and gastrin receptors. The contrasting functional properties of A-57696 at guinea pig CCK-A and NCI-H345 CCK-B/gastrin receptors and the existence of several CCK-A- and CCK-B-selective antagonists should facilitate our understanding of the structural and conformational requirements for binding and activation of these multiple CCK/gastrin receptors.

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[1] Lin *et al.*, unpublished data.
[2] Barrett *et al.*, manuscript in preparation.

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