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Cyclic cholecystokinin analogues with high selectivity for central receptors

(guinea pig/receptor binding/brain/pancreas/cholecystokinin octapeptide)

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ABSTRACT **Taking as a model the N-terminal folding of the cholecystokinin tyrosine-sulfated octapeptide [CCK-8; Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2] deduced from conformational studies, two cyclic cholecystokinin (CCK) analogues were synthesized by conventional peptide synthesis: Boc-D-Asp-Tyr(SO_3H)-Ahx-D-Lys-Trp-Ahx-Asp-Phe-NH_2 [compound I (Ahx, 2-aminohexanoic acid)] and Boc-γ-D-Glu-Tyr(SO_3H)-Ahx-D-Lys-Trp-Ahx-Asp-Phe-NH_2 (compound II). The binding characteristics of these peptides were investigated on brain cortex membranes and pancreatic acini of guinea pig. Compounds I and II were competitive inhibitors of [^{3}H]Boc[Ahx^{28,31}]CCK-(27–33) binding to central CCK receptors and showed a high degree of selectivity for these binding sites (compound I: K_i for pancreas/K_i for brain, 179; compound II: K_i for pancreas/K_i for brain, 1979). This high selectivity was associated with a high affinity for central CCK receptors (compound I: K_i, 5.1 nM; compound II: K_i, 0.49 nM). Similar affinities and selectivities were found when ^{125}I Bolton–Hunter-labeled CCK-8 was used as a ligand. Moreover, these compounds were only weakly active in the stimulation of amylase release from guinea pig pancreatic acini (EC_{50} > 10,000 nM) and were unable to induce contractions in the guinea pig ileum (to 10^{-6} M). The two cyclic CCK analogues, therefore, appear to be synthetic ligands exhibiting both high affinity and high selectivity for central CCK binding sites. These compounds could help clarify the respective role of central and peripheral receptors for various CCK-8-induced pharmacological effects.**

The C-terminal octapeptide of cholecystokinin, CCK-8 [Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2], is a hormonal regulator of pancreatic secretion (1–3) that also can induce gallbladder contraction (1, 4, 5) and increase gut motility (4). Moreover, CCK-8 is present in high concentrations in the mammalian brain (6–9), where it could be a neurotransmitter or neuromodulator, as suggested from various biochemical and pharmacological studies (10–16). Extensive binding studies have clearly shown that the ligand specificities differ greatly for central and peripheral receptors (17–20), and irreversible labeling experiments have revealed entities with different molecular weights for pancreas and brain binding sites (21). Nevertheless, the respective roles of both types of receptors in many CCK-8-induced pharmacological responses are still controversial, especially when peripheral or even intracerebroventricular routes of administration have been used (22, 23).

Potent ligands, highly specific for either class of receptors, are therefore essential for understanding the physiological role of CCK. Moreover, CCK is colocalized with dopamine in the mesolimbic pathway (24) and could modulate dopaminergic transmission (11, 25). Consequently, in preliminary studies CCK-8 was suggested to exhibit neuroleptic properties in the human and thus was proposed as a relevant compound for the treatment of various psychiatric disorders (26, 27). However, in more extensive clinical studies, no significant antipsychotic effect was demonstrated (28–32). Clearly centrally selective, long-acting CCK agonists and antagonists that cross the blood–brain barrier would have application for more rigorous conclusive clinical trials.

The existence of several types of CCK receptors implies different biologically active conformations for the native peptide. Thus, constrained structures could be designed to favor recognition of one particular receptor. This approach has been successfully undertaken recently, leading to nonpeptidic antagonists that are highly specific for peripheral CCK receptors (33, 34). For enkephalins (35, 36), probes specific to the several opioid receptors were obtained by cyclizing the peptide to mimic the favorable conformation of native peptide (37, 38).

Studies on CCK-8 by ^{1}H NMR, fluorescence transfer, and energy calculations (39, 40) have shown that the sulfated peptide exists under folded conformations in various solvents, including water, with its N-terminal residues in equilibrium between a β-turn including the residues Asp-26 Tyr(SO_3H)-27 Met-28 Gly-29 and a γ-turn including Tyr(SO_3H)-27 Met-28 Gly-29. From such findings we synthesized constrained CCK analogues mimicking the N-terminal β-turn by cyclization between residues in positions 26 and 29. Instead of native CCK-8 [CCK-(26–33)], the parent compound chosen for cyclization was Boc-[Ahx^{28,31}]CCK-(27–33) because we had previously found the structural modifications introduced in this analogue to increase its chemical and enzymatic stability. Moreover, this molecule retains the full biological properties of CCK-8 (41).

In this paper, we report the binding and pharmacological properties of two cyclic compounds characterized by an internal amide bond between the side-chain amino group of a D-Lys-29 residue and either the β-carboxyl group of a D-Asp-26 residue (compound I) or the α-carboxyl group of a D-Glu-26 residue (compound II). These peptides are promising cyclic CCK analogues that exhibit both high affinity and, more interestingly, high selectivity for central CCK receptors of guinea pig (42).

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Abbreviations: CCK, cholecystokinin; CCK-8, cholecystokinin tyrosine-sulfated octapeptide; Boc, *t*-butoxycarbonyl; K_i, inhibitor dissociation constant; FAB, fast atom bombardment; ^{125}I-BHCCK-8, ^{125}I Bolton–Hunter-labeled CCK-8; sf, selectivity factor(s) determined from K_i for pancreas/K_i for brain; Ahx, 2-aminohexanoic acid.

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MATERIALS AND METHODS

Peptide Synthesis. The cyclic analogues I and II (Fig. 1) were synthesized by conventional peptide synthesis using a fragment condensation method schematized in Fig. 2 (43). This type of synthesis requires only two amino-protecting groups [benzyloxycarbonyl (Z) and *t*-butoxycarbonyl (Boc)] and two kinds of carboxyl protection (methyl and benzyl ester). The N-terminal pentapeptide (Boc-Xaa(OBzl)-Tyr-Ahx-D-Lys(Z)-Trp-OCH_3 was obtained by a stepwise synthesis. In this peptide Xaa is a D-Asp or D-Glu residue. The latter amino acid was linked to the tyrosine residue by its γ-carboxyl group (referred to in the text as γ-D-Glu). After removal of the protecting groups of D-Asp-26 (or γ-D-Glu-26) and D-Lys-29 residues, cyclization was achieved by means of diphenylphosphoryl azide (DPPA) (44, 45) using a dilute (5×10^{-3} M) solution of the precursor. A 50% yield of the corresponding cyclic pentapeptides was obtained, and the structures were confirmed by fast atom bombardment (FAB)-MS.

The final cyclic octapeptides were obtained by condensation of the fragments Boc-Xaa-Tyr-Ahx-D-Lys-Trp-OH with the C-terminal tripeptide H-Ahx-Asp(OBzl)-Phe-NH_2. Finally, after deprotection of the Asp-32 residue, the tyrosine was sulfated using a freshly prepared SO_3–pyridine complex in dimethylformamide/pyridine mixture. Structures of the final peptides and of all intermediates were confirmed by ^1H NMR spectroscopy. Compounds I and II (Na salt form) were characterized by amino acid analysis on an LKB Biochrom 4400 analyzer after hydrolysis in 6 M HCl for 24 hr at 110°C. Mass spectra were recorded on a double-focusing VG 70-250 instrument (VG Instruments, Le Chesnay, France). The FAB ionization was obtained with FAB saddle field source (Ion Tech, Teddington, U.K.) operated with xenon at 8 kV and 1 mA.

Purity of compounds I and II was checked by HPLC (Waters apparatus) on a μBondapak C_{18} reversed-phase column with 25 mM triethylammonium phosphate buffer, pH 6.5/CH_3CN (63:37) as eluent (flow rate, 1.2 ml/min), with UV (210 nm) detection, and by TLC using Merck plates precoated with F 254 silica gel, in the following solvent systems: A, ethyl acetate/pyridine/acetic acid/H_2O (40:20:6:11); B, 1-butanol/acetic acid/H_2O (4:1:1).

Results for the two compounds were as follows. (*i*) Boc-D-Asp-Tyr(SO_3Na)-Ahx-D-Lys-Trp-Ahx-Asp(Na)-Phe-NH_2 (compound I): R_f(A), 0.48 and R_f(B), 0.45; HPLC (retention time, t_R = 11.6 min); FAB-MS (MH^+), 1306. Amino acid analysis: Asp, 1.93; Ahx, 2.0; Tyr, 0.93; Phe, 0.96; and Lys, 0.93. (*ii*) Boc-γ-D-Glu-Tyr(SO_3Na)-Ahx-D-Lys-Trp-Ahx-Asp(Na)-Phe-NH_2 (compound II): R_f (A), 0.48 and R_f (B), 0.45; HPLC (t_R = 13.6 min); FAB-MS (MH^+), 1320. Amino acid analysis: Asp, 0.92; Glu, 0.94; Ahx, 2.0; Tyr, 0.94; Phe, 1.01; and Lys, 0.99.

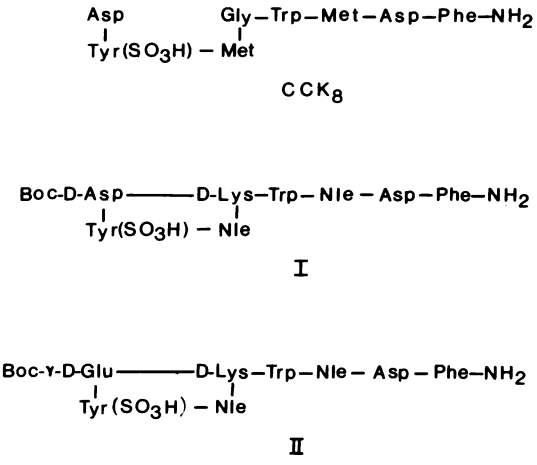

FIG. 1. Structural formulas of CCK-8 compound I, and compound II. The CCK-8 sequence has been drawn to represent the N-terminal β-turn seen in solution. Nle, 2-aminohexanoic acid (Ahx).

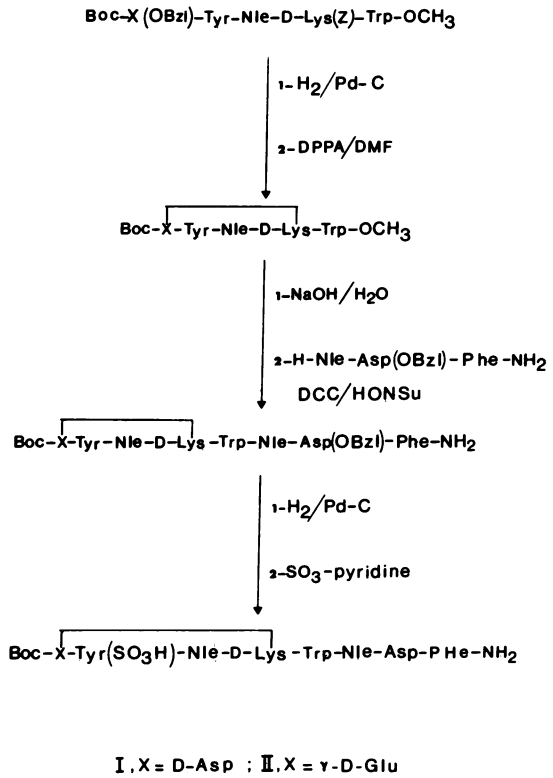

FIG. 2. Scheme for the synthesis of compounds I and II. Nle, Ahx; z, benzyloxycarbonyl; Pd-C, palladium-charcoal; DPPA, diphenylphosphoryl azide; DCC, *N, N'*-dicyclohexylcarbodiimide; HONSu, *N*-hydroxysuccinimide.

Binding Studies. *Guinea pig brain cortex membranes.* Male guinea pigs (250–300 g) were killed by decapitation; the brain cortex was dissected on ice and homogenized in 50 mM Tris·HCl/5 mM $MgCl_2$, pH 7.4. The homogenate was incubated for 30 min at 35°C, centrifuged for 35 min at 100,000 × *g*, and the resulting pellet was rehomogenized and centrifuged under the same conditions. The final pellet was resuspended in the buffer of the binding assay (see below).

Binding experiments with [^3H]Boc[$Ahx^{28,31}$]CCK-(27–33) (100 Ci/mmol; 1 Ci = 37 GBq) (46, 47) were done as described (48). Incubations (60 min, 25°C, final vol to 1 ml) were done in 50 mM Tris·HCl, pH 7.4/5 mM $MgCl_2$ containing bacitracin at 0.2 mg/ml in the presence of brain membranes (0.6 mg of protein per ml), [^3H]Boc[$Ahx^{28,31}$]CCK-(27–33) (0.2 nM), and increasing concentrations of the corresponding competitors. The incubation was stopped by filtration using Whatman G/FB filters presoaked in 50 mM Tris·HCl, pH 7.4 containing bovine serum albumin (1 mg/ml). Nonspecific binding was measured in the presence of 10^{-6} M CCK-8. Displacement curves were fitted by linear regression analysis of the Hill transformation, and inhibitor dissociation constant (K_i) values were calculated according to the Cheng–Prusoff equation, with a K_d value of 0.13 nM for [^3H]Boc[$Ahx^{28,31}$]CCK-(27–33).

The competitive aspect of the binding of compounds I and II was assessed by Scatchard analysis of saturation curves of [^3H]Boc[$Ahx^{28,31}$]CCK-(27–33) (0.05 nM–1 nM) in the presence of 6 nM of compound I or 0.6 nM of compound II.

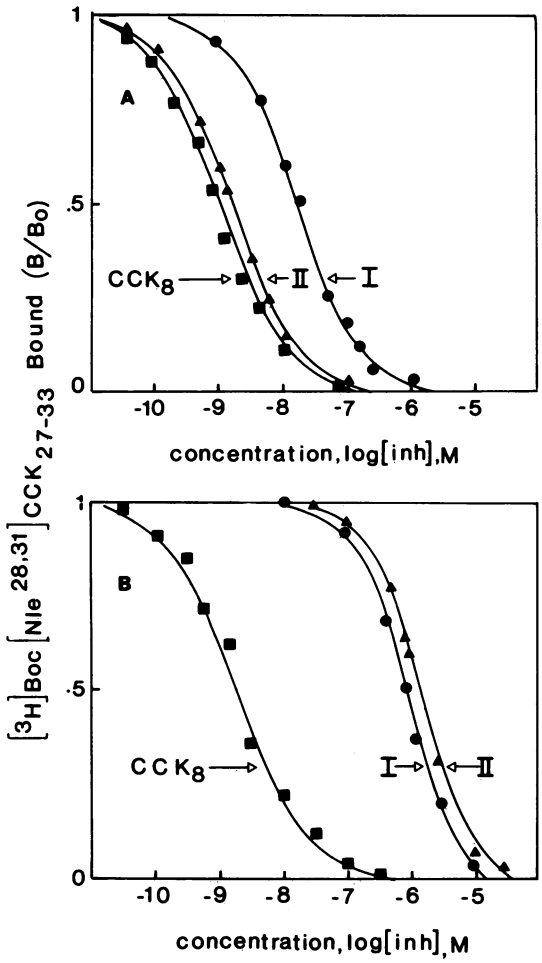

FIG. 3. Inhibition of [^{3}H]Boc[Ahx^{28,31}]CCK-(27–33) binding to guinea pig brain cortex membranes (*A*) and pancreatic acini (*B*) by CCK-8 and cyclic analogues. ■, CCK-8; ●, compound I; and ▲, compound II. B/B_o, ratio of binding with the corresponding concentration of inhibitor over binding without inhibitor. Each point represents the mean value of triplicate determinations, and the experiment was done three times. Nle, Ahx; inh, inhibitor.

^{125}I Bolton–Hunter-labeled CCK-8 (^{125}I-BHCCK-8) (Amersham, 2000 Ci/mmol) was displaced on the same membrane preparation under the conditions described by Chang and Lotti (34) for guinea pig cortical brain membranes at a protein concentration of 0.1 mg/ml and a radioiodinated ligand concentration of 15 pM.

Guinea pig pancreatic acini. Acini were prepared as reported (2), with the following modifications. The standard incubation medium [Hepes-buffered Ringer's solution supplemented with essential vitamin and amino acid mixtures, bovine serum albumin (2 g/liter), trypsin inhibitor (3 mg/liter)] also contained bacitracin (0.5 g/liter). Pancreatic lobules obtained by collagenase digestion of the tissue were then dispersed by successive aspirations through a P5000 Gilson pipette, and the isolated acini were washed four times in the standard incubation medium by successive decantations.

Displacement experiments with both [^{3}H]Boc[Ahx^{28,31}]-CCK-(27–33) and ^{125}I-BHCCK-8 were done in the standard incubation medium without bovine serum albumin. [^{3}H]Boc[Ahx^{28,31}]CCK-(27–33) (0.5 nM) was incubated for 20 min at 37°C with the acini suspension (final dilution, 4 g of original wet weight per 100 ml), ^{125}I-BHCCK-8 (10 pM) was incubated for 40 min at 37°C with acini (final dilution, 0.8 g of original wet weight per 100 ml), in the presence of increasing concentrations of competitors. Incubation was terminated as described for brain membranes.

In vitro *bioassays.* Amylase release from pancreatic acini was measured after incubation for 30 min at 37°C in the presence of CCK-8 or CCK analogues, as described (49). Amylase activity was determined using the Phadebas amylase test (Pharmacia).

Contractile activity of guinea pig ileum was done according to Hutchinson and Dockray (50).

Antagonist activities of compounds I and II were investigated using CCK-8 as an agonist in both assays. In pancreatic acini, the dose-dependent stimulation of amylase release induced by CCK-8 was monitored in the presence of increasing concentrations of the analogues (10^{-9}–10^{-5} M). Likewise, the contractions of the guinea pig ileum caused by CCK-8 (3×10^{-9} M) were measured in the presence of compounds I or II (10^{-9}–10^{-6} M), added 2 min before CCK-8.

RESULTS AND DISCUSSION

The two cyclic CCK analogues I and II were compared with CCK-8 for ability to displace the specific binding of [^{3}H]Boc[Ahx^{28,31}]CCK-(27–33) (46–48) from CCK receptors on guinea pig brain membranes and pancreatic acini. Results were also compared with those obtained with the more widely used ligand, ^{125}I-BHCCK-8 (34, 51–53).

In the brain, compounds I and II inhibited, with a high affinity, the specific binding of [^{3}H]Boc[Ahx^{28,31}]CCK-(27–33) in a concentration-dependent manner (Fig. 3). Compound II (K_i, 0.49 nM) was found to be more potent than compound I (K_i, 5.1 nM) and almost equipotent to CCK-8 (K_i, 0.30 nM). The same order of affinities was found when ^{125}I-BHCCK-8 was used (CCK-8: IC_{50}, 0.42 nM; compound I: IC_{50}, 10.2; compound II: IC_{50}, 0.92) (Table 1).

In contrast to their strong interaction with central CCK binding sites, compounds I and II were poor ligands for pancreatic receptors (Fig. 3, Table 1), as shown by displacement of both the tritiated (CCK-8: K_i, 0.9 nM; compound I: K_i, 910 nM; compound II: K_i, 970 nm) and the iodinated

Table 1. Potency (K_i) of compounds I and II in inhibiting specific binding of [^{3}H]Boc[Ahx^{28,31}]CCK-(27–33) and ^{125}I-BHCCK-8 in guinea pig brain membranes and pancreatic acini

	[^{3}H]Boc[Ahx^{28,31}]CCK-(27–33)		^{125}I-BHCCK-8	
Compound	Brain, K_i in nM	Pancreas, K_i in nM	Brain, IC_{50} in nM	Pancreas, IC_{50} in nM
CCK-8	0.30 ± 0.07	0.91 ± 0.26	0.42 ± 0.08	1.0 ± 0.25
I	5.10 ± 0.88	910 ± 140	10.20 ± 1.33	1600 ± 300
II	0.49 ± 0.03	970 ± 160	0.92 ± 0.02	1530 ± 460

[^{3}H]Boc[Ahx^{28,31}]CCK-(27–33) was used at 0.2 nM in brain and 0.5 nM in pancreas. ^{125}I-BHCCK-8 was used at 15 pM in brain and 10 pM in pancreas. Values represent mean ± SEM of three separate experiments done in triplicate.

(CCK-8: IC_{50}, 1.0 nM; compound I: IC_{50}, 1600 nM; compound II: IC_{50}, 1530 nM) ligands.

These results clearly show the high selectivity of these cyclic analogues, and especially compound II, for central CCK receptors, as illustrated by their selectivity factors (sf) determined from K_i for pancreas/K_i for brain obtained with either [^{3}H]Boc[Ahx^{28,31}]CCK-(27–33) (compound I: sf, 179; compound II: sf, 1979) or ^{125}I-BHCCK-8 (compound I: sf, 157; compound II: sf, 1663).

To determine whether cyclic peptides I and II interacted competitively or noncompetitively with central CCK receptors, the binding of [^{3}H]Boc[Ahx^{28,31}]CCK-(27–33) was analyzed according to Scatchard (54) in the presence of these compounds. Fig. 4 shows that the presence of compound I and compound II decreased the K_d values of the tritiated probe without modifying the binding capacity. This result suggests that compounds I and II interact competitively with the tritiated ligand at the level of the central CCK receptors, with K_i values of 6.0 ± 0.3 nM and 0.46 ± 0.03 nM, respectively.

The weak interaction of the two cyclic analogues with the peripheral CCK receptors was further examined by assessing their potency in two *in vitro* peripheral bioassays—i.e., stimulation of amylase release from pancreatic acini and induction of contractions in the guinea pig ileum.

In agreement with their low affinity for pancreatic CCK receptors, compounds I and II were only weakly active in stimulating amylase release from pancreatic acini (Fig. 5) (CCK-8: EC_{50}, 0.13 nM; compound I: EC_{50} > 10,000 nM; compound II: EC_{50} > 10,000 nM). Similarly, these compounds were unable to induce the contractions of guinea pig ileum when used at concentrations up to 10^{-6} M (EC_{50} of CCK-8 in this assay was 0.68 nM). Note that in the amylase release test, compound I was slightly more potent than compound II, despite the similar affinities displayed by these two peptides for pancreatic receptors. Nevertheless, the weak peripheral activity of compound II was clearly lower by at least a factor of 100,000 than that of CCK-8. In addition, no antagonistic activity was detected until a concentration of 10^{-6} M for the two peptides in both bioassays.

Thus, a high selectivity for central CCK receptors has been obtained for the cyclic CCK analogues I and II.

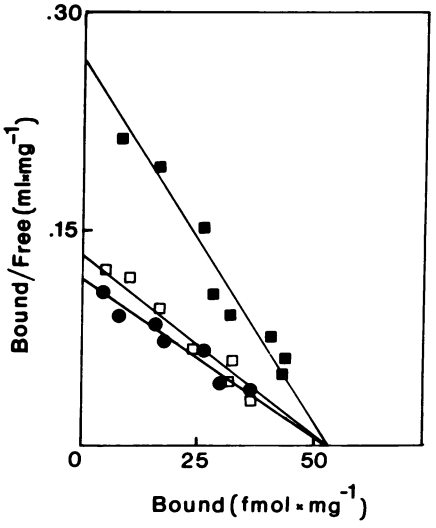

FIG. 4. Scatchard analysis of specific [^{3}H]Boc[Ahx^{28,31}]CCK-(27–33) binding to guinea pig cortex brain membranes in the absence (■) and presence of 6 nM of compound I (□) or 0.6 nM of compound II (●). Each point represents the mean value of triplicate determinations. This experiment was done three times. The mean K_d values were 0.20 ± 0.02 nM in control and in the presence of compounds I and II were 0.40 ± 0.03 nM and 0.46 ± 0.02 nM, respectively.

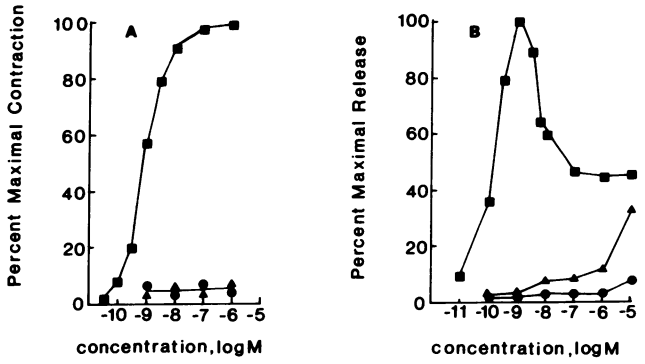

FIG. 5. Potencies of CCK-8 (■), compound I (●), and compound II (▲) in two peripheral bioassays. (*A*) Contractile activity of guinea pig ileum. Maximal contraction is expressed as the percentage of maximal stimulation induced by 500 nM of acetylcholine. Each point is the mean value of four experiments. (*B*) Stimulation of amylase release from guinea pig pancreatic acini. Results are expressed as percentage of maximal stimulation induced by CCK-8. Each point is the mean value of three separate experiments done in triplicate.

Furthermore, a 10-fold increase in selectivity towards brain CCK binding sites occurred by replacement of the D-Asp residue in compound I by a γ-D-Glu residue in compound II. The improvement in the sf, which reached between 1500 and 2000 in compound II, was essentially achieved through an increased affinity for central CCK receptors. This could relate to an increased flexibility of the cyclic moiety that better conformationally adapts the Tyr(SO_3H) residue to the central binding sites. In support of this hypothesis, the isomer of compound II obtained by cyclization through formation of an amide bond between the side-chain carboxyl and amino groups of D-Glu-26 and D-Lys-29 residues [Boc-D-Glu-Tyr(SO_3H)-Ahx-D-Lys-Trp-Ahx-Asp-Phe-NH_2] displayed an affinity (K_i, 6.3 nM) and a selectivity (sf, 160) similar to that of compound I.

Interestingly, such a selective modulation in the affinity for brain binding sites also occurred when the D-Asp residue of compound I was replaced by an L-Asp residue because the previously described analogue Boc-L-Asp-Tyr(SO_3H)-Ahx-D-Lys-Trp-Ahx-Asp-Phe-NH_2 (43) exhibited a 10-fold specificity towards central receptors, combined with an affinity for pancreatic receptors similar to that of compounds I and II.

The cyclization of the N-terminal part of CCK therefore opens the possibility of selectively modulating the interactions with central CCK receptors by small changes directed towards the cyclic portion of the molecule. Structure–activity studies on such cyclic compounds, combined with conformational investigations, could better define the prerequisite structure for selective interaction with central CCK receptors.

Cyclic peptide compounds I and II are extremely selective synthetic ligands for brain CCK receptors of guinea pig and, accordingly, could be valuable for investigating CCK function in the central nervous system.

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